

Folate bioavailability from breads and a meal assessed with a human stable-isotope area under the curve and ileostomy model^{1–3}

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ABSTRACT

Background: Recent data revealed differences in human absorption kinetics and metabolism between food folates and folic acid supplements and fortificant.

Objective: The objective was to determine folate bioavailability after ingestion of breads or a breakfast meal fortified with either 5-CH₃-H₄ folate or folic acid by using a stable-isotope area under the curve (AUC) and ileostomy model.

Design: In a randomized crossover trial, healthy ileostomists ($n = 8$) ingested single doses of whole-meal bread that contained ≈ 450 nmol (200 μg) of either (6S)-[¹³C₅]5-CH₃-H₄ folate or [¹³C₅]folic acid or a breakfast meal that contained ≈ 450 nmol (200 μg) [¹³C₅]folic acid. We collected blood from the subjects during 12 h postdose for assessment of plasma kinetics. Nonabsorbed folate was assessed from labeled folate contents in stomal effluent 12 and 24 h postdose.

Results: The median (range) plasma AUC_{0–12} (AUC from 0 to 12 h after ingested dose) of 66 nmol · h/L (34–84 nmol · h/L) after ingestion of bread that contained (6S)-[¹³C₅]5-CH₃-H₄ folate was significantly greater ($P < 0.001$) than that after ingestion of [¹³C₅]folic acid in fortified bread [28 nmol · h/L (15–38 nmol · h/L)] and a fortified breakfast meal [26 nmol · h/L (15–60 nmol · h/L)]. Both labeled doses resulted in increases of plasma [¹³C₅]5-CH₃-H₄ folate. However, the kinetic variables C_{max} (maximum plasma concentration) and T_{max} [time (min) of maximum plasma concentration] varied after ingestion of the different folate forms. The stomal folate content was $< 10\%$ of the ingested dose and did not vary significantly after ingestion of test foods that contained (6S)-[¹³C₅]5-CH₃-H₄ folate [median (range): 13 nmol (10–31 nmol)] or [¹³C₅]folic acid [median (range): 25 nmol (8–42 nmol)] ($P = 0.33$).

Conclusions: Our data confirm differences in plasma absorption kinetics for reduced folates and synthetic folic acid administered with the test foods. Stomal folate contents indicated almost complete bioavailability of labeled folate from the breads or breakfast meal. *Am J Clin Nutr* 2010;92:532–8.

INTRODUCTION

Good folate status is associated with a number of health benefits. Therefore, mandatory fortification of staple foods with synthetic folic acid has been introduced in several countries to compensate for folate intakes below the recommended intake (1). It is commonly assumed that supplemental folic acid has a higher bioavailability and stability than food folates (1). Recent data in humans revealed differences in absorption kinetics and metabolism between (mainly reduced) food folates and folic acid

supplements or fortificant (2). After consumption of folic acid–fortified foods, folic acid has been observed in plasma (3, 4). Current opinions regarding the role of folic acid (eg, in dementia) are contradictory (4), and therefore it is important to identify any differences in metabolism between folic acid and food folates.

Short-term folate bioavailability is often assessed by using folate plasma concentrations and biokinetic methods such as the area under the curve (AUC) approach (2, 5–12) or urinary folate excretion (9, 13–16). Another approach is to quantify non-absorbed folate in human feces (13, 17–19) or in stomal effluent of ileostomy volunteers (6–8). A combination of different methods may be used to strengthen results, eg, a combined human plasma AUC and ileostomy model (6–8).

A major drawback of the AUC method is that peripheral plasma folate concentrations are affected by hepatic first-pass retention and the subject's folate status (2). Hence, presaturation of folate body stores (5–7, 16) is commonly used to improve sensitivity in human-bioavailability studies, but this does not reflect physiologic conditions. Another limitation is that without the use of extrinsic-labeled doses (9, 12–15) or intrinsic-labeled doses (2), the differentiation of endogenous folate that derives from body stores and folate from the dose in peripheral plasma, urine, feces, or stomal effluent is not possible.

In this study, a stable-isotope AUC and ileostomy model was used 1) to determine folate plasma kinetics and assess the nonabsorbed fraction after ingestion of single doses of bread fortified with either 5-CH₃-H₄ folate or folic acid and 2) to compare folate plasma kinetics and assess the nonabsorbed fraction after ingestion of a single dose of a folic acid–fortified complete breakfast meal with those after ingestion of folic acid–fortified bread.

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² Supported by the Swedish Research Council Formas. Lantmännen (Järna, Sweden) carried out the bread baking. Unlabeled folate standards were donated by Merck Eprova AG (Schaffhausen, Switzerland) and Convatec (Bromma, Sweden) donated parts of the ileostomy bags.

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SUBJECTS AND METHODS

Study population

Eight apparently healthy ileostomists (3 men and 5 women) were recruited. The median (range) age of the volunteers was 54 y (39–66 y), and the median (range) body mass index (in kg/m²) was 32 (19–33). Volunteers did not use medication or vitamin supplements that might affect folate metabolism. At a health check before the study, the following variables were assessed for normal range: blood status (hematocrit, hemoglobin, mean cell volume, red and white blood cell count, and individual white blood cells), liver status (serum aspartate transaminase and serum alanine transaminase), blood lipids (LDL:HDL-cholesterol ratio and serum triglycerides), iron status (serum ferritin, serum transferrin, and serum transferrin saturation), as well as fasted serum concentrations of glucose, folate, total homocysteine, and vitamin B-12. Blood pressure and body mass index were recorded. Median (range) concentrations of volunteers were as follows: serum folate: 17 nmol/L (15–45 nmol/L); serum total homocysteine: 9 μmol/L (7–23 μmol/L); and serum vitamin B-12: 362 pmol/L (152–1076 pmol/L). Volunteers were non-smoking subjects. One volunteer used snuff. All volunteers had had a conventional well-established ileostomy for 7–43 y. For 7 volunteers, the maximal intestinal resection was 20 cm. For one volunteer, the resection was larger (only 1.7 m intestine left).

Study protocol

This study had a randomized crossover design with 3 independent study days separated by a washout period of 2–5 wk. On each study day after an overnight fast, volunteers received a single dose of the test food (bread or a complete breakfast meal) fortified with ≈450 nmol (200 μg) of either [¹³C₅]5-CH₃-H₄ folate or [¹³C₅]folic acid (**Table 1**). Collection of blood and stomal effluent samples was strictly standardized at 12 h post-dose (7).

During the study day (until 12 h postdose), volunteers received a standardized low-folate and low-fat lunch (4 h and 30 min postdose) and snack (7 h and 15 min postdose). Access to water was not restricted (7). Upon removal of the cannula after collection of blood at *T*₇₂₀ [time (min) after ingestion of dose] on each study day, volunteers received a 3-course dinner with unlimited access to beverages and foods.

Before the study, volunteers were provided with a list of folic acid-fortified foods and instructed to avoid the foods for 4 d before each study day and to avoid alcohol for 1 d before each study day. The study protocol was approved by the Regional Ethical Review Board in Uppsala (Sweden). The trial was carried out at the Centre for Clinical Research and the Department of Public Health and Clinical Medicine, Umeå, Sweden, from March to July 2008 (17 wk).

Test compounds, test foods, and other foods

Stable-isotope-labeled (6S)-Ca-[¹³C₅]5-CH₃-H₄ folate and [¹³C₅]folic acid were purchased from Merck Eprova (Schaffhausen, Switzerland). Doses were corrected for the actual standard concentration, which was 73 ± 3% of the weight for [¹³C₅]5-CH₃-H₄ folate and 89 ± 6% of the weight for [¹³C₅]folic acid, as measured by spectroscopy [by using the conditions

for [¹³C₅]5-CH₃-H₄ folate (extinction coefficient: 32; λ: 290 nm; pH 7.0) and for [¹³C₅]folic acid (extinction coefficient: 27; λ: 282 nm; pH 7.0)] (20). The content of unlabeled folate content was <1% as verified by using liquid chromatography–mass spectrometry (LC-MS) (21).

Fortified whole-meal breads (8% dietary fiber) were prepared by adding, by weight, (6S)-[¹³C₅]5-CH₃-H₄ folate (to a final concentration of 1155 μg/100 g bread) or [¹³C₅]folic acid (to a final concentration of 568 μg/100 g bread) to the dough fluid before mixing with flour as previously described (21). Breads were baked at the Lantmännen test bakery (Järna, Sweden) and immediately frozen and stored at –20°C (21). The folate content in the breads was quantified before and after the trial, and the amount of intact (6S)-[¹³C₅]5-CH₃-H₄ folate and folic acid recovered after bread baking was 38 ± 5% and 76 ± 10%, respectively. The labeled folate content in the bread fortified with [¹³C₅]5-CH₃-H₄ folate was 441 ± 54 μg labeled folate/100g (≈1000 nmol) and in the bread fortified with [¹³C₅]folic acid was 430 ± 38 μg labeled folate/100g (≈975 nmol). The unlabeled folate (ie, the sum of folate expressed as folic acid) content in the breads was 38 μg unlabeled folate/100 g (86 nmol). Other food items were purchased in local stores in Umeå, Sweden.

Single doses of ≈450 nmol fortificant were ingested within 19 min in the following forms: 1) 2 slices (in total 47–49 g, including crust) of [¹³C₅]5-CH₃-H₄ folate bread with pear sauce (15 g; Kiviks Musteri AB, Kivik, Sweden) and a glass of water (150 mL); 2) 2 slices (47–49 g, including crust) of [¹³C₅]folic acid-fortified bread with pear sauce and a glass of water (150 mL); or 3) 2 slices (47–49 g, including crust) of [¹³C₅]folic acid-fortified bread as part of a meal consisting of liver pâté (15 g, 41% liver, 14% fat; Scan, Johanneshov, Sweden), orange juice (130 mL; JO-Bolaget, Stockholm, Sweden), cornflakes (12 g, nonfortified; Änglamark, Solna, Sweden), and fermented milk (150 mL, 3% fat; filmjölk; Norrmejerier, Umeå, Sweden). The amount of unlabeled folate (ie, the sum of folate expressed as folic acid, measured from a composite breakfast sample collected and prepared on one of the study days) in the breakfast was 118 μg unlabeled folate/portion (266 nmol). The energy and fat contents (calculated) in a portion of test foods (1 and 2) were 534 kJ (128 kcal) and 3 g and in the breakfast meal (3) were 1383 kJ (330 kcal) and 6 g.

The standardized low-folate and low-fat lunch consisted of hard wheat bread (70 g; tunnbröd; Gene, Bredbyn, Sweden), margarine (20 g, 38% fat; Becel, Solna, Sweden), smoked ham (40 g, 72% meat; NyhlénsHugosons, Umeå, Sweden), and horse meat (40 g, 72% meat; NyhlénsHugosons). The standardized low-folate and low-fat snack consisted of 2 crisp rolls (10 g; Pågen, Malmö, Sweden) with pear sauce (15 g). Volunteers had free access to water, apple juice, and lingonberry drink (JOKK, Norrmejerier, Sweden) during the meals. A composite sample of the lunch, snack, and beverages (150 mL apple juice and 150 mL lingonberry drink) contained 32 ± 2 μg (73 nmol) unlabeled folate (analyzed; the sum of folate expressed as folic acid), 2329 kJ (556 kcal; calculated), and 13 g fat (calculated).

The contents of labeled and unlabeled folate in doses and other food samples were measured in triplicate by using LC-MS as described previously (21). Briefly, samples were homogenized in phosphate buffer (0.1 mol/L, pH 6.1, that contained 2% sodium ascorbate and 0.1% 2,3-dimercaptopropanol), and thermostable

TABLE 1

Kinetic variables of labeled and unlabeled 5-CH₃-H₄ folate in plasma and stomal effluent after single doses of test foods fortified with stable, isotopically labeled [¹³C₅]5-CH₃-H₄ folate or [¹³C₅]folic acid¹

	[¹³ C ₅]5-CH ₃ -H ₄ folate-fortified bread (n = 8)	[¹³ C ₅]Folic acid-fortified bread (n = 8)	Breakfast with [¹³ C ₅]folic acid-fortified bread (n = 8)	P
[¹³ C ₅] Dose (nmol) ²	464 (450–467) ³	451 (448–472)	462 (448–471)	0.61 ⁴
[¹³ C ₅] AUC _{0→12} (nmol · h/L) per nmol (dose) ⁵	0.15 (0.07–0.54)	0.07 (0.05–0.17)	0.06 (0.03–0.20)	<0.01 ⁶
[¹³ C ₅] AUC _{0→∞} (nmol · h/L) per nmol (dose) ⁵	0.20 (0.10–0.24)	0.11 (0.08–0.18)	0.10 (0.05–0.25)	<0.01 ⁶
Relative [¹³ C ₅] AUC _{0→12} of [¹³ C ₅] AUC _{0→∞} (%)	73 (60–88)	50 (38–74)	56 (40–71)	<0.01 ⁶
[¹³ C ₅] C _{max} (nmol/L)	10.6 (6.3–16.5)	3.1 (1.7–4.5)	3.3 (2.1–5.7)	<0.01 ⁶
[¹³ C ₅] T _{max} (min)	40 (40–90)	150 (90–240)	240 (120–240)	<0.01 ⁶
[¹³ C ₅] Stomal excretion (% of dose 24 h postdose) ⁷	2.7 (2.1–6.7)	5.4 (1.9–6.3)	5.6 (1.7–9.0)	0.15 ⁶
Unlabeled folate content in test food, lunch, and snack (nmol) ⁸	113 ± 1	118 ± 2	339 ± 8	<0.01 ⁴
C ₀ , 5-CH ₃ -H ₄ folate (nmol/L)	13.4 (7.0–27.3)	16.2 (9.1–40.7)	14.7 (5.2–95.1)	0.83 ⁶
Unlabeled stomal excretion per 12 h (nmol) ⁹	99 (52–178)	106 (43–148)	103 (46–166)	0.53 ⁶

¹ AUC_{0→12}, area under the plasma concentration time curve from 0 to 12 h; AUC_{0→∞}, area under the plasma concentration time curve from 0 h to infinity; C_{max}, maximum plasma concentration; T_{max}, time (min) of maximum plasma concentration; C₀, fasted plasma concentration before ingestion of dose. Conversion factors from nanomoles per liter to nanograms per milliliter and nanomoles to micrograms were 0.465 ([¹³C₅]5-CH₃-H₄ folate), 0.460 (5-CH₃-H₄ folate), and 0.441 (folic acid).

² [¹³C₅]5-CH₃-H₄ folate or [¹³C₅]folic acid content provided by study dose.

³ Median; range in parentheses (all such values).

⁴ Differences in variables between the 3 study days were measured by using Kruskal-Wallis test (calculated with the Minitab 15.1.0.0 program; Minitab Ltd, Coventry, United Kingdom).

⁵ Dose normalized (AUC/dose) for [¹³C₅]5-CH₃-H₄ folate.

⁶ Differences in responses to test foods between the 3 study days were measured by using Q statistics designed for crossover trials with ≥3 treatments and the same number of periods (24) (calculated with the Minitab 15.1.0.0 program; Minitab Ltd).

⁷ As [¹³C₅]5-CH₃-H₄ folate after ingestion of [¹³C₅]5-CH₃-H₄ folate-fortified bread and [¹³C₅]folic acid and traces of [¹³C₅]5-CH₃-H₄ folate after ingestion of [¹³C₅] folic acid-fortified bread and breakfast.

⁸ Mean ± SD sum (n = 3) of H₄ folate, 5-CH₃-H₄ folate, 5-HCO-H₄ folate, and 10-HCO folic acid.

⁹ Sum of unlabeled individual folate.

α-amylase was added (15 μL thermostable α-amylase/g sample) before extraction (12 min) in a boiling-water bath. After deconjugation with rat serum (37°C, 2 h), samples were purified by using strong anion exchange solid-phase extraction (SAX SPE) (19). H₄ folate, 5-CH₃-H₄ folate, [¹³C₅]5-CH₃-H₄ folate, 5-HCO-H₄ folate, 10-HCO folic acid, folic acid, and [¹³C₅]folic acid were quantified by using LC-MS with external calibration (21). Interassay CVs during food analysis (n = 4) for individual folate forms were as follows: H₄ folate: 6%; 5-CH₃-H₄ folate: 2%; [¹³C₅]5-CH₃-H₄ folate: 16%; 5-HCO-H₄ folate: 16%; 10-HCO folic acid: 11%; and [¹³C₅]folic acid: 15%. Folic acid was not detected in the breads.

Collection and preparation of plasma and stomal effluent samples for folate determination

Sample collection and analyses of screening variables were carried out according to routine procedures (Norrlands University Hospital, Umeå, Sweden). At beginning of each study day, a fasting predose blood sample was withdrawn into coded sterile 4.5 mL EDTA-coated tubes (BD Diagnostics; Plymouth, United Kingdom) when volunteers were cannulated. After ingestion of the test food or meal, postdose blood samples were withdrawn at 20, 40, 60, 90, 120, 180, 240, 360, 480, 600, and 720 min by research nurses at the Centre for Clinical Research, Umeå, Sweden. Tubes were stored on ice in the dark and centrifuged within 30 min (2000 g, 4°C, 13–15 min; Sigma 2–16KC; Sigma, Osterode am Harz, Germany). Plasma was transferred to cryotubes (Nunc, Langensfeld, Germany) and immediately stored at –20°C. Volunteers changed their ileostomy bags immediately

before ingestion of the test meal and every 2 h thereafter until 12 h postdose. Between 12 and 24 h, postdose ileostomy bags were changed when needed. Bags were sealed, immediately frozen on dry ice in the dark, and stored at –20°C (7).

Plasma samples were thawed at room temperature in the dark. Deuterium-labeled internal standards (d₄-H₄ folate, d₄-5-CH₃-H₄ folate, d₄-5-HCO-H₄ folate, and d₄-folic acid), which were synthesized as previously described (22), were added in amounts commensurate with the expected sample folate concentration (target peak area ratio of 0.5–4 for standard and analyte). Samples were purified by SAX SPE (Discovery DSC-Ph SPE tube 100 mg 1 mL; Supelco, Bellefonte, PA; or Strata SAX SPE tube 100 mg 1 mL; Phenomenex, Aschaffenburg, Germany) and eluted by using 0.1 M sodium acetate trihydrate that contained 5% sodium chloride and 1% ascorbic acid as previously described (23).

Ileostomy samples were weighed, thawed at room temperature in the dark, homogenized, pooled into day (0–12 h) and night (12–24 h) samples and stored at –20°C under an argon atmosphere. Ileostomy samples were lyophilized (Modell P8K-E-54–5; Piatkowski, Munich, Germany) by increasing the temperature stepwise from –20°C to 5°C (400 μbar) and finally to 20°C (0 μbar) (at 24 h) and, thereafter, stored at –20°C. On the day of analysis, buffer [0.02 M, 2% (wt:vol) ascorbic acid, 1.4% (vol:vol) mercaptoethanol; 10 mL] and internal deuterium-labeled folate standards were added to the sample (0.5 g).

Samples were incubated with protease (2 mg; type XIV bacterial from *Streptomyces griseus*; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 4 h. After enzyme inactivation (5 min in a boiling-water bath), folates were deconjugated by the

addition of 2 mL chicken-pancreas deconjugase suspension [0.2 mg/mL chicken pancreas (Sigma-Aldrich) dissolved in 0.1 mol/L phosphate buffer (pH 7.0) that contained 1% wt:vol ascorbic acid] and 150 μ L rat serum (Biozol, Eching, Germany) before purification with SAX SPE (Discovery DSC-SAX SPE tube 500 mg 3 mL; Supelco; Strata SAX SPE tube 500 mg 3 mL; Phenomenex) as described elsewhere (23). All extracts were stored in HPLC vials at -20°C until LC-MS/mass spectrometry analysis.

Intact folates were quantified in plasma and ileostomy samples by a stable-isotope dilution assay (23) with an HPLC (LC-20A prominence; Shimadzu Corporation, Kyoto, Japan) coupled to an API 4000 Q-Trap LC-MS/mass spectrometry system (Applied Biosystems, Foster City, CA). Folates were separated on Nucleosil C_{18} columns (for plasma samples: 150×2.00 mm, $3 \mu\text{m}$; Phenomenex; and for ileostomy samples: 250×3.00 mm, $5 \mu\text{m}$; Macherey Nagel, Düren, Germany) by using 1% (vol:vol) acetic acid and 0.1% (vol:vol) formic acid in acetonitrile for gradient elution. The contents of H_4 folate, 5- $\text{CH}_3\text{-H}_4$ folate, [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate, 5-HCO- H_4 folate, 10-HCO folic acid, folic acid, and [$^{13}\text{C}_5$]folic acid were quantified by using response curves with linear ranges exceeding the substance ratios of 3:1 to 1:3 (d_4 -labeled standard:analyte). The limit of detection (23) of individual folate forms was 1 nmol/L in plasma and 6 nmol/100 g in stomal effluent samples [the median (range) weight of 12-h postdose stomal effluents was 282 g (79–570 g)]. The intra- and interassay CV of folate analyses was $<5\%$ in plasma samples and $<20\%$ in stomal effluent samples. Each sample was prepared in duplicate and quantified by duplicate injection.

Kinetic and statistical calculations

Area under the plasma concentration time curve from 0 to 12 h ($\text{AUC}_{0 \rightarrow 12}$) was calculated by using the trapezoidal method (24) and corrected for the dose of labeled folate. Because [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate concentrations were above baseline (>0 nmol) at the last blood sampling (T_{720}) on all study days (Figure 1), Area under the plasma concentration time curve extrapolated from 12 h to infinity ($\text{AUC}_{0 \rightarrow \infty}$) was extrapolated by log-linear regression analysis by using plasma concentrations in the elimination phase (24). The positive $\text{AUC}_{0 \rightarrow 12}$ of unlabeled plasma 5- $\text{CH}_3\text{-H}_4$ folate was also calculated (6). Nonabsorbed folate was estimated by quantifying the content of labeled [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate and [$^{13}\text{C}_5$]folic acid in 12 and 24-h postdose stomal effluent.

Data were expressed as medians and ranges. Response variables were not normally distributed; therefore, nonparametric methods were used. Differences between the 3 study days in dose-normalized $\text{AUC}_{0 \rightarrow 12}$, dose-normalized $\text{AUC}_{0 \rightarrow \infty}$, C_{max} (maximum plasma concentration), T_{max} , [time (min) of maximum plasma concentration] and labeled and unlabeled stomal folate content (Table 1) were analyzed by using Q statistics (25). The Kruskal-Wallis test was used to compare fasting plasma concentrations (of unlabeled folate). Wilcoxon's signed-rank test was used to compare within-day changes in unlabeled 5- $\text{CH}_3\text{-H}_4$ folate [ie, the folate concentration in fasting blood sample compared with final blood sample (T_{720})]. The Minitab 15.1.0.0 program (Minitab Ltd, Coventry, United Kingdom) was used for statistical tests. A 2-sided $P < 0.05$ was regarded as statistically significant.

RESULTS

A stable-isotope AUC and ileostomy model was used to assess folate-absorption kinetics after single doses of the isotope-labeled test foods, bread, or a complete breakfast meal (Table 1). The only labeled folate form that exceeded the limit of detection in postdose plasma was [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate after ingestion of [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate and [$^{13}\text{C}_5$]folic acid. After ingestion of bread fortified with [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate, the median (range) $\text{AUC}_{0 \rightarrow 12}$ of [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate in volunteers was 66 nmol \cdot h/L (34–84 nmol \cdot h/L). After ingestion of the [$^{13}\text{C}_5$]folic acid-fortified bread and breakfast meal, the median (range) $\text{AUC}_{0 \rightarrow 12}$ was only about one-half that value (ie, 28 nmol \cdot h/L (15–38 nmol \cdot h/L) and 26 nmol \cdot h/L (15–60 nmol \cdot h/L), respectively. After correction for ingested doses (Table 1), the same trend was observed for dose-normalized $\text{AUC}_{0 \rightarrow 12}$ and $\text{AUC}_{0 \rightarrow \infty}$. The median $\text{AUC}_{0 \rightarrow 12}$ relative to $\text{AUC}_{0 \rightarrow \infty}$ was 56% for all meals (Table 1). T_{max} and C_{max} of [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate varied significantly between the 3 test foods (Figure 1). The median (range) concentration of unlabeled 5- $\text{CH}_3\text{-H}_4$ folate in fasting plasma (C_0) was 14 nmol/L (5–95 nmol/L) and did not vary significantly between study days (Table 1). The dominant folate form in fasting plasma samples ($n = 24$) was 5- $\text{CH}_3\text{-H}_4$ folate, which amounted to 92%, whereas 7% folic acid and 2% H_4 folate were also quantified. However, because the concentrations of folic acid and H_4 folate in plasma samples were around the limit of quantification (1 nmol/L), only unlabeled 5- $\text{CH}_3\text{-H}_4$ folate concentrations were used for calculations.

Plasma data ($\text{AUC}_{0 \rightarrow 12}$, C_{max} , and T_{max} , data not shown) for unlabeled folate were not significantly different between test days, despite the higher unlabeled folate content in the breakfast (150 μg folate compared with 50 μg folate for days when fortified bread was consumed). The median (range) increase in the unlabeled folate concentration ($C_{\text{max}} - C_0$) on test days was 3 nmol/L (2–13 nmol/L), and the T_{max} was 210 min (40–720 min). At the end of each study day (T_{720}), median 5- $\text{CH}_3\text{-H}_4$ folate concentrations were not significantly different from fasting concentrations ($+1 \pm 16$ nmol/L; $P = 0.48$).

During 24 h postdose after ingestion of bread fortified with [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate, a median range of 13 nmol (10–31 nmol) [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate was observed in the collected stomal effluents, whereas after ingestion of the [$^{13}\text{C}_5$]folic acid-fortified bread and breakfast meal, a median (range) of 25 nmol (8–37 nmol) labeled folate was observed. The stomal content of labeled folate was not significantly different between the 3 study days ($P = 0.15$) and comprised $<10\%$ of oral doses (Table 1).

A total (range) of 98% (92–100%) of labeled folate in stomal effluent was observed during the first 12 h postdose. After ingestion of test foods containing [$^{13}\text{C}_5$]folic acid, traces of [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate [median (range): 0.6 nmol (0.1–5.0 nmol)] were observed in 15 of 16 stomal effluent samples collected during the first 12 h postdose. In effluent samples collected during the night, only 7 of 16 contained traces of [$^{13}\text{C}_5$]5- $\text{CH}_3\text{-H}_4$ folate [median (range): 0 nmol (0–0.43 nmol)].

The median (range) amount of unlabeled folate excreted with stomal effluent was 103 nmol (43–178 nmol)/12 h stomal effluent and did not differ significantly between study days (Table 1). The median (range) pattern of unlabeled folate forms quantified in stomal effluent was 38% (31–61%) 10-HCO folic acid, 28%

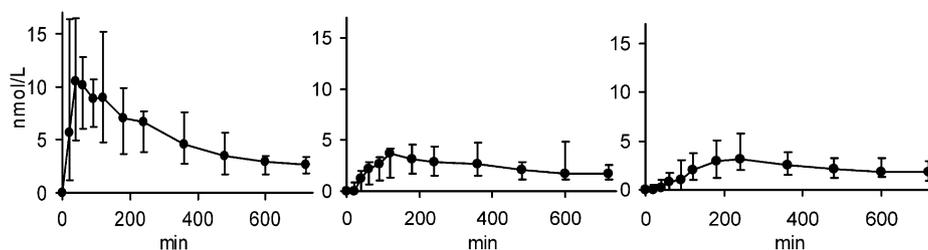


FIGURE 1. Median (range) concentration of plasma [$^{13}\text{C}_5$]5- CH_3 - H_4 folate after ingestion of bread fortified with [$^{13}\text{C}_5$]5- CH_3 - H_4 folate (left), bread fortified with [$^{13}\text{C}_5$]folic acid (middle), and a breakfast with bread containing 450 nmol of [$^{13}\text{C}_5$]folic acid (right). $n = 8$.

(16–34%) H_4 folate, 26% (21–49%) % 5- CH_3 - H_4 folate, 6% (3–14%) 5- HCO - H_4 folate, and 1% (1–2%) folic acid.

DISCUSSION

The short-term plasma kinetics of folate and folic acid have been extensively studied (2, 5–12). However, the current trial was unique in combining an isotopic-labeling technique (2, 9, 12, 14, 15) and an AUC and ileostomy model (6–8) to enable folate from the ingested dose to be distinguished from endogenous folate in plasma and stomal effluent samples.

Before appearing in peripheral plasma, absorbed folic acid is bioconverted into 5- CH_3 - H_4 folate, which is a saturable step (12). Because only labeled 5- CH_3 - H_4 folate was detected in plasma, the bioconversion of given single doses of 450 nmol [$^{13}\text{C}_5$]folic acid appeared to be complete. However, the requirement to convert absorbed [$^{13}\text{C}_5$]folic acid may partly explain differences in absorption kinetics (Table 1) that caused a significant delay in T_{max} observed at a time where sampling was less frequent (every 60 min compared with every 20 min). Hence, the estimation of C_{max} and the resulting AUC after ingestion of bread containing [$^{13}\text{C}_5$]5- CH_3 - H_4 folate is probably more accurate, whereas the C_{max} and resulting AUC after ingestion of folic acid are likely to be underestimated.

Wright et al (2, 12) and Konings et al (8) reported a T_{max} of <1 h after ingestion of oral doses of reduced folate forms, which was substantially faster than after ingestion of folic acid supplements (2–3 h). Our results (Table 1) confirmed their findings. Wright et al (12) explained differences in absorption kinetics by assuming that folic acid is not bioconverted in the enterocytes (2) but in the liver. Because liver enzymes are postulated to have a greater affinity for oxidized folic acid than reduced folates (2), more folic acid would be retained by the liver as result of the first-pass effect, and thus, less folic acid would appear in peripheral plasma. In the current trial, plasma AUCs after ingestion of [$^{13}\text{C}_5$]folic acid–fortified test foods were $\approx 50\%$ smaller than plasma AUCs after a dose of [$^{13}\text{C}_5$]5- CH_3 - H_4 folate–fortified bread, which supports the hypothesis that a greater amount of folic acid is retained by the liver. Wright et al (12) also observed an increase of unlabeled 5- CH_3 - H_4 folate after an oral dose of labeled folic acid and 5- HCO - H_4 folate pharmaceuticals and interpreted their data to be a result of the displacement of endogenous folates. Because labeled folate doses in the current study were administered with food that contained unlabeled endogenous folate, it was not possible to distinguish whether the increase of unlabeled plasma 5- CH_3 - H_4 folate was caused by the ingestion of unlabeled food folate or the displacement of endogenous hepatic folates.

The bioavailability of food folate is incomplete (6, 8, 16), and several food-related factors have been suggested to reduce bioavailability [eg, a high content of folate polyglutamates (8), dietary fiber (8), and folate-binding proteins (6) in the food]. Therefore a whole-meal test food was included into the study that reflected rather physiologic conditions, whereas in most absorption trials, single test foods or isolated folate supplements were administered (2, 3, 5–9, 11, 12, 15, 16, 26). However, our findings of similar estimated plasma responses after ingestion of [$^{13}\text{C}_5$]folic acid as part of a complete breakfast meal or as bread alone suggest that any positive or negative effects on folic acid bioavailability are negligible.

The whole-meal bread matrix did not seem to inhibit the bioavailability of added folate, as indicated by the low content of labeled folate (2–9% of the dose/24 h) in the stomal effluent (Table 1). According to our data, the content of intact labeled folate in stomal effluent did not significantly differ between test days and was estimated to <10% of the administered doses (bearing in mind a high interassay CV of 20% for folate analysis in stomal effluent samples), which suggested a high absorption of the administered doses. This result does not necessarily indicate a folate absorption of 90%, because our method did not account for possible folate-degradation products, but it confirms the findings of a high absorption of orally administered radiolabeled folic acid reported by others (13, 17, 19) who measured 10–21% of radiolabel in human postdose stool samples.

Incomplete stomal effluent collection would result in an overestimated bioavailability but could not explain the high bioavailability in the current trial ($\approx 90\%$) because 99% (92–100%) of labeled folate in stomal effluent was observed within 12 h postdose. This implies that future stomal effluent sample collection could be simplified for volunteers by avoiding collection overnight.

On the basis of data on folate excretion with stomal effluent, the bioavailabilities of [$^{13}\text{C}_5$]5- CH_3 - H_4 folate and [$^{13}\text{C}_5$]folic acid when ingested as food fortificants were similar. However, because of the lower stability of 5- CH_3 - H_4 folate in combination with the higher cost during synthesis, 5- CH_3 - H_4 folate may not be suitable for food-fortification purposes.

Aufreiter et al (26) observed increased plasma folate concentrations after cecal infusion of stable isotopically labeled folate in humans, which showed the occurrence of folate absorption in the colon. Data from our ileostomy trial indicated that <10% of the administered labeled folate doses would reach the colon. We did not expect substantial effects on bioavailability data in case of partial absorption.

It was estimated that 30–50% of folate in human-infant stools originates from dietary sources (18). In the current trial, <20% of

the folate content in stomal effluent was isotopically labeled, whereas ≈ 60 – 80% of the ingested folate supplied by test foods and snacks during study days (0–12 h postdose) was labeled. Consequently, only about one-third of the folate in stomal effluent was derived from recently ingested food folate. Because ileostomists lack a colon with microbial folate synthesis, and the influence of bacteria in the effluent was minimized by frequent bag changes and immediate freezing, bacterial folate synthesis should have been negligible, and therefore lysed enterocytes and gastrointestinal secretions (27), for example, might have contributed to folate content. Furthermore, it is possible that labeled folate fortificants were less entrapped in the food matrix than were endogenous food folates and, therefore, were more easily absorbed.

After ingestion of test foods with [$^{13}\text{C}_5$]folic acid, minor amounts of [$^{13}\text{C}_5$]5- CH_3 - H_4 folate, which corresponded to $\approx 1\%$ of the given dose, were detected in stomal effluent. Contamination of fortificants during the baking and preparation of test foods could be ruled out because of the standardized preparation procedures and according to analyses of breads. It seems likely that the detected [$^{13}\text{C}_5$]5- CH_3 - H_4 folate originated from bile because 73% of absorbed folic acid has been estimated to be retained in the human liver (2). Furthermore, folate excretion via bile was assumed to be rapid because the C_{max} in bile folate was reported to be 75–120 min after oral administration of radioisotopically labeled folic acid (28). The reabsorption of bile folates in humans is nearly complete (96%) as reported by Lin et al (13), which might explain the low content of [$^{13}\text{C}_5$]5- CH_3 - H_4 folate in stomal effluents after ingestion of [$^{13}\text{C}_5$]folic acid.

This study had several limitations. One weakness was the estimation of labeled folate doses. Doses were calculated by using data from LC-MS analysis with dienzyme treatment (20) of breads (441 μg [$^{13}\text{C}_5$]5- CH_3 - H_4 folate/100g and 430 μg [$^{13}\text{C}_5$] folic acid/100 g bread) with an analytic recovery of 74% for the sum of folates. With the use of an accredited microbiological assay with trienzyme treatment (interassay CV: 18%; National Food Administration Sweden), the total folate content was 618 μg folate/100g in the bread fortified with [$^{13}\text{C}_5$]5- CH_3 - H_4 folate, 494 μg folate/100g in the [$^{13}\text{C}_5$]folic acid-fortified bread, and 41 μg folate/100 g in nonfortified bread. We chose to estimate folate bioavailability by using doses on the basis of direct LC-MS quantification.

Other limitations of the study were the different sampling intervals at T_{max} for [$^{13}\text{C}_5$]5- CH_3 - H_4 folate and [$^{13}\text{C}_5$]folic acid and the 12-h collection of plasma that corresponded to $<60\%$ of $\text{AUC}_{0 \rightarrow \infty}$ on study days with ingestion of [$^{13}\text{C}_5$]folic acid-fortified foods (Table 1). Plasma AUC proved to be a valuable indicator of bioavailability as long as the postdose plasma measurement period was long enough to capture 80% of the total AUC (29). However, the benefits of prolonged study days should be carefully weighed against the inconvenience and potential loss of volunteers.

Studies in folate bioavailability on the basis of plasma AUC data would not necessarily require ileostomy volunteers. However, previous short-term absorption studies reported differences in plasma absorption kinetics between reduced folates and folic acid and explained this by different hepatic metabolism (2, 12). Therefore, it is important when the absorption of oxidized and reduced folates are compared to account for nonabsorbed folate. This could be assessed by the use of radiolabeled folate doses or

the ileostomy model. Because only a few folate ileostomy trials were carried out (6–8), we cannot confidently evaluate the appropriateness of the ileostomy model. To our knowledge, there is no evidence in the literature that physiologic changes in the gut linked to folate have to be expected. However, we cannot fully exclude adaptation processes because data from one volunteer (with a substantially larger intestinal resection than others) did not deviate significantly.

In conclusion, this stable-isotope AUC and ileostomy trial with single isotope-labeled test doses provided data on the bioavailability of reduced and oxidized folates ingested with test foods or in a complete meal. Our data confirm previous findings that absorption kinetics for reduced folates and folic acid differ greatly in humans (12), the bioavailability of folic acid fortificant ingested with bread or in a complete breakfast meal is similar, and probably only 30–50% of excreted folate originates from recently ingested dietary sources (18).

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