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Physicochemical effect of pH and antioxidants on mono- and triglutamate forms of 5-methyltetrahydrofolate, and evaluation of vitamin stability in human gastric juice: Implications for folate bioavailability

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Abstract

This report examines the physico-chemical properties of mono and triglutamate forms of 5-methyltetrahydrofolate$_n$ ($5CH_3$–H_4–$PteGlu_n$) in human gastric juice and under artificial conditions, typical of the upper gastrointestinal tract.

Degradation of $5CH_3$–H_4–$PteGlu_n$ to 5-methyldihydrofolate$_n$ ($5CH_3$–5,6-$H_2PteGlu_n$) and on to the C_9–N_{10} scission product *p*-aminobenzoylglutamate (P-ABG), and the salvage of $5CH_3$–H_4–$PteGlu_n$ from $5CH_3$–5,6-$H_2PteGlu_n$ was examined with emphasis on the influence of pH and ascorbic acid. At pH 3.5, physiological levels of ascorbic acid (50 μMol/l) salvage both $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ from acid labile $5CH_3$–5,6-$H_2PteGlu_n$ and prevent loss of vitamin activity as indicated by P-ABG_n formation. This process is less efficient at pH 7.0, although under such conditions $5CH_3$–5,6-$H_2PteGlu_n$ is rendered relatively stable.

Ascorbic acid (50 μMol/l) reduces degradation of $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ at both pH 3.5 and 7.0. However, irrespective of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ are both more stable at pH 3.5 than pH 7.0. There is a clear differential between mono- and polyglutamate stability, since, irrespective of pH or the presence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ is inherently more stable than $5CH_3$–$H_4PteGlu_3$. While ascorbic acid clearly stabilises mono- and triglutamate forms of methylfolate in human gastric juice, a factor in gastric juice removes any differential instability as seen in buffer solution. We speculate that this endogenous factor could be either a binding protein or some other antioxidant.

These *in vitro* findings provide information that may be useful in evaluating the *in vivo* bioavailability of natural polyglutamate forms of the vitamin.

Keywords: 5-Methyltetrahydrofolate; Folic acid; Vitamin; Dietary folic acid; Vitamin C; Vitamin bioavailability; Vitamin absorption

1. Introduction

Naturally occurring food folates are in the form of reduced folylmono- or polyglutamates, while oxidised folate in the form of pteroylmonoglutamate (PteGlu) is synthetic and does not occur in nature. Despite this, PteGlu is a major component of our daily micronutrient intake since it is often consumed in the form of a supplement or when fortified in foods (Gregory, 2001; Lucock, 2004). Bioavailability is estimated as 50% for naturally occurring food folate (Sauberlich, Kretsch, Skalah, Johnson, & Talyor, 1987) and up to 100% for synthetic folate when consumed on an empty stomach (Gregory, 1997).

Ascertaining factors that influence the bioavailability of natural folates is extremely important because this water soluble group of B-vitamins is one of our most significant

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micronutrients for maintaining health and wellbeing: It is an essential co-factor for various one-carbon transfer reactions that play an important role in nucleotide biosynthesis, methylation reactions, including those involved in both gene expression and the amelioration of vasculotoxic homocysteine (Shane, 1995).

Natural food folates are highly sensitive to heat and cooking can result in destruction of 50–95% of the folate content of food (Northwestern University, 2004). The degree of loss is influenced by environmental factors such as pH, O_2 content, metal ion concentrations and antioxidant levels (Keagy, 1985). As an example, the stability (Lucock, Priestnall, et al., 1995), cellular uptake (Chiao, Roy, Tolner, Yang, & Sirotnak, 1997) and enzymatic deconjugation of native folates is pH sensitive (Bhandari, Gregory, Renuart, & Merritt, 1990). Therefore, physiological conditions or medications that alter the pH of the stomach or proximal small intestine could impair folate absorption (Gregory, 2001; Lucock, Green, et al., 1995; Lucock, Priestnall, et al., 1995).

The reduced folylpolyglutamates, such as substituted or unsubstituted dihydro- and tetrahydro-forms that are contained in foods and in cells, are extremely unstable (Blakeley, 1969). They are reduced at the 5, 6, 7 and 8 positions of the pteridine ring which renders the molecule sensitive to oxidative cleavage at the C_9–N_{10} bond. Degradation yields a pteridine residue and *p*-aminobenzoylglutamate (P-ABG) (Murphy, Boyle, Weir, & Scott, 1978; Murphy, Keating, Boyle, Weir, & Scott, 1976). These C_9–N_{10} scission products are biologically inactive and cannot be reconverted into any useful folate form (Scott, 2001). Although folates with substituents at the N-5 or N-10 position decrease the tendency for scission, they are still susceptible to oxidative chemical reactions, and consequently, loss of activity (Blakeley, 1969). Although the stability of one of the main food folates (5-methyltetrahydrofolate) is influenced by pH, the presence of ascorbic acid has a profound protective effect (Lucock, Green, Hartley, & Levene, 1993; Lucock, Green, et al., 1995; Lucock, Priestnall, et al., 1995). Ascorbic acid is an antioxidant whose reducing property helps to bring about and maintain this particular folate's functional molecular state (Davies, Austin, & Partridge, 1991; Lucock, Green, et al., 1995; Lucock, Priestnall, et al., 1995). Where stomach histology is normal, ascorbic acid is present at high concentrations in gastric juice. Under these conditions a concentration gradient exists from the gastric lumen down to plasma in the order of approximately $5 > 1$ (Lucock, Green, et al., 1995; Lucock, Priestnall, et al., 1995).

In the present study, HPLC with photodiode array (PAD), fluorescence and electrochemical detection were used to analyse the physico-chemical properties of mono and polyglutamate forms of 5-methyltetrahydrofolate$_n$ ($5CH_3$–H_4–$PteGlu_n$) under conditions typical of the upper gastrointestinal tract. We describe the first account of the differential stability of $5CH_3$–H_4–$PteGlu_1$ and $5CH_3$–H_4–$PteGlu_3$ at critical pH values found in the gastric lumen. This has been achieved by evaluating the pH dependent effect of ascorbic acid concentration on the degradation and salvage of these folates in milieu that mimics the gastrointestinal environment and human gastric juice. In particular, we focused on the influence of pH and ascorbic acid on the degradation of $5CH_3$–H_4–$PteGlu_n$ to 5-methyldihydrofolate$_n$ ($5CH_3$–5,6-$H_2PteGlu_n$) and on to the C_9–N_{10} scission product P-ABG$_n$, and the salvage of $5CH_3$–H_4–$PteGlu_n$ from $5CH_3$–5,6-$H_2PteGlu_n$. We discuss the implications of our findings in the context of the currently perceived view of folate bioavailability.

2. Materials and methods

2.1. Chemicals

(6*R*,*S*)-5-Methyl-5,6,7,8-tetrahydropteroylmono-L-glutamic acid, calcium salt [$5CH_3$–H_4–PteGlu], (6*R*,*S*)-5-methyl-5,6-dihydrofolic acid, ammonium salt [$5CH_3$–5,6-H_2PteGlu], *para*-aminobenzoyl-L-glutamic acid (P-ABG), (6*R*,*S*)-5-methyl-5,6,7,8-tetrahydropteroyldi-γ-L-glutamic acid trihydrochloride [$5CH_3$–H_4–$PteGlu_2$] and (6*R*,*S*)-5-methyl-5,6,7,8-tetrahydropteroyltri-γ-L-glutamic acid trihydrochloride [$5CH_3$–H_4–$PteGlu_3$] were purchased from Dr. B. Schircks, Jona, Switzerland. L-Ascorbic acid, sodium salt was purchased from Sigma Chemical Co. Ltd., (Poole, Dorset, UK). Analar grade ethylenediaminetetra-acetic acid disodium salt and Aristar grade potassium dihydrogen orthophosphate were purchased from Merck (Lutterworth, Leics, UK). HiPerSolv orthophosphoric acid was purchased from Merck (Kilsyth, Vic, Australia). Methanol (HPLC grade) was purchased from Lab-Scan (Thailand).

2.2. Solutions

All aqueous solutions were prepared from filtered water purified by a Milli-Q system (Millipore, Sydney, Australia). Stock aliquots of 1 mg/ml $5CH_3$–H_4–$PteGlu_1$, $5CH_3$–H_4–$PteGlu_2$, $5CH_3$–H_4–$PteGlu_3$, $5CH_3$–5,6-H_2PteGlu and P-ABG were made up in water containing no antioxidant and immediately frozen at −85 °C. Master working solutions were made up at 500 picomoles/μl in water, dispensed into small aliquots and again immediately frozen at −85 °C. Working solutions for experimentation and injection onto the liquid chromatograph were typically 25 pMol/μl (5 μl injection represents 125 picomoles on column). Fresh stock ascorbic acid was made up at 1 Mol/l and immediately frozen at −85 °C. This was used to generate a concentration of ascorbic acid that equates to a level typical of gastric juice when added to a given folate vitamer (i.e. 50 μMol/l).

2.3. Equipment and chromatographic conditions

A Varian Pro Star HPLC system (Varian, North Ryde, Australia) was used, and configured as follows: A Rheodyne manual injector was added to a Model 430 autosam-

pler. A Model 510 column oven was set at 25 °C. Two detectors were placed in series according to need; either (1) A Model 335 photodiode array detector followed by a Model 363 fluorescence detector or (2) a Model 335 photodiode array detector followed by an ESA Coulochem III electrochemical detector (Analytical Technologies, North Ryde, Australia).

The chromatographic separation is similar to that previously reported (Lucock, Green, et al., 1995). Briefly, a Waters Associates Nova-Pak phenyl stainless-steel cartridge column (15 cm × 3.9 mm. i.d.; 4 μM spherical bonded silica) was protected by a LiChro-CART, LiChrospher – 5 μM C18 guard column (E.Merck, Darmstadt, Germany). The mobile phase consisted of 15% (v/v) methanol in 0.05 M KH_2PO_4 (pH adjusted to 3.5 with orthophosphoric acid). The filtered mobile phase was run at 0.8 ml/min at 25 °C.

PAD was used to discriminate between different oxidation states and scission products of methylfolate. Evolving chromatograms were monitored at 280 nm with PAD data collected between 200 and 400 nm. Fluorimetric detection was set at an excitation λ of 310 nm and emission λ of 352 nm. Coulometric electrochemical detection was used to evaluate and compare the voltage–current profiles of mono-, di- and triglutamyl forms of methylfolate in an attempt to show any difference in the effect of oxidative stress upon these three molecular forms of native dietary folate. Hydrodynamic voltammagrams were generated from −150 mV to +450 mV. A palladium reference electrode was used, resulting in a negative shift of approximately 300 mV over that typical for a silver/silver chloride electrode. The unusual characteristics of a palladium reference therefore explain the difference in hydrodynamic voltammagrams obtained here, when compared to earlier work by the authors using a silver/silver chloride reference (Lucock, Hartley, & Smithells, 1989).

2.4. Kinetics

To investigate effects near physiological pH, folate solutions (±ascorbic acid) were made up in pH 3.5 and 7.0 phosphate buffer (0.05 M). Mono- and triglutamyl $5CH_3$–H_4–PteGlu stability kinetics were then examined in these artificial milieu in the presence and absence of 50 μMol/l exogenous ascorbic acid. The dose–response relationship between folic acid stability and ascorbic acid concentration was characterised, as was the equilibrium between salvage of $5CH_3$–5,6-H_2PteGlu$_n$ to $5CH_3$–H_4–PteGlu$_n$ and irreversible degradation to P-ABG$_n$. Quantitative differences between mono- and polyglutamate forms of folic acid were then evaluated.

Folate degradation/salvage was also studied in human gastric juice (GJ) aspirate (pH 2.25). Kinetics were investigated in 'normal' pH 2.25 GJ containing only endogenous ascorbic acid, which was used as the medium to study mono- and triglutamyl $5CH_3$–H_4–PteGlu stability kinetics in the presence and absence of 50 μMol/l exogenous ascorbic acid. Briefly, crude filtered GJ stored at −85 °C was thawed and centrifuged at 13,000*g* for 2 min. 940–950 μl of the resulting supernatant at +4 °C was spiked with either 50 μl of 500 picomoles/μl folate ($5CH_3$–H_4–PteGlu$_1$, $5CH_3$–5,6-H_2PteGlu$_1$, $5CH_3$–H_4–PteGlu$_3$) or 50 μl of 500 picomoles/μl ($5CH_3$–H_4–PteGlu$_1$, $5CH_3$–5,6-H_2PteGlu$_1$, $5CH_3$–H_4–PteGlu$_3$) plus 10 μl of 5 mMol/l ascorbate.

Timed aliquots (2.5 μl being equivalent to 62.5 picomoles of 'undegraded' folate on column) were injected onto the HPLC at regular intervals until any reaction was complete. GJ blanks, with or without ascorbic acid but containing no folates, were also injected over the same time period. All GJ and non-GJ incubations were carried out at 37 °C in the dark.

Ascertaining chromatographic stability and quantitative validation was achieved by periodic injection of 125 picomoles of a solution containing a composite mixture of $5CH_3$–H_4–PteGlu$_1$, $5CH_3$–5,6-H_2PteGlu$_1$ and P-ABG$_1$ in water at +4 °C. The identity of all reaction products was verified on the basis of retention time and a spectral library comparison.

3. Results

Fig. 1 shows the reversed-phase chromatographic separation of equimolar P-ABG$_1$, $5CH_3$–H_4–PteGlu$_1$ and $5CH_3$–5,6-H_2PteGlu$_1$ (each compound represents 125 pmoles on column). Fig. 2 shows the separation of $5CH_3$–H_4–PteGlu$_1$ and $5CH_3$–H_4–PteGlu$_3$, again, each compound represents 125 picomoles on column. The compounds of interest and their degradation products are easily identified by their spectral characteristics, which are given in Fig. 3.

The effect of pH on the degradation of $5CH_3$–H_4–PteGlu$_1$ and $5CH_3$–H_4–PteGlu$_3$ (±50 μMol/l ascorbic acid) is given in Table 1, which reports the time in minutes at which 50% of the initial concentration of each vitamer remains in an artificial buffered milieu.

As a further measure of the degradation reaction of $5CH_3$–H_4–PteGlu$_1$ and $5CH_3$–H_4–PteGlu$_3$ (±50 μMol/l ascorbic acid), the time at which 50% of the maximal potential product, either P-ABG (C_9–N_{10} bond scission product) or $5CH_3$–5,6-H_2PteGlu (mono or triglutamate oxidation product) has formed is given in Table 2. This information is helpful in ascertaining the influence of pH and oxidative stress on methylfolate stability and hence its potential bioavailability.

Table 3 provides a measure of the degradation reaction of $5CH_3$–5,6-H_2PteGlu$_{(n)}$ to P-ABG$_{(n)}$ (irreversible loss of vitamin activity via C_9–N_{10} bond scission), versus salvage of $5CH_3$–H_4–PteGlu$_{(n)}$ from $5CH_3$–5,6-H_2PteGlu$_{(n)}$ due to the presence of ascorbic acid and an appropriate pH. Again, the time at which 50% of the maximal potential product is formed in the presence and absence of ascorbic acid (±50 μMol/l), and at pH 3.5 and 7.0, is shown. Also shown is the time at which only 50% of the $5CH_3$–5,6-H_2PteGlu$_{(n)}$ substrate remains.

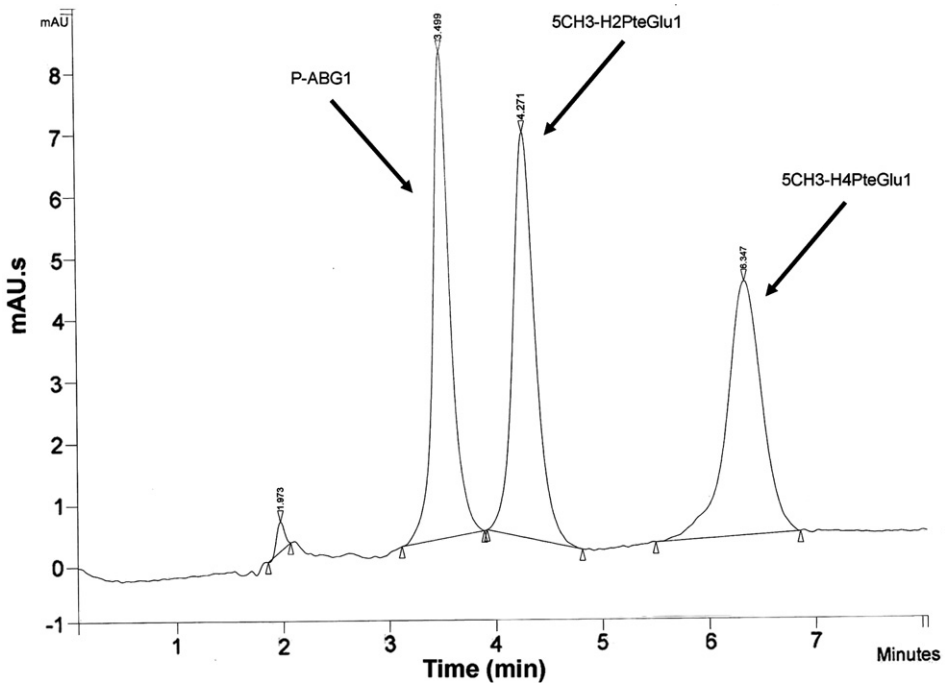

Fig. 1. Reversed-phase (ion-suppression) chromatographic separation of equimolar $5CH_3$–H_4–$PteGlu_1$ and its degradation products, $5CH_3$–5,6-$H_2PteGlu_1$ and P-ABG_1. Each compound represents 125 picomoles on column. Method is as previously described (Lucock, Green, et al., 1995).

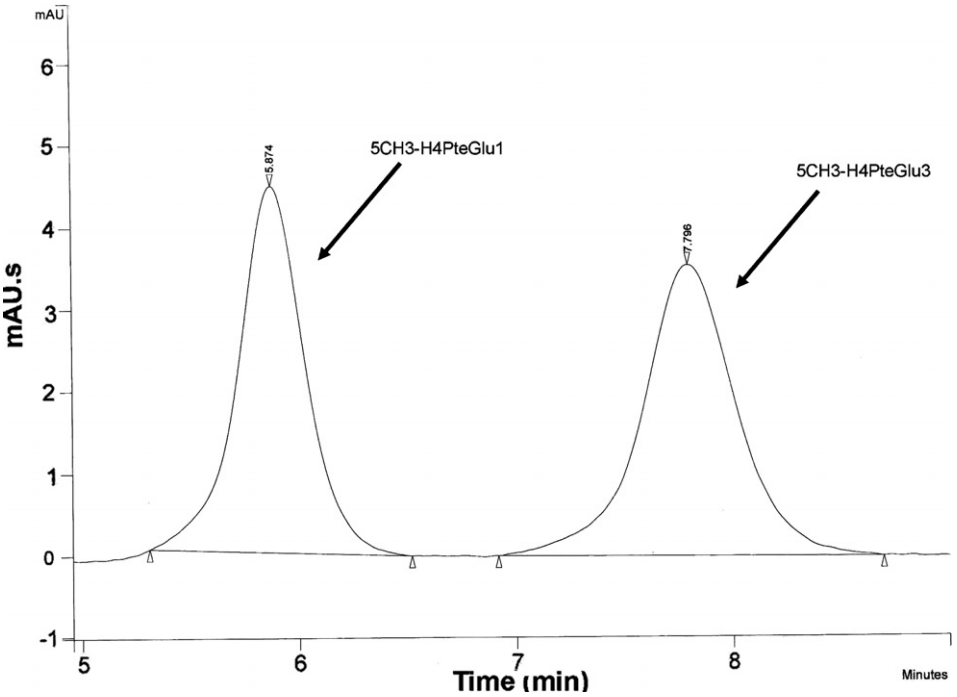

Fig. 2. Reversed-phase (ion-suppression) chromatographic separation of equimolar monoglutamyl $5CH_3$–H_4–$PteGlu_1$ and its triglutamate form, $5CH_3$–H_4–$PteGlu_3$, again, each compound represents 125 picomoles on column. Method based on that described previously (Lucock, Green, et al., 1995).

Although the above experiments were conducted at ±50 μMol/l ascorbic acid, a low to moderate concentration for the vitamin within gastric milieu, experiments were also performed to evaluate the effect of different ascorbic acid concentrations on the degradation and salvage of $5CH_3$–H_4–$PteGlu_{(n)}$.

Table 4 and Fig. 4 show the influence of ascorbic acid concentration on the degradation of $5CH_3$–5,6-$H_2PteGlu_1$ to P-ABG_1 and the salvage of $5CH_3$–5,6-$H_2PteGlu_1$ back to $5CH_3$–$H_4PteGlu_1$ (system contains 125 picomoles of folate). The pace of $5CH_3$–$H_4PteGlu_1$ formation is so rapid that it was necessary to limit measurement of the rate to the

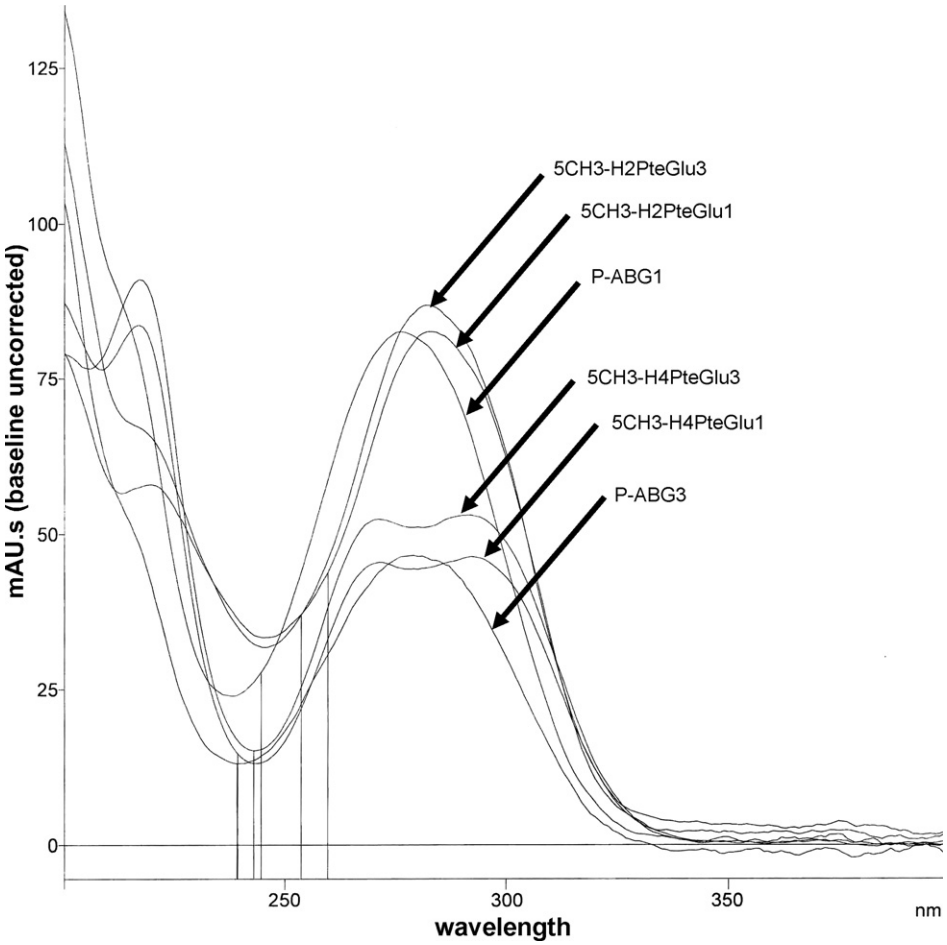

Fig. 3. Monoglutamyl $5CH_3$–H_4–$PteGlu_1$ and its triglutamate form $5CH_3$–H_4–$PteGlu_3$, along with their degradation products, are easily identified by their spectral characteristics.

Table 1
The effect of pH on the degradation of $5CH_3$–H_4–$PteGlu_1$ and $5CH_3$–H_4–$PteGlu_3$ (±50 μMol/l ascorbic acid) providing the time at which only 50% of the initial concentration of each vitamer remains in the artificial buffered milieu

	pH 3.5		pH 7.0	
	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$
+ Ascorbic acid	1183 min	538 min	426 min	241 min
− Ascorbic acid	682 min	212 min	133 min	83 min

Table 2
Time at which 50% of the maximal potential product, either P-ABG (C_9–N_{10} bond scission product), or $5CH_3$–5,6-H_2PteGlu (mono or triglutamate oxidation product) has formed from degradation of $5CH_3$–H_4–$PteGlu_1$ and $5CH_3$–H_4–$PteGlu_3$ (±50 μMol/l ascorbic acid in an artificial buffered milieu)

Substrate	pH 3.5		pH 7.0	
	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$
Product 1	P-ABG_1	P-ABG_3	P-ABG_1	P-ABG_3
+ Ascorbic acid	2404 min	1231 min	Negligible	Negligible
− Ascorbic acid	672 min	488 min	Negligible	Negligible
Product 2	$5CH_3$–5,6-$H_2PteGlu_1$	$5CH_3$–5,6-$H_2PteGlu_3$	$5CH_3$–5,6-$H_2PteGlu_1$	$5CH_3$–5,6-$H_2PteGlu_3$
+ Ascorbic acid	Negligible	Negligible	392 min	192 min
− Ascorbic acid	Negligible	Negligible	94 min	112 min

Table 3
Degradation reaction of $5CH_3$–5,6-$H_2PteGlu_{(n)}$ to P-$ABG_{(n)}$ (irreversible loss of vitamin activity via C_9–N_{10} bond scission), versus salvage of $5CH_3$–H_4–$PteGlu_{(n)}$ from $5CH_3$–5,6-$H_2PteGlu_{(n)}$ due to the presence of ascorbic acid and an appropriate pH in an artificial buffered milieu: Time at which 50% of the maximal potential product is formed in the presence and absence of ascorbic acid (±50 μMol/l), and at pH 3.5 and 7.0

Substrate	pH 3.5		pH 7.0	
	$5CH_3$–5,6-$H_2PteGlu_1$ Remaining substrate	$5CH_3$–5,6-$H_2PteGlu_3$ Remaining substrate	$5CH_3$–5,6-$H_2PteGlu_1$ Remaining substrate	$5CH_3$–5,6-$H_2PteGlu_3$ Remaining substrate
+ Ascorbic acid	4.5 min	Too rapid to measure accurately	671 min	781 min
− Ascorbic acid	5.7 min	Too rapid to measure accurately	938 min	1229 min
Product 1	P-ABG_1	P-ABG_3	P-ABG_1	P-ABG_3
+ Ascorbic acid	Trace formation	Trace formation	No P-ABG (forms unknown product)	No P-ABG (forms unknown product)
− Ascorbic acid	6.1 min	4.3 min	No P-ABG (forms unknown product)	No P-ABG (forms unknown product)
Product 2	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$	$5CH_3$–H_4–$PteGlu_1$	$5CH_3$–H_4–$PteGlu_3$
+ Ascorbic acid	4.5 min	Too rapid to measure accurately	Equilibrium in favour of $5CH_3$–H_4–$PteGlu_{(n)}$ formation, but concomitant degradation of reduced folate complicates enumeration*	Equilibrium in favour of $5CH_3$–H_4–$PteGlu_{(n)}$ formation, but concomitant degradation of reduced folate complicates enumeration*
− Ascorbic acid	No formation	No formation	No formation	No formation

The table also provides the time at which only 50% of the $5CH_3$–5,6-$H_2PteGlu_{(n)}$ substrate remains.
* For comprehensive details, see effect of ascorbic acid concentration below.

Table 4
Effect of ascorbic acid concentration on the degradation of $5CH_3$–5,6-$H_2PteGlu_1$ to P-ABG_1 and salvage of $5CH_3$–5,6-$H_2PteGlu_1$ back to $5CH_3$–$H_4PteGlu_1$ (the artificial buffered milieu contains 125 picomoles of folate)

Ascorbic Acid (μMol/l)	Rate of $5CH_3$–$H_4PteGlu_1$ salvage	$5CH_3$–5,6-$H_2PteGlu_1$ remaining	Rate of $PABG_1$ formation indicating loss of vitamin activity
$5CH_3$–$H_4PteGlu_1$ salvage at pH 3.5: initial formation rate (first minute) picomoles/min			
1000	96.1	23.5	5.4
500	73.0	46.7	5.3
400	78.9	35.9	10.2
300	56.0	57.9	11.1
200	39.5	74.8	10.7
150	44.5	69.2	11.3
100	24.6	91.7	8.7
50	23.2	96.4	5.4
25	8.3	110.1	6.6
0	0	119.4	5.6

first minute of the reaction. Shortly after this time point, conversion to the reduced form of methylfolate is approaching completion at the higher concentrations of ascorbic acid.

Although no graph is given, Table 5 clearly shows that, unlike the rapid and saturable salvage of reduced $5CH_3$–$H_4PteGlu_1$ from oxidised $5CH_3$–5,6-$H_2PteGlu_1$ at pH 3.5, at pH 7.0 the rate of salvage is greatly reduced with a rate that remains proportional to the ascorbic acid concentration over the measured time course, which in all cases represented the time required to achieve the maximum measured level of reduced methylfolate ($r = 0.9961$; $p = 0.0003$; 95% CI $0.9396 \rightarrow 0.9990$ for rate of $5CH_3$–$H_4PteGlu_1$ formation versus ascorbic acid concentration).

The variability in decay rate has been determined by replicate measurements of the amount of folate remaining at a time point when the rate of decay in the absence of exogenous ascorbic acid was maximal (250 min in either pH 3.5 buffer or pH 2.25 gastric juice, and 85 min in pH 7.0 buffer). The mean remaining folate (%), SD and CV at these time points are given in Table 6, along with the p value from a student's t-test to illustrate the significant difference that exists between $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ decay rates at a given pH.

Results showing the physico-chemical properties of native folates in gastric juice compared to an artificial milieu are presented in Fig. 5. These graphs clearly show that:

(i) Ascorbic acid reduces the degradation of $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ at both pH 3.5 and 7.0.
(ii) Irrespective of pH or the presence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ is inherently more stable than $5CH_3$–$H_4PteGlu_3$.
(iii) Irrespective of the presence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ are both more stable at pH 3.5 than at pH 7.0.
(iv) While ascorbic acid clearly stabilises mono- and triglutamate forms of methylfolate in gastric juice, there appears to be a factor in the gastric juice that removes the differential instability between mono- and polyglutamate forms of folate. This endogenous factor could be a binding protein or other antioxidant.

Considerable amounts of natural folate in our diet are likely to exist as $5CH_3$–5,6-$H_2PteGlu_1$ due to the impact of excess cooking and/or storage. The relative amount of oxidised to reduced methylfolate in our food will influence bioavailability, due to the differential effect of gastric acid upon tetra- and dihydro forms of the vitamin. Fig. 6 illustrates how important the ascorbic acid concentration of

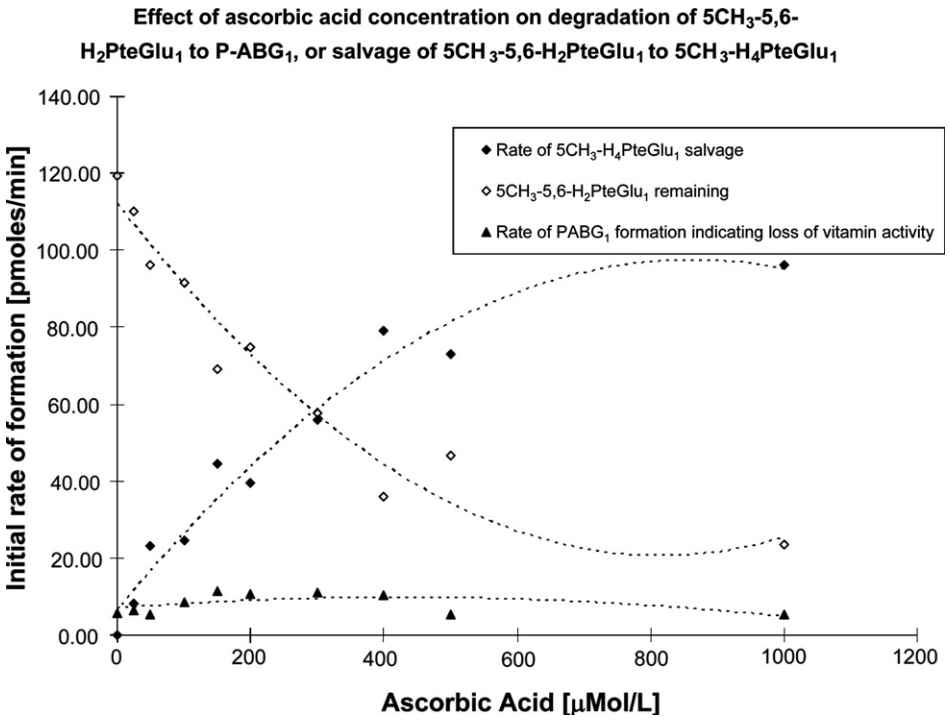

Fig. 4. The influence of ascorbic acid concentration on (i) the degradation of $5CH_3$–5,6-$H_2PteGlu_1$ to P-ABG_1 and (ii) salvage of $5CH_3$–5,6-$H_2PteGlu_1$ back to $5CH_3$–$H_4PteGlu_1$. The artificial buffered milieu contained 125 picomoles of $5CH_3$–5,6-$H_2PteGlu_1$.

Table 5
At pH 7.0, in an artificial buffered milieu containing 125 picomoles of folate, the rate of $5CH_3$–$H_4PteGlu_1$ salvage from oxidised $5CH_3$–5,6-$H_2PteGlu_1$ is much reduced compared to pH 3.5 (see Table 4) with a rate that remains proportional to ascorbic acid concentration over the measured time course

Ascorbic acid (μMol/l)	Rate of $5CH_3$–$H_4PteGlu_1$ salvage
$5CH_3$–$H_4PteGlu_1$ salvage from $5CH_3$–5,6-$H_2PteGlu_1$ at pH 7.0: formation rate (picomoles/min)	
1000	0.15
400	0.072
200	0.037
50	0.017
0	0

In all cases, this time course represented the time required to achieve the maximum measured level of reduced methylfolate ($r = 0.9961$; $p = 0.0003$; 95% CI 0.9396 → 0.9990 for rate of $5CH_3$–$H_4PteGlu_1$ formation versus ascorbic acid concentration).

gastric juice is in the salvage of oxidised $5CH_3$–5,6-$H_2PteGlu_1$ back to $5CH_3$–$H_4PteGlu_1$. It also shows how easily $5CH_3$–5,6-$H_2PteGlu_1$ can be lost in gastric juice by C_9–N_{10} bond scission. Once C_9–N_{10} bond scission has occurred, folate activity is irreversibly lost.

At a concentration of 50 μMol/l exogenous ascorbic acid, $5CH_3$–5,6-$H_2PteGlu_1$ is salvaged back to $5CH_3$–$H_4PteGlu_1$ in the gastric juice, however, substantially more is lost via C_9–N_{10} bond scission to P-ABG_1. Clearly, ascorbic acid concentration is a very important component in folate bioavailability.

It would seem that $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ exhibit a differential stability that may reflect increased sensitivity to oxidative stress for polyglutamate forms of the vitamin. This may be an extremely important finding since polyglutamates are the predominant folate

Table 6
Variability in decay rate as determined by replicate measurements of the amount of folate remaining at a time point when the rate of decay in the absence of exogenous ascorbic acid was maximal (250 min in either pH 3.5 buffer or pH 2.25 gastric juice, and 85 min in pH 7.0 buffer)

	$5CH_3$–$H_4PteGlu_1$ pH 3.5	$5CH_3$–$H_4PteGlu_3$ pH 3.5	$5CH_3$–$H_4PteGlu_1$ pH 2.25 – gastric juice	$5CH_3$–$H_4PteGlu_3$ pH 2.25 – gastric juice	$5CH_3$–$H_4PteGlu_1$ pH 7.0	$5CH_3$–$H_4PteGlu_3$ pH 7.0
Percentage $5CH_3$–$H_4PteGlu_n$ remaining at 250 min (pH 3.5 buffer/gastric juice) or 85 min (pH 7.0 buffer)						
Mean % remaining	89.7	78.1	66.5	79.4	82.0	71.9
n	15	11	11	11	9	9
SD	5.88	5.74	7.00	5.18	5.20	5.50
CV (%)	6.55	7.35	10.53	6.53	6.35	7.64
Student's *t*-test (*p*)	0.0002		0.0002		0.0011	

The mean remaining folate (%), SD and CV at these time points are given along with the *p* value from a student's *t*-test to illustrate the significant difference that exists between $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ decay rates at a given pH.

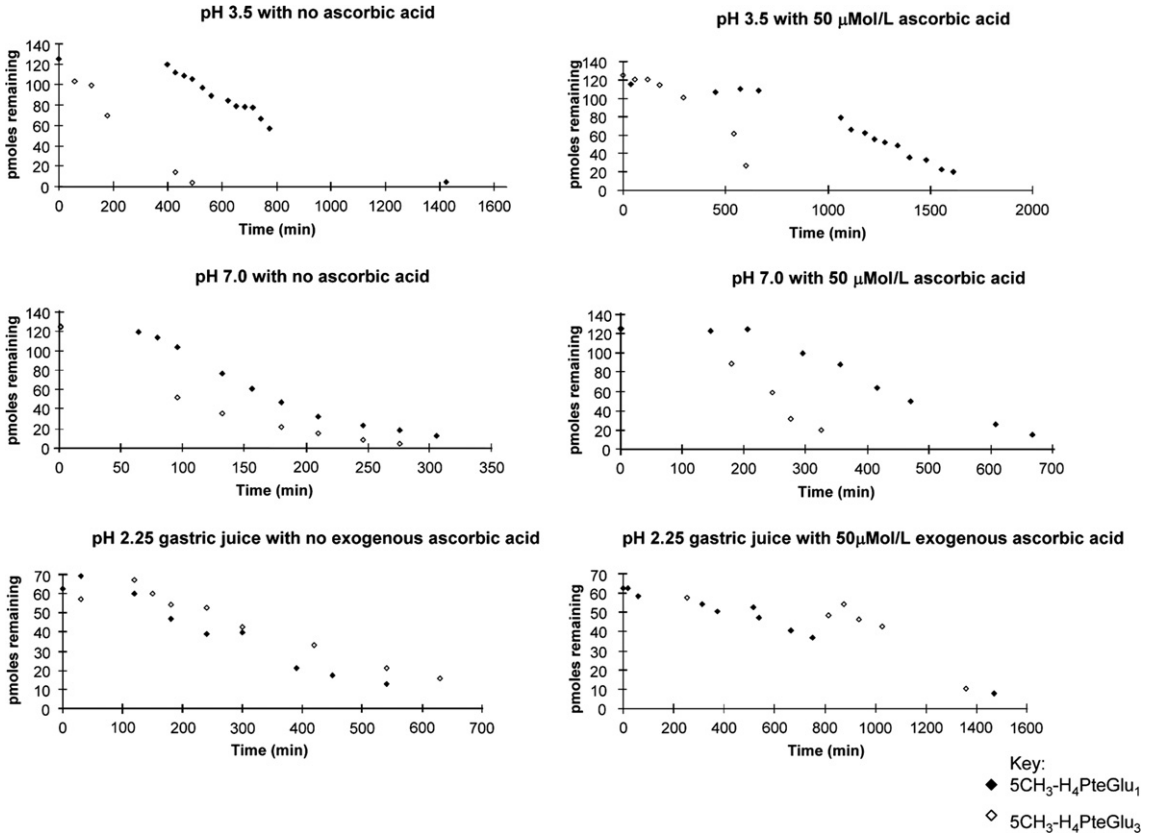

Fig. 5. Physico-chemical properties of native monoglutamyl $5CH_3$–H_4–$PteGlu_1$ and its triglutamate form, $5CH_3$–H_4–$PteGlu_3$ in human gastric juice compared to an artificial milieu: Ascorbic acid reduces the degradation of $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ at both pH 3.5 and 7.0. However, irrespective of pH or the presence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ is inherently more stable than $5CH_3$–$H_4PteGlu_3$. In the presence or absence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ are both more stable at pH 3.5 than at pH 7.0. Although ascorbic acid clearly stabilises mono- and triglutamate forms of methylfolate in gastric juice, a factor in the gastric juice removes the differential instability between mono- and polyglutamate forms.

forms found in food. In order to further characterise this phenomenon, the voltage–current profile (hydrodynamic voltammagram) for $5CH_3$–$H_4PteGlu_1$, $5CH_3$–$H_4PteGlu_2$ and $5CH_3$–$H_4PteGlu_3$ have been ascertained. Fig. 7 shows the normalised voltage–current profiles. Clearly, the profiles for the various polyglutamate forms of methylfolate are almost identical, as is the case with their spectral properties (Lucock, Ng, Veysey, & Yates, 2005). Although the optimum signal current is generated between −100 and +300 mV, the most selective analysis with a palladium reference electrode will likely occur between −100 and +100 mV.

4. Discussion

Although a considerable body of knowledge now exists on the bioavailability of folic acid, little work has been done on the pH sensitivity of the natural labile form of the vitamin within the gastrointestinal environment, particularly with respect to molecular integrity. Any such work has been carried out on monoglutamyl forms of the vitamin. To our best knowledge, this is the first study to look at the pH sensitivity of polyglutamyl forms of natural folic acid.

Our findings show that under mildly acidic, typical postprandial conditions (pH 3.5), physiological levels of ascorbic acid can salvage both $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ from acid labile $5CH_3$–5,6-$H_2PteGlu_n$, and prevent loss of vitamin activity as indicated by P-ABG_n formation. This process is less efficient under neutral conditions (pH 7.0), although as Table 3 shows, under such conditions, clearly, $5CH_3$–5,6-$H_2PteGlu_n$ is rendered relatively stable, although in this form, it apparently cannot enter one-carbon metabolism (Ratanasthien, Blair, Leeming, Cooke, & Melikian, 1977).

These findings support earlier work (Lucock, Green, et al., 1995; Lucock, Priestnall, et al., 1995), but also provide additional information that may be of critical importance in evaluating the bioavailability of natural polyglutamate forms of the vitamin. Even moderate physiologically equivalent levels at 50 μMol/l ascorbic acid reduce the degradation of $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ at both pH 3.5 and 7.0. However, irrespective of the presence of ascorbic acid, $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ are both more stable at pH 3.5 than at pH 7.0. There is a clear differential effect between mono- and polyglutamates with respect to stability, since, irrespective of pH or the presence of ascorbic acid, $5CH_3$–H_4

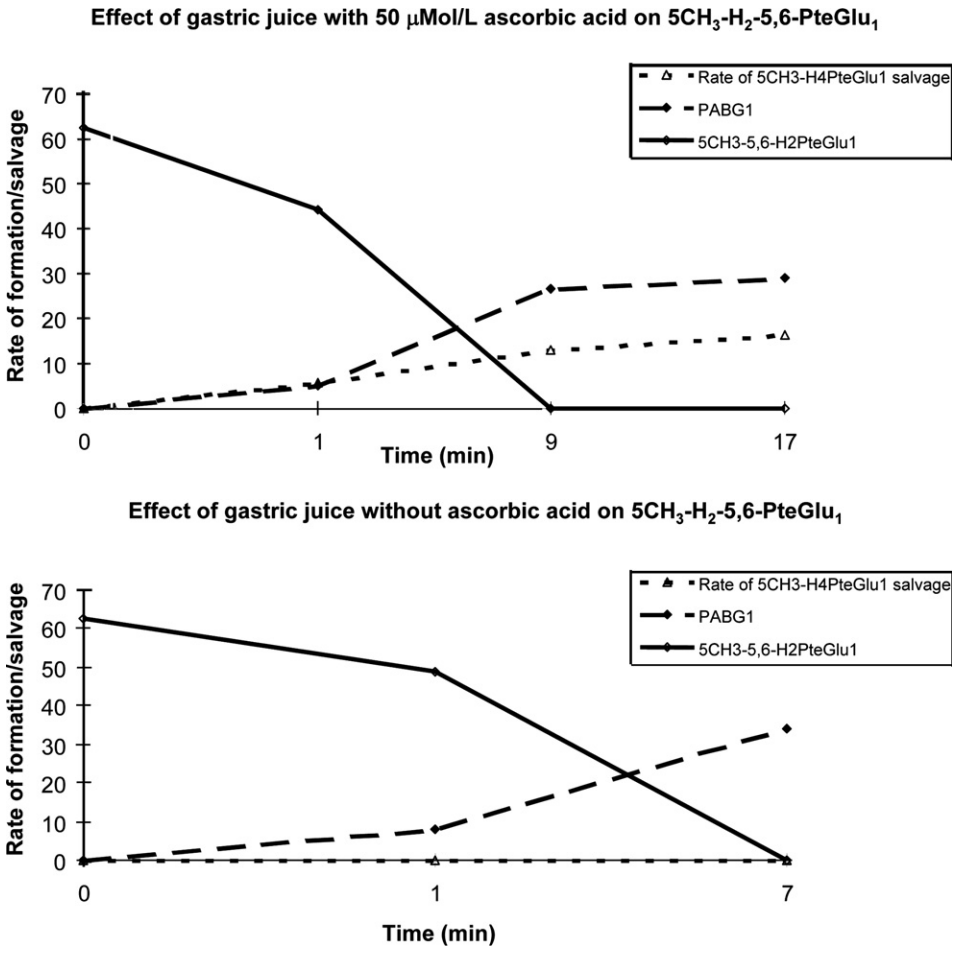

Fig. 6. This figure shows how important endogenous ascorbic acid, which is maintained against a concentration gradient within the stomach lumen, is in the salvage of oxidised $5CH_3$–5,6-$H_2PteGlu_1$ back to $5CH_3$–$H_4PteGlu_1$ in gastric juice. It also shows how easily $5CH_3$–5,6-$H_2PteGlu_1$ can be lost in gastric juice by C_9–N_{10} bond scission. Once C_9–N_{10} bond scission has occurred, folate activity is irreversibly lost. At 50 μMol/l exogenous ascorbic acid, $5CH_3$–5,6-$H_2PteGlu_1$ is salvaged back to $5CH_3$–$H_4PteGlu_1$in the gastric juice, although much is lost via C_9–N_{10} bond scission to P-ABG_1. Clearly, active secretion of ascorbic acid into the gut lumen is a very important component in folate bioavailability.

$PteGlu_1$ is inherently more stable than $5CH_3$–$H_4PteGlu_3$. The results from gastric juice are extremely interesting; while ascorbic acid clearly stabilises mono- and triglutamate forms of methylfolate in gastric juice, there appears to be a factor in the gastric juice that removes the differential instability between mono- and polyglutamate forms of folate seen in buffer solution. We speculate that this endogenous factor could be either a binding protein or some other antioxidant.

This report focuses on the physico-chemical properties of methylfolate, the main natural form of the vitamin found in food. However, substantial dietary folate is now obtained from highly stable, synthetic PteGlu as either discretionary supplements, or via government-mandated fortification. There are potential concerns related to the mandated use of PteGlu at a population level, and for this reason it is still important to understand all the issues surrounding natural methylfolate bioavailability. The absorption and biotransformation process for PteGlu is saturated at doses in the region of 400 μg. Therefore, at or below this dose, humans can metabolize synthetic PteGlu into natural $5CH_3$–$H_4PteGlu_1$ with 100% efficiency. However, at doses above 400 μg PteGlu, the synthetic form of the vitamin is transported into the blood in a fashion that is directly proportional to dose (Kelly, McPartlin, Goggins, Weir, & Scott, 1997; Lucock, Wild, Smithells, & Hartley, 1989). The effects of a lifetime's exposure to unmetabolized xenobiotic PteGlu when mandatory fortification is undertaken is unclear – there may well be no health risks, however, we cannot know this for certain. The long-term biological effects of government-mandated PteGlu fortification of grain products at a national level are therefore unknown. Excess exposure to PteGlu may lead to an antimetabolite effect; mask the detection of B_{12} deficiency, particularly in the elderly; modify therapeutic control of epilepsy by anticonvulsant drugs; influence antifolate cancer chemotherapy or modulate risk for cancer (Charles, Ness, Campbell, Davey Smith, & Hall, 2004; Lucock, 2006; Ulrich, Bigler, Bostick, Fosdick, & Potter, 2002; Ulvik et al., 2001). Furthermore, evidence exists that periconceptional folate may increase the prevalence of the potentially deleterious C677T-MTHFR TT genotype, which is associated with

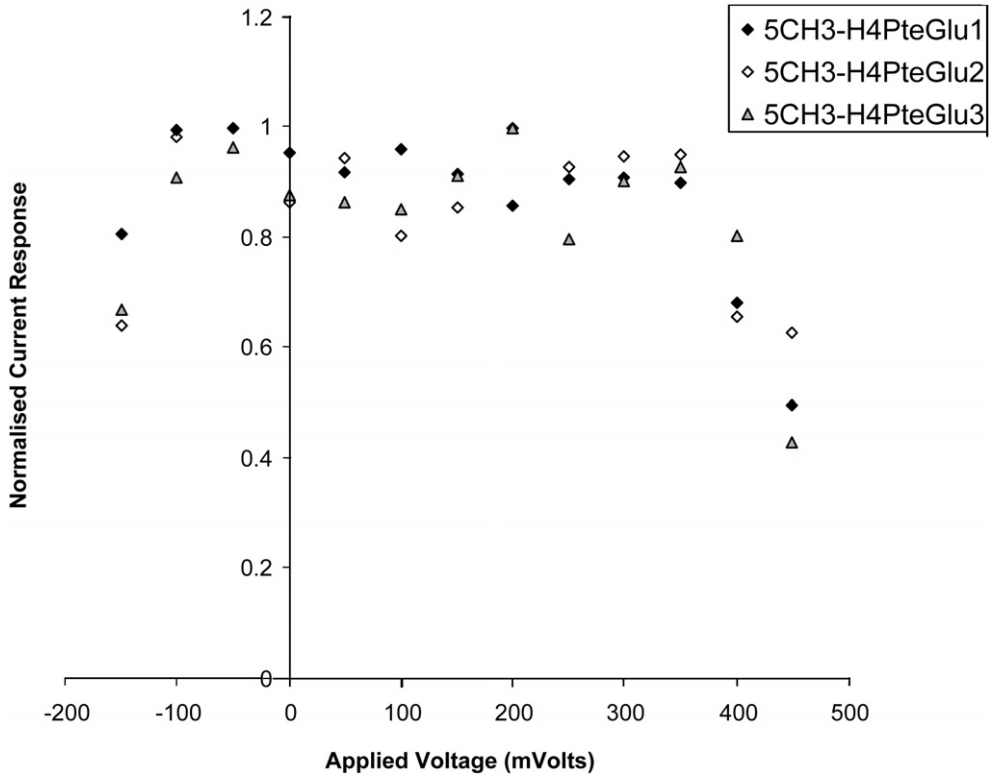

Fig. 7. Normalised voltage–current profiles (hydrodynamic voltammagrams) for $5CH_3$–$H_4PteGlu_1$, $5CH_3$–$H_4PteGlu_2$ and $5CH_3$–$H_4PteGlu_3$. Profiles for the various mono and polyglutamate forms of methylfolate are almost identical, as is the case with their spectral properties (Lucock et al., 2005). The optimum signal current is generated between −100 and +300 mV, although the most selective analysis with a palladium reference electrode will likely occur between −100 and +100 mV.

higher homocysteine levels (Isotalo, Wells, & Donnelly, 2000; Lucock & Yates, 2005; Munoz-Moran, Dieguez-Lucena, Fernandez-Arcas, Peran-Mesa, & Reyes-Engel, 1998; Reyes-Engel et al., 2002).

In spite of these putative negative aspects, it is important to recognize that there are clear benefits to receiving PteGlu in preventing spina bifida and other neural tube defects and lowering homocysteine. Despite this, some further clarity is required in explaining the outcome of a recent extremely large clinical study where it was shown that treatment with folic acid, vitamin B_{12} and vitamin B_6 failed to lower the risk of recurrent cardiovascular disease after acute myocardial infarction. A harmful effect from combined B vitamin treatment was suggested, indicating that such treatment should not be recommended (Bonaa et al., 2006). There is also an interesting debate at present on the role of folate in the occurrence of twin births.

Although the mandatory use of PteGlu has been adopted widely, clearly there is still good reason to learn about the factors determining the bioavailability of this critical nutrient in its natural form(s). The differential lability of $5CH_3$–$H_4PteGlu_1$ and $5CH_3$–$H_4PteGlu_3$ is a novel finding, as is the influence of gastric juice upon these two vitamers. While such subtleties likely play a role in overall folate bioavailability, there are many other factors that are also important. One of the most recent is the G80A-reduced folate carrier single nucleotide polymorphism (SNP), which influences the absorption and cellular translocation of dietary folate (Dufficy et al., 2006). Another SNP that might potentially influence folate bioavailability, but which is not as common as the G80A-reduced folate carrier SNP, is the C1561T-glutamate carboxypeptidase (folate deconjugase) SNP. The reason for a possible functional effect with respect to this SNP is that folate deconjugation is essential prior to the intestinal absorption of this critical micronutrient. Natural folate bioavailability is therefore likely to be dependent upon both key nutrient-nutrient and gene-nutrient interactions. The results presented here raise interesting questions and beg further research to better understand folate bioavailability.

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