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Recovery Correction Technique for NMR Spectroscopy of Perchloric Acid Extracts using DL-valine-2,3- d_2 : Validation and Application to 5-Fluorouracil-induced Brain Damage

Ryutaro NAKAGAMI^{1,2,3†}, Masayuki YAMAGUCHI^{1†*}, Kenji EZAWA^{1,2†}, Sadaaki KIMURA¹,
Shusei HAMAMICHI¹, Norio SEKINE², Akira FURUKAWA², Mamoru NIITSU⁴,
and Hirofumi FUJII¹

¹*Division of Functional Imaging, Research Center for Innovative Oncology,
National Cancer Center Hospital East Kashiwanoha
6-5-1, Kashiwa, Chiba 277-8577, Japan*

²*Graduate School of Human Health Sciences, Tokyo Metropolitan University*

³*Research Fellow of the Japan Society for the Promotion of Science*

⁴*Department of Radiology, Saitama Medical University*

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Purpose: We explored a recovery correction technique that can correct metabolite loss during perchloric acid (PCA) extraction and minimize inter-assay variance in quantitative ¹H nuclear magnetic resonance (NMR) spectroscopy of the brain and evaluated its efficacy in 5-fluorouracil (5-FU)- and saline-administered rats.

Methods: We measured the recovery of creatine and DL-valine-2,3- d_2 from PCA extract containing both compounds (0.5 to 8 mM). We intravenously administered either 5-FU for 4 days (total, 100 mg/kg body weight) or saline into 2 groups of 11 rats each. We subsequently performed PCA extraction of the whole brain on Day 9, externally adding 7 μ mol of DL-valine-2,3- d_2 . We estimated metabolite concentrations using an NMR spectrometer with recovery correction, correcting metabolite concentrations based on the recovery factor of DL-valine-2,3- d_2 . For each metabolite concentration, we calculated the coefficient of variation (CEV) and compared differences between the 2 groups using unpaired *t*-test.

Results: Equivalent recoveries of DL-valine-2,3- d_2 ($89.4 \pm 3.9\%$) and creatine ($89.7 \pm 3.9\%$) in the PCA extract of the mixed solution indicated the suitability of DL-valine-2,3- d_2 as an internal reference. In the rat study, recovery of DL-valine-2,3- d_2 was $90.6 \pm 9.2\%$. Nine major metabolite concentrations adjusted by recovery of DL-valine-2,3- d_2 in saline-administered rats were comparable to data in the literature. CEVs of these metabolites were reduced from 10 to 17% before to 7 to 16% after correction. The significance of differences in alanine and taurine between the 5-FU- and saline-administered groups was determined only after recovery correction (0.75 ± 0.12 versus 0.86 ± 0.07 for alanine; 5.17 ± 0.59 versus 5.66 ± 0.42 for taurine [μ mol/g brain tissue]; $P < 0.05$).

Conclusion: A new recovery correction technique corrected metabolite loss during PCA extraction, minimized inter-assay variance in quantitative ¹H NMR spectroscopy of brain tissue, and effectively detected inter-group differences in concentrations of brain metabolites between 5-FU- and saline-administered rats.

Keywords: *quantitative NMR spectroscopy, brain, perchloric acid extraction, recovery correction*

*Corresponding author, Phone & Fax: +81-4-7134-6832,
E-mail: masyamag@east.ncc.go.jp

†These authors equally contributed to this paper.

Introduction

^1H magnetic resonance spectroscopy (MRS) can provide insight into changes in brain metabolism in various brain disorders. In Alzheimer's disease, for instance, ^1H MRS can detect the reduction of *N*-acetylaspartate (NAA) in the cortical grey matter and the white matter, where either a loss of neuronal components or disruption of neuronal function, or both, takes place.¹ In survivors of breast cancer treated with high dose chemotherapy, ^1H MRS can also detect reduction in NAA in the centrum semiovale, which may reflect brain damage induced by the chemotherapy.² In addition, MRS-detectable metabolites, including NAA, choline, and myoinositol, have been reported as putative diagnostic indicators of these brain disorders, but this requires pathological validation.¹

Quantitative nuclear magnetic resonance (NMR) spectroscopy of the brain is a reasonable approach for validating *in vivo* ^1H MRS observation.^{3–7} Quantitative NMR spectroscopy traditionally involves perchloric acid (PCA) extraction of brain metabolites because PCA can quickly deactivate enzymes to minimize postmortem changes in metabolite profiles. Accordingly, profiles similar to those *in vivo* can be obtained in NMR spectroscopy even in excised non-perfused brain samples.

Although PCA extraction is an established technique, it is somewhat limited with respect to metabolite yield. Some metabolites are inevitably removed with protein and potassium perchlorate (KClO_4) precipitate during extraction,^{3,6} and large inter-assay variance in metabolite yield is common, presumably because of massive KClO_4 precipitate.⁸ These limitations would most likely result in serious error when researchers aim to validate the quantity of metabolites measured with *in vivo* ^1H MRS by comparing it to that measured with *in vitro* ^1H NMR spectroscopy. Thus, a correction technique is needed that avoids quantification error from metabolite loss and minimizes the large variance in metabolite yield. Traditional techniques utilized a ratio of metabolites, dividing the signal strength of a metabolite by that of an internal standard, such as a ratio of NAA to creatine (Cr), assuming no change in the quantity of the internal standard among individuals regardless of their health. This assumption is no longer considered valid because the quantity of the internal standard depends on each individual and various conditions. Furthermore, these techniques do not take into account the influence of metabolite loss or inter-assay variance in metabolite yield on quantification. Rather, they cancel out these sources of error based

on the inappropriate assumption.

In this study, we explored a technique that can correct metabolite loss during the PCA treatment and minimize inter-assay variance in quantitative ^1H NMR spectroscopy of the brain. To the best of our knowledge, however, such technique has not been reported. To this end, we tried to identify an internal reference compound that can monitor metabolite recovery in each PCA extract. Such a compound would require 1) low molecular weight and water solubility, 2) a recovery yield similar to that of native tissue metabolites, such as Cr and NAA, 3) a simple singlet peak, and 4) a chemical shift range where important MRS-detectable brain metabolites such as lactate, NAA, glutamine, glutamate, creatine, choline, and myoinositol do not resonate. If such an internal reference compound is artificially added to brain samples before PCA extraction, the difference in quantity of the reference compound before and after the PCA extraction could indicate the recovery of this marker.

To demonstrate the effectiveness of this correction technique, we conducted a quantitative ^1H NMR spectroscopy study of the brain obtained from rats administered either 5-fluorouracil (5-FU) or saline. The former served as an animal model of chemotherapy-induced brain damage.^{9–11} Because there exist particular constraints in sampling human brain tissue, animal research is a practical approach to chemically analyze brain samples involved with chemotherapy-induced brain damage. It is well known that 5-FU can alter brain metabolism. Previous studies have demonstrated that 5-FU catabolites, particularly fluoroacetate and fluorocitrate, affect metabolic pathways related to alanine and taurine metabolism in the brain.^{12–15} According to our premise, the correction technique would sensitively detect the difference in brain metabolite concentrations between these 2 groups of rats.

Materials and Methods

Chemicals

We purchased perchloric acid (60%, 24-1051-5; Sigma Aldrich Japan, Tokyo, Japan), 3-(trimethylsilyl)-propionic-2,2,3,3- d_4 acid (TSP- d_4 , 269913; Isotec Inc., Miamisburg, OH, USA), DL-valine-2,3- d_2 (Val- d_2 , D-2920; C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada), creatine anhydrous (Cr, Fluka WA13057; Sigma-Aldrich Corp., Buchs, Switzerland), *N*-acetylaspartate (NAA, Fluka 00920; Sigma-Aldrich Corp.), phosphocholine chloride calcium (P0378; Sigma-Aldrich Corp., St. Louis, MO, USA), deuterium oxide (D_2O , 1.13366.0100; Merck KGaA, Darmstadt, Germany),

and 5-fluorouracil (Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan).

Preparation of mixed solutions of creatine and Val- d_2 , as well as creatine, N-acetylaspartate, and phosphocholine

We dissolved 10.49 g of Cr and 9.54 g of Val- d_2 in 10 mL of deionized water to yield a mixed solution of 8-mM Cr and 8-mM Val- d_2 and serially diluted the solution with deionized water to obtain solutions of 4, 2, one, and 0.5 mM. We added 10 mL of 6% ice-cold perchloric acid to these one-mL solutions containing 8, 4, 2, one, and 0.5 μ mol of Cr and Val- d_2 ($n = 4$ for each concentration level). We also prepared one-mL solutions ($n = 3$) containing 6.67 mM of phosphocholine, 20 mM of Cr, and 20 mM of NAA. We performed PCA extraction as described below for these mixed solutions to determine the recovery of the dissolved metabolites.

Animals

Our institutional animal experimental committee approved the study protocol. We purchased 22 male Wistar rats (aged 8 weeks, 180 to 200 g) from Japan SLC (Hamamatsu, Japan). They were housed two per cage under controlled environmental conditions (12-hour light/12-hour dark cycles, $22 \pm 2^\circ\text{C}$ room temperature, and 50% humidity) and fed *ad libitum*. After a week of acclimatization, we randomly assigned 11 rats to each of 2 groups. The first group received 25 mg/kg body weight (BW) of 5-FU for 4 consecutive days (total dose, 100 mg/kg BW) via their tail veins, and the second group received an equivalent volume of saline for 4 days. Nine days after 5-FU or saline administration, we killed the rats by decapitation, immediately froze the heads with liquid nitrogen in a basin, and stored them in a freezer at -80°C . The next day, we excised the whole brain of each rat on ice to avoid thawing, weighed it, and stored it at -80°C for several days until the PCA extraction.

Perchloric acid extraction

We pulverized the brain using dedicated equipment (TK-CM20; Tokken Inc., Kashiwa, Japan) that consisted of a stainless cell (height, 61 mm; outer diameter, 30 mm; inner diameter, 24 mm; volume, 20 mL), a stainless crusher (cylinder; height, 25 mm; diameter, 23.7 mm), and a detachable shaft. We placed the frozen brain and crusher in the cell, which had been cooled with liquid nitrogen, cooled the cell in a basin of liquid nitrogen for several minutes, pulled it out, and struck it against a rubber floor typically 150 times at room temperature while

shaking the shaft attached to it. Approximately every 30 times (approximately 30 s), we cooled the cell with liquid nitrogen for several minutes to keep the cell at low temperature. After a cryogenic mill (Cryo-Press; Microtec Co., Ltd., Funabashi, Japan) was introduced, we used this equipment, and quickly pulverized the brain typically within one minute. We placed the frozen brain into a pre-cooled stainless cell (height, 31 mm; outer diameter, 40 mm; inner diameter, 30 mm; volume, 9 mL), put a pre-cooled stainless crusher (height, 41 mm; diameter, 29.7 mm) on the cell, and crushed it using an air compressor. Before and after crushing, we poured liquid nitrogen onto the brain to keep it at a low temperature. We then transferred the pulverized brain into a 50-mL tube and subjected it to PCA extraction.

We applied the pulverized brain into 10 volumes of ice-cold perchloric acid (6%) containing 7 μ mol Val- d_2 . We homogenized the brain (NS-310E or NS-310EII; Microtec Co., Ltd., Funabashi, Japan), cleaned the homogenizer shaft with one mL of deionized water to collect the residual sample and added it to the homogenate, centrifuged the homogenate at 3000 rpm and 4°C for 20 min (5930; Kubota Corp., Tokyo, Japan), and recovered the supernatant to obtain water-soluble extract. To recover the residual metabolites in the precipitate, we re-extracted them with one mL of ice-cold perchloric acid (6%). The extract was alkalized by adding 5 N potassium hydroxide (KOH) to precipitate KClO_4 and then neutralized to $\text{pH } 7.0 \pm 0.1$ with hydrochloric acid and/or KOH (0.05 to one N). To remove KClO_4 salt, we centrifuged the neutralized extract at 3000 rpm and 4°C for 10 min and recovered the supernatant. To collect residual metabolites in the precipitate, we added one mL of deionized water, mixed them, centrifuged the mixture at 3000 rpm and 4°C for 10 min, recovered another supernatant, and added it to the extract. The extract was stored in a refrigerator at 4°C overnight to promote further KClO_4 precipitation. The next day, we again measured the pH of the extract and adjusted it to 7.0 if necessary. The extract was centrifuged at 3000 rpm at 4°C for 10 min to further remove KClO_4 . We added ion-exchange resin (Chelex 100 Resin, 142-2832; Bio-Rad Laboratories, Inc., Hercules, CA, USA; 0.5 g/10 mL extract fluid) to the extract and mixed them at 4°C using a tube rotator (MACSmix MX001; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) to remove paramagnetic ions. Then, we filtered the mixture through a filtration funnel to remove the ion-exchange resin and recovered the extract in a 40- to 80-mL freeze-dry bottle. We cleaned the funnel

with one mL of deionized water to recover the residual sample and added it to the extract. The extract was frozen and lyophilized with a freeze-dry machine (FDU-810; Tokyo Rikakikai Co., Ltd., Tokyo, Japan) for typically 24 hours. After lyophilization, we dissolved the lyophilized sample with 600 μ L of D₂O containing 1.5- to 3.0- μ mol TSP-*d*₄, centrifuged it at 12000 $\times g$ at room temperature for one min (Mx-305; Tomy Seiko Co., Ltd., Tokyo, Japan) to precipitate undissolved KClO₄, recovered supernatant in a 5-mm NMR tube (EC 57; Shigemi Co., Ltd., Tokyo, Japan), and neutralized it to pD 7.0 $\pm$ 0.4 with sodium deuterioxide (NaOD) or deuterium chloride (DCl).

During the PCA extraction of the mixed solution containing Cr and Val-*d*₂, we applied the solution (one mL) to 10 mL of ice-cold perchloric acid (6%). For the mixed solution of Cr, NAA, and phosphocholine, we applied one mL of the solution to 4 mL of ice-cold perchloric acid (25%). Subsequently, we performed the extraction procedure described above except for pulverization and homogenization.

NMR measurements

All NMR spectra were acquired using a standard-bore NMR spectrometer (AVANCE III 400; Bruker BioSpin AG, Fällanden, Switzerland) equipped with a tunable multinuclear probe (PA BBO 400S1 BBF-H-D05 ZPLUS; Bruker BioSpin) operating at 400 MHz for ¹H. We obtained ¹H NMR spectra of the Cr and Val-*d*₂ mixed solutions as well as PCA extracts of the brain using a single pulse sequence with solvent suppression (zgpr; Bruker BioSpin). The parameters consisted of 9-s repetition time (TR), 90° flip angle, 20.6-ppm spectral width, and 16 accumulations. This repetition time was unlikely to induce errors in metabolite quantification related to signal saturation because our preliminary experiment demonstrated no significant differences in metabolite quantities between measurements with TRs of 9 and 20 s. Nine seconds also seemed sufficient for the relaxation of the longitudinal magnetization of Val-*d*₂ methyl protons, whose typical T₁ relaxation time was 1.3 s. For the ¹H NMR spectra of the Cr, phosphocholine, and NAA solution, we used a single pulse sequence without solvent suppression (zg; Bruker BioSpin) with a delay of 20 s, 90° flip angle, and 4 excitations. Subsequently, we acquired 64K (65536) data points with a spectral width of 8223.7 Hz. Data were Fourier-transformed without zero-filling and with 0.3-Hz line broadening to obtain NMR spectra with a spectral resolution of 0.25 Hz. This spectral resolution seemed sufficient to resolve metabolite peaks, including those of

TSP-*d*₄ Si(CH₃)₃, Val-*d*₂ CH₃, and Cr N-CH₃, whose full width half maximum was typically 0.8 to 1.9 Hz in PCA extracts of the brain. Spectra were automatically phased and the baseline flattened using the manufacturer's standard software (TopSpin version 2.1; Bruker BioSpin). We set the chemical shift value of the trimethylsilyl resonance of TSP to 0 ppm and determined chemical shifts of each metabolite relative to the TSP trimethylsilyl resonance. We measured integrals of each metabolite using the TopSpin software and compared the integral of each metabolite resonance with that of TSP trimethylsilyl resonance to determine the quantities of metabolites. In the rat brain study, we measured 9 metabolites, including lactate, alanine, NAA, glutamate, glutamine, total Cr (creatine and phosphocreatine), choline compounds, taurine, and myoinositol, which are observable in *in vivo* MRS of rat brains at 9.4 tesla.¹⁶

Analysis

We determined peak integrals of Val-*d*₂ methyl resonance at 1.04 ppm and TSP trimethylsilyl resonance at 0 ppm and then calculated the quantity of Val-*d*₂ using the following equation:

$$\begin{aligned} &(\text{quantity of Val-}d_2 [\mu\text{mol}]) \\ &= 3 \times (\text{peak integral of Val-}d_2 \text{ CH}_3) \\ &\quad \times (\text{known quantity of TSP } [\mu\text{mol}]) \\ &\quad / (\text{peak integral of TSP (CH}_3)_3). \end{aligned} \quad [1]$$

We determined the recovery of Val-*d*₂ by dividing the values obtained before and after PCA extraction and expressed it in percent. Then, we corrected the quantity of each metabolite using the equation below assuming that the recovery of each metabolite was equivalent to that of Val-*d*₂:

$$\begin{aligned} &\text{Metabolite}_{\text{corrected}} [\mu\text{mol}] \\ &= \text{Metabolite}_{\text{postPCA}} [\mu\text{mol}] \\ &\quad \times 100 / \text{Recovery}_{\text{Val-}d_2} [\%], \end{aligned} \quad [2]$$

where Metabolite_{corrected} represents the corrected quantity of metabolite after taking the recovery of Val-*d*₂ into account, Metabolite_{postPCA} signifies the quantity of metabolite after PCA extraction, and Recovery_{Val-*d*₂} represents the recovery of Val-*d*₂ as mentioned above. For the Val-*d*₂ and Cr solution study, we determined the recoveries of Val-*d*₂ as well as Cr and corrected the quantity of Cr using Eq. [2]. For the Cr, phosphocholine, and NAA mixed solution study, we determined the differences in recovery between phosphocholine and Cr as well as between NAA and Cr. For the rat brain analysis, we calculated metabolite concentrations

in the brain samples by dividing $\text{Metabolite}_{\text{corrected}}$ by the wet weight of the brain [g]. All values are expressed as means $\pm$ standard deviation (SD). We also calculated the coefficient of variation (CEV).

Statistical tests

In the Val- d_2 and Cr solution study, we investigated the correlation between the known quantity of Cr applied before PCA extraction and the quantity of Cr corrected with Eq. [2] ($\text{Cr}_{\text{corrected}}$) and performed linear regression between these quantities and calculated the coefficient of determination (R^2). We also investigated the correlation between the known quantity of Cr and the quantity of Cr after PCA extraction ($\text{Cr}_{\text{post PCA}}$) and performed linear regression between these 2 quantities, and we compared the difference in metabolite concentration in the brain between the 5-FU- and saline-administered rat groups using unpaired t -test and commercially available software (SPSS, version 19; IBM, Tokyo, Japan). $P < 0.05$ was determined significant.

Results

Table 1 shows the recovery of Val- d_2 and Cr after PCA extraction and the quantity of Cr corrected by Val- d_2 recovery. The average of Val- d_2 recoveries ($n = 20$, Val- d_2 ranged from 0.5 to 8.0 μmol) was $89.4 \pm 3.9\%$, whereas that of Cr was $89.7 \pm 3.9\%$. Creatine values ($\text{Cr}_{\text{post PCA}}$) were highly correlated with those before PCA extraction ($\text{Cr}_{\text{pre PCA}} = 0.8846 \times \text{Cr}_{\text{pre PCA}} + 0.0295$, 95% confidence interval [CI] for the slope, 0.8594 to 0.9098; 95% CI for the intercept, -0.0745 to 0.1335 , $R^2 = 0.9999$). After the correction with Val- d_2 recovery, creatine values ($\text{Cr}_{\text{corrected}}$) became equivalent to

those before the extraction ($\text{Cr}_{\text{pre PCA}}$). These corrected creatine values were also highly correlated with those before extraction ($\text{Cr}_{\text{corrected}} = 0.9969 \times \text{Cr}_{\text{pre PCA}} + 0.0109$, 95% CI for the slope, 0.9901 to 1.0037; 95% CI for the intercept, -0.0173 to 0.0390 , $R^2 = 1$). The CEVs of the creatine quantity ranged from one to 8% before correction and were reduced to one to 2% after correction, indicating the reduction of inter-assay variance.

The difference in recovery was $0.3 \pm 0.8\%$ between phosphocholine and Cr and $0.3 \pm 1.2\%$ between NAA and Cr, indicating that the recoveries of phosphocholine and NAA were equivalent to that of Cr in the PCA extract.

The 5-FU-administered brain samples weighed 1.77 ± 0.05 g and the saline-administered samples, 1.89 ± 0.15 g. Figure 1 shows representative NMR spectra of brain PCA extracts of control and 5-FU-treated rats. The addition of excessive Val- d_2 produced significant methyl peaks at 0.97 and 1.04 ppm (Fig. 2). Visual inspection of the NMR spectra revealed no apparent difference in metabolic profiles between normal and 5-FU-treated brains. Tables 2 and 3 summarize brain metabolite concentrations in the 5-FU- and saline-administered groups with and without correction. The recovery of Val- d_2 was $90.6 \pm 9.2\%$. After the correction, standard deviations as well as CEVs of the metabolite concentrations were reduced for all metabolites in both groups except for alanine in the 5-FU-treated group. Statistical tests for detecting differences in metabolite concentrations between the 2 groups revealed that P -values were also decreased after the correction in all metabolites. Alanine and taurine concentrations in the 5-FU-administered group were significantly lower than those in the saline-administered group ($P = 0.008$

Table 1. Recovery of DL-valine-2,3- d_2 (Val- d_2) and creatine (Cr) after perchloric acid (PCA) extraction, and the quantity of Cr corrected by Val- d_2 recovery

Applied Val- d_2 or Cr (μmol)	N	Val- d_2 _{post PCA} (μmol)	Val- d_2 _{recovery} (%)	Cr _{post PCA} (μmol)	CEV (%)	Cr _{recovery} (%)	Cr _{corrected} (μmol)	CEV (%)
0.5	4	0.43 ± 0.03	86.2 ± 6.5	0.44 ± 0.04	8.0	87.4 ± 7.0	0.51 ± 0.01	2.0
1.0	4	0.92 ± 0.02	91.6 ± 2.4	0.91 ± 0.02	1.7	91.2 ± 1.6	1.00 ± 0.01	1.9
2.0	4	1.81 ± 0.00	90.5 ± 0.2	1.83 ± 0.02	1.4	91.5 ± 1.2	2.02 ± 0.03	1.4
4.0	4	3.59 ± 0.12	89.8 ± 3.0	3.59 ± 0.14	3.9	89.8 ± 3.5	4.00 ± 0.04	0.9
8.0	4	7.11 ± 0.34	88.8 ± 4.2	7.09 ± 0.32	4.5	88.6 ± 4.0	7.98 ± 0.08	1.0

Val- d_2 _{post PCA} and Cr_{post PCA} are the quantities of Val- d_2 and Cr after PCA extraction.

Val- d_2 _{recovery} and Cr_{recovery} are the recovery of Val- d_2 and Cr.

Cr_{corrected} is the amount of Cr corrected with Val- d_2 recovery using Eq. [2] in Materials and Methods.

CEV, coefficient of variation (standard deviation [SD] $\times$ 100/mean).

Fig. 1. Representative ^1H nuclear magnetic resonance (NMR) spectra of the rat brain after administration of 5-fluorouracil (5-FU; upper) and saline (lower). 1, 3-(trimethylsilyl)-propionic acid (TSP); 2, DL-valine-2,3- d_2 (Val- d_2); 3, lactate; 4, alanine; 5, N-acetylaspartate (NAA); 6, glutamate; 7, glutamine; 8, creatine (Cr) + phosphocreatine; 9, choline compounds; 10, taurine; 11, myoinositol.

Fig. 2. ^1H nuclear magnetic resonance (NMR) spectra of the rat brain with (upper) and without (lower) addition of DL-valine-2,3- d_2 (Val- d_2). Note large methyl peaks indicative of Val- d_2 at 0.97 and 1.04 ppm. In contrast, native valine produces small doublet peaks at 0.98 and 1.01 ppm and at 1.04 and 1.06 ppm. The lower spectrum was derived from an age-matched normal Wistar rat.

Table 2. Brain metabolite concentrations WITH recovery correction in 5-fluorouracil (5-FU)- and saline-administered groups

	5-FU-administered group		Saline-administered group		<i>t</i> -values	<i>P</i> -values	Literature data	
	($\mu\text{mol/g}$)	CEV	($\mu\text{mol/g}$)	CEV			(μmol/g)	
Lactate	4.92 ± 0.38	7.7	5.36 ± 0.67	12.5	1.905	0.071	3.16 ± 0.13 ³	1.46 ± 0.05 ⁶
Alanine	0.75 ± 0.12	15.6	0.86 ± 0.07	7.8	2.924*	0.008	0.57 ± 0.02 ⁶	0.48 ¹⁷ 0.65 ¹⁸
N-acetylaspartate	6.97 ± 0.58	8.3	7.39 ± 0.52	7.0	1.772	0.092	6.51 ± 0.22 ⁶	
Glutamate	9.51 ± 0.67	7.0	9.76 ± 0.69	7.0	0.874	0.392	9.92 ± 0.64 ³	8.64 ± 0.32 ⁶
Glutamine	2.77 ± 0.87	31.3	3.33 ± 0.41	12.2	1.945	0.066	5.18 ± 0.20 ⁶	5.02 ¹⁷ 5.6 ¹⁸
Total Cr (Cr + PCr)	8.27 ± 0.75	9.1	8.61 ± 0.65	7.6	1.139	0.268	7.48 ± 0.33 ³	7.74 ± 0.24 ⁶
Choline compounds	1.85 ± 0.14	7.6	1.92 ± 0.14	7.2	1.110	0.280	1.28 ± 0.04 ⁶	
Taurine	5.17 ± 0.59	11.5	5.66 ± 0.42	7.3	2.254*	0.036	4.6 ¹⁷ 6.6 ¹⁸	
Myoinositol	6.63 ± 0.71	10.7	6.93 ± 0.58	8.3	1.063	0.300	n/a	

Metabolites are expressed as mean ± standard deviation (SD).

CEV, coefficient of variation (SD × 100/mean); Cr, creatine; PCr, phosphocreatine

*indicates statistical significance ($P < 0.05$)

Rat brain myoinositol concentration is not available.

Table 3. Brain metabolite concentrations WITHOUT recovery correction in 5-fluorouracil (5-FU)- and saline-administered groups

	5-FU-administered group		Saline-administered group		t-values	P-values
	($\mu\text{mol/g}$)	CEV	($\mu\text{mol/g}$)	CEV		
Lactate	4.56 ± 0.44	9.6	4.71 ± 0.65	13.7	0.631	0.535
Alanine	0.69 ± 0.10	14.0	0.76 ± 0.13	16.5	1.553	0.136
N-acetylaspartate	6.47 ± 0.69	10.6	6.52 ± 0.91	14.0	0.154	0.879
Glutamate	8.84 ± 1.03	11.6	8.59 ± 0.89	10.4	0.616	0.545
Glutamine	2.60 ± 0.95	36.5	2.93 ± 0.43	14.5	1.053	0.305
Total Cr (Cr + PCr)	7.67 ± 0.81	10.5	7.61 ± 1.16	15.3	0.124	0.902
Choline compounds	1.72 ± 0.19	10.9	1.69 ± 0.24	14.2	0.289	0.776
Taurine	4.80 ± 0.65	13.6	4.99 ± 0.69	13.7	0.686	0.501
Myoinositol	6.16 ± 0.82	13.3	6.11 ± 0.86	14.1	0.146	0.886

Metabolites are expressed as mean $\pm$ standard deviation (SD).

CEV, coefficient of variation ($\text{SD} \times 100/\text{mean}$); Cr, creatine; PCr, phosphocreatine

for alanine and 0.036 for taurine, unpaired *t*-test).

Discussion

We propose herein a simple and practical recovery correction technique for quantitative ^1H NMR spectroscopy of PCA extracts of the brain. Our rat brain study demonstrated the efficacy of the technique in clarifying the differences in metabolite quantities between normal and abnormal groups. The proposed technique reduced variances in metabolite quantity in each group, probably by removing inter-assay errors, and allowed us to detect inter-group differences that were veiled before correction. The correction technique also elevated the average quantities of metabolites in each group, but the elevation for each metabolite did not differ significantly between the 5-FU and saline groups. Furthermore, we noted no systematic error in metabolite quantities before and after correction in the 2 groups (Bland-Altman analysis, data not shown). Therefore, it was unlikely that unknown bias related to the correction procedure itself produced the inter-group differences. It was thought that this correction technique could enhance the diagnostic performance of quantitative ^1H NMR spectroscopy in discriminating pathologic brain conditions not only for rat but also for human brain PCA extracts. The enhanced diagnostic performance could enable the validation of *in vivo* ^1H MRS findings in not only chemotherapy-induced brain damage but also other disorders, such as Alzheimer's disease, in which NAA is reported as a potential diagnostic marker.¹

We think that our PCA extraction and NMR quantification was optimal. Our observed metabolite recovery was comparable to that reported by

the groups of Veech (90 to 102%) and Le Belle (58 to 88%) after PCA extraction in rat brains, and the precorrected coefficients of variation in the quantities of brain metabolites, including Cr, choline compounds, NAA, alanine, and lactate, were also comparable between our study and that of LeBelle.⁶ Metabolite concentrations in the saline-administered rat brain were also comparable to those published in previous studies.^{3,5,6,17,18} The exception included lactate and alanine concentrations, which were 3.6 and 1.4 times higher in our study than those reported by Le Belle and colleagues.⁶ This discrepancy was probably caused by our different procedures for freezing brain tissues. Le Belle's group froze brains *in situ* by pouring liquid nitrogen directly onto the exposed skull, whereas we froze the skull in a liquid nitrogen basin immediately after decapitation, which may have rendered the brain anoxic for several minutes until it was completely frozen. Such anoxia can explain the elevated levels of lactate and alanine in our study.

We chose Val- d_2 as an internal reference compound for monitoring metabolite recovery because it is a water-soluble and low-molecular-weight metabolite and gives rise to singlet peaks. As we demonstrated, Val- d_2 can be recovered after PCA extraction as efficiently as Cr, which is widely accepted as an internal reference compound when estimating relative metabolite quantity. As shown in Fig. 1, the chemical shift ranges of Val- d_2 are distant from those of important MRS-detectable brain metabolites. These characteristics fulfill the requirements for an internal reference compound as mentioned. In addition, Val- d_2 allows accurate measurement of the recovered internal reference

compound with minimal interference with native compounds because Val- d_2 gives rise to 2 singlet peaks in the region where branched-chain amino acids (BCAAs, including valine, leucine, and isoleucine) methyl peaks resonate. Since these native BCAA concentrations in the brain tissues (e.g., 0.06 to 0.07 $\mu\text{mol/g}$ for valine)^{17,18} are miniscule enough to generate only small peaks in that chemical shift region, we generated large methyl peaks indicative of Val- d_2 by adding excessive amounts, approximately 50 to 60 times more than native amounts of valine, to the rat brain sample (approximately 1.8 to 1.9 g in weight). The addition of the large amount of Val- d_2 can reduce errors in the integral measurement of Val- d_2 , ignoring the contribution of these native BCAA peaks to Val- d_2 peaks.

On the contrary, the addition of a large amount of external Val- d_2 has a drawback. This apparently interferes with NMR observation of methyl resonances from native valine, leucine, and isoleucine, BCAAs known to play important roles in regulating nitrogen metabolism, glutamine synthesis, and energy production in the brain.^{19,20} Therefore, an NMR study to analyze such BCAA metabolism could become impossible using our proposed technique; hence, this is a major limitation of the technique. A compound with chemical shift range wherein no native low molecular weight metabolites resonate would be ideal, but we contend that Val- d_2 is a practical and readily available internal reference compound.

To quantify the reference compound, we used TSP- d_4 , a well known chemical shift reference, as a second internal standard compound. The known quantity of TSP- d_4 can also be utilized as an internal reference for calibrating the signal strength of protons in NMR samples. Trimethylsilyl compounds, including TSP- d_4 , are not used to monitor metabolite recovery because they are easily volatile during PCA extraction (more precisely during lyophilization) so that their recovery yields are lower than those in native tissue metabolites.

With the proposed recovery correction technique, we demonstrated a reduction in alanine and taurine after 5-FU administration. Alanine is produced by reductive amination of pyruvate and associated with glycolysis, and taurine has osmoregulatory, antioxidant, and membrane-stabilizing properties and can influence cognition.¹⁵ Metabolites associated with 5-FU catabolism, specifically fluoroacetate and fluorocitrate, have been shown to affect metabolic pathways related to alanine and taurine in the brain.¹⁴ It is well documented that fluoroacetate or fluorocitrate administration reduces taurine levels in the rat brain.^{12–15} In addition, we observed the 5-FU dose-dependent (0 to 125 mg of 5-FU per kg

body weight) reduction of brain taurine concentrations in rats (unpublished data). Lahiri and associates reported the reduction of brain alanine production after fluoroacetate administration.¹² Therefore, we contend that the reduction in taurine and alanine concentrations that we observed in *in vitro* NMR spectroscopy probably reflected the reduction of the corresponding metabolites in the rat brain. The reduction in taurine and alanine concentrations in the brain after 5-FU administration warrants further study to investigate the relevance of metabolic pathways related to these metabolites and symptoms in 5-FU-induced brain damage.

In clinical practice, 5-FU is well known to cause brain damage; 5-FU-induced leukoencephalopathy^{21,22} and a condition commonly called “chemo-brain” are examples. The latter produces long-term and subtle symptoms related to working memory, executive functioning, and processing speed that lead to deterioration of quality of life in a subgroup (17 to 34%) of cancer survivors treated with chemotherapy.^{23–25} Presently, no diagnostic indicator of chemotherapy-induced brain damage, including 5-FU-induced leukoencephalopathy and “chemo-brain,” has been established. Our data indicate that quantitative ^1H NMR spectroscopy could help determine such a diagnostic indicator of chemotherapy-induced brain damage by demonstrating changes in levels of some NMR-detectable metabolites. We think that the changes in these NMR detectable metabolites could also be examined in patients non-invasively using *in vivo* MR spectroscopy, thereby providing useful information for diagnosis of chemotherapy-induced brain damage in clinical practice.

Conclusion

We explored a new recovery correction technique that can correct metabolite loss in PCA extraction and minimize inter-assay variance in quantitative ^1H NMR spectroscopy of the brain. Our approach effectively detected inter-group differences in concentrations of brain metabolites between 5-FU- and saline-administered rats.

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