

## Single oral doses of $^{13}\text{C}$ forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies

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Single  $^{13}\text{C}_6$ -labelled doses of pteroylmonoglutamic acid (PteGlu; 634 nmol) or 5-formyltetrahydrofolic acid (431–569 nmol) were given to fasted adult volunteers, and the rise in total and  $^{13}\text{C}$ -labelled plasma 5-methyltetrahydrofolic acid metabolite monitored over 8 h by HPLC and liquid chromatography–MS. The dose-adjusted area under the curve (AUC) for total (labelled plus unlabelled) plasma 5-methyltetrahydrofolic acid following a 5-formyltetrahydrofolic acid test dose was 155 % that obtained following a PteGlu test dose. Surprisingly, an average 60 and 40 % of the total plasma 5-methyltetrahydrofolic acid response to [ $^{13}\text{C}_6$ ]PteGlu and [ $^{13}\text{C}_6$ ]5-formyltetrahydrofolic acid, respectively, was unlabelled; an observation never before reported. Short-term kinetics of plasma [ $^{13}\text{C}_6$ ]5-methyltetrahydrofolic acid showed a slower initial rate of increase in plasma concentration and longer time to peak following an oral dose of [ $^{13}\text{C}_6$ ]PteGlu compared with that for an oral dose of [ $^{13}\text{C}_6$ ]5-formyltetrahydrofolic acid, while the [ $^{13}\text{C}_6$ ]5-methyltetrahydrofolic acid AUC for [ $^{13}\text{C}_6$ ]5-formyltetrahydrofolic acid was 221 % that for [ $^{13}\text{C}_6$ ]PteGlu. These data indicate that PteGlu and 5-formyltetrahydrofolic acid, which are thought to be well absorbed (about 90 %) at physiological doses, exhibit dramatically different rates and patterns of plasma response. A limitation in the rate of reduction of PteGlu before methylation could result in slower mucosal transfer of [ $^{13}\text{C}_6$ ]5-methyltetrahydrofolic acid derived from [ $^{13}\text{C}_6$ ]PteGlu into the plasma. This, when coupled with an observed similar plasma clearance rate for [ $^{13}\text{C}_6$ ]5-methyltetrahydrofolic acid metabolite derived from either folate test dose, would yield a comparatively smaller AUC. These findings suggest potential problems in interpretation of absorption studies using unlabelled or labelled folates where the rate of increase, the maximum increase, or the AUC, of plasma folate is employed for test foods (mainly reduced folates) v. a ‘reference dose’ of PteGlu.

### Pteroylmonoglutamic acid: Folate: Absorption: Plasma: Stable isotopes

Folate is a generic term for B-group vitamins found widely in foodstuffs, mainly reduced methyl and formyl folates (Perry, 1971), that have nutritional properties and chemical structures similar to those of pteroylmonoglutamic acid (PteGlu) (Scott & Weir, 1976). PteGlu is the synthetic form of the vitamin that is used extensively for food fortification purposes and supplements, but which does not occur naturally in significant amounts. Folates are crucial for methionine and nucleotide biosynthesis (Shane, 1995). Periconceptual supplementation of women with PteGlu has been shown to significantly reduce the incidence and recurrence of neural tube defects, such as spina bifida (Anonymous, 1991; Czeizel & Dudas, 1992). Marginal folate deficiency is also associated with elevated plasma homocysteine, an emerging risk factor for vascular

diseases (Bousey *et al.* 1995), epigenetic factors such as cytosine-phosphate-guanosine dinucleotide methylation-associated transcription factor-binding (Nephew & Huang, 2003) and uracil-induced genomic instability (Blount *et al.* 1997). Either manipulation of the diet or fortification of food with physiological levels of PteGlu may be expected to reduce the prevalence of these folate-related diseases. However, the bioavailability of different folate vitamers is not well understood. Is folate status increased best by the intake of folate supplements, fortified foods or natural food folates? Is optimal folate status easily achievable in countries that do not permit the PteGlu fortification of foodstuffs?

Methods for the assessment of folate bioavailability in human subjects include the comparison of the serum or

**Abbreviations:** AUC, area under the curve; 5- $\text{CH}_3\text{H}_4\text{PteGlu}$ , 5-methyltetrahydrofolic acid; 5- $\text{CHOH}_4\text{PteGlu}$ , 5-formyltetrahydrofolic acid; 5MTHF, 5-methyltetrahydrofolic acid; PteGlu, pteroylmonoglutamic acid.

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plasma response to a single oral dose relative to that of a PteGlu reference dose (Gregory, 1997, 2001). This may entail either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over 2 to 3 h (Perry & Chanarin, 1970, 1972; Brown *et al.* 1973; Lucock *et al.* 1989; Bower *et al.* 1993; Kelly *et al.* 1997), or measurement of the rise in plasma folate concentration (the area under the curve (AUC) of the increase in plasma folate concentration above fasting baseline level) over 6 h or more (Markkanen, 1968, 6 h AUC; Bailey *et al.* 1988, 8 h AUC; Fenech *et al.* 1999, 7 h AUC; Prinz-Langenohl *et al.* 1999, 10 h AUC). In respect of methodological aspects in vitamin bioavailability testing, the AUC is accepted as a valuable indicator of bioavailability provided the post-dosing plasma measurement test period is long enough to capture  $\geq 80\%$  of the whole AUC (Pietrzik *et al.* 1990).

Because of the potential benefits of sensitivity and specificity, it is increasingly common to study the absorption and metabolism of folates in human subjects by using stable-isotope-labelled folates (Wolfe *et al.* 2001). Our group has used such an approach to assess the relative absorption of two different folate vitamers; PteGlu and 5-formyltetrahydrofolic acid (5-CHOH<sub>4</sub>PteGlu). This way, folate can be followed and differentiated from the natural store of folate already present in the body. Thus, more accurate estimates of 'relative absorption' should be able to be calculated as a result.

During the course of these studies it became apparent that there are a number of problems in interpreting comparative folate absorption based on short-term plasma folate responses, even when folate test doses are labelled. The present paper presents the evidence for this statement, which emerged in a human volunteer study. Human plasma 5-methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu) AUC excursion profiles were followed over a period of 8 h following ingestion of single oral physiological doses of <sup>13</sup>C<sub>6</sub>-labelled PteGlu or (6S-) <sup>13</sup>C<sub>6</sub>-labelled 5-CHOH<sub>4</sub>PteGlu. Folate-binding affinity columns were used to isolate extracted plasma folate and, following HPLC analysis of folate concentrations, a newly developed liquid chromatography-MS analytical method was used to determine the proportions of <sup>13</sup>C-labelled and unlabelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu (Hart *et al.* 2002).

## Materials and methods

### Materials

Purified [<sup>2</sup>H<sub>2</sub>]PteGlu was synthesised using a previously published procedure (Gregory, 1990), stored frozen at  $-18^{\circ}\text{C}$ , and used as the internal standard. Proton NMR, HPLC and mass spectrometric results gave a purity of  $>99\%$  and an isotopic enrichment of  $>95\%$ . Purified [<sup>13</sup>C<sub>6</sub>]PteGlu with an isotope enrichment of 96% was synthesised by the method of Maunder *et al.* (1999), and then encapsulated in gelatin capsules ('softgels') and stored at  $+4^{\circ}\text{C}$  (Finglas *et al.* 2002b). These capsules release their contents rapidly once in contact with acid stomach contents and the bioavailability of encapsulated water- and acid-soluble compounds (such as folates), as measured by

plasma appearance, has been shown to be indistinguishable from oral aqueous solutions (Seager *et al.* 1988). Purified (6S-) [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu was synthesised using a previously published enzymic-chemical procedure (Moran *et al.* 1986), and stored frozen, as a solid, at  $-18^{\circ}\text{C}$ . The purified [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu was checked by HPLC analysis, u.v. spectrum and MS. The mass spectrum gave an isotopic enrichment of  $>94\%$ . There were no detectable impurities and contaminants from the HPLC chromatogram, and u.v. spectra gave an estimated purity  $>98\%$ . Intermittently, as required, a batch of oral doses of [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu were prepared. Approximately 10  $\mu\text{mol}$  solid was weighed out and dissolved in 0.5 ml NaOH solution (0.1 mol NaOH/l). High-grade water was then added to obtain a solution of about 1.2  $\mu\text{mol/ml}$ . This was then dispensed, in doses of 0.5 ml (about 600 nmol), into 2.0 ml plastic screw-capped microfuge tubes. One dose was retained for spectroscopic analysis, with folate concentration being calculated after further dilution in PBS (0.1 mol/l, pH 7.0) using a molar extinction coefficient (E(mol, 10 mm)) of 37 200 (Blakley, 1969). The residual doses were temporarily stored frozen at  $-18^{\circ}\text{C}$  until required, and used within 2 weeks.

### Human study design

The present work forms part of an ongoing crossover, within-subject, study comparing the absorption of <sup>13</sup>C<sub>6</sub>-labelled folate isolates or <sup>15</sup>N-intrinsically labelled spinach folates in a group of healthy male and female adults. The study was approved by the Norwich and District Ethics Committee (Norfolk & Norwich Area Health Care Trust). After giving written consent, a 12 h fasting blood sample was taken and analysed at the haematology department of a local hospital for full-blood-count, blood glucose, erythrocyte-folate, serum B<sub>12</sub>, urea and electrolytes, and liver function tests. If all results were inside normal assay ranges, volunteers then attended test days where, following an overnight fast, a baseline (time zero) blood sample (10 ml) was taken and where they were then given a single oral dose of [<sup>13</sup>C<sub>6</sub>]PteGlu (634 nmol) or (6S-) [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu (431–569 nmol). The dose of [<sup>13</sup>C<sub>6</sub>]PteGlu was administered in gelatin capsules washed down with a glass of bottled 'still' mineral water. The thawed dose of (6S-) [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu was added to a glass of 25 ml bottled 'still' mineral water and then drunk by the volunteer. The glass was quickly rinsed out twice with further water, which was also drunk. A timer was started after the test doses had been completely swallowed and rinsed down. Volunteers were always allowed access to water, and were given a light lunch only after a 4 h post-dosing venous blood sample had been obtained. With few exceptions, the sub-set of volunteers reported in the present paper did not complete both <sup>13</sup>C<sub>6</sub>-labelled PteGlu and 5-CHOH<sub>4</sub>PteGlu test days. The present paper therefore reports essentially independent results from ten volunteers (age 30.9 (SE 1.3) years; BMI 25.7 (SE 1.3) kg/m<sup>2</sup>) who had (6S-) [<sup>13</sup>C<sub>6</sub>]5-CHOH<sub>4</sub>PteGlu as the test dose and twelve volunteers (age 31.1 (SE 1.2) years; BMI 25.4 (SE 1.3) kg/m<sup>2</sup>) who had [<sup>13</sup>C<sub>6</sub>]PteGlu as the test dose.

### Blood sampling and storage

Venous blood samples (10 ml) were taken by cannula at time zero and at eleven further time points over an 8 h period following each test dose; 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 and 8 h. Blood samples were transferred immediately into tubes containing K<sub>2</sub>-EDTA (Sarstedt Ltd, Beaumont Leys, Leics., UK) and mixed gently. Samples were centrifuged (1500 g, 10 min), plasma removed and frozen immediately over solid CO<sub>2</sub>, and then stored at -30°C until analysis.

### Sample preparation, folate extraction and analysis

Following storage, plasma samples were mixed thoroughly and 5 ml transferred into 50 ml screw-top glass centrifuge tubes. Extraction buffer (20 ml, 0.1 M-K<sub>2</sub>HPO<sub>4</sub>, pH 7.0; containing 0.05 M-sodium ascorbate and 0.01 M-mercaptoethanol as antioxidants) and an internal standard (0.1 ml [<sup>2</sup>H<sub>2</sub>]PteGlu; 2 µg/ml) were added. The tubes were then flushed with N<sub>2</sub>, capped, and the sample mixed thoroughly before being placed into a boiling water bath for 10 min for proteins to precipitate. The samples were cooled over ice, precipitates agitated, then centrifuged for 10 min (1000 g). The supernatant fraction was removed and stored in a clean tube. Extraction buffer (15 ml) was added to the pellet and thoroughly mixed. The sample was re-centrifuged and the wash supernatant fraction removed and combined with the first supernatant fraction. All folate from each extracted plasma sample was purified on an affinity column using folate-binding protein and eluted with HCl (0.1 mol/l) into a 5 ml volumetric flask containing ascorbic acid solid (10 mg), mixed thoroughly, and transferred in three roughly equal portions to microfuge tubes (Hart *et al.* 2002). These folate isolates were temporarily stored in liquid N<sub>2</sub> (with no loss of potentially labile 5-CH<sub>3</sub>H<sub>4</sub>-PteGlu, even after several months) and subsequently analysed, utilising a cooled (4°C) autoinjector, in negative ion mode by liquid chromatography-MS as previously described (Hart *et al.* 2002). Selected ion monitoring was conducted on the [M-H]<sup>-</sup> ion: m/z 458 for analysing 5-CH<sub>3</sub>H<sub>4</sub>PteGlu; m/z 464 [M + 6-H]<sup>-</sup> to determine 5-CH<sub>3</sub>-H<sub>4</sub>PteGlu derived from the label dose; m/z 444 for analysis of <sup>2</sup>H<sub>4</sub>PteGlu internal standard; m/z 446 and 478 to confirm there was no evidence of unmetabolised labelled PteGlu or 5-CHOH<sub>4</sub>PteGlu originating from the oral test doses. Total plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu concentrations were analysed (100 µl injection) by HPLC utilising a cooled (7°C) autoinjector (with no loss of potentially labile 5-CH<sub>3</sub>H<sub>4</sub>PteGlu, even after 30 h). Chromatographic separation was on a 250 mm × 4.6 mm internal diameter column packed with LiCrosphere 100 RP C<sub>18</sub> (Merck Ltd, Poole, Dorset, UK), performed isocratically at 1.0 ml/min with a mobile phase consisting of phosphoric acid (0.05 mol/l)-acetonitrile (9:1, v/v) with fluorescence detection for 5-CH<sub>3</sub>H<sub>4</sub>-PteGlu (excitation at 292 nm and emission at 356 nm) and u.v. absorbance (288 nm) for both PteGlu and 5-CHOH<sub>4</sub>PteGlu. HPLC quantification was against external standards. Stock folate solutions were made up in borate buffer (0.05 mol/l, pH 9.3) containing 0.4% (v/v) mercaptoethanol, and samples were further diluted in phosphate

buffer (0.1 mol/l, pH 7.0; van den Berg *et al.* 1994) before true folate concentrations were established using appropriate molar extinction coefficients (Blakley, 1969). When required, sub-samples of stock solutions (that had been stored in liquid N<sub>2</sub>) were thawed and diluted to suitable concentrations with HCl (0.1 mol HCl/l containing 2 mg/ml ascorbic acid as an antioxidant); the same matrix as used for isolated plasma samples. Plasma concentrations of unlabelled and <sup>13</sup>C<sub>6</sub>-labelled 5-CH<sub>3</sub>H<sub>4</sub> PteGlu were calculated using selected ion monitoring ratios (Hart *et al.* 2002) (see Appendix 1). Step 1 involves calculating the fraction of each source in the sample. Step 2 involves multiplying the measured total 5-CH<sub>3</sub>H<sub>4</sub>PteGlu concentration by the derived fraction of each source to give the actual plasma concentrations of unlabelled and <sup>13</sup>C<sub>6</sub>-labelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu.

### Area under the curve calculations of the rise in plasma folate above initial fasting baseline concentration

The AUC was calculated using the trapezoidal method, and then adjusted to an oral dose equivalent to 600 nmol folate. For each test dose, in each volunteer, both the 'total' and the 'unlabelled' plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC (the AUC of the rise in plasma folate concentration) were calculated after deduction of each individual's baseline (time zero) concentration from the concentration values obtained for each of their post-absorption time points. The <sup>13</sup>C<sub>6</sub>-labelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC was calculated without adjustment, since there was no excess label at baseline (time zero).

### Statistics

Originally, our ongoing study (of which the volunteers reported on in the present paper form a part) had a cross-over within-subject design. However, partly because of the intrusive nature of 8 h cannulations, most subjects reported here did not complete both <sup>13</sup>C<sub>6</sub>-labelled PteGlu and 5-CHOH<sub>4</sub>PteGlu test days. Hence, for statistical purposes, the data that are reported in the present paper are treated as independent observations. For PteGlu and 5-CHOH<sub>4</sub>PteGlu test meals, plasma total, labelled and unlabelled AUC, unlabelled:labelled ratio and total:labelled ratio were examined by the Kolmogorov-Smirnov one-sample continuous distribution test. As distributions were not significantly different from normal, each parameter for the two test doses was compared by parametric Student's unpaired *t* test for: (i) total plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC; (ii) plasma <sup>13</sup>C<sub>6</sub>-labelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC; (iii) plasma unlabelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC; (iv) plasma unlabelled:labelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC ratio; (v) plasma total:labelled 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC ratio. Linear regression analysis was used to assess associations (*r*<sup>2</sup>) with either fasting plasma baseline folate concentration, or erythrocyte-folate concentration. It was also used, within subject, to assess association between labelled and unlabelled plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC response.

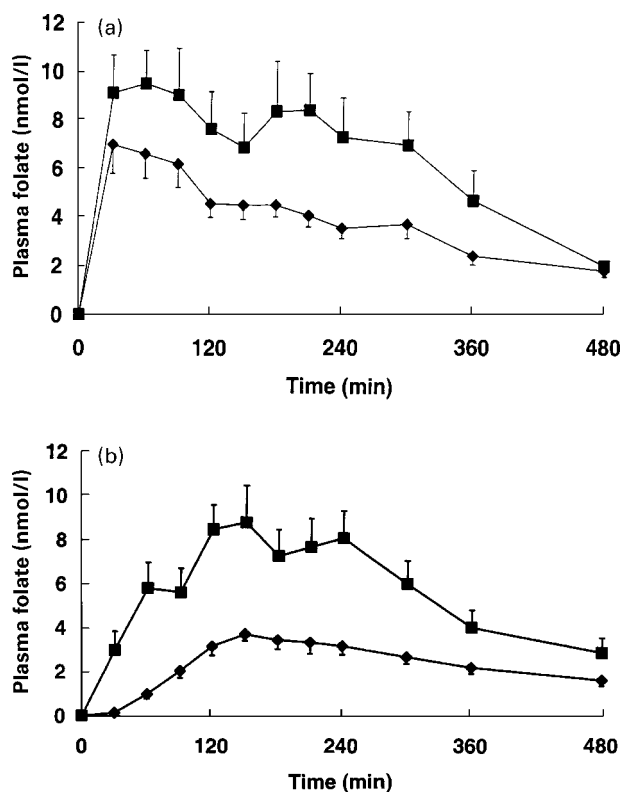
## Results

Selected ion monitoring indicated that neither of the test doses appeared unmetabolised in the plasma as PteGlu or 5-CHOH<sub>4</sub>PteGlu.

The total plasma response to both labelled PteGlu and 5-CHOH<sub>4</sub>PteGlu test doses was significantly greater than their labelled plasma response. This indicates that there was a significant appearance of unlabelled folate in the plasma following both test folates (Fig. 1).

The labelled plasma response to PteGlu was slower, and peaked much later than the response to the 5-CHOH<sub>4</sub>PteGlu test dose ( $T_{\max}$  173 (SE 10) v. 41 (SE 9) min respectively).  $T_{\max}$  is the time between the administration of the dose and the occurrence of the peak plasma concentration of labelled 5MTHF.

Table 1 shows (i) 'total', (ii) 'labelled', and (iii) 'unlabelled' plasma AUC responses, together with the (iv) unlabelled:labelled and total:labelled AUC ratios to the PteGlu and 5-CHOH<sub>4</sub>PteGlu test doses.



**Fig. 1.** (a), Plasma response to (6*S*-) [<sup>13</sup>C<sub>6</sub>]5-formyltetrahydrofolic acid (5-CHOH<sub>4</sub>PteGlu). Mean values with their standard errors of total (■) and <sup>13</sup>C<sub>6</sub>-labelled (◆) plasma 5-methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu) areas under the curve (AUC) of the rise in plasma folate concentration in response to an oral test dose (*n* 10) of (6*S*-) [<sup>13</sup>C<sub>6</sub>]CHOH<sub>4</sub>PteGlu. AUC responses are adjusted to an oral dose of 600 nmol. The unlabelled response, not of test-dose origin, is the difference between the two responses shown. (b), Plasma response to [<sup>13</sup>C<sub>6</sub>]pteroylmonoglutamic acid (PteGlu). Mean values with their standard errors of total (■) and <sup>13</sup>C<sub>6</sub>-labelled (◆) plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC of the rise in plasma folate concentration in response to an oral test dose (*n* 12) of [<sup>13</sup>C<sub>6</sub>]PteGlu. AUC responses are adjusted to an oral dose of 600 nmol. The unlabelled response, not of test-dose origin, is the difference between the two responses shown.

On average, the 'total' plasma AUC response to 5-CHOH<sub>4</sub>PteGlu was 155 % of the response to PteGlu; a significant trend for difference ( $P=0.062$ ).

In comparison, the average plasma 'labelled' AUC response to 5-CHOH<sub>4</sub>PteGlu was 221 % of the response to PteGlu; a significant difference ( $P<0.001$ ).

In contrast to the 'total' and 'labelled' response, the average plasma 'unlabelled' AUC response to 5-CHOH<sub>4</sub>PteGlu was the same as the response to PteGlu; no significant difference ( $P=0.864$ ).

Plasma 'unlabelled' and 'labelled' AUC varied greatly between volunteers but an 'unlabelled' plasma response was always present. Notably, there was no significant association, within subject, between 'unlabelled' and 'labelled' plasma AUC responses to either PteGlu ( $r^2$  0.039;  $P=0.541$ ) or 5-CHOH<sub>4</sub>PteGlu ( $r^2$  0.166;  $P=0.243$ ) test doses.

A comparison of the plasma 'unlabelled':'labelled' AUC ratios for the two oral folate test doses indicated that the mean ratio for PteGlu (1.43) was more than double, and significantly different ( $P=0.012$ ), than the mean ratio for 5-CHOH<sub>4</sub>PteGlu (0.61).

A comparison of the plasma 'total':'labelled' AUC ratios for the two oral folate test doses indicated that the mean ratio for PteGlu (2.27) was significantly different ( $P=0.027$ ) from the mean ratio for 5-CHOH<sub>4</sub>PteGlu (1.47).

Table 2 shows the results of linear regression analysis between both labelled and unlabelled plasma AUC responses to PteGlu and 5-CHOH<sub>4</sub>PteGlu test doses with (a) fasting plasma and (b) erythrocyte-folate concentration. It also shows that there was no relationship between fasting plasma and erythrocyte-folate concentrations. Neither 'unlabelled' nor 'labelled' plasma AUC responses were significantly associated ( $r^2$ ) with volunteer fasting baseline erythrocyte-folate concentrations. Furthermore, neither the 'unlabelled' plasma AUC response to both test folates, nor the 'labelled' plasma AUC response to the 5-CHOH<sub>4</sub>PteGlu test dose, were significantly associated ( $r^2$ ) with volunteer fasting baseline plasma concentrations. However, interestingly, there was an extremely high degree of association between the 'labelled' plasma AUC response to the PteGlu test dose, and volunteers' fasting baseline plasma folate concentration ( $r^2$  0.880;  $P<0.001$ ).

## Discussion

The size of the oral folate test doses used in the present study did not exceed any threshold for the reported direct appearance of unmetabolised PteGlu in the plasma (Kelly *et al.* 1997); consequently no evidence of unmetabolised PteGlu or 5-CHOH<sub>4</sub>PteGlu appearing in any of the analysed samples was found.

Results indicated that a significant proportion of the total plasma AUC response to an oral load of labelled PteGlu or labelled 5-CHOH<sub>4</sub>PteGlu did not originate directly from the oral test doses because it was not labelled (Fig. 1(a) and (b)). Approximately 40 % (range 7 to 64 %) of the plasma response to the dose of labelled 5-CHOH<sub>4</sub>PteGlu, and approximately 60 % (range 38 to 75 %) of the plasma response to the dose of labelled PteGlu did not

**Table 1.** Plasma 5-methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu) area under the curve (AUC) responses and unlabelled:labelled and total:labelled ratios in fasting healthy adult volunteers following an oral dose of either [<sup>13</sup>C<sub>6</sub>]pteroylmonoglutamic acid (PteGlu) or (6S-) [<sup>13</sup>C<sub>6</sub>]5-formyltetrahydrofolic acid (5-CHOH<sub>4</sub>PteGlu)  
(Mean values with their standard errors)

	5-CH <sub>3</sub> H <sub>4</sub> PteGlu AUC* (pmol × min/ml per 600 nmol dose)				Statistical significance of difference between oral [ <sup>13</sup> C <sub>6</sub> ]PteGlu and (6S-)[ <sup>13</sup> C <sub>6</sub> ]5-CHOH <sub>4</sub> PteGlu test doses†
	Oral dose [ <sup>13</sup> C <sub>6</sub> ]PteGlu (n 12)		Oral dose (6S-) [ <sup>13</sup> C <sub>6</sub> ]5-CHOH <sub>4</sub> PteGlu (n 10)		
	Mean	SE	Mean	SE	
Total AUC	2437	300	3771	646	P=0.062 (trend)
<sup>13</sup> C <sub>6</sub> -labelled AUC	1149	128	2542	187	P<0.001
Unlabelled AUC	1497	257	1589	503	P=0.864 (NS)
Ratio of unlabelled:labelled	1.43	0.23	0.61	0.17	P=0.012
Ratio of total:labelled	2.27	0.26	1.47	0.20	P=0.027

\*All AUC responses are adjusted to an oral dose of 600 nmol, and are over the post-dosing period from 0 to 480 min.

† Student's unpaired t test.

originate from the labelled oral test doses (Table 1). This result has not been demonstrated previously.

The 'unlabelled' plasma AUC response cannot be as a result of metabolic cleavage and loss of label from the test folates during subsequent metabolism. The underlying parent structure of all monoglutamate folate compounds that exhibit a common vitamin activity is based on the parent structure of PteGlu (pteroyl-L-monoglutamic acid). The pteroylmonoglutamate molecule consists of an aromatic pteridine ring linked through a methylene bridge to para-amino-benzoic acid, and then to one L-glutamic acid residue. Cleavage between the pteridine ring and benzoic-acid ring (generating a pteridine residue+para-aminobenzoylglutamic acid), between the benzoic-acid ring and glutamic acid (generating ptericoic acid+glutamic acid), or between both (generating a pteridine residue+para-aminobenzoyl residue+glutamic acid), will result in molecules with no vitamin activity that human metabolism cannot reassemble. Hence, the classification of folates as 'vitamins'. It is thus self-evident that the benzene ring of our labelled test folates that carries the six <sup>13</sup>C-carbons cannot be removed and substituted with a non-labelled

ring without irreversible destruction of the vitamin folate molecule.

The large, and highly variable, displacement of endogenous folate is problematic in interpreting absorption studies using 'unlabelled' folates because the portion of plasma response due to oral *v.* endogenous folate cannot be determined. There was no relationship between this 'unlabelled' folate response and folate status. Furthermore, there does not appear to be a simple relationship between oral and endogenous response as there was no significant association, within subject, between 'unlabelled' and 'labelled' plasma AUC response to either 'labelled' PteGlu or 'labelled' 5-CHOH<sub>4</sub>PteGlu test doses. It could be speculated that the larger variation in unlabelled plasma folate response may be due to variable absorptive epithelial cell exposure to folate intake in the day-or-so immediately preceding the test days, and a knock-on perturbation effect. The latter effect would result in the previously absorbed 'cold' folate being pushed out of the epithelial cells into the body-proper to generate an unlabelled folate plasma response that is completely unpredictable, and unrelated to the absorption of any labelled test dose. If unlabelled

**Table 2.** Linear regression analysis of the association ( $r^2$ ) between unlabelled or <sup>13</sup>C<sub>6</sub>-labelled plasma 5-methyltetrahydrofolic acid (5-CH<sub>3</sub>H<sub>4</sub>PteGlu) area under the curve (AUC) response in fasting healthy adult volunteers, following an oral dose of either [<sup>13</sup>C<sub>6</sub>]pteroylmonoglutamic acid (PteGlu) or (6S-) [<sup>13</sup>C<sub>6</sub>]5-formyltetrahydrofolic acid (5-CHOH<sub>4</sub>PteGlu), and fasting plasma folate concentration or fasting erythrocyte-folate concentration

Test meal...	Oral dose [ <sup>13</sup> C <sub>6</sub> ]PteGlu (n 12)		Oral dose (6S-) [ <sup>13</sup> C <sub>6</sub> ]5-CHOH <sub>4</sub> PteGlu (n 10)	
	Fasting plasma folate	Fasting erythrocyte folate	Fasting plasma folate	Fasting erythrocyte folate
Unlabelled AUC per 600 nmol dose				
$r^2$	0.000	0.007	0.066	0.004
P	0.990 (NS)	0.805 (NS)	0.505 (NS)	0.878 (NS)
<sup>13</sup> C <sub>6</sub> -labelled AUC per 600 nmol dose				
$r^2$	0.880	0.022	0.226	0.174
P	<0.001	0.990 (NS)	0.196 (NS)	0.265 (NS)
Fasting plasma (oral dose PteGlu)				
$r^2$		0.032		
P		0.600 (NS)		
Fasting plasma (oral dose 5-CHOH <sub>4</sub> PteGlu)				
$r^2$				0.102
P				0.442 (NS)

folate was mainly a result of hepatic biliary excretion then one may have expected the 'unlabelled' AUC to be related to the initial fasting plasma folate concentration of volunteers, because it too is a function of enterohepatic recirculation (Steinberg *et al.* 1979). Such a relationship was not observed.

The oral dose of 5-CHOH<sub>4</sub>PteGlu induced larger 'total' plasma AUC responses than equivalent oral doses of PteGlu (Table 1). If the AUC of an unlabelled oral test folate is compared with the AUC of an equivalent unlabelled dose of PteGlu (i.e. using PteGlu as the 'reference' dose) then this would have indicated that the 'relative bioavailability' of 5-CHOH<sub>4</sub>PteGlu was 155% that of PteGlu. Similarly, the 'relative bioavailability' of 5-CHOH<sub>4</sub>PteGlu calculated when using only the plasma 'labelled' AUC responses was 221% that of PteGlu. Under the conditions of the present trial in which relatively small folate test doses (within the physiological range) were given to fasting volunteers, one would expect that a high proportion (approximately 90%) of the dose would be absorbed (Bhandari & Gregory, 1992; Clifford *et al.* 1998). Thus, differences in plasma kinetics and AUC as seen in the present study are almost assuredly due to differences in rate or extent of conversion to 5-CH<sub>3</sub>H<sub>4</sub>PteGlu, displacement of tissue folates, and release into the circulation. These data suggest that a reduced folate such as (6S-) 5-CH<sub>3</sub>H<sub>4</sub>PteGlu or (6S-) 5-CHOH<sub>4</sub>PteGlu would be a more appropriate 'reference' dose in bioavailability studies of natural folates, especially comparisons carried out 'within volunteer'.

A review by Selhub *et al.* (1983) concluded that the intestine is capable of both reduction and one-carbon substitution (methylation) of physiological doses of folate before absorbed folate is transported from the mucosal to the serosal side. It is therefore expected that physiological oral doses of both PteGlu and 5-CHOH<sub>4</sub>PteGlu will only result in the transfer of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu into the hepatic portal vein. Though an extensive hepatic uptake of absorbed folate has been noted (Gregory, 2001), this, logically, should be the same in response to both these oral test folates and calculation of 'relative absorption' should still be valid. However, a comparison of labelled plasma AUC responses indicated that the 'initial rate' and 'time to peak' response to PteGlu test doses was slower and longer, respectively, than that to 5-CHOH<sub>4</sub>PteGlu test doses (Fig. 1(a) and (b)). Arguably, a slower mucosal transfer of labelled 5-methyltetrahydrofolic metabolite, derived from labelled PteGlu, to the plasma (possibly due to a limitation in the rate of initial reduction of PteGlu to H<sub>2</sub>-PteGlu, before further reduction to H<sub>4</sub>-PteGlu, subsequent 1-carbon addition and re-arrangement to 5-CH<sub>3</sub>-H<sub>4</sub>-PteGlu; Mathews & Huennekens, 1963) when coupled with a similar plasma clearance rate to 5-methyltetrahydrofolic metabolite derived from 5-CHOH<sub>4</sub>PteGlu would result in a comparatively smaller 'labelled' plasma AUC.

The lack of association (Table 2), with one exception, between labelled or unlabelled plasma AUC responses to PteGlu or 5-CHOH<sub>4</sub>PteGlu test doses, and either volunteer fasting baseline plasma or erythrocyte-folate concentrations, indicates that plasma AUC responses cannot be

predicted using either of these criteria. The exceptional observation of a high degree of association ( $r^2$  0.880;  $P < 0.001$ ) between the labelled plasma AUC response to a labelled PteGlu oral test dose and volunteers' fasting baseline plasma folate concentration could be real, and could eventually provide an insight into the specific metabolism of PteGlu. Alternatively, the association could be an artifact of having undertaken a total of ten statistical comparisons (i.e. including testing of associations between 'unlabelled' and 'labelled' plasma response to both test folates). So, without an experimental '*a priori*' intention to make such comparisons, the associations should, strictly speaking, be left to future examination. However, since fasting baseline plasma folate concentration is maintained by enterohepatic recirculation (Steinberg *et al.* 1979), it is too tempting to avoid the speculation that this strong relationship suggests that absorbed PteGlu is reduced and methylated in the liver, and not the mucosa, as has been the accepted wisdom for at least the past two decades (Selhub *et al.* 1983).

In summary, the present paper reports that after an oral folate test dose there is a large and unexpected concurrent displacement of tissue 5-CH<sub>3</sub>H<sub>4</sub>PteGlu into the plasma. This makes comparisons of the relative bioavailability of unlabelled reduced folates (i.e. from test food doses), *v.* unlabelled PteGlu reference doses, ambiguous. It is also reported that there is a large difference in plasma 'labelled' response to a 'labelled' naturally occurring reduced folate compared with 'labelled' unreduced PteGlu. This difference would lead to spurious conclusions in bioavailability studies based on comparisons of oral doses of reduced 'labelled' folate (for example, labelled food folate) *v.* a 'labelled' PteGlu reference dose. It could be speculated that this phenomenon may be due to a slower rate of mucosal processing of PteGlu compared with 5-CHOH<sub>4</sub>PteGlu. This may result in a slower transfer of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu metabolite to the plasma, which will, when coupled with a similar clearance rate to that of 5-CH<sub>3</sub>H<sub>4</sub>PteGlu metabolite derived from oral 5-CHOH<sub>4</sub>PteGlu, result in comparatively smaller labelled plasma AUC. Contrary to current theory, it is suggested that absorbed physiological doses of PteGlu mainly enter the hepatic portal vein unmodified with immediate removal to the liver for biotransformation, with subsequent plasma 5-CH<sub>3</sub>H<sub>4</sub>PteGlu AUC response being entirely a function of enterohepatic recirculation.

Thus, neither comparison of 'total' or 'labelled' plasma AUC responses appears to give a reliable index of 'relative bioavailability'. It is concluded that currently held views on the absorption, metabolism and subsequent tissue distribution of folates (which suggest that the bioavailability of oral test doses can be estimated by contrasting the plasma response with that from an equivalent 'reference dose' of PteGlu), need re-evaluation.

Two potential solutions to this problem are suggested. First, an adaptation could be made to the oral and intravenous dual-label stable-isotope 'urinary excretion ratio' protocol of Gregory *et al.* (1992), using isotopically labelled (6S-) 5-CH<sub>3</sub>H<sub>4</sub>PteGlu as the intravenous dose, rather than isotopically labelled PteGlu. This adaptation to the method may overcome differences in the kinetics

of urinary excretion of oral and intravenous doses that have been observed when using labelled PteGlu as the intravenous dose (Gregory *et al.* 1992; Rogers *et al.* 1997; Finglas *et al.* 2002b). However, this manoeuvre may eventually prove to be only a partial solution and have to be restricted to the estimation of the 'relative absorption' of oral doses of 'natural' reduced folates and not PteGlu (as either supplement or fortificant). Second, one could mathematically model plasma-labelled response data (entrance and clearance rates) using a single-compartment model with estimates for the size of pool mass and volume, and (perhaps) first-pass liver effect. The advantages of this approach may be at least to obtain an accurate estimate of 'relative bioavailability', if not absolute absorption. Metabolic models are currently being investigated (Finglas *et al.* 2002a).

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### References

- Anonymous (1991) Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **338**, 131–137.
- Bailey LB, Barton LE, Hillier SE & Cerda JJ (1988) Bioavailability of mono and polyglutamyl folate in human subjects. *Nutr Rep Int* **38**, 509–518.
- Bhandari SD & Gregory JF (1992) Folic acid 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and in vivo kinetics in rats. *J Nutr* **122**, 1847–1854.
- Blakley RL (1969) The biochemistry of folic acid and related peridines. In *Frontiers of Biology*, vol. 13, pp. 92–94 [A Neuberger and EL Tatum, editors]. London: North-Holland.
- Blount BC, Mack MM & Wehr CM, *et al.* (1997) Folate deficiency causes uracil misincorporation into human DNA and chromosome breakage: Implications for cancer and neuronal damage. *Proc Natl Acad Sci USA* **94**, 3290–3295.
- Bousey CJ, Beresford SA, Omenn GS & Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease – probable benefits of increasing folic acid intakes. *JAMA* **274**, 1049–1057.
- Bower C, Stanley FJ, Croft M, De Clerk NH, Davis RE & Nicol DJ (1993) Absorption of pteroylpolyglutamates in mothers of infants with neural tube defects. *Br J Nutr* **69**, 827–834.
- Brown JP, Scott JM, Foster FG & Weir DG (1973) Ingestion and absorption of naturally occurring pteroylmonoglutamates (folates) in man. *Gastroenterology* **64**, 223–232.
- Clifford AJ, Arjomand A, Dueker SR, Schneider PD, Buchholz BA & Vogel JS (1998) The dynamics of folic acid metabolism in an adult given a small tracer dose of <sup>14</sup>C-folic acid. *Adv Exp Med Biol* **445**, 239–251.
- Czeizel AE & Dudas I (1992) Prevention of the first occurrence of neural tube defects by periconceptual vitamin supplementation. *N Engl J Med* **327**, 32–35.
- Fenech M, Noakes N, Clifton P & Topping D (1999) Aleurone flour is a rich source of bioavailable folate in humans. *J Nutr* **129**, 1114–1119.
- Finglas PM, Dainty JR, Hart D, Wolfe C, Wright AJ, Wright D, Southon S & Gregory JF (2002a) Modelling of labelled absorbed folates using plasma in humans. *FASEB J* **16**, A748.
- Finglas PM, Withöft CM, Vahteristo L, *et al.* (2002b) Use of an oral/intravenous dual-label stable-isotope protocol to determine folic acid bioavailability from fortified cereal grain foods in women. *J Nutr* **132**, 936–939.
- Gregory JF (1990) Improved synthesis of [<sup>3</sup>H, <sup>5</sup>-<sup>2</sup>H<sub>2</sub>] folic acid: extent and specificity of deuterium labelling. *J Agric Food Chem* **38**, 1073–1076.
- Gregory JF (1997) Bioavailability of folate. *Eur J Clin Nutr* **51**, Suppl. 1, S54–S59.
- Gregory JF (2001) Case study: Folate bioavailability. *J Nutr* **131**, Suppl. 4, 1376S–1382S.
- Gregory JF, Bhandari SD, Bailey LB, Toth JP, Baumgartner TG & Cerda JJ (1992) Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *Am J Clin Nutr* **55**, 1147–1153.
- Hart DJ, Finglas PM, Wolfe CA, Mellon FA, Wright AJ & Southon S (2002) Determination of 5-methyltetrahydrofolate (<sup>13</sup>C-labelled and unlabelled) in human plasma and urine by combined liquid chromatography mass spectrometry (LC/MS). *Anal Biochem* **305**, 206–213.
- Kelly P, McPartlin J, Goggins M, Weir DG & Scott JM (1997) Unmetabolized folic acid in serum: acute studies in subjects consuming fortified food and supplements. *Am J Clin Nutr* **65**, 1790–1795.
- Luccock MD, Wild J, Smithells RW & Hartley R (1989) In vivo characterization of the absorption and biotransformation of pteroylmonoglutamic acid in man: a model for future studies. *Biochem Med Metab Biol* **42**, 30–42.
- Markkanen T (1968) Absorption tests with natural folate material in controls and in gastrectomized patients. *Am J Clin Nutr* **21**, 473–481.
- Mathews CK & Huennekens FM (1963) Further studies on dihydrofolate reductase. *J Biol Chem* **238**, 3436–3442.
- Maunder P, Finglas PM, Mallet AI, Mellon FA, Razaque MA, Ridge B, Vahteristo L & Withöft C (1999) The synthesis of folic acid, multiply labelled with stable isotopes, for bioavailability studies in human nutrition. *J Chem Soc Perkin Trans 1*, 1311–1323.
- Moran RG, Keyomasri K & Colman PD (1986) Preparation of (6S)-5-formyltetrahydrofolate labelled at high specific activity with <sup>14</sup>C- and <sup>2</sup>H-. *Meth Enzymol* **122**, 309–313.
- Nephew KP & Huang TH-M (2003) Epigenetic gene silencing in cancer initiation and progression. *Cancer Lett* **190**, 125–133.
- Perry J (1971) Folate analogues in normal mixed diets. *Br J Nutr* **21**, 435–441.
- Perry J & Chanarin I (1970) Intestinal absorption of reduced folate compounds in man. *Br J Haematol* **18**, 329–339.
- Perry J & Chanarin I (1972) Observations on folate absorption with particular reference to folate polyglutamate and possible inhibitors to its absorption. *Gut* **13**, 544–550.
- Pietrzik K, Hages M & Remer T (1990) Methodological aspects in vitamin bioavailability testing. *J Micronutr Anal* **7**, 207–222.
- Prinz-Langenohl R, Bronstrup A, Thorand B, Hages M & Pietrzik K (1999) Availability of food folate in humans. *J Nutr* **129**, 913–916.
- Rogers LM, Pfeiffer CM, Bailey LB & Gregory JF (1997) A dual-label stable-isotopic protocol is suitable for determination of

- folate bioavailability: Evaluation of urinary excretion and plasma folate kinetics of intravenous and oral doses of [13C5] and [2H2]folic acid. *J Nutr* **127**, 2321–2327.
- Scott JM & Weir DG (1976) Folate composition, synthesis and function in natural materials. *Clinics in Haematology* **5**, 547–568.
- Seager H, Aulton ME & Morton FSS (1988) Drug delivery systems; soft gelatin capsules. In: *Pharmaceutics. The Science of Dosage Form Design*, pp. 330–340. [ME Aulton, editor]. London: Churchill Livingstone.
- Selhub J, Dhar GJ & Rosenberg IH (1983) Gastrointestinal absorption of folates and antifolates. *Pharmacol Ther* **20**, 397–418.
- Shane B (1995) Folate chemistry and metabolism. In *Folate in Health and Disease*, pp. 1–22 [LB Bailey, editor]. New York, NY: Marcel Dekker.
- Steinberg SE, Campbell CL & Hillman RS (1979) Kinetics of the normal folate enterohepatic cycle. *J Clin Invest* **64**, 83–88.
- Van den Berg H, Finglas PM & Bates C (1994) FLAIR Intercomparisons on serum and red cell folate. *Int J Vitam Nutr Res* **64**, 288–293.
- Wolfe CA, Finglas PM, Hart D, Wright AJA & Southon S (2001) Isotopic methods to detect food folates. *Innovat Food Sci Emerg Technol* **1**, 297–302.

## Appendix 1

There are two possible sources of  $^{13}\text{C}$  in the  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  in our plasma samples: the tracer (the  $^{13}\text{C}_6$ -labelled  $5\text{-CH}_3\text{H}_4\text{PteGlu}$ ; 5MTHF) and the tracee (unlabelled  $5\text{-CH}_3\text{H}_4\text{PteGlu}$ ). The mass spectrometer is set up to measure the largest fraction of the  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  coming from the tracer (mass 464;  $M + 6$ ) and the largest fraction coming from the tracee (mass 458;  $M + 0$ ). The response for either  $M + 0$  or  $M + 6$  will depend on the number of  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  molecules present in the sample that have come from the two sources. Examining each source separately, the number of  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  molecules in the sample, measured in the  $M + 6$  channel, that have come from the tracer (defined as  $M + 6_{\text{sampletracer}}$ ) is proportional to the number of mol of the tracer present in the sample (defined as  $\text{mol}_{\text{tracer}}$ ) and the fraction of the tracer that is actually present at mass 464 (defined as  $M + 6_{\text{tracer}}$ ). Therefore:

$$M + 6_{\text{sampletracer}} \propto M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}},$$

and, in a similar fashion, the number of molecules of  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  in the sample, measured in the  $M + 6$  channel, that have been derived from the tracee (unlabelled) source is:

$$M + 6_{\text{sampletracee}} \propto M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}.$$

The overall contribution from the two sources to the total number of  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  molecules, measured in the  $M + 6$  channel, will be a linear combination of the two equations above and is defined as  $M + 6_{\text{sample}}$ . Therefore, the number of molecules of  $5\text{-CH}_3\text{H}_4\text{PteGlu}$  in the sample that have been derived from the tracer (labelled) and tracee (unlabelled) source is:

$$M + 6_{\text{sample}} \propto M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}, \quad (1)$$

and, similarly for  $M + 0_{\text{sample}}$ ,

$$M + 0_{\text{sample}} \propto M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}. \quad (2)$$

On dividing (2) by (1):

$$\frac{M + 6_{\text{sample}}}{M + 0_{\text{sample}}} = \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. \quad (3)$$

The ratio  $M + 6_{\text{sample}}/M + 0_{\text{sample}}$  is defined as  $M+6/M+0R$ . Therefore:

$$M+6/M+0R = \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. \quad (4)$$

Similarly for the  $M + 0_{\text{sample}}/M + 0_{\text{sample}}$  ratio:

$$M+0/M+0R = \frac{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. \quad (5)$$

If the denominator in equations 4 and 5 is set to be a constant,  $k$ , then:

$$1/k = M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}. \quad (6)$$

Equations 4 and 5 can now be rewritten:

$$M+6/M+0R = M + 6_{\text{tracer}} \times k \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times k \times \text{mol}_{\text{tracee}}; \quad (7)$$

$$M+0/M+0R = M + 0_{\text{tracer}} \times k \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times k \times \text{mol}_{\text{tracee}}. \quad (8)$$

Equations 7 and 8 can be represented in matrix notation:

$$\begin{bmatrix} M+6/M+0R \\ M+0/M+0R \end{bmatrix} = \begin{bmatrix} M + 6_{\text{tracer}} & M + 6_{\text{tracee}} \\ M + 0_{\text{tracer}} & M + 0_{\text{tracee}} \end{bmatrix} \times \begin{bmatrix} k \times \text{mol}_{\text{tracer}} \\ k \times \text{mol}_{\text{tracee}} \end{bmatrix}, \quad (9)$$

or,

$$R = A \times x. \quad (10)$$

Solving for  $x$ :

$$x = A \times R, \quad (11)$$

where

$$x = \begin{bmatrix} k \times \text{mol}_{\text{tracer}} \\ k \times \text{mol}_{\text{tracee}} \end{bmatrix}.$$



In terms of mol fractions:

$$\begin{aligned} &\text{mole fraction of tracer (i.e. } 5\text{MTHF}_{\text{fractiontracer}}) \\ &= \frac{k \times \text{mol}_{\text{tracer}}}{k \times \text{mol}_{\text{tracer}} + k \times \text{mol}_{\text{tracee}}}, \end{aligned} \quad (12)$$

$$\begin{aligned} &\text{mole fraction of tracee (i.e. } 5\text{MTHF}_{\text{fractiontracee}}) \\ &= \frac{k \times \text{mol}_{\text{tracee}}}{k \times \text{mol}_{\text{tracer}} + k \times \text{mol}_{\text{tracee}}}. \end{aligned} \quad (13)$$

It is now a simple task to calculate the quantity of the

5-CH<sub>3</sub>H<sub>4</sub>PteGlu that has come from the tracer or tracee in any plasma sample because the total 5-CH<sub>3</sub>H<sub>4</sub>PteGlu (defined as 5MTHF<sub>total</sub>) in the sample is known from an HPLC measurement.

$$\begin{aligned} \text{Quantity of tracer} &= \\ &= \frac{5\text{MTHF}_{\text{fractiontracer}} \times 5\text{MTHF}_{\text{total}}}{5\text{MTHF}_{\text{fractiontracer}} + 5\text{MTHF}_{\text{fractiontracee}}}; \end{aligned} \quad (14)$$

$$\begin{aligned} \text{Quantity of tracee} &= \\ &= \frac{5\text{MTHF}_{\text{fractiontracee}} \times 5\text{MTHF}_{\text{total}}}{5\text{MTHF}_{\text{fractiontracer}} + 5\text{MTHF}_{\text{fractiontracee}}}. \end{aligned} \quad (15)$$