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RESEARCH PAPER

[6S]-5-methyltetrahydrofolate increases plasma folate more effectively than folic acid in women with the homozygous or wild-type 677C→T polymorphism of methylenetetrahydrofolate reductase

R Prinz-Langenohl[1], S Brämswig[1], O Tobolski[1]*, YM Smulders[2], DEC Smith[2], PM Finglas[3] and K Pietrzik[1]

[1]*Department of Nutrition and Food Sciences, Human Nutrition II, Pathophysiology, University of Bonn, Germany,* [2]*Department of Internal Medicine, Institute of Cardiovascular Research, VU University Medical Center, Amsterdam, the Netherlands, and* [3]*Institute of Food Research, Norwich, UK*

Background and purpose: 5,10-Methylenetetrahydrofolate reductase (MTHFR) is responsible for the synthesis of 5-methyltetrahydrofolate (5-MTHF). The 677C→T mutation of MTHFR reduces the activity of this enzyme. The aim of this study was, first, to compare pharmacokinetic parameters of [6S]-5-MTHF and folic acid (FA) in women with the homozygous (TT) and wild-type (CC) 677C→T mutation, and second, to explore genotype differences. The metabolism of [6S]-5-MTHF and FA was evaluated by measuring plasma folate derivatives.
Experimental approach: Healthy females (TT, $n = 16$; CC, $n = 8$) received a single oral dose of FA (400 μg) and [6S]-5-MTHF (416 μg) in a randomized crossover design. Plasma folate was measured up to 8 h after supplementation. Concentration-time-profile [area under the curve of the plasma folate concentration vs. time (AUC)], maximum concentration (C_{max}) and time-to-reach-maximum (t_{max}) were calculated.
Key results: AUC and C_{max} were significantly higher, and t_{max} significantly shorter for [6S]-5-MTHF compared with FA in both genotypes. A significant difference between the genotypes was observed for t_{max} after FA only ($P < 0.05$). Plasma folate consisted essentially of 5-MTHF irrespective of the folate form given. Unmetabolized FA in plasma occurs regularly following FA supplementation, but rarely with [6S]-5-MTHF.
Conclusions and implications: These data suggest that [6S]-5-MTHF increases plasma folate more effectively than FA irrespective of the 677C→T mutation of the MTHFR. This natural form of folate could be an alternative to FA supplementation or fortification.
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Keywords: [6S]-5-methyltetrahydrofolate; folic acid; MTHFR polymorphism; folate derivatives; humans

Abbreviations: AUC, concentration–time-profile; C_{max}, the maximum concentration; CC, CC genotype of the 677C→T mutation of 5,10-methylenetetrahydrofolate reductase FA, folic acid (pteroylmono-L-glutamic acid); 5,10-CHTHF, 5,10-methenyltetrahydrofolate; [6S]-5-MTHF, [6S]-5-methyltetrahydrofolate; MTHFR, 5,10-methylenetetrahydrofolate reductase (E.C. 1.5.1.20); NTD, neural tube defect; RBC, red blood cell; THF, tetrahydrofolate; t_{max}, the time-to-reach-the-maximum; TT, TT genotype of the 677C→T mutation of 5,10-methylenetetrahydrofolate reductase

Introduction

Numerous studies have shown that supplementation with folic acid (FA), the synthetic form of the B-group vitamin, reduces the risk of a neural tube defect (NTD)-affected pregnancy (reviewed by Scholl and Johnson, 2000; Smith *et al.*, 2008). FA supplementation of about 400 μg·day^{-1} in the

Correspondence: Dr R Prinz-Langenohl, Institute of Nutrition and Food Sciences, Human Nutrition II, Pathophysiology, University of Bonn, Endenicher Allee 11-13, D-53115, Bonn, Germany. E-mail: r.prinz@uni-bonn.de
*Current address: Siebengebirgsstraße, Cologne, Germany.
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periconceptional period is therefore recommended by several health authorities for all women of childbearing age as primary prevention of NTD (CDC, 1992; Commission of the European Communities, 1993).

FA is the stable synthetic form of the vitamin used in drugs, supplements and fortified food, and needs to be reduced within the cell to tetrahydrofolate (THF) in order to become metabolically active. 5-Methyltetrahydrofolate (5-MTHF) is the folate derivative normally found in the circulation, and in addition, is the predominant type of folate present in food. 5-MTHF is also available commercially as the natural form [6*S*]-5-MTHF or the racemic mixture [6*RS*]-5-MTHF.

The enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is responsible for the irreversible reduction of 5,10-methylenetetrahydrofolate to 5-MTHF, which is needed for the conversion of homocysteine to methionine via donation of a one-carbon group. A variant in the MTHFR gene causes the replacement of cytosine with thymine at nucleotide 677 (677C→T polymorphism), resulting in the amino acid alanine being replaced by valine. In the European population, up to 12% are homozygous (TT), 43% heterozygous (CT) and 45% wild-typed (CC) for that polymorphism (Brattström *et al.*, 1998; Gudnason *et al.*, 1998; Koch *et al.*, 1998; Meisel *et al.*, 2001; Klerk *et al.*, 2002; Meleady *et al.*, 2003). In the TT genotype, the enzyme activity *in vitro* is reduced by approximately 75% compared with that of the wild type (Kang *et al.*, 1988; Frosst *et al.*, 1995), and this is associated with an elevated plasma level of homocysteine as a result of a decreased production of 5-MTHF; this is especially noticeable when the folate levels are low (Brattström *et al.*, 1998; Gudnason *et al.*, 1998; Klerk *et al.*, 2002). Furthermore, the 677C→T variant of the MTHFR gene has been identified as a genetic risk factor for NTD (Whitehead *et al.*, 1995; Christensen *et al.*, 1999; van der Put and Blom, 2000), and may account for up to 19% of NTD cases (Ou *et al.*, 1996; Shields *et al.*, 1999).

Recent studies in humans have shown that 5-MTHF is at least as effective as FA in increasing plasma folate, red blood cell folate or in lowering homocysteine in healthy and diseased adults. Most of the studies, however, did not take into account the genotype of the 677C→T mutation of the MTHFR (Venn *et al.*, 2002; Houghton *et al.*, 2006; 2009), excluded the TT-genotype (Pentieva *et al.*, 2004), or worked with a small number of homozygous participants in the different treatment groups (Litynski *et al.*, 2002; Fohr *et al.*, 2002; Venn *et al.*, 2002; Lamers *et al.*, 2004; 2006; Willems *et al.*, 2004). Therefore, data on the effect of [6*S*]-5-MTHF or FA on plasma folate in the TT genotype are rare. In addition, the folate compounds were given in unphysiologically high doses in some studies, and/or in form of a racemic mixture of 5-MTHF.

Unlike [6*S*]-5-MTHF, FA needs to be reduced and substituted with one-carbon residues, a process involving MTHFR, before entering the systemic circulation as 5-MTHF. Therefore, FA might have a smaller effect compared with [6*S*]-5-MTHF on plasma folate in individuals with reduced MTHFR activity like those with the TT genotype.

Hence, the aim of the present study was, first, to compare pharmacokinetic parameters by administering a physiological, single oral dose of [6*S*]-5-MTHF and FA to women of childbearing age with either the homozygous (TT genotype) or wild-type (CC genotype) 677C→T mutation of MTHFR, and, second, to explore genotype differences. Pharmacokinetic parameters included the concentration–time profile [area under the curve of the plasma folate concentration vs. time (AUC)], the maximum concentration (C_{max}) and the time-to-reach-maximum (t_{max}) of the total plasma folate concentration.

In addition, we evaluated the short-term absorption and initial metabolism of FA and [6*S*]-5-MTHF *in vivo* by measuring plasma concentrations of the folate derivatives FA, 5-MTHF, tetrahydrofolate (THF) and 5,10-methenyltetrahydrofolate (5,10-CHTHF).

Methods

Subjects

Healthy female volunteers TT or CC genotyped for the 677C→T mutation of the MTHFR were recruited from a volunteer database existing from former studies conducted at the Institute of Nutrition and Food Sciences, Human Nutrition II, Pathophysiology, University of Bonn, Germany. Women of childbearing age with a body mass index (BMI) between 17–25 $kg \cdot m^{-2}$, haematological and clinical chemistry parameters within the normal range, adequate folate status [plasma folate > 6.8 nM, red blood cell (RBC) folate > 317 nM] and vitamin B-12 status (plasma vitamin B-12 > 110 pM), were eligible for participation. In addition, all participants were using reliable contraception. The main exclusion criteria were organic or mental disease, medical treatments interfering with folate metabolism (i.e. methotrexate, sulphasalazine, salicylic acid, antiepileptic drugs), pregnancy or lactation, and abuse of alcohol or drugs. The subjects were instructed to maintain their regular dietary habits for a 4-week period before intervention and for the duration of the study, but to abstain from the intake of vitamin supplements and food fortified with FA. The study was approved by the Ethics Committee of the Medical Association of Hamburg, Germany, and conducted in accordance with the Helsinki Declaration. All participants gave written informed consent.

Design

The study was a randomized, double-blind study with crossover design (Figure 1). The clinical part of this study included three examination days (screening, study day I, study day II). Screening took place 12 days before the first study day. The study days were separated by a 'wash-out period' of 6 days. The study treatment consisted of an immediate-release film-coated tablet, containing 400 μg FA or equimolar amounts of 416 μg [6*S*]-5-MTHF. Participants were randomized to one of two treatment sequences: either [6*S*]-5-MTHF on study day I and FA on study day II, or FA on study day I and [6*S*]-5-MTHF on study day II. Randomization was stratified according to the 677C→T MTHFR polymorphism to ensure equal distribution of the two genotypes (TT/CC) in the two sequences. On the study days, a fasting blood sample (12 h overnight fast) was drawn in the morning (0 min). Directly after collection, the subjects received either FA or [6*S*]-5-MTHF as a single oral dose with 200 mL of tap water. Further blood samples were taken after ingestion of the tablets for a period of 8 h (30, 60, 90,

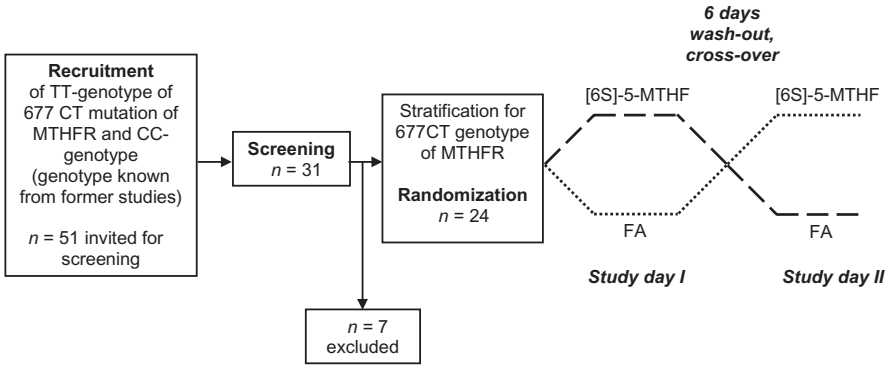

Figure 1 Study flow chart. MTHFR, methylenetetrahydrofolate reductase; [6S]-5-MTHF, [6*S*]-5-methyltetrahydrofolate; FA, folic acid; TT, TT genotype of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase; CC, CC genotype of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase.

120, 180, 240, 360, 480 min). The subjects were given a standardized diet three times during the study day (2, 4, 6 h after test dose administration) in accordance with the requirements for FA/folate bioavailability studies (Prinz-Langenohl *et al*., 1999). This diet consisted of a folate-free diet drink (85 g powder mixed in 240 mL tap water, 437 kcal per portion). The diet drink was a commercially available nutritional supplement (Scandishake Mix Vanille). Additionally, the subjects were allowed to drink 200 mL mineral water or black coffee with the diet drink. Tolerance and safety were monitored by questionnaires on each study day and by routine laboratory variables on study day II.

Blood samples

Blood samples for kinetic analysis were drawn from the median cubital vein into heparin-coated tubes and centrifuged within 15 min at 3000× *g* for 10 min. Plasma was separated and stored at –80°C until analysis within 3 months after completion of the study. Blood samples for the measurement of the plasma folate derivatives (FA, 5-MTHF, THF and 5,10-CHTHF) were collected in EDTA coated tubes, centrifuged at 4°C within 15 min (1000× *g*, 10 min) and stored at –80°C.

Analyses

Total plasma folate was measured using an immunoassay kit for the Immulite 2000 analyzer (intra-assay CV < 5.4%; inter-assay CV < 8.3%). The assay is based on a two-cycle, on board sample treatment of the plasma sample. The sample, along with ligand-labelled folic acid, is first treated with dithiothreiotol and then with sodium hydroxide/potassium cyanide. The pre-treated sample is transferred to a second reaction tube containing a murine anti-folate binding protein antibody-coated polystyrene bead and folate binding protein. During a 30 min incubation, folic acid released from binding proteins in the sample competes with ligand-labelled folic acid for binding with folate-binding protein. The bead is washed, and alkaline phosphatase labelled anti-ligands bind to the ligand-labelled folate that was bound to the bead during the first incubation. The unbound enzyme conjugate is removed by centrifugal wash. Substrate is added, and the procedure continues as a typical immune reaction. Plasma folate was measured after manually diluting all samples with the assay-specific folate specimen diluent (1:5). To avoid between-run variation, all samples from each subject were analysed in one run at the end of the study. Routine laboratory variables were measured immediately after venipuncture at screening and at the end of the second study day by the central laboratory of the University Hospital, Bonn. The determination of plasma FA, 5-MTHF, THF and 5,10-CHTHF was conducted at the VU University Medical Centre (Deptartment of Clinical Chemistry, Amsterdam, the Netherlands). Plasma samples were transferred by courier to Amsterdam in a cold-box over solid carbon dioxide. The concentration of the various folates was measured with the use of liquid chromatography tandem mass spectrometry (LC-MS-MS) as described by Kok *et al*. (2004) (5-MTHF: intra-assay CV < 3.8%, inter-assay CV < 1.7%; FA: intra-assay CV < 3.2%, inter-assay CV < 7.1%). Briefly, [$^{13}C_5$]-5-MTHF was added to the plasma samples as an internal standard. Plasma samples were purified using folate-binding protein affinity columns, followed by a concentration step. LC-MS-MS was performed with a triple quadrupole tandem mass spectrometer (API 3000) in the positive ion mode following multiple reactions monitoring of precursor fragment transitions for relevant types of folate. Duplicate injections were performed on all samples, and standards were run in duplicate within each run.

Statistics

This study has the character of a pilot study. Sample size could only be estimated, as published data on [6S]-5-MTHF in healthy TT genotyped subjects were not sufficient for calculation. On the basis of the data of Prinz-Langenohl *et al*. (2003), obtained in females heterozygous for the 677C→T mutation of MTHFR, it was estimated that 16 TT volunteers eligible for analysis would be sufficient to compare the pharmacokinetic parameters of [6S]-5-MTHF with those of FA with adequate precision. In order to explore possible genetically influenced differences in pharmacokinetic parameters of the two vitamin preparations, a total of 8 CC subjects were included additionally in the study. Data are expressed as means ± SD, where *n* equals the number of subjects. Analysis of pharmacokinetic parameters was done with paired samples

t-test to quantify the effect of FA and [6S]-5-MTHF on the total plasma folate concentration. The mean AUC observed within each treatment and the treatment difference in the mean AUC are shown with 95% confidence intervals. The test was performed using a two-sided type I error rate of 5%. To calculate AUC for one subject and one treatment, the change from baseline was calculated for each of the nine time points (baseline = '0 min' assessment). From these values, the AUC was calculated using the trapezoidal rule. AUC values have been presented in nM, that is adjusted for time. To assess the relative bioavailability of the different treatments, individual ratios of the pharmacokinetic parameter AUC were obtained and expressed as a percentage. For sensitivity analysis, an analysis of covariance (ANCOVA) on AUC was performed to evaluate treatment effects, with treatment sequence, genotypes and pre-dose plasma folate as covariates taking into account within-subject correlations. A descriptive summary was produced for total plasma folate concentrations and concentrations of FA, 5-MTHF, THF and 5,10-CHTHF.

All analyses were performed using SAS (version 9.1.3; SAS Inc., Heidelberg, Germany).

Materials

[6S]-5-MTHF (Metafolin: calcium salt of [6S]-5-MTHF) was obtained from Merck Selbstmedikation GmbH (Darmstadt, Germany). The diet drink Scandishake Mix Vanille was from Nutricia Nahrungsmittel GmbH&CoKG (Wien, Austria). The heparin and EDTA coated tubes were from Sarstedt (Nümbrecht, Germany). The immunoassay kit for the Immulite 2000 analyzer was from Diagnostic Products Corporation Biermann GmbH (Bad Nauheim, Germany), and the triple quadruple tandem mass spectrometer (API 3000) was from Applied Biosystems (Foster City, AC, USA).

Results

Subject characteristics

Thirty-one women were screened for the study. Seven of whom did not fulfil the inclusion criteria and were excluded from the study. All 24 randomized subjects (TT, $n = 16$, CC, $n = 8$) completed the study and were eligible for the analysis of the full data set. Screening characteristics of these subjects are presented in Table 1. No significant differences were found between the genotype and treatment sequence groups with respect to plasma vitamin B12-concentration, red blood cell and total plasma folate concentration, height, weight, BMI and vital signs. Due to the inclusion criteria (plasma folate > 6.8 nM, RBC folate > 317 nM), our group showed a relatively high folate status.

The study treatment was well tolerated by the volunteers. No treatment related adverse events were observed.

Total plasma folate

Figure 2 presents the mean total plasma folate concentrations on the study days before and after application of the test dose in the TT group. The mean absolute change in plasma folate concentrations from the baseline value (0 min) was consistently higher with [6S]-5-MTHF than with FA throughout the 480 min follow-up. As seen in Figure 2, the absorption of [6S]-5-MTHF is different from that of FA, whereas the rate of clearance is similar. The change in the mean total plasma folate concentrations with the different treatments was almost the same in the CC group (see Figure 3).

Table 1 Characteristics of the population studied at screening (12 days before the first study day)

	TT (n = 16)	*CC (n = 8)*
Age (years)	28.1 ± 2.7	27.3 ± 2.1
Height (cm)	169.6 ± 6.4	171.0 ± 6.4
Weight (kg)	63.0 ± 8.5	63.8 ± 8.8
BMI ($kg \cdot m^{-2}$)	21.8 ± 1.9	21.8 ± 2.4
Plasma vitamin B-12 (pM)	266.9 ± 115.7	279.8 ± 149.5
Red blood cell folate (nM)	1109.4 ± 629.1	982.5 ± 374.3
Total plasma folate (nM)	17.1 ± 6.6	25.6 ± 9.0

No significant differences were observed between the groups (one-factor ANOVA). The data are presented as the arithmetic mean ± SD. (all such values). BMI, body mass index; CC, CC genotype of the 677C→T mutation of 5,10-methylenetetrahydrofolate reductase; TT, TT genotype of the 677C→T mutation of 5,10-methylenetetrahydrofolate reductase.

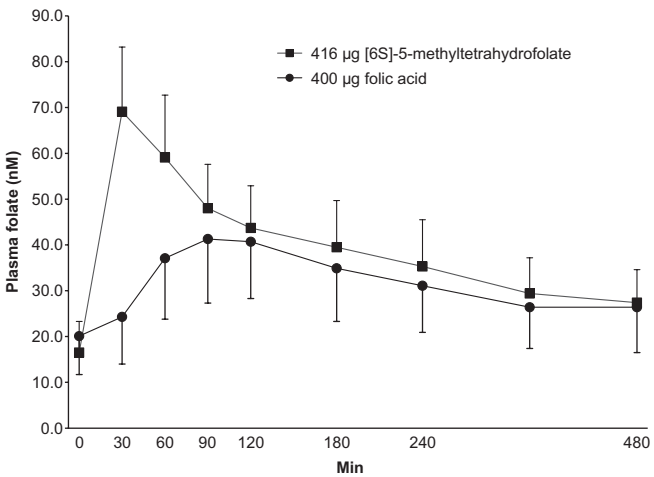

Figure 2 Arithmetic mean total plasma folate concentrations over time after a single oral dose of folic acid or [6S]-5-methyltetrahydrofolate in the TT genotype ($n = 16$) of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase. Bars represent SD.

Pharmacokinetic variables

Table 2 shows a summary of the results of the pharmacokinetic parameters. In the TT group, both the mean AUC and C_{max} for the total plasma folate concentration were significantly higher with [6S]-5-MTHF than with FA ($P < 0.0001$). After administration of [6S]-5-MTHF, the AUC was twice as high as the AUC after administration of FA. The maximum plasma folate concentrations, $C_{max,}$, was nearly doubled with [6S]-5-MTHF compared with FA. The mean t_{max} of the total plasma folate concentrations was significantly shorter with [6S]-5-MTHF than with FA.

Similar results for the pharmacokinetic variables AUC and C_{max} after administration of [6S]-5-MTHF and FA were seen in

Table 2 Pharmacokinetic variables of plasma folate concentration in women with the TT (n = 16) or CC (n = 8) genotype of the 677C→T mutation of MTHFR

		FA	*[6S]-5-MTHF*	P
AUC (nM) adjusted for time	TT	10.8 ± 2.9 (9.3–12.4)	21.4 ± 4.6 (19.0–23.9)	<0.0001
	CC	11.9 ± 2.9 (9.5–14.3)	19.2 ± 4.5 (15.4–22.9)	0.0012
C_{max} (nM)	TT	43.4 ± 12.4 (36.8–50.0)	71.1 ± 13.5 (63.9–78.3)	<0.0001
	CC	48.0 ± 13.5 (36.7–59.3)	66.5 ± 12.7 (55.9–77.1)	0.0006
t_{max} (min)	TT	119.4 ± 69.2[§] (82.5–156.3)	33.3 ± 10.9 (27.5–39.1)	0.0002
	CC	79.0 ± 27.9 (55.6–102.3)	36.9 ± 14.2 (25.0–48.7)	0.0134

The data are presented as the arithmetic mean ± SD, with the 95% CI in parentheses. Student.s *t*-test was used to assess differences between the variables.
[§]Significantly different from t_{max} after FA application in the CC genotype, P = 0.0217.
[6S]-5-MTHF, [6S]-5-methyltetrahydrofolate; AUC, concentration–time profiles of total plasma folate concentration; CC, CC genotype of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase; FA, folic acid; TT, TT genotype of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase.

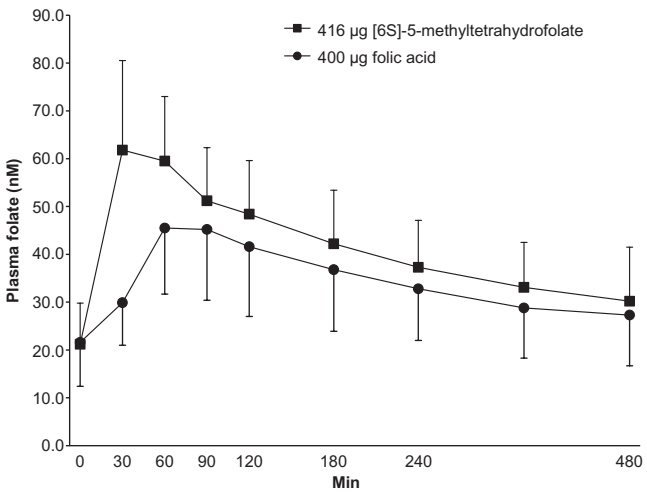

Figure 3 Arithmetic mean total plasma folate concentrations over time after a single oral dose of folic acid or [6S]-5-methyltetrahydrofolate in the CC genotype (n = 8) of the 677C→T mutation of the 5,10-methylenetetrahydrofolate reductase. Bars represent SD.

the CC subjects (see Table 2). In the CC group, the mean AUC and Cmax were also significantly higher (P < 0.005) after application of [6S]-5-MTHF compared with FA. The mean t_{max} was shorter with [6S]-5-MTHF than with FA (P < 0.05).

Significant differences in the pharmacokinetic parameters between the TT and CC genotype only existed with respect to t_{max} after FA application. Mean t_{max} was longer in the TT group than in the CC group.

In both genotypes, the AUC-ratios ([6S]-5-MTHF/FA) showed a higher relative bioavailability after a single dose of [6S]-5-MTHF compared with a single dose of FA [TT: 200.95%, 95% confidence interval (CI) 169.61–232.3%; CC: 159.2%, 95% CI 126.54–191.87%].

ANCOVA yielded no statistically significant treatment sequence, genotype or predose plasma folate effect, but a statistically significant treatment effect was present (P < 0.0001).

Plasma folate derivatives

5-MTHF was the predominant derivative in all subjects, at all time points, irrespective of the genotype. Trace amounts of 5,10-CHTHF were detected in five subjects at different time points in concentrations ≤3 nM. THF was detected in two subjects (≤4.0 nM). FA was seen in 18 of 24 subjects after FA application (TT, n = 13, CC, n = 5). The peak plasma folic acid concentration was 14.3 ± 6.1 nM, and usually occurred at either 90 or 120 min after FA administration. An incidental FA peak was observed in 2 of 24 subjects after [6S]-5-MTHF supplementation (TT, n = 1, CC, n = 1; plasma folic acid peak 21.4 and 6.1 nM, respectively).

In the fasting blood sample of the first study day, FA was not detected in the plasma of any of the participants. This indicates a good compliance to the protocol, as the consumption of any FA-containing food or supplements 4 weeks before intervention was not allowed.

Discussion

Our data indicate that [6S]-5-MTHF is more effective at increasing plasma folate compared with FA in a short-term protocol with single-oral dose application in the physiological range, irrespective of the participants' genotype of the 677C→T mutation of the MTHFR. This conclusion is based on the apparent AUC values, which were significantly higher with [6S]-5-MTHF than with FA, and the resulting higher relative bioavailability of [6S]-5-MTHF in both genotypes. There were no genotyped-induced differences in pharmacokinetic parameters, except for the t_{max}, which was significant longer after FA application in the TT compared with the CC group.

Our results differ from those from existing studies, as we focused on healthy young women TT-genotyped and used biologically equimolar amounts of FA and the natural folate form [6S]-5-MTHF in a physiologically low dose.

Willems *et al*. (2004) showed that 5-MTHF had a higher bioavailability compared with FA in TT and CC genotyped older cardiovascular patients in a similar type of study. However, in their study, a high dose (5 mg) of the racemic mixture [6RS]-5-MTHF was used. In addition, their study might have missed the nutritional aspect because the [6R]-isomer is presumed to be biologically inactive. Also, adverse effects of the [6R]-isomer on storage cannot be excluded (Mader *et al*., 1995; Willems *et al*., 2004). Lamers *et al*. (2004), Venn *et al*. (2002) and Fohr *et al*. (2002) also considered the genotype of the MTHFR in their long-term

studies comparing 5-MTHF with FA with respect to increasing plasma or RBC folate and/or reducing homocysteine concentrations. However, the randomization procedure stratified for genotype resulted in a very small number of the TT genotype in the different treatment groups, consequently not allowing TT genotype-specific conclusions to be reached from these studies.

In contrast to Pentieva *et al*. (2004), we showed that the short-term bioavailability of [6S]-5-MTHF is higher than that of FA in the CC genotype. However, Pentieva *et al*. (2004) used the FA presaturation model, which could alter the folate-binding protein capacities, as indicated by the study of Houghton *et al*. (2009).

In our study, the plasma folate concentrations in both genotypes peaked at a significantly higher level and within a shorter period of time with [6S]-5-MTHF compared with FA. Moreover, the t_{max} for FA was significantly longer in subjects with the TT rather than the CC genotype. This observation might be due to differences in metabolism of the two forms of the vitamin in the different genotypes. [6S]-5-MTHF is the folate derivative that is biologically active and can be stored in the body. Thus, the administration of [6S]-5-MTHF may induce a change in plasma folate concentration directly without any first-pass effect or indirectly by displacing 5-MTHF stored in the liver in both genotypes. Similar to [6S]-5-MTHF, some of the FA administered may appear directly in the systemic circulation without biotransformation in the mucosal cell or liver. Unmetabolized FA in plasma was seen in our study. However, the majority of the FA given orally was presumably taken up by mucosal cells and hepatocytes, where it is converted to 5-MTHF, which induces a direct or indirect increase in plasma folate, as described above. Therefore, the prolonged t_{max} in the TT genotype after FA intake could be explained by the reduced activity of the MTHFR.

The methodological approach used in our study only describes the appearance and clearance of total plasma folate after application of [6S]-5-MTHF and FA. It is not clear whether the increase in plasma folate is derived from the oral dose itself or induced by tissue redistribution as previously postulated by other workers (Wright *et al*., 2007). Further studies using a protocol with labelled folates/FA are required to give a clearer insight into the variations of plasma folate concentration and biotransformation of oral doses of [6S]-5-MTHF and FA in subjects with different genotypes. In addition, the urinary excretion of FA and [6S]5-MTHF should be considered in further studies to explore possible genotype differences.

In contrast to other authors (Harmon *et al*., 1996; Molloy *et al*., 1997; Klerk *et al*., 2002; de Bree *et al*., 2003), we did not find genotype differences in plasma or RBC folate at screening. This might be due to the study's inclusion criteria eliminating subjects with low folate status.

In the present study, FA appeared in the circulation almost always after FA application (18 of 24 volunteers), but only occasionally (2 of 24 volunteers) after administration of [6S]-5-MTHF. We suggest that [6S]-5-MTHF displaces small amounts of FA taken previously (weeks or months ago), and which is still tightly bound to (liver) folate-binding proteins. The high FA peak after [6S]-5-5MTHF (21.4 nM) was indeed from a volunteer who received FA on the first and [6S]-5-MTHF on the second study day.

As shown in other studies, unmetabolized FA is detected in the systemic circulation of man (Kelly *et al*., 1997) and in breast milk (Houghton *et al*., 2009) even after low oral doses of FA. Kalmbach *et al*. (2008) reported an increase of FA in plasma from a population-based sample of Americans among non-supplement and supplement users after the mandatory low-dose FA fortification of grain products was introduced in the USA and Canada in 1998 (Food and Drug Administration, 1996). Unmetabolized FA in blood is thought to interfere with the metabolism, transport and functions of the natural folates in the human body (Smith *et al*., 2008), and it possibly masks the haematological manifestations of unrecognized vitamin B12 deficiency, thereby predisposing people to irreversible neurological damage (Kelly *et al*., 1997). The involvement of FA in reduced natural killer cell cytotoxicity is under discussion (Troen *et al*., 2006).

In conclusion, using a short-term protocol with equimolar test dose, [6S]-5-MTHF is more effective at increasing total plasma folate concentrations compared with FA in subjects with the TT and CC genotype of the 677C→T mutation of MTHFR. We found a significantly higher AUC after application of [6S]-5-MTHF compared with FA in both genotypes. The differences in t_{max} after application of FA between the TT and CC genotype might be due to the reduced activity of MTHFR. In our study, unmetabolized FA in plasma occured regularly after supplementation with FA. This phenomenon was only occasionally observed with [6S]-5-MTHF supplementation. As [6S]-5-MTHF is not known to have any potential adverse effects, this natural and biologically active form of folate could be an alternative to FA supplementation. The feasibility of enriching food with [6S]-5-MTHF requires further study.

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Conflicts of interest

None of the authors had any personal or financial conflicts of interest with the sponsoring institution.

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