Folate absorption from folate-fortified and processed foods using a human ileostomy model

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Data on folate absorption from food from validated human studies using physiological folate doses are still needed to estimate dietary requirements and to formulate recommendations. The aim of the present work was to study the effects from fortified and processed foods on folate absorption in ileostomy volunteers (n 9) using the area under the plasma concentration curve (AUC) and kinetic modelling. Using a standardized single-dose protocol, dairy products fortified with a candidate fortificant (6S)-5-methyltetrahydrofolate ((6S)-5-CH3-H4folate), folic acid-fortified bread and a dessert crème containing natural yeast folate polyglutamates were compared with folate supplements. Absorbed folate was estimated by AUC and a kinetic model, and non-absorbed folate by ileostomal folate excretion. Median apparent absorption from test foods ranged from 55 to 86%. Added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased. After added folate-binding proteins (FBP) significantly reduced folate absorption from dairy products, as in the absence of FBP, AUC–dose-corrected ratios were increased and ileal folate excretion decreased.

An optimal folate status is linked to several health-protective effects, e.g. diminished risk for neural tube defects (Honein et al. 2001; Liu et al. 2004) and spontaneous abortions (George et al. 2002), decreased risk of occlusive vascular diseases (Wald et al. 2002) and improved cognitive or mental functions (Seshadri et al. 2002). These reported health benefits have led to increased folate intake recommendations in the USA and some European and Nordic countries (Yates et al. 1998; Becker et al. 2004).

Information about the extent to which certain foods could contribute to increased folate intake is still incomplete. However, some in vitro and in vivo trials aimed to determine the effects of food matrix on folate absorption, e.g. folic acid-fortified cereal-based foods (Pfeiffer et al. 1997; Malinow et al. 1998; Johansson et al. 2002) or dairy products which contain folate-binding proteins (FBP; Arkbåge et al. 2003; Verwei et al. 2003). Different human models have been used to determine long-term (Malinow et al. 1998; Johansson et al. 2002; Vahteristo et al. 2002) or short-term (Pfeiffer et al. 1997; Prinz-Langenohl et al. 1999; Finglas et al. 2002; Konings et al. 2002; Witthöft et al. 2003) folate bioavailability or absorption. Long-term protocols are tedious, and also most short-term protocols have certain requirements and limitations as reviewed elsewhere (Witthöft et al. 1999; Gregory, 2001), e.g. lack of sensitivity demanding high test doses or presaturation of volunteers’ body stores as recommended for dual-label stable-isotope protocols (Pfeiffer et al. 1997; Rogers et al. 1997). The area under the plasma concentration curve (AUC) technique (Prinz-Langenohl et al. 1999; Konings et al. 2002) is commonly used to estimate folate absorption by comparing a single oral dose of test food with a known dose of a pharmaceutical folate preparation. This concept was questioned as it was hypothesized that oxidized folic acid and reduced folates have different sites of initial metabolism resulting in a greater liver sequestering of folic acid (Wright et al. 2003).

Abbreviations: AUC, AUClow–1800, area under the (plasma concentration) curve, superscript time range (in min); Cpf, plasma folate concentration, subscript defines time (in min); FBP, folate-binding proteins; (6S)-5-CH3-H4folate, (6S)-5-methyltetrahydrofolate; t, t50, time (point), subscript defines time (in min) of folate concentration in plasma.

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The present study was carried out to determine effects from differently fortified and processed foods on folate absorption using the AUC technique and a new kinetic modelling method (Kok et al. 2004; Wright et al. 2005) in human ileostomy volunteers and forgoing body store presaturation (Witthöft et al. 2003). Test foods were differently processed dairy and cereal products, which were fortified with folic acid or a new candidate fortificant (6S)-5-methyltetrahydrofolate ((6S)-5-CH₃-H₄folate) or natural yeast folate polyglutamates. Furthermore, effects from dairy FBP on folate absorption and the fate of FBP during in vivo gastro-intestinal passage were studied.

Material and methods

Subjects

Nine subjects were recruited (eight males, one female), apparently healthy based on routine haematological and biochemical measurements and a physical examination. They had a mean age of 62 (SD 9.3, range 51–79) years, a mean BMI of 28.9 (SD 4.3, range 22.6–38.4) kg/m², were non-smokers, and did not use any medication or vitamin supplements affecting folate metabolism. They underwent protocolectomy 12–37 years earlier as a result of ulcerative colitis with a maximal resection of 5–10 cm (except one volunteer: 25 cm) and did not use any medication or vitamin supplements affecting folate metabolism. The ileum was distal ileum and possessed a conventional well-established ileostomy with no signs of inflammation. Volunteers were screened for fasting serum folate, serum cobalamin and erythrocyte folate concentrations to ensure normal folate and vitamin B₁₂ status. The protocol was approved by the Ethical Committee of Umeå University Hospital.

Study design

All volunteers underwent nine independent study days each 2–4 weeks apart in random order. They received, after overnight fast, either a single dose of test food or a pharmaceutical preparation of the naturally occurring diastereoisomer (6S)-5-methyltetrahydrofolate ((6S)-5-CH₃-H₄folate) or folic acid (Table 1). On one day they received no folate to allow for estimation of baseline folate excretion into stomal effluent. During the several months’ long trial, volunteers’ folate status was standardized by presaturation of body stores with a daily dose of 0.96 mg folic acid from day 9 to day 2 prior to each study day (Witthöft et al. 2003). A standardized low-folate and low-fat lunch (Witthöft et al. 2003) was consumed at 4 h 5 min post-dose, providing 2556 kJ, 13.6 g fat and 18.1 µg folate. A snack of 8 g unsalted rice-cake and 15 g pasteurized apple crème, providing 163 kJ, 0.2 g fat and 3.4 µg folate was consumed at 7 h 5 min post-dose.

Table 1. Pharmaceutical preparations and test foods

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Folate dose/portion*</th>
<th>Further details</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Intraduodenal injection</td>
<td>Injection solution (1 ml) prepared from (6S)-Ca-5-CH₃-H₄ folate (Merck Eprova AG, Schaffhausen, Switzerland) according to Witthöft (2003).</td>
</tr>
<tr>
<td>O</td>
<td>Pharmaceutical preparation</td>
<td>Gelatine capsule by Merck Eprova AG.</td>
</tr>
<tr>
<td>C</td>
<td>Pharmaceutical preparation</td>
<td>Gelatine capsule by Merck Eprova AG.</td>
</tr>
<tr>
<td>U</td>
<td>Fermented milk</td>
<td>Commercial fermented milk product (Filmjölk™; Arla Foods, Stockholm, Sweden; 0.5% fat), addition of (6S)-Ca-5-CH₃-H₄ folate injection solution 30 min prior to consumption; 400 g/portion.</td>
</tr>
<tr>
<td>F</td>
<td>Fermented milk with FBP</td>
<td>Commercial fermented milk product (Filmjölk™; Arla Foods, Stockholm, Sweden; 0.5% fat), addition of (6S)-Ca-5-CH₃-H₄ folate injection solution and whey protein concentrate WPC 65 (Arla Foods) 30 min prior to consumption; providing 156–442 nmol FBP/400 g portion.</td>
</tr>
<tr>
<td>P</td>
<td>Pasteurized milk with FBP</td>
<td>Pasteurized skimmed milk (0.5% fat) with strawberry taste, fortified with 262 nmol FBP/portion by addition of whey protein concentrate, WPC 65 (Arla Foods). Prior to consumption, the milk was defrosted in a refrigerator overnight and mixed (400 g/portion).</td>
</tr>
<tr>
<td>B</td>
<td>Bread</td>
<td>Wheat bread, fortified with folic acid (Merck Eprova), baked by Cerealia (Järna, Sweden), stored at –20 °C. The bread was thawed in a refrigerator overnight, the crust removed and portions of 50 g weighed out 20 min prior to consumption, providing 12 µg (26 nmol) endogenous 5-CH₃-H₄ folate/portion.</td>
</tr>
<tr>
<td>Y</td>
<td>Yeast crème</td>
<td>Lemon mousse ‘fresta’ (citronfromage; Ekströms, Procordia Food, Eslov, Sweden) with yeast flakes (Edelhefe-Flocken auf Melasse Basis, Tartex + Dr Ritter GmbH, Freiburg, Germany); portions (about 170 g) were prepared 30–45 min prior to consumption.</td>
</tr>
</tbody>
</table>

(6S)-5-CH₃-H₄ folate, (6S)-5-methyltetrahydrofolate; FBP, folate-binding proteins.

* Concentrations in µg as free 5-CH₃-H₄ folic acid.
† Range for n 8, another = 433 µg/921 nmol.
‡ Range for n 8, another = 111 µg/241 nmol.
Folate absorption was estimated by plasma AUC and a kinetic model from venous blood samples collected 10 min pre-dose and at 20, 40, 60, 90, 120 min and 3, 4, 6, 8 and 10 h post-dose. Non-absorbed folate was estimated from folate excretion into stomal effluent samples, collected every second hour over 10 h post-dose. Urine samples from spontaneous bladder emptying during 10 h post-dose were used to ensure that ingested doses did not exceed the kidney threshold. Detailed information of sample collection and storage is given elsewhere (Withthöft et al. 2003).

Sample pretreatment for folate analysis

Procedures for extraction and purification of plasma and ileostomy samples by strong anion exchange solid-phase extraction and urine samples by affinity chromatography using bovine FBP (Scripps Laboratories, Cincinnati, OH, USA) are described elsewhere (Withthöft et al. 2003). Individual urine samples derived from each subject were pooled beforehand for each test day. Procedures for extraction, deconjugation using hog kidney acetone powder (Sigma Chemical Co., St Louis, MO, USA) and strong anion exchange solid-phase extraction purification of aliquots from food samples (dairy products 5 g, dessert crème with yeast 3·5 g, freeze-dried homogenized lunch and snack samples 2 g) have been described earlier (Withthöft et al. 2003). To ensure complete deconjugation of folate polyglutamates in the yeast dessert crème, rat serum (Scanbur, Sollentuna, Sweden) was used according to Patring et al. (2005). Freeze-dried bread samples (2 g) were extracted by a tri-enzyme method using thermostable α-amylase (Megazyme International, Cork, Ireland) and protease (Sigma Chemical Co.) according to Johansson et al. (2002).

Folate quantification by HPLC

5-CH₃-H₄folate content in test foods, pharmaceutical folate preparations and human samples was quantified by reverse-phase HPLC according to Jastrzębowa et al. (2003) using a HP 1100 series system equipped with a multi-wavelength detector and a fluorescence detector (Agilent Technologies, Waldbronn, Germany) and a Zorbax SB C8, 150 £ 4·6 mm, 5 µm (Agilent Technologies, Palo Alto, CA, USA) column. External calibration (n 8) was carried out using the standards (Eprova AG, Schaffhausen, Switzerland) (6S)-H₄folate, (6S)-5-CH₃-H₄folate, (6S)-5-HCO-H₄folate at 290/356 nm (fluorescence detector) and folic acid at 290 and 300 nm (multiwavelength detector). The limits of quantification were 0·5 ng/ml for H₄folate, 0·3 ng/ml for 5-CH₃-H₄folate, 4 ng/ml for 5-HCO-H₄folate and 4 ng/ml for folic acid. Calibration was linear over a range of 0·5–100 ng/ml for H₄folate, 0·3–100 ng/ml for 5-CH₃-H₄folate, 4–200 ng/ml for 5-HCO-H₄folate and 4–200 ng/ml for folic acid. Intra-assay CV and relative recoveries for 5-CH₃-H₄folate were: CV of 11·0 % (n 4), 84–105 % recovery in stomal effluent samples and CV of 6·9 % (n 4), 86–94 % recovery in plasma samples, including sample preparation and all analytical steps. An in-house plasma control sample and a milk sample as control for stomal effluent samples were carried through all procedures of sample extraction and purification, resulting in CV of 6·2 % (n 41, plasma) and 5·5 % (n 35, milk) of 5-CH₃-H₄folate concentrations. For folic acid in bread a CV of 0·9 % (n 3) and for 5-CH₃-H₄folate in yeast crème a CV of 7·5 % (n 3) were observed. Spiking of urine samples with 5-CH₃-H₄folate prior to affinity purification resulted in recoveries of 92–111 % (Withthöft et al. 2003). The day-to-day repeatability for affinity procedures and subsequent 5-CH₃-H₄folate quantification resulted in CV of 6·8 % (n 3, urine) and 4·5 % (n 18, standard solution).

Folate-binding protein quantification

FBN concentrations in dairy products and stomal effluent samples were determined by a two-site ELISA developed for milk according to Höjer-Madsen et al. (1986) with minor modifications as published by Wigertz et al. (1997) using rabbit anti-bovine FBP 24739 (State Serum Institute, Copenhagen, Denmark), FBP calibrant (Central Hospital Hillerød, Hillerød, Denmark) and the software KinetiCalc 4, version 2·5 for Windows (Bio-Tek Instruments, Winooski, VT, USA). A whey protein concentrate, containing 65 % protein (WPC 65; Arla Foods, Göteborg, Sweden), was included as in-house reference material into every analysis. The CV between runs did not exceed 15 %.

Kinetic and statistical calculations

Non-absorbed folate from oral doses was estimated by 10 h post-dose stomal effluent. Absorbed folate was estimated using plasma folate net increase above baseline concentrations (pre-dose). When plasma concentration fell below the pre-dose level, the increment was taken as zero. The (positive) AUC0–∞ from t₀ to infinity was calculated for each subject using linear and logarithmic trapezoidal rules for ascending and descending plasma concentrations up to the last time-point. If folate concentrations at the last blood sampling point (Cₖ₉) were still above baseline concentrations (C₀), the AUC beyond Cₖ₉ to infinity (AUC∞–Cₖ₉) was extrapolated by log-linear regression analysis using the last three to five plasma concentration data points (choosing the best fit by correlation coefficients).

Relative folate absorption from test foods was compared using AUC–dose-corrected ratios (AUCTestfood/DoseTestfood (h ng/ml) per mol)) to normalize for differences in individual test portions.

Apparent folate absorption was estimated by assuming a zero-order absorption process in a single compartment model as described by Kok et al. (2004) using the following equations for all test foods and doses except bread:

\[ C = \frac{M}{VT_k} \left(1 - e^{-k(t - t_{lag})}\right) \quad (0 < t < t_{max}) \]  
\[ C = \frac{M}{VT_k} \left(1 - e^{-kT} e^{-(t - t_{lag}) - T}\right) \quad (t > t_{max}) \]  

where M is the mass of dose absorbed, t_{lag} is the time during which the plasma enrichment remains at baseline, t_{max} is the time at which the 5-CH₃-H₄folate concentration is a maximum in the plasma, T is the time period for absorption (t_{max} – t_{min}), C is the 5-CH₃-H₄folate concentration in the sampled (plasma) compartment, V is the distribution volume of 389 ml/kg body weight as estimated by Loew et al. (1987) and k is the elimination rate constant. By fitting the above equations to the
5-CH₃-H₄folate curve (above C₀) over time, M can be calculated. For bread, folate absorption was estimated using the first-order absorption process using the Bateman function:

\[ C = \frac{M K_a}{V (K_a - K_c)} (e^{-K_c t} - e^{-K_a t}) \]  

(3)

where C is the concentration in the sampled compartment, M is the quantity of the dose that is absorbed, V is the distribution volume (389 ml/kg body weight) and Kₐ and Kₑ are rate constants of absorption and elimination, respectively. The apparent folate absorption was calculated according to: apparent absorption (%) = 100 × M/Dose_total.

All calculations were made using Office Excel 97,SR or 2003 SP1 (Microsoft, Redmond, WA, USA). All statistical analyses were made using Minitab release 13.32 (Minitab Ltd, Coventry, UK). Continuous variables are presented as median and range.

Normal plots of the residuals after fitting linear models showed that log-transformed response variables: AUC–dose-corrected ratios, apparent folate absorption and relative folate excretion with stomal effluent, were approximately normally distributed. Tukey’s method was used to control the simultaneous experimental error when performing pair-wise comparison among the treatments. When comparing the intramuscular injection (day I) with the oral treatments Dunnett’s method was used to control the simultaneous experimental error. Wilcoxon signed rank test was used to compare effects of treatments P and F (see Table 1 for treatments) on relative FBP excretion in ileostomal effluent. A two-sided P value less than 0.05 was considered significant in all analyses.

Nutrient content in standardized low-folate and low-fat lunch and snack was calculated using the software MATs den flexible, version 2.2; Rudans lättdata, Västera˚s, Sweden.

Results
Effects of ingested doses on folate content in plasma, urine and ileostomal effluent

After ingestion of test foods and pharmaceutical preparations containing 5-CH₃-H₄folate and folic acid, post-dose plasma 5-CH₃-H₄folate concentrations increased above fasted baseline levels, but no folic acid was detected. AUC–dose-corrected ratios after intramuscular injection of pharmaceutical (65)-5-CH₃-H₄folate (day I) were greater than AUC on days B, P, F (P<0.0001) and U (P=0.0074), borderline greater than on day C (P=0.0581) and similar to days O (P=0.2898) and Y (P=0.2360). When no folate dose was given to volunteers (day N), no clear increase and subsequent decrease of plasma 5-CH₃-H₄folate concentrations over time was observed. Resulting AUC from t₀ to tₕ00 had for all volunteers a mean size of below 10 % of the AUC on day I (data not shown), and were not taken into account for further calculations. AUC–dose-corrected ratios after ingestion of fermented milk without FBP (U) and yeast dessert crème (Y) were higher compared to the other foods (Table 2). This is similar when estimating apparent folate absorption (Table 3). Apparent absorption from fermented milk without FBP (U) is similar to yeast crème (Y) (P=0.9891), and both are significantly larger than from pasteurized milk with FBP (P=0.0137 and P=0.0056, respectively). Apparent folate absorption from bread (B) tends to be larger than from pasteurized milk (P=0.067).

Only small quantities of intact 5-CH₃-H₄folate from below 1 to 20 µg were excreted into urine during 10 h post-dose (data not shown). Highest amounts of intact 5-CH₃-H₄folate excreted into urine corresponded on three occasions to a maximum of 8 %, and on all other occasions to below 5 % of the given dose.

After ingestion of test foods containing 5-CH₃-H₄folate, only this folate form was found in stomal effluents and no other folate forms were detected. On day N (baseline), when no folate dose was given, only negligible quantities of 5-CH₃-H₄folate (1.6–6.0 µg/10h) were excreted, being in the same magnitude as absolute 5-CH₃-H₄folate excretion after intramuscular injection (I) (0.7–11.2 µg/10h) and after ingestion of folic acid-fortified bread (B) (1.7–15.4 µg/10h, n 8, for one volunteer peak masked). Relative 5-CH₃-H₄folate excretion increased significantly after ingestion of all test foods containing 5-CH₃-H₄folate (F, P, U, Y and O, all P<0.0001) compared with the intramuscular injection (I),

Table 2. Area under the plasma concentration curve (AUC)—dose-corrected ratios of plasma 5-methyltetrahydrofolate (5-CH₃-H₄folate) after absorption of 5-CH₃-H₄ folate and folic acid from test foods* (Median values and range for nine subjects)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AUC–dose-corrected ratio (ng/ml per mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.099ab</td>
</tr>
<tr>
<td>O</td>
<td>0.065cd</td>
</tr>
<tr>
<td>C</td>
<td>0.075ab</td>
</tr>
<tr>
<td>U</td>
<td>0.053ab</td>
</tr>
<tr>
<td>F</td>
<td>0.030ab</td>
</tr>
<tr>
<td>P</td>
<td>0.020b</td>
</tr>
<tr>
<td>Y</td>
<td>0.039ab</td>
</tr>
<tr>
<td>P</td>
<td>0.143a</td>
</tr>
</tbody>
</table>

* For details of treatments and procedures, see Table 1 and p. 182.

Table 3. Apparent 5-methyltetrahydrofolate (5-CH₃-H₄folate) and folic acid absorption (% of dose) from test foods using kinetic modelling of plasma concentration curves* (Median values and range for nine subjects)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Apparent absorption (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U</td>
<td>86a</td>
</tr>
<tr>
<td>F</td>
<td>62ab</td>
</tr>
<tr>
<td>P</td>
<td>55a</td>
</tr>
<tr>
<td>B</td>
<td>74ab</td>
</tr>
<tr>
<td>Y</td>
<td>80a</td>
</tr>
</tbody>
</table>

* For details of treatments and procedures, see Table 1 and p. 182.

Discontinuous variables with unlike superscript letters were significantly different (P<0.05) (Tukey pair-wise comparison among treatments on log-transformed AUC–dose-corrected ratios).
being significantly higher on days P and Y compared with day U (P=0.0214 and P=0.00371, respectively) and day O (P=0.0058 and P=0.0111, respectively) (Table 4). After ingestion of folic acid-fortified bread (B), some folic acid traces up to approximately 17 µg/10 h were excreted in stomal effluent, and after pharmaceutical folic acid (C) 1–13 µg/10 h were excreted. This is only a rough estimate due to folate concentrations below the limit of quantification in some of the ileostomal fractions.

**Effects from dairy processing and presence of folate-binding proteins on folate absorption**

The presence of FBP in dairy products affected folate absorption. AUC–dose-corrected ratios were significantly increased on day U after ingestion of fermented milk without FBP compared to days F (P=0.0243) and P (P=0.0001). Median AUC–dose-corrected ratios for both dairy products containing FBP did not differ significantly (P=0.5877). However, apparent folate absorption on day U was only significantly increased compared to day P (P=0.0137), but not to day F (P=0.6224). Plasma results were complemented by data on relative ileostomal folate excretion, which increased significantly on day P (P=0.0214) compared to day U, but not on day F (P=0.7152) (Table 4).

After *in vivo* gastrointestinal passage of dairy products fortified with FBP (P and F), FBP was found in ileostomal effluents (Table 5), being significantly higher on day P than day F (P=0.0009). On days without FBP ingestion, e.g. days U and N, no FBP was detected in post-dose effluents, as controlled for four volunteers (data not shown).

**Effect of ingested folate form on extent of absorption**

Folate absorption by means of AUC–dose-corrected ratios did not differ significantly (P=0.9940) after oral ingestion of pharmaceutical preparations of (6S)-5-CH₃-H₄folate (O) and folic acid (C). Also total folate excretion after ingestion of both folate forms was similar. After ingestion of 5-CH₃-H₄folate (O), 2–74 µg 5-CH₃-H₄folate were found in stomal effluent during 10 h post-dose, and after ingestion of folic acid (C), 3–41 µg 5-CH₃-H₄folate and an additional 1–13 µg of folic acid were excreted.

Absorption of different folate forms as fortificant, (6S)-5-CH₃-H₄folate monoglutamate in fermented milk (U) compared to folic acid in wheat bread (B) compared to yeast 5-CH₃-H₄folate polyglutamates as ‘bio-fortificant’ (Y), differed significantly when expressed as AUC–dose-corrected ratios. Y was significantly more absorbed than U (P=0.0001) and B (P<0.0001), and B significantly less than U (P=0.0384). After ingestion of folic acid as fortificant within a bread matrix (B), AUC–dose-corrected ratios were significantly smaller (P=0.0041) compared with a supplement (C). Ileostomal folate excretion was estimated to be 4–24 µg on day B and 1–13 µg on day C. (6S)-5-CH₃-H₄folate given as supplement (O) was similarly absorbed as when given as fortificant in fermented dairy matrix (without FBP, U), based on AUC–dose-corrected ratios (P=0.7822).

**Discussion**

**Effects of dairy processing and presence of folate-binding proteins on folate absorption**

New information on effects of presence of FBP on folate absorption in human subjects was provided by the present study. Plasma results demonstrated that (6S)-5-CH₃-H₄folate, a candidate compound for food fortification and the dominant native food folate form, is bioavailable from all tested dairy products on folate absorption in human subjects was provided by the present study. Plasma results demonstrated that (6S)-5-CH₃-H₄folate, a candidate compound for food fortification and the dominant native food folate form, is bioavailable from all tested dairy products.

<table>
<thead>
<tr>
<th>Treatment†</th>
<th>Median‡</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>4†</td>
<td>0–32</td>
</tr>
<tr>
<td>P</td>
<td>24*</td>
<td>3–43</td>
</tr>
</tbody>
</table>

*For details of treatments and procedures, see Table 1 and p. 182.† No folate-binding proteins found in ileostomal effluent on other days (N, U).‡ Median with unlike superscript letters significantly different (P<0.009) (Wilcoxon signed rank test).

*Table 4. Relative excretion of 5-methyltetrahydrofolate (5-CH₃-H₄folate) (% of dose) with stomal effluent over 10 h post-dose* (

(Median values and range for nine subjects)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Median†</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2†</td>
<td>0–5</td>
</tr>
<tr>
<td>O</td>
<td>8</td>
<td>2–39</td>
</tr>
<tr>
<td>U</td>
<td>7</td>
<td>4–25</td>
</tr>
<tr>
<td>F</td>
<td>16</td>
<td>5–29</td>
</tr>
<tr>
<td>P</td>
<td>20</td>
<td>13–51</td>
</tr>
<tr>
<td>Y</td>
<td>23</td>
<td>13–37</td>
</tr>
</tbody>
</table>

*For details of treatments and procedures, see Table 1 and p. 182.† Median for Y (n=8) as peak for one volunteer masked, median for I (n=8) as amount for one volunteer traces only.

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Arkbäe et al. (2003) reported a significantly decreased bioaccessibility, from yoghurt fortified with FBP, of both 5-CH₃-H₄folate and folic acid. In agreement, we conclude that dairy products might be a suitable matrix for folate fortification if no active FBP is present.

Folate fortificants and supplements

Folate absorption from pharmaceutical folic acid and (6S)-5-CH₃-H₄folate supplements did not differ significantly, but more folic acid was absorbed as a supplement than from a bread matrix (Table 2). However, as median apparent absorption from folic acid-fortified bread was high at 74% (Table 3), bread is a suitable matrix for folic acid fortification. Data from a previous study (Johansson et al. 2002) showed that folic acid fortification of bread results in a significant improvement of volunteers’ folate status after just 4 weeks of intervention.

The interest of the comparison of the already-established folic acid fortification practice of cereal-based food with alternative fortificants and matrices, e.g. by using as a new candidate fortificant the biologically active form of (6S)-5-CH₃-H₄folate in dairy matrices, or natural yeast folate polyglutamates for ‘bio-fortification’. Folic acid is used as food fortificant because it is inexpensive and relatively stable, but, in contrast to reduced folates, a high intake can delay diagnosis of an underlying vitamin B₁₂ deficiency. Around 80% of all folate ‘fortificants’, yeast polyglutamates from dessert crème (Y), (6S)-5-CH₃-H₄folate from fermented milk without FBP (U) and folic acid from bread, were absorbed (Table 3). Median apparent absorption of yeast folate from the dessert crème in the present study (86%) was much higher than the estimate of folate bioavailability of a yeast drink of 59% in the intervention study of Hannon-Fletcher et al. (2004). Our HPLC method, allowing quantification of four different folate forms, might have led to an underestimation of the total folate content in that particular test food when other folate forms were present. In theory, this could result in an overestimation of folate absorption. As high folate-producing yeast strains could be an alternative for folate enrichment, future investigation of this interesting matrix is warranted.

Critical appraisal of the ileostomy—area under the curve model: limitations and advantages

The model enables the direct estimation, by comparison of AUC—dose-corrected ratios and kinetic models, of the extent of folate absorption after ingestion of different test foods.

Some noise in plasma folate concentrations was visible in the form of minor AUC on day N with no test food application, which may reflect effects from fasting, enterohepatic circulation and ingestion of the standardized low-folate, low-fat meals on plasma folate levels. We decided not to correct for them when interpreting plasma results due to strict standardization of the study protocol (regarding sampling, fasting periods and test food ingestion), as we expect possible confounding effects to be similar on all days. Another possible confounder regarding plasma data is the hepatic first pass effect (Pfeiffer et al. 1997; Rogers et al. 1997). In line with their recommendation, we presaturated, and therefore standardized, volunteers’ body stores. Using the plasma AUC approach (Prinz-Langenohl et al. 1999; Konings et al. 2002), folate absorption from a test food is usually estimated by comparison with an oral reference dose of folic acid; but hereby it is not guaranteed that the oral reference dose is completely absorbed. To overcome this problem, the concept of an intramuscular reference dose was developed (Withthöft et al. 2003), where relative absorbed folate from an oral test dose was estimated using a reference dose of (6S)-5-CH₃-H₄folate administered by intramuscular injection (day I). Using labelled folate compounds, Wright et al. (2003, 2005) observed concurrent displacement of endogenous (unlabelled) liver folates after an oral folate test dose and hypothesized differences in metabolism of oxidized and reduced folates. It was suggested earlier that different folate forms (oxidized compared to reduced) and administration (oral compared to intravenous injection) could result in different handling in the body (Finlas et al. 2002). This may lead to the conclusion that the quantification of absorbed folate from a test food by comparison with any reference dose might be unsuitable when no labelled compounds are used. Therefore, we decided to avoid estimation of relative folate absorption by a reference dose, but rather determine effects of processed and fortified food on folate absorption by direct comparison of AUC—dose-corrected ratios.

Plasma results are complemented by data from ileostomal folate excretion, and estimated absorbed (by AUC) and non-absorbed (by stomal excretion) folate should in theory amount to approximately 100% (Withthöft et al. 2003). Overestimation of total recovery could be caused by overestimating plasma AUC due to a bad curve fit when extrapolating or during kinetic modelling, when estimating the distribution volume V using the factor of 389 ml/kg body weight from Loew et al. (1987), which was estimated after a single intravenous dose of oxidized folic acid of pharmacological magnitude. Underestimation of the model’s overall recovery can mainly be caused by incomplete collection of ileostomal effluent.

The small quantities of 5-CH₃-H₄folate in 10h post-dose urine are in line with earlier findings (Pfeiffer et al. 1997; Withthöft et al. 2003). Thus, the given doses can be considered to be of physiological size and that the kidney threshold was not reached.

In conclusion, this new human model was used to compare folate absorption from differently processed and fortified foods. As each volunteer was randomly participating in the nine strictly standardized study days, intra-individual as well as inter-individual comparison of folate absorption was possible. The presented model would be strengthened by combination with stable-isotope techniques, as differentiation of plasma folate deriving from the exogenous dose and from endogenous body stores is of importance when studying folate absorption and elimination kinetics by AUC.

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References


