

The Short-Term Bioavailabilities of [6S]-5-Methyltetrahydrofolate and Folic Acid Are Equivalent in Men¹

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ABSTRACT The natural folate derivative, 5-methyltetrahydrofolate ([6S]-5-MTHF), could be an option for supplementation and fortification but its bioavailability remains unclear. This study compared the bioavailability of [6S]-5-MTHF with that of folic acid (FA) by measuring plasma folate responses after a single ingestion of equivalent doses of the two folate forms. In a double-blind, crossover study, 13 men (presaturated with FA) received in random order each of the following treatments administered orally at 1-wk intervals: 1) placebo capsule; 2) 500 μ g FA capsule; and 3) 500 μ g [6S]-5-MTHF capsule. Plasma total folate concentrations were measured before and up to 10 h after each treatment ($n = 10$ samples per treatment). Plasma folate concentrations increased significantly (compared with baseline) from 0.5 to 5 h after both folate treatments. The maximum plasma folate response did not differ between the two treatments (mean \pm SEM, 33.4 ± 3.9 vs. 31.8 ± 3.9 nmol/L, $P = 0.7$, for FA and [6S]-5-MTHF, respectively) and typically occurred in individuals between 0.5 and 3 h postprandially. The area under the plasma folate response curve was significantly greater after both folate treatments compared with placebo, and the response did not differ between the treatments. These results indicate that the short-term bioavailabilities of [6S]-5-MTHF and FA are equivalent. Supplementation with the natural folate derivative could have all the beneficial effects associated with FA, but without the potential disadvantage of masking the anemia of vitamin B-12 deficiency. *J. Nutr.* 134: 580–585, 2004.

KEY WORDS: • [6S]-5-methyltetrahydrofolate • folic acid • plasma folate • short-term folate bioavailability

Mandatory folic acid (FA)³ fortification of enriched grain products has been in place in the United States and Canada since 1998, with the primary aim of decreasing the prevalence of neural tube defects (1,2). It was also recognized that the increased FA intake might have some other potentially beneficial effects, such as reducing the risk of developing cardiovascular disease (via homocysteine lowering) (3) and possibly cancer (4). Evidence for the effectiveness of the FA fortification program has been provided by reports showing a substantial increase in folate status and a lowering of homocysteine in subjects of the Framingham cohort (5,6). Of greater importance, there has been a decline in the rate of neural tube defects reported in the United States and Canada since the start of fortification (7,8). Despite these findings, the authorities in many other countries are choosing not to introduce similar fortification legislation because of concerns of poten-

tially adverse effects of FA, in particular the concern that high intakes of FA may mask the hematological abnormalities related to vitamin B-12 deficiency in older people and therefore delay its timely diagnosis and treatment. This would allow the associated neurological dysfunction to progress to irreversible subacute combined degeneration of the spinal cord. There is even some evidence that high levels of FA may stimulate the neurological complications of vitamin B-12 deficiency to progress at a faster rate (9,10).

FA is the synthetic, oxidized, chemically stable form of the vitamin that is used for supplementation and fortification. After ingestion, FA is reduced and methylated via one-carbon substitution to 5-methyltetrahydrofolate (5-MTHF) in the intestinal cells; under normal circumstances, only this folate form enters the circulation. From here, 5-MTHF is taken up by cells and can be retained only after it has been converted, in the presence of vitamin B-12, to tetrahydrofolate (THF), which is then polyglutamated. However, there is some evidence from both acute and chronic studies that the metabolic capacity of intestinal cells to reduce FA may be limited and that if FA is ingested in higher doses, unmetabolized FA may appear in the circulation (11–13). Although the clinical relevance of this remains to be established, the appearance of unmetabolized FA may be a concern because it can enter cells

¹ Funded by EU project "Nutraceuticals for a healthier life: n-3 polyunsaturated fatty acids and 5-methyltetrahydrofolate" (QLRT-1999-00888) and supported by Abbot GmbH, Weisbaden-Delkenheim, Germany.

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³ Abbreviations used: AUC, area under the curve; FA, folic acid; [6S]-5-MTHF, [6S]-5-methyltetrahydrofolate; Rmax, maximum plasma folate response; THF, tetrahydrofolate.

directly where it is acted on by dihydrofolate reductase, converted to dihydrofolate, then THF, thus enabling the formation of polyglutamate. In this way, FA can by-pass the normal vitamin B-12-dependent control step and be metabolized and utilized for DNA biosynthesis even in vitamin B-12-deficient cells, thereby allowing full remission of the anemia of vitamin B-12 deficiency. Therefore, one potential advantage of 5-MTHF over FA is that if ingested in large amounts by vitamin B-12-deficient individuals, it would not mask the anemia because in the absence of vitamin B-12, it would effectively remain metabolically "trapped" in the same form within cells (14).

However, understanding of the bioavailability of 5-MTHF within the physiologic range of doses is quite limited. Only a few studies have addressed the issue of relative bioavailability of 5-MTHF and FA and their results are inconsistent. Early short-term studies based on monitoring blood folate response after a single ingestion of 5-MTHF or FA showed consistently higher serum folate responses to 5-MTHF than to FA (12,15). More recent acute studies demonstrated variable results with respect to the relative bioavailability of the two folate forms in healthy women depending on the administration (16) or not (17) of a presaturation regime with FA, supporting the idea that folate presaturation of tissues may be required to accurately assess the full extent of the plasma folate response. Theoretically, studies involving labeled folate derivatives should facilitate very accurate determinations of the bioavailability of reduced folate forms relative to FA; however, one recent study (18) highlighted a potential limitation even with this approach arising from the displacement of unlabeled tissue folate (not originating from the oral test doses) into the plasma. The results of long-term interventions with 5-MTHF and FA also were inconsistent. Fohr et al. (13) demonstrated that FA is more effective than 5-MTHF in lowering plasma homocysteine (a functional marker of folate status), whereas Venn et al. showed similar plasma and RBC folate responses to both folate forms (19) and more effective plasma homocysteine lowering after 5-MTHF than after FA supplementation (20).

Thus, the question of whether FA and 5-MTHF have equivalent bioavailabilities requires further investigation. The aim of the present study was to compare the bioavailability of 5-MTHF with that of FA by measuring plasma folate responses after the single ingestion of equivalent doses of the two folate forms in folate saturated subjects.

SUBJECTS AND METHODS

Subjects. Male volunteers ($n = 21$) aged 18–45 y were recruited through advertisements among the staff and student population of the University of Ulster at Coleraine. All recruited volunteers were interviewed using a short questionnaire regarding general health, supplement and medicine use, and blood samples were taken for screening blood tests. Subjects were excluded from participation in the study if they had a history of hepatic, gastrointestinal, renal, vascular, hematological, or neuropsychiatric disease, were taking FA-containing supplements or medicines known to interfere with folate metabolism, were homozygous for 677C→T (so-called thermolabile) variant of the methylenetetrahydrofolate reductase (MTHFR) gene, had high plasma homocysteine concentrations, or had deficient status of folate or vitamin B-12. These criteria were implemented to limit the inclusion of subjects in the study who could potentially give an abnormal response to the ingested folate (FA or 5-MTHF). The University of Ulster Research Ethical Committee granted approval for the study. All volunteers gave informed signed consent in accordance with the declaration of Helsinki.

Study design and treatments. In a crossover design, subjects received three different treatments in random order, with an interval

of at least 1 wk between each test day. The treatments were as follows: 1) placebo capsule; 2) FA capsule, 500 μg ; and 3) 5-MTHF capsule, containing the biologically active isomer [6S]-5-MTHF, 500 μg . Subjects took the treatment capsules under supervision mid-way through their breakfast, early in the morning after an overnight fast. Folic acid, [6S]-5-MTHF-Ca (21) and placebo capsules were provided by BASF Aktiengesellschaft.

To minimize interindividual differences in baseline plasma folate concentrations, a presaturation regimen with FA was administered before the start of the study (5 mg/d FA for 1 wk, followed by 2 FA-free days before treatment) and during the intervals between treatments (5 mg/d FA for 5 d, followed by 2 FA-free days). To monitor compliance, subjects were provided with FA in a 7-d tablet organizer box (Carepac) and asked to return the box at each visit; any missed tablets were recorded.

During each of the test days, subjects consumed an identical, specially prepared low-folate diet (Table 1). The low-folate diet included breakfast, two snacks, and lunch, and provided 65% of energy requirements for men with a sedentary lifestyle (22). The lunch meal was prepared in advance in one batch and kept frozen at -20°C for the duration of the study. To decrease the folate content, all of the ingredients of the lunch meal were boiled three times, with the water discarded after each boiling. The dish was then made more palatable by seasoning and stir-frying before serving. A duplicate portion of the low-folate diet consumed by the subjects on the test days (breakfast, two snacks, and a lunch dish) was analyzed for folate content. The folate content of placebo, FA and [6S]-5-MTHF capsules was also determined as described below.

Blood sampling and analysis. Subjects were cannulated and blood was taken before the administration of the capsules and afterwards at the following intervals over a period of 10 h: 0.5, 1, 1.5, 2, and 2.5 h (before midmorning snack); 3 and 5 h (before lunch); 7 h (before afternoon snack); and 10 h. Blood samples were collected into foil wrapped Monoject tubes with EDTA (Sherwood). The samples

TABLE 1

Food, energy and nutrient composition of the low folate diet provided to subjects during each test day

Food	
Breakfast	
White bread, Hovis, g	90
Margarine, Golden Cow, g	10
Strawberry jam, Robertson's, g	15
Mineral water, Vittel, mL	400
Midmorning snack	
Plain chocolate, Tesco, g	50
Sprite, Bottlers, mL	330
Lunch	
Chicken breast, g	130
Carrots, g	80
Celery, g	50
Onion, g	60
Mushrooms, g	40
Colman's Chicken Chasseur mix, g	10
Sunflower oil, Tesco, mL	5
Pasta, Buitoni, g	300
Afternoon snack	
Plain chocolate, Tesco, g	50
Sprite, mL	330
Energy and nutrient composition ¹	
Energy, kJ	6918
Protein, g	66.1
Fat, g	50.9
Carbohydrates, g	244.1
Total folate, ² μg	87

¹ Energy and nutrient contents (excluding folate) of all meals and snacks were calculated (38).

² The folate content was analyzed in duplicate meal samples according to the procedure of Tamura et al. (28).

were immediately placed on ice and plasma was separated by centrifugation ($2000 \times g$, 10 min) within 30 min and stored at -80°C until analysis. All samples collected from each subject (10×3 treatments) were analyzed in one assay to remove interassay measurement variability.

The folate concentration in plasma and RBC, and the folate content of the 3 types of capsules were determined using a *Lactobacillus casei* microbiological assay (23), adapted to a microtiter plate procedure and carried out by a robotic system (Hamilton). The calibration of the assay was performed using FA (Sigma Chemical) as a standard. Under the conditions of the assay in our laboratory (pH 6.7 of the assay medium), *L. casei* showed equivalent responses to FA and [6S]-5-MTHF. The interassay CV in folate concentration of quality control samples was 5.5% ($n = 48$). Plasma total homocysteine was measured by Abbott Imx FPIA (24). Plasma vitamin B-12 concentration was analyzed by the method of Kelleher and Broin (25). Quality control was provided by repeated analysis of small aliquots of pooled plasma and erythrocyte lysate samples covering a broad range of values. DNA was extracted from frozen whole blood (-20°C) by incubating with proteinase K (Gibco Life Technologies) as described in detail by Kawasaki and Erlich (26). The MTHFR 677C \rightarrow T genotype was identified by PCR amplification followed by HinF1 restriction digestion (27).

The total folate content of the low-folate diet was determined in duplicate meals by microbiological assay (23) after thermal extraction and trienzyme treatment (α -amylase, protease, and folate conjugase) according to the procedure of Tamura et al. (28).

Statistical analysis. The differences in plasma folate concentrations between baseline and each of the time points over 10 h within one treatment were tested by repeated-measures ANOVA followed by Dunnett's post-hoc test. Differences among treatments were evaluated by repeated-measures ANOVA and Scheffé's post-hoc test. Plasma folate response vs. time was plotted, and the maximum plasma folate response (R_{max}) after both folate treatments was determined by inspection of individual values at different time points. The area under the curve (AUC) for plasma folate response was determined by the trapezoidal method (29) and the differences in AUC among the 3 treatments were evaluated by 1-way ANOVA followed by Scheffé's post-hoc test. All statistical analysis were performed using the Data-Desk, version 6 (Data Descriptions) and Microsoft Excel 97 SR-1 (Microsoft). Differences with P -values < 0.05 were considered significant.

RESULTS

Folate analysis of capsules and meals. Just before the start of the study, there was no detectable folate in the placebo capsules ($n = 10$ capsules) and folate contents were 515 ± 24 μg /FA capsule ($n = 10$) and 506 ± 18 μg /[6S]-5-MTHF capsule ($n = 10$).

The low-folate diet consumed by the subjects during the 10-h treatment period had a total folate content of 87 μg (based on duplicate meal analysis) (Table 1). The breakfast (during which treatments were administered) contained only 15 μg folate, each of the snacks provided 6 μg folate, and the lunch provided the remainder, 60 μg folate.

Plasma folate response to treatment with FA or [6S]-5-MTHF. On the basis of screening 21 subjects who were initially recruited, 13 were identified as eligible to take part in the study. The baseline characteristics of this group are shown in Table 2. Plasma concentrations of folate, vitamin B-12, total homocysteine, and RBC folate of all participants were within the normal reference ranges.

We examined plasma folate concentrations at baseline and at different time points during the 10-h test period after treatments (Table 3). Plasma folate concentrations did not differ among the three treatments at baseline. After ingestion of both FA and [6S]-5-MTHF capsules, plasma folate increased at 0.5 h and remained significantly higher than baseline for up to 5 h after both treatments. After ingestion of the placebo

TABLE 2

Subject characteristics and baseline data¹

Category	Reference range	Mean \pm SD
Age, y	—	25.6 \pm 5.5
BMI, kg/m ²	20–25	23.1 \pm 2.0
MTHFR genotype, CC/CT ²	—	9/4
Plasma folate, ³ nmol/L	6.80–45.32	22.7 \pm 8.2
Red cell folate, ³ nmol/L	340–2266	1612 \pm 465
Plasma total homocysteine, ⁴ $\mu\text{mol/L}$	≤ 15.0	8.5 \pm 2.0
Plasma B-12, ³ pmol/L	111–738	386.1 \pm 150.0

¹ $n = 13$.

² Heterozygous (CT) and wild-type (CC) genotypes for the 677C \rightarrow T (thermolabile) variant of MTHFR.

³ Laboratory reference ranges used in the Vitamin Research Laboratory, Trinity College Dublin.

⁴ Cut-off for hyperhomocysteinemia defined by Kang et al. (39).

capsule, plasma folate did not differ from baseline. Plasma folate concentration tended to decrease by 10 h after treatments ($P = 0.4$ and 0.7 for FA and [6S]-5-MTHF, respectively) but was significantly lower than baseline at 10 h only after the placebo treatment ($P < 0.0001$).

The plasma folate response to each of the three treatments was calculated on an individual basis by subtracting the baseline plasma folate concentration from the plasma folate value at each time point, and the responses were compared among treatments (Fig. 1). After ingestion of the FA capsule, R_{max} was established between 0.5 and 2 h (most commonly at 1.5 h) postprandially. After administration of the [6S]-5-MTHF capsule, R_{max} was observed between 0.5 and 3 h (most commonly at 2 h postprandially). R_{max} values did not differ in response to FA or [6S]-5-MTHF (33.4 ± 3.9 vs. 31.8 ± 4.1 nmol/L).

The AUC for plasma folate response, calculated up to 7 h postprandially, was estimated to be 145.8 ± 16.1 , 141.6 ± 10.7 , and -27.0 ± 15.9 h \times nmol/L after the FA, 5-MTHF, and placebo treatments, respectively. The AUC after ingestion of either of the folate capsules was greater than that after the placebo ($P < 0.0001$). The AUCs in response to FA or [6S]-5-MTHF treatments did not differ ($P = 0.9$).

DISCUSSION

The results of the current study indicate that the bioavailabilities of FA and [6S]-5-MTHF are similar in folate presaturated men after a single ingestion of equivalent doses of these folate forms. This conclusion is based on the almost identical pattern and magnitude of the plasma folate response to FA or [6S]-5-MTHF, measured before and up to 10 h after the oral administration of equal doses of these folates. In both cases, plasma folate concentrations increased significantly from 30 min to 5 h post-treatment; neither the maximum plasma folate response nor the AUC differed between the [6S]-5-MTHF and FA treatments.

Plasma folate response after folate treatment is a result of the rate and extent of intestinal folate absorption, tissue uptake, and urinary and biliary excretion of the circulating folates. The similar plasma folate response after ingestion of FA and [6S]-5-MTHF in our study suggests that there is a general equivalence in these various processes between the two folate forms. This is consistent with the findings by others indicating that the intestinal transport process does not show a particular preference for any of the folate forms (30). The dose of folate used in the current study, i.e., 500 μg , is close to

TABLE 3

Plasma folate concentrations at baseline and during the 10-h test periods in men after [6S]-5-MTHF, folic acid, and placebo treatment administered in random order at weekly intervals¹

Treatment	Time, h									
	0	0.5	1	1.5	2	2.5	3	5	7	10
	nmol/L									
[6S]-5-MTHF, 500 µg	58.4 ± 5.0	83.5 ± 6.5*	85.8 ± 4.9*	82.9 ± 5.2*	90.2 ± 4.6*	83.5 ± 4.9*	79.0 ± 4.8*	79.4 ± 5.2*	64.6 ± 4.1	47.7 ± 4.1
Folic acid, 500 µg	59.1 ± 6.5	81.2 ± 6.4*	91.5 ± 6.0*	92.6 ± 7.8*	86.5 ± 7.5*	83.0 ± 6.9*	80.9 ± 7.4*	77.2 ± 6.0*	59.9 ± 4.8	47.1 ± 4.1
Placebo	67.1 ± 6.7	63.8 ± 4.9	65.6 ± 5.2	61.5 ± 5.7	63.9 ± 5.5	65.0 ± 6.3	62.0 ± 5.3	66.0 ± 4.6	57.8 ± 3.8	44.8 ± 3.9*

¹ Values are means ± SEM, *n* = 13; * different from time 0, *P* < 0.05.

the daily recommended supplemental level for women (400 µg) for the prevention of first occurrence of neural tube defects (31,32).

Previous studies compared the bioavailabilities of 5-MTHF and FA using various approaches. Like the current study, some of these have used a single ingestion of [6S]-5-MTHF or FA followed by monitoring the plasma folate response for various lengths of time (2–8 h) (12,15–17); however, verification of the folate dose used to confirm the equivalence of the two treatments was not always reported. Consistent with our findings are the results of a recent short-term study that demonstrated a similar response to 400 µg FA or [6S]-5-MTHF in folate presaturated women based on the measurement of plasma folate concentrations over the course of 8 h after treatments (16). In contrast to these findings are the results of some earlier studies indicating 135–218% higher responses to [6S]-5-MTHF compared with FA (12,15). However, the validity of these conclusions is questionable given the lack of placebo treatment and particularly, the short period of monitoring (2–3 h), which was insufficient to quantify the full plasma folate response. In the current investigation, we used numerous time points (10 in the course of 10 h), which allowed us to monitor as fully as possible the magnitude of the folate peak and the changes in plasma folate concentrations until their return to the baseline level. In addition, we fed the subjects a standardized, specially prepared low-folate diet on

each test day and treatments were administered mid-way through consuming the breakfast. The possible confounding effect of the diet during the test days was not always taken into account in the earlier published studies; for example, Perry and Chanarin (12) allowed the volunteers to have their own nonstandardized breakfast (of unknown folate content). The low-folate standardized diet we used avoided fasting and ensured that the conditions were physiological and identical for each of the 3 test days. We are confident that the amount of folate in this standard diet did not affect the folate response because there was no increase in plasma folate concentrations as a result of the placebo treatment. Furthermore, in some of the previous studies, there was a considerable difference in baseline plasma folate between treatments (15), which could have biased the response toward a particular treatment. In an attempt to overcome this potential problem, we administered the 3 treatments to subjects in random order. By minimizing the effects of these possible confounding factors, the current study provides a more precise estimation of the overall response to treatment with FA and [6S]-5-MTHF compared with these earlier studies.

A second approach to evaluating the bioavailability of natural folate derivatives involves the administration of oral, or the combination of oral and intravenous, doses of stable isotope folate forms with subsequent monitoring of isotope excretion ratios of urinary folates or plasma folate responses

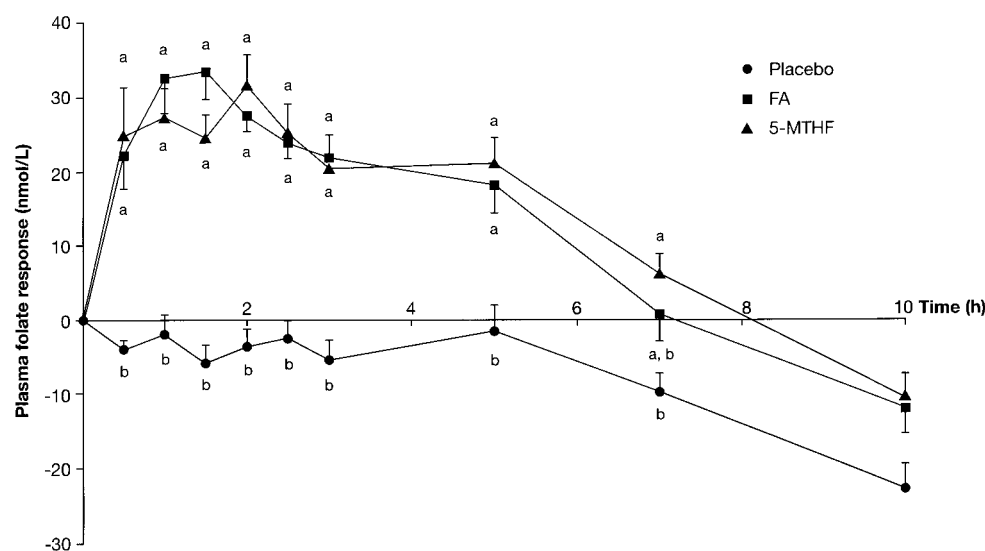


FIGURE 1 Plasma folate response (corrected for baseline values) in men after treatment with 500 µg 5-MTHF, 500 µg folic acid, or placebo administered in random order at weekly intervals. Values are means ± SEM, *n* = 13. Means without a common letter at a time differ, *P* < 0.05).

(with or without folate presaturation) (18,33). These studies generally suggest that the bioavailability of FA differs from that of the natural derivatives, in contrast with the current results. In theory, acute experiments with labeled folate derivatives should allow very precise calculations of relative folate bioavailability. In practice, however, the interpretation of certain studies using this approach may potentially be limited. Some of these potential limitations include the following: administration of the folate derivatives in different doses with subsequent mathematical adjustment of the response to an equivalent dose (18,33); the displacement of some of the administered labeled folate into the unsaturated body pools as was recently reported (18); and the fasting of subjects for >16 h (18) which may provoke an increase of unlabeled plasma folate by diminishing bile secretion into the gut, reported to be a major elimination route for folates (34).

Recent studies examined relative folate bioavailability by studying long-term responses (serum folate, RBC folate, or plasma homocysteine) after intervention periods of 8–24 wk (13,19,20). One such study (13), using a racemic mixture (biologically active [S] and nonactive [R] forms) of 5-MTHF, was inherently problematic because the folate derivatives were not administered at equivalent doses. The responses to treatments, therefore, are not comparable. However, our results are in good agreement with the findings of a recent long-term (24-wk) study in women, which demonstrated similar plasma and RBC folate (19) responses to low-dose (100 μg) FA or [6S]-5-MTHF. This report (19), together with the results of the current study, suggests an equivalent bioavailability of the 2 folate forms in the range of 100–500 $\mu\text{g}/\text{d}$.

Supplementation with [6S]-5-MTHF is unlikely to have the adverse effects of high intakes of FA, in particular, the effect of masking the anemia of vitamin B-12 deficiency. This is especially important in light of recent reports showing that the fortified products on the U.S. market since the introduction of mandatory FA fortification are actually overfortified with FA (35), resulting in a current FA intake that is twice that initially projected (36,37). This means that the potential for unmetabolized FA to appear in the plasma and tissues of consumers may be even greater. Therefore, considering the equivalent bioavailability of [6S]-5-MTHF and FA in the current study and in a recent long-term study (19), 5-MTHF may be a better alternative than FA for supplementation and fortification. However, the prohibitively high cost of producing [6S]-5-MTHF must be considered before it can be viewed as a viable alternative for fortification purposes. More importantly, all studies on the prevention of neural tube defects were carried out with FA; thus, there is only theoretical evidence that 5-MTHF would also be effective. For ethical reasons, a clinical trial to prove the effectiveness of 5-MTHF in the prevention of neural tube defects in humans is not feasible.

In summary, this study demonstrates equivalence in the bioavailabilities of FA and [6S]-5-MTHF after a single ingestion of the two folate forms. The implications are that the natural folate derivative could have all the beneficial effects associated with FA, but without the potential disadvantage of masking the anemia of vitamin B-12 deficiency. Importantly, [6S]-5-MTHF is a natural folate derivative, a normal constituent of the body, and safety and tolerance of high doses are not issues of concern. Although the current protocol cannot replace the valuable information provided by long-term intervention studies, it can overcome the practical difficulties encountered in the execution of long-term studies which are, by their nature, expensive, labor intensive, and, unless carefully monitored, often plagued by subjects' noncompliance. Future

studies, currently underway in this center, will extend the current findings by comparing the response to 5-MTHF and FA at different doses within the physiologic range of intake (100–400 $\mu\text{g}/\text{d}$).

ACKNOWLEDGMENT

We wish to acknowledge the cooperation of the volunteers who participated in the study.

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