

A human model to determine folate bioavailability from food: a pilot study for evaluation

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Abstract

Background: Knowledge about folate bioavailability from food is essential for the estimation of dietary requirements. Yet, there is a lack of data obtained from validated human studies performed with physiological folate doses.

Objective: In this pilot study, a new model for the determination of folate absorption is developed and validated.

Design: Under strictly standardized procedures, two healthy ileostomy volunteers consumed single portions of test foods or an oral dose of a pharmaceutical folate preparation of the natural folate diastereomer (6S)-5-methyltetrahydrofolate. Relative folate absorption from oral doses versus an intramuscular injection of the same pharmaceutical preparation was determined using postdose plasma folate concentration curves. Non-absorbed folate was estimated by postdose folate excretion into stomal effluent.

Results: Estimated by plasma areas under the curve, relative folate absorption ranged from 47 to 67% for oral doses from the test foods strawberries and broccoli and the pharmaceutical (6S)-5-methyltetrahydrofolate preparation. During 10 h postdose, 19–44% of the dietary folate was excreted with the stomal effluent. Varying gut passage times were observed for different food matrices by determining ileostomal folate excretion in 2 h intervals. Around 90% of the folate from the oral doses was recovered in the collected body fluids, plasma and stomal effluent, by 10 h postdose, independent of the size of the administered folate doses of 200 or 400 µg (0.4 or 0.9 µmol).

Conclusion: The results imply that this model provides a suitable tool to estimate folate bioavailability from foods.

Keywords: *Folate absorption; folate in ileostomal effluent; ileostomists; plasma folate kinetics; urinary folate excretion*

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Introduction

An optimal folate status is linked to a diminished risk for neural tube defects and spontaneous abor-

tions (1), low serum homocysteine levels (elevated concentrations are associated with an increased risk of occlusive vascular diseases), some forms of cancer (2) and improved cognitive or mental functions (3). This new concept of health-protective effects is reflected in recent recommendations of the US Food and Nutrition Board (4) and latest editions of several European and the Nordic Nutritional Recommendations (5).

However, information about the extent to which certain foods contribute to the increased folate intake is still incomplete. This emphasizes the need

Abbreviations: 5-HCO-H₄folate, 5-formyl-tetrahydrofolate, 5-CH₃-H₄folate, 5-methyl-tetrahydrofolate, AUC, area under the (plasma concentration) curve, AUC^{0→600}, superscript indicates time range (in minutes), *C*, *C*₂₀, *C*_{max}, concentration, suffix defines time (in minutes) or at maximum, *D*, dose, GC, gas chromatography, HPLC, high-performance liquid chromatography, H₄folate, tetrahydrofolate, *k*, *k*₂₁, *k*₂₂, (biokinetic) elimination constants, MS, mass spectrometry, PteGlu, pteroylglutamic acid, SAX, strong anion exchange, SPE, solid-phase extraction, *t*, *t*₂₀, *t*_{max}, time (-point), suffix defines time (in minutes) or at maximum, *V*, distribution volume in the body

for a critical evaluation of dietary folate sources and of the impact of food-processing techniques on folate content and human folate bioavailability.

Several human models have been developed including long- and short-term protocols to determine either folate absorption or bioavailability. Long-term protocols usually investigate the impact of repeated folate ingestion on folate status, e.g. by feeding fortified foods, natural food folates or folic acid supplements (6, 7). Subsequently, the folate status is often determined by plasma or erythrocyte concentrations; the latter reflects the folate status over the past 3 months. More recent studies also include serum homocysteine (8, 9). However, long-term studies are intensive in respect to both cost and time, and reflect the total diet rather than effects of a single food source.

Short-term protocols often compare a single oral folate dose from a pharmaceutical folate preparation with fortified or natural food. Often, postdose plasma or urinary folate concentrations are determined (10–13). The area under the plasma response curve (AUC) or urinary folate excretion allows the determination of relative folate bioavailability from food versus pharmaceutical preparations. A drawback is that the application of a single folate dose of physiological magnitude can result in a minimal plasma or urine response. Therefore, pharmacological doses are often used that do not reflect physiological conditions (7, 10, 14, 15).

Stable isotope protocols to study folate bioavailability were introduced by Gregory and co-workers (14, 16), who studied bioavailability from isotopically labelled folates in fortified foods or as pharmaceutical preparations using high-performance liquid chromatography (HPLC) and subsequent gas chromatographic–mass spectrometric (GC-MS) techniques. This dual-label oral/intravenous approach has been applied by European researchers to study relative folic acid bioavailability from some cereal grain foods (17). An advantage of the use of stable isotope protocols is that isotopically labelled folate from the dose can be differentiated from endogenous body folate. However, procedures are intensive and costly regarding sample preparation techniques, provision of labelled folates and MS equipment, and therefore not commonly applicable.

A new approach to determine folate bioavailability was used in a study by Konings et al. (18), who monitored apparent folate absorption from spinach in healthy volunteers with ileostomy by the simple

balance of consumed and excreted dietary folate. Konings' study was the first one to compare direct folate absorption from stomal effluent data with relative folate absorption from plasma AUC.

The present pilot study was carried out to develop and evaluate a new human ileostomy model for the determination of folate bioavailability from single test food doses (broccoli and strawberries) versus pharmaceutical folate doses. Using several parameters, the absorbed amount of folate was determined directly and indirectly. Relative folate absorption was estimated using short-term postabsorption plasma kinetics after single oral doses of test food compared with a reference dose of the naturally occurring diastereomer (6S)-5-methyltetrahydrofolate [(6S)-5-CH₃-H₄folate] by intramuscular injection. Folate excretion into urine was evaluated as a parameter for relative folate absorption. Folate excretion into the ileostomal effluent reflects non-absorbed folate, and by collecting individual fractions every 2 h postdose, not only total folate absorption but also a time pattern of intestinal passage can be determined.

Furthermore, the volunteers' compliance to the strictly standardized study protocol with respect to sampling times, dose ingestion and fasting periods was tested before the application of the model in a main trial.

Material and methods

Subjects

Three subjects were recruited, of whom two completed the study. These two were male ileostomists aged 55 and 77 years, and were apparently healthy based on routine haematological and biochemical measurements and a physical examination. They had a body mass index of 26.7 and 27.3 kg m⁻², were non-smokers and did not use any medication or vitamin supplements. Subjects were screened for fasting serum folate (>3.4 nmol l⁻¹), serum cobalamin (>96 pmol l⁻¹) and erythrocyte folate (>215 nmol l⁻¹) concentrations to ensure normal folate and vitamin B₁₂ status. They had undergone proctocolectomy 23 and 25 years earlier as a result of ulcerative colitis; at most, 10–15 cm of the distal ileum had been resected. Neither subject had undergone further surgical procedures since the initial surgery, with establishment of conventional ileostomy. Both had well-established and functioning ileostomies with no signs of inflammation since

the operation. The protocol was approved by the ethics committee of the Umeå University Hospital and was fully explained to the participants before acquiring written informed consent.

Study design

Volunteers participated in four strictly standardized independent study days (each 2–4 weeks apart) in a random order. On three occasions they received either a single oral portion of each test food (strawberries, day S; or broccoli, day B) or a pharmaceutical preparation of (6S)-5-CH₃-H₄folate (day O) after an overnight fast. On one occasion, volunteers received an intramuscular injection of (6S)-5-CH₃-H₄folate (day I) as a reference dose. One volunteer, attended a fifth study day, on which no folate (day N) was given to account for baseline data regarding folate excretion into stomal effluent and the diurnal folate profile in plasma throughout the study day. Each study day was strictly standardized with respect to sampling procedures and times.

As the trial lasted for several months, the volunteers' folate status was standardized to avoid influences from the liver first-pass effect during absorption. Volunteers' folate body stores were saturated by a daily dose of 0.96 mg folic acid from day 9 to day 2 before each study day, similar to procedures described and used by Gregory and co-workers (14, 16, 19).

Preparation of test foods and diets

Test foods were given as single portions; either 400 g strawberries or 300 g steamed broccoli. Strawberries of the cultivar Camorosa were immediately frozen after collection and cleaning. For consumption, they were thawed covered overnight in a refrigerator. The broccoli was a frozen vegetable product (Signum®) available on the Swedish market. A portion of 300 g was microwaved at 590 W for 7.5 min after the addition of 22.5 g water and 0.9 g NaCl. Strawberries and broccoli contained $50.7 \pm 9.4 \mu\text{g } 100 \text{ g}^{-1}$ ($n = 3$) and $64.4 \pm 5.9 \mu\text{g } 100 \text{ g}^{-1}$ ($n = 3$) 5-CH₃-H₄folate, respectively.

A low-folate/low-fat lunch snack (4 h 5 min postdose) and dinner (10 h 15 min postdose) was consumed on every study day. Lunch consisted of 72 g wheatbread (tunnbröd), 20 g margarine (40% fat), 40 g smoked ham (3% fat), 40 g cucumber, 40 g roast beef and 400 g apple juice, providing the volunteers with 611 kcal, 13.6 g fat and 22 μg folate,

calculated by the software MATs 4.05 (2001, MATs den Flexible; Rudans lättdata, Sweden). Dinner consisted of 125 g Falu sausage, 225 g pasta, 15 g ketchup, 5 g mustard, 300 g apple juice, 27 g chocolate cookie and 150 g coffee, providing 894 kcal, 33.5 g fat and 17 μg folate.

Preparation of pharmaceutical doses

(6S)-Ca-5-CH₃-H₄folate (Knoll-BioResearch/BASF Pharma, S Antonio, Switzerland) was produced according to GLP/pharmaceutical standards and tested by the manufacturer for chemical and microbiological purity (according to Ph. Eur. FDA and USP 23; endotoxin concentration $< 0.3 \text{ EE mg}^{-1}$). Aliquots of 1.5 mg (6S)-Ca-5-CH₃-H₄folate (corresponding to 2.55 μmol after correction for Ca and water content) were weighed into amber glass ampoules under sterile conditions and stored at -20°C until use.

The intramuscular solutions were prepared 20 min before injection by dissolving the ampoule content with 3 ml sterile isotonic NaCl solution for injection (Fresenius Kabi, Uppsala, Sweden), and 1 ml (corresponding to 0.87 μmol or 400 μg Ca-5-CH₃-H₄folic acid) was injected intramuscularly through a sterile filter (Millex-GS, non-pyrogen, 0.22 μm ; Millipore, France). The injected volume was determined by weighing the syringe before and after injection (four decimals). Aliquots of the remaining solution were stored at -20°C for 5-CH₃-H₄folate quantitation by HPLC, containing $392.3 \mu\text{g ml}^{-1}$ (853.8 nmol, 0.9978 g injected) and $378.6 \mu\text{g ml}^{-1}$ (823.9 nmol, 0.9826 g injected) for volunteer 1 and 2, respectively. The oral dose was prepared similarly 10 min before consumption by pipetting 1 ml of the (6S)-Ca-5-CH₃-H₄folate injection solutions [of $396.5 \mu\text{g ml}^{-1}$ (862.9 nmol) and $369.7 \mu\text{g ml}^{-1}$ (804.6 nmol)] into a glass of tap water (100 ml).

Collection and treatment of samples

Venous blood samples were collected via an intravenous catheter, inserted according to routine procedures into the arm vein. Blood was collected into 5 ml K₃-EDTA Vacutainers at defined time-points 10 min predose and at 20, 40, 60, 90, 120 min and 3, 4, 6, 8 and 10 h postdose. The samples were stored on ice, excluded from light, and centrifuged at 1500 g for 10 min under cooling within 30 min. The plasma was transferred into 1.5 ml vials and

stored at -20°C in portions of approximately 1 ml until folate analysis.

Subjects collected urine for 10–12 h after the oral dose on spontaneous bladder emptying into individual 500 ml plastic bottles containing 2 g ascorbic acid. The time of collection was noted down. Volume was determined by weighing. After thorough mixing, two aliquots of approximately 50 ml (if available) were stored frozen at -20°C for folate analysis in 125 ml bottles. Overnight collection of urine (12–24 h past intervention) was carried out repeatedly into a single 1 litre bottle containing 10 g ascorbic acid, and samples were treated as described above.

Ileostomy bags were changed every 2 h postdose over a period of 10–12 h. Exact time-points of bag change were noted. Locked bags were immediately frozen on dry ice. The content was quantified by weighing. During postdose overnight collection bags were changed at the volunteers' convenience, preferably as often as possible. Bags were stored frozen at -20°C until analysis.

Chemicals and standards

Tetrahydrofolic acid [(6R,S)-H₄folate, trihydrochloride salt], 5-methyltetrahydrofolic acid [(6R,S)-5-CH₃-H₄folate, calcium salt] and 5-formyltetrahydrofolic acid (6-R,S)-5-HCO-H₄folate, calcium salt were obtained from Dr Schircks Laboratories (Jona, Switzerland). Purity of standards was controlled by molar extinction as described by van den Berg et al. (20). All chemicals were of HPLC quality or p.a. grade and purchased from E. Merck (Darmstadt, Germany) or Sigma Chemical Co. (St Louis, MO, USA). Hog kidney deconjugase was prepared from 10 g hog kidney acetone powder (Sigma) and dialysed overnight (21). The dialysate was stored in portions at -20°C until use. Bovine folate binding protein (cat. no. F0524) was purchased from Scripps Laboratories (San Diego, CA, USA), and agarose affinity gel 10 and poly-prep chromatography columns from BioRad Laboratories (Richmond, USA). Deionized water was of Milli-Q grade.

Sample pretreatment

Plasma samples were thawed in the dark and centrifuged for 5 min at 10 000 rpm (Eppendorf 4517C). Samples were purified by solid-phase extraction (SPE) using strong anion exchange (SAX) columns (LiChrolut 200 mg, Merck) and a

vacuum manifold. Columns were prepared by flushing with 2.5 ml of methanol and 2.5 ml of water; 0.5 ml of plasma was applied. The column was washed with 2 ml deionized water and eluted with 2.5 ml 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) mercaptoethanol. The eluted volume was quantified by weighing. Samples were transferred into amber autosampler vials, overlaid with nitrogen, loaded onto the cooled autosampler (8°C) and analysed within 12 h.

Ileostomy samples were thawed overnight at 5°C , sheltered from light, in the original ileostomy bags and homogenized for 120 s with an Ultra-Turrax T25. Aliquots of 4 g were added to 20 ml 0.1 M potassium phosphate buffer, pH 6.0, 2.0% (w/v) ascorbic acid and 0.1% (v/v) mercaptoethanol in screw-capped vials under nitrogen atmosphere. Strawberries were crunched in a bag while half-frozen, and 20 g mashed material was added 1:1 (w/w) to the phosphate buffer and homogenized for 60 s. Then, 4 g of the homogenate was added to 20 ml phosphate buffer in screw-capped vials. Broccoli was thawed and roughly cut, and 15 g was homogenized with 30 g phosphate buffer for 120 s in an extraction vial. All samples were extracted under nitrogen atmosphere for 12 min in a boiling water bath, then cooled on ice in the dark to room temperature, and the pH was adjusted to 4.9 with 1.5 M phosphoric acid. Hog kidney deconjugase preparation was added (2 ml to ileostomy samples, 3 ml to food samples), and the samples were overlaid with nitrogen and incubated for 3 h at 37°C in a shaking water bath. Samples were heated for 5 min in a boiling water bath to deactivate the enzyme, cooled on ice and centrifuged at 15 000 g and 5°C . The supernatant was collected and the pellet resuspended in approximately 10 ml 0.1 M potassium phosphate buffer, pH 6.0, 0.1% (v/v) mercaptoethanol and centrifuged as above. Supernatants were combined, made up to a volume of 50 ml, mixed and stored as subsamples at -20°C until purification.

Extracts from food samples were purified by SPE using SAX columns (Isolute 500 mg, International Sorbent Technology) and an elution buffer of 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (v/v) mercaptoethanol according to Jastrebova et al. (21). When purifying ileostomy samples the volumes were modified: 1.0 ml sample was applied to SAX,

a first portion of 0.5 ml was discarded after elution and a second portion of 3 ml was collected and quantified by weighing (three decimals). Samples were transferred into amber vials, overlaid with nitrogen, loaded onto the cooled autosampler (8°C) and analysed within 12 h.

Aliquots of the remaining (6S)-Ca-5-CH₃-H₄folate solutions in isotonic NaCl (for injection and oral application) were thawed and diluted 1:1000 into 10 ml 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid and 0.1% (w/v) mercaptoethanol. Aliquots were transferred into amber vials, overlaid with nitrogen, loaded onto the cooled autosampler (8°C) and quantified within 12 h by HPLC.

To 55 ml urine, 5.5 ml of 0.5 M potassium phosphate buffer, pH 7.0, was added; if necessary, the pH was adjusted to pH 7.0 with 1 M NaOH. The samples were left for 30 min at room temperature, sheltered from light, for salt precipitation and then filtered (Munktell V120H).

Affinity chromatography columns containing 0.5 mg folate binding protein (Scripps, USA) in 2 ml agarose gel per column were prepared according to Konings (22). For urine purification, the columns were equilibrated with 5–10 ml 0.1 M K₂HPO₄, pH 7. Desalted urine (20–40 ml) was loaded onto each affinity column. The columns were washed with 5 ml 0.05 M K₂HPO₄, pH 7. Samples were eluted with 10 ml 0.02 M trifluoroacetic acid/0.01 M dithiothreitol into a volumetric flask containing 60 µl 1M piperazine and 0.2% Na ascorbate (200 µl of a freshly prepared 10% (w/v) Na ascorbate solution) according to Karilouto et al. (23). Samples were transferred into amber vials, loaded onto the cooled autosampler (8°C) and analysed within 12 h by HPLC. Affinity columns were washed with 15 ml 0.1 N HCl and re-equilibrated with 15–20 ml 0.1 M K₂HPO₄, pH 7.

Folate quantitation

Test foods, pharmaceutical folate preparations and human samples were quantified by HPLC as described by Jastrebova et al. (21) on a HP 1100 series system equipped with a gradient quaternary pump, a cooled autosampler (8°C), a thermostatically controlled column compartment (23°C), a diode array and a fluorescence detector. The system was controlled by Chemstations software (Rev A.07.01 [682] Hewlett-Packard 1990–1995). The analytical column was Zorbax SB C8, 150 × 4.6, 5

µm (Agilent Technologies). The mobile phase consisted of 0.03 M phosphate buffer, pH 2.3, using a linear gradient with acetonitrile starting at 6% with a lag time of 5 min, rising to 25% within 20 min. The flow rate was 0.4 ml min⁻¹ with a total run time of 33 min. The fluorescence detector (FL) was set to ex/em = 290/356 nm and the diode-array detector to 290 nm. External calibration was carried out using the standards H₄folate, 5-CH₃-H₄folate and 5-HCO-H₄folate (Schircks, Jona, Switzerland) by the FL-detector. Peak identity was confirmed by comparison of retention time and relative peak area in both detectors. The limits of quantification were 0.5 ng ml⁻¹ for H₄folate, 0.3 ng ml⁻¹ for 5-CH₃-H₄folate and 4 ng ml⁻¹ for 5-HCO-H₄folate.

Sample preparation and folate quantitation of stomal effluent (four replicates) resulted in coefficients of variation of 6.8% for H₄folate and 11.4% for 5-CH₃-H₄folate (no 5-HCO-H₄folate detected). Addition of 6–1300 ng 5-CH₃-H₄folate per 1 g stomal effluent (equal to 15–100% of initial 5-CH₃-H₄folate concentrations in sample) resulted in recoveries of 84–105%. Addition of 30–360 ng H₄folate per 1 g stomal effluent (equal to 50–100% of initial H₄folate concentration in sample) resulted in recoveries of 77–91%. The coefficient of variation (CV) of plasma purification and quantitation was 6.9% (*n* = 4) for 5-CH₃-H₄folate. Addition of 12.5 and 20 ng ml⁻¹ 5-CH₃-H₄folate to plasma resulted in recoveries of 94 and 86%, respectively.

Spiking of urine samples with 0.5–1 ng ml⁻¹ 5-CH₃-H₄folate (34–67% of initial folate concentration) before desalting and affinity purification resulted in recoveries of 92–111%. A 5-CH₃-H₄folate solution [1 ng ml⁻¹, in 1.0% Na ascorbate (w/v), pH 7.0] was included in every batch of samples for affinity chromatography, and applied systematically onto each column in turn, resulting in a CV of 4.5% (*n* = 18). 5-CH₃-H₄folate concentrations in a urine sample, desalted, purified and analysed in triplicate, resulted in a CV of 6.8%. Four urine samples, prepared with one sample each on 2 days, varied from 1.7 to 10.2%.

Kinetic calculations

Non-absorbed folate from the oral dose was estimated by quantification of total folate excretion into stomal effluent over time. To express the net increase in plasma folate above baseline concentrations, the baseline (predose) folate concentration (*C*₀) was subtracted from each postdose folate

concentration ($C_{20} - C_{600}$). The (positive) AUC was calculated according to:

$$\text{AUC}^{1 \rightarrow T} (\text{h} * \text{ng ml}^{-1}) = \{[(C_0 + C_1)/2 * (t_1 - t_0)] \\ + [(C_1 + C_2)/2 * (t_2 - t_1)] + \dots \\ + [(C_{T-1} + C_T)/2 * (t_T - t_{T-1})]\} \quad (1)$$

where $C_T - C_{T-1} \geq 0$.

When folate concentrations at the last blood sampling (C_{600}) were above baseline concentrations (C_0), the theoretical AUC ($\text{AUC}^{600 \rightarrow \infty}$) was estimated by extrapolation:

$$\text{AUC}^{600 \rightarrow \infty} = C_{600}/k_{\text{el}} \quad (2)$$

where the elimination constant was estimated as (24):

$$k_{\text{el}} = -(dc/dt) = -[(C_{600} - C_{480})/(t_{600} - t_{480})]. \quad (3)$$

The relative amount of absorbed folate from single oral doses compared with (6S)-5-CH₃-H₄folic acid administered by intramuscular injection was calculated individually for each volunteer using the (positive) AUC by the equation:

$$\% \text{ folate}_{\text{absorbed}} = [(\text{AUC}_{\text{Testfood}}/\text{Dose}_{\text{Testfood}})/ \\ (\text{AUC}_{\text{Injection}}/\text{Dose}_{\text{Injection}})] \\ \times 100. \quad (4)$$

Calculations were done using Microsoft Excel 97.SR.

Results

Plasma folate concentrations and area under the curve

The volunteers' predose 5-CH₃-H₄folate plasma concentrations on individual study days did not differ throughout the 6 month study period. Mean concentrations were $3.6 \pm 0.4 \text{ ng ml}^{-1}$ ($7.8 \pm 0.9 \text{ nmol l}^{-1}$, CV = 11%) and $7.3 \pm 0.7 \text{ ng ml}^{-1}$ ($15.9 \pm 1.5 \text{ nmol l}^{-1}$, CV = 9%) for volunteer 1 and 2, respectively. Only 5-CH₃-H₄folate was detected in plasma. After application of 5-CH₃-H₄folate from pharmaceutical preparations (orally or by intramuscular injection) and from test foods, plasma concentrations increased at C_{max} to $2.5 - 14.9 \text{ ng ml}^{-1}$ ($5.4 - 32.4 \text{ nmol l}^{-1}$) above predose concentrations (Table 1). Plasma kinetic parameters from individual study days for both volunteers are summarized in Table 1. Individual folate plasma profiles are shown in Fig. 1, indicating a longer intestinal passage for complex food matrices than for pharmaceutical preparations, shown by a delayed t_{max} .

After no folate application (day N), plasma 5-CH₃-H₄folate concentrations (volunteer 1) remained at $4.0 \pm 0.3 \text{ ng ml}^{-1}$ ($8.7 \pm 0.7 \text{ nmol l}^{-1}$, CV = 6.8%, $n = 11$) from $t = 0 \text{ min}$ to $t = 600 \text{ min}$, and did not rise visibly under strictly standardized procedures, including a low-folate/low-fat lunch snack.

For technical reasons, blood collection had to be finalized after 600 min. For both volunteers, plasma folate levels decreased mostly to predose concentrations 8–10 h after ingestion of test food. After administration of pharmaceutical preparations (both orally and by intramuscular injection), which were twice as high as food samples, final plasma folate concentrations were still increased by $0.9 - 3.0 \text{ ng ml}^{-1}$ ($2.0 - 6.5 \text{ nmol l}^{-1}$) above predose concentrations. In these cases, the theoretical $\text{AUC}^{0 \rightarrow \infty}$ was estimated by extrapolation, comprising 7–11% of the total AUC (Table 1). Relative bioavailability for test foods and pharmaceutical preparations, calculated by extrapolated AUCs, ranged from 47 to 64% and from 53 to 67% for volunteer 1 and 2, respectively.

Ileal folate excretion

Folate forms detected in stomal effluent were 5-CH₃-H₄folate and H₄folate. In Table 2, 5-CH₃-H₄folate excretion into stomal effluent is shown for 10 h, 12 h and 24 h postdose for each volunteer and test day. On the injection day I and day N, no 5-CH₃-H₄folate was administered via the intestine. Only $2 - 5 \text{ } \mu\text{g}$ ($4 - 11 \text{ nmol}$) 5-CH₃-H₄folate was found in stomal effluent over 10–12 h postdose during the strictly standardized study day. 5-CH₃-H₄folate excretion on these occasions was similar for both volunteers. After application of oral folate doses, however, stomal 5-CH₃-H₄folate excretion increased, ranging from $40 - 150 \text{ } \mu\text{g}$ ($87 - 325 \text{ nmol}$) 10 h^{-1} to $55 - 160 \text{ } \mu\text{g}$ ($120 - 350 \text{ nmol l}^{-1}$) 24 h^{-1} postdose. 5-CH₃-H₄folate excreted with stomal effluent 10 h postdose corresponded to about 20–45% of the oral doses. Fig. 2 shows the 5-CH₃-H₄folate for both volunteers in five subsequent 2 h fractions of stomal effluent, indicating a longer intestinal passage time of food samples than pharmaceutical preparations by delayed release of 5-CH₃-H₄folate into stomal effluent.

Approximately $1 - 2 \text{ } \mu\text{g}$ ($2.2 - 4.5 \text{ nmol}$) H₄folate per hour was excreted with the stomal effluent, amounting to $10 \text{ } \mu\text{g}$ (22.5 nmol) 10 h^{-1} to $35 \text{ } \mu\text{g}$ (78.6 nmol) 24 h^{-1} . No H₄folate was given with the

Table 1. Kinetic parameters of 5-CH₃-H₄folate in plasma after single doses of test food or pharmaceutical 5-CH₃-H₄folate preparations

Parameter	Volunteer 1					Volunteer 2			
	N	I	O	B	S	I	O	B	S
Study day:									
Folate dose (µg) ^a	0	391	397	193	200	372	370	193	202
C ₀ (ng ml ⁻¹) ^b	3.8	3.5	3.0	3.8	4.1	6.8	7.9	6.7	7.9
C _{max} (ng ml ⁻¹) ^c	4.4	18.3	7.1	6.2	7.0	21.3	14.1	10.5	12.6
ΔC (ng ml ⁻¹) ^d	0.6	14.9	4.1	2.5	2.9	14.5	6.1	3.8	4.7
t _{max} (min)	240	22	40	244	60	60	47	242	242
C ₆₀₀ (ng ml ⁻¹)	4.0	4.3	4.0	3.7	4.6	8.1	9.0	7.4	6.5
AUC ^{0→600} (h * ng ml ⁻¹) ^e	1.8	47.7	20.3	11.9	14.4	64.2	39.9	18.3	22.1
AUC ^{0→∞} (h * ng ml ⁻¹)	–	51.3	24.7	–	16.9	66.0	43.9	–	–
Rel. absorption (%) ^f	–	–	47	47	64	–	67	53	62

Random order of individual study days for each subject at intervals of 2–4 weeks: N: no folate application; O: oral application of (6S)-5-CH₃-H₄folate with 100 ml tap water; I: intramuscular injection of (6S)-5-CH₃-H₄folate; B: ingestion of 300 g steamed broccoli; S: ingestion of 400 g thawed strawberries.

^aCalculated as free (6S)-5-CH₃-H₄folic acid.

^bC₀: predose plasma concentration; C₆₀₀: plasma concentration 600 min postdose (subscript defines time of blood taking in minutes).

^cC_{max}: maximum plasma concentration; t_{max}: time (min) of max plasma concentration.

^dMaximum increase calculated as C_{max} – C₀, concentration at t_{max}.

^eAUC: positive area under the concentration curve above basal 5-CH₃-H₄folate concentration at t = 0 before dose; AUC^{0→600} from t = 0 to t = 600 min; AUC^{0→∞} extrapolated as described in Material and Methods.

^fRelative absorption (%) = [(AUC_{Testfood}/Dose_{Testfood})/(AUC_{Reference}/Dose_{Reference})] × 100 (after extrapolation of AUC).

test doses. H₄folate excretion was constant over time and similar for both volunteers (data not shown).

Urinary folate excretion

Small quantities of 5-CH₃-H₄folate were quantified in urine (Table 2). Excreted urinary folate during 12 h or 24 h postdose comprised <1–3% of the ingested dose. For volunteer 1, 5-CH₃-H₄folate excretion over 12 h postdose was higher on days with folate application (days I, O, B, S) than on day N, but this pattern was not observed in 24 h urine. The individual fractions of urine collected 0–12 h postdose contained variable amounts of folate without any time-dependent pattern (data not shown). For example, the total amount of 3.7 µg (8.1 nmol) 5-CH₃-H₄folate excreted by volunteer 2 on day I derived from six fractions containing 0.1–1.5 µg (0.2–3.3 nmol) each. A total of 2.1 µg (4.6 nmol) excreted on day S derived from six fractions containing 0.1–0.7 µg (0.2–1.5 nmol) each (data not shown). Tentatively, for 12 h postdose, urinary 5-CH₃-H₄folate excretion followed the order I > O > B = S. Urinary folate excreted overnight (12–24 h postdose) was constant, ranging from 1.1 to 1.3 µg (2.4–2.8 nmol) and from 2.1 to 2.9 µg (4.6–6.3 nmol) for volunteers 1 and 2, respectively. For both volunteers, no clear dose-related 5-CH₃-H₄folate excretion was observed.

Relative and absolute folate absorption

Agreement between findings on relative folate absorption based on plasma AUC and non-absorbed folate into ileostomal effluent can be assessed by determination of the mean folate recovery of the human model, which amounts to approximately 90% over 10 h postdose without consideration of the minimal urinary folate excretion. Recovered folate from administered doses amounted to 85, 87 and 95% for volunteer 1 and 86, 73 and 106% for volunteer 2 on test days O, B and S, based on data from extrapolated plasma AUCs (Table 1) and folate excretion into stomal effluent 10 h postdose (Table 2).

Discussion

This new human model was developed and evaluated for the determination of folate bioavailability from food. Relative folate absorption was estimated by plasma AUCs after ingestion of a test food versus a reference dose. In contrast to other studies, in which an oral dose of a folic acid pharmaceutical was given as a reference dose (11, 18), the biological form (6S)-5-CH₃-H₄folate, which is given by intramuscular injection, was chosen. Volunteers' body stores were standardized by saturation before each test day to minimize the hepatic first-pass uptake affecting the plasma response. Folate excretion into stomal effluent was used as parameter to assess

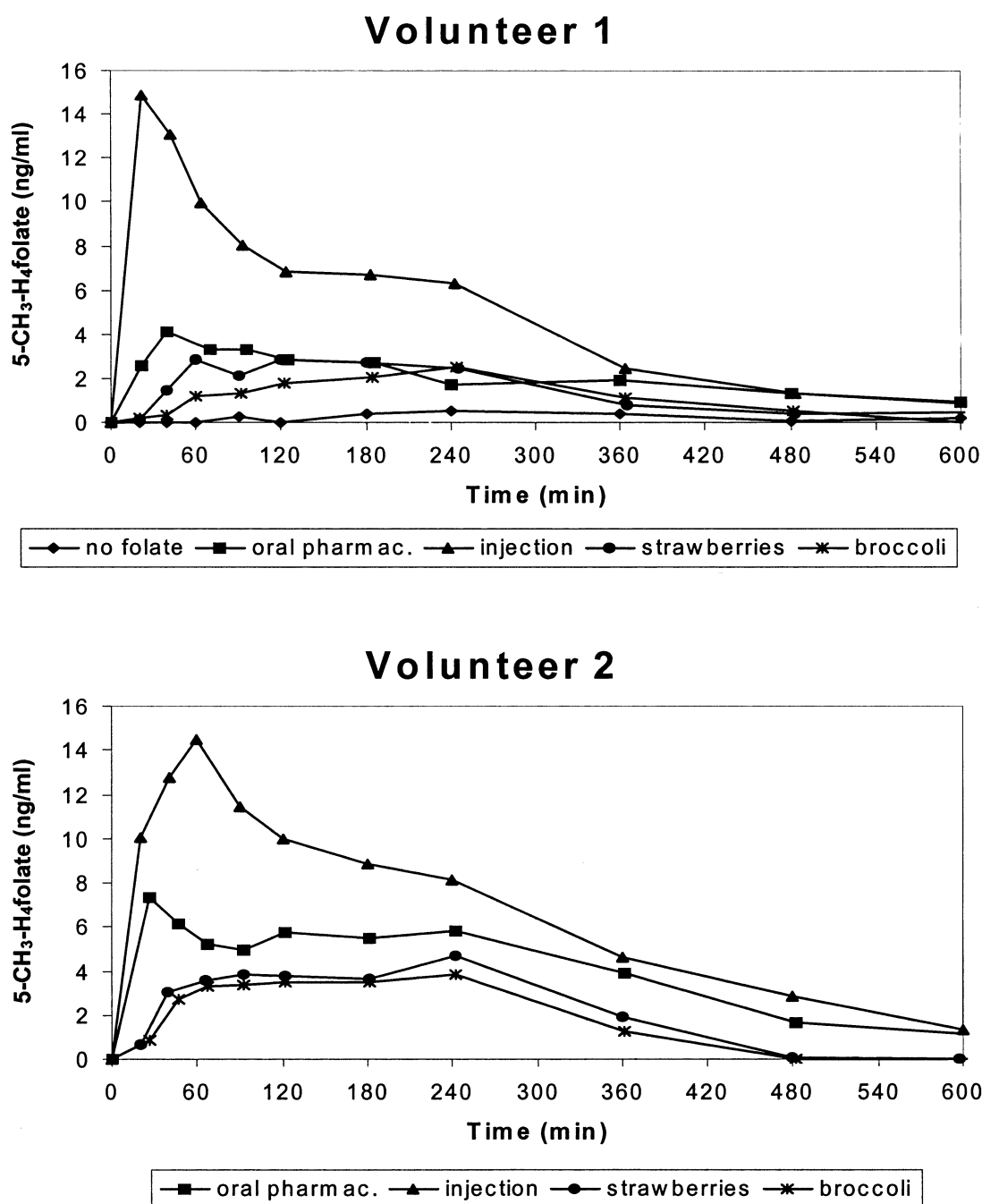


Fig. 1. 5-CH₃-H₄folate plasma concentration curves after single folate doses of test food and pharmaceutical 5-CH₃-H₄folate preparations. Random order of individual study days for each subject at intervals of 2–4 weeks; oral application of (6S)-5-CH₃-H₄folate with 100 ml tap water, intramuscular injection of (6S)-5-CH₃-H₄folate; ingestion of 300 g steamed broccoli, ingestion of 400 g thawed strawberries; 5-CH₃-H₄folate concentrations are given as $C - C_0$ (after subtraction of basal predose 5-CH₃-H₄folate concentration).

absolute folate absorption from given doses. By introducing one study day without folate application, basic ileal folate excretion from bile was accounted for.

Estimation of the AUC, which is appropriate for pharmacokinetic studies involving drugs, is not

perfectly suited to studying the bioavailability of nutrients, which are present in the circulating blood and stored in the body. Nevertheless, this model is used in many studies on folate bioavailability (7, 10, 11). Scott et al. (15) pointed out that the hepatic first-pass effect and enterohepatic circulation are

Table 2. Excretion of 5-CH₃-H₄folate in stomal effluent and urine after single doses of test food or pharmaceutical 5-CH₃-H₄folate preparations

Parameter	Volunteer 1					Volunteer 2			
	N	I	O	B	S	I	O	B	S
Study day:									
Folate dose (μg) ^a	0	391	397	193	200	372	370	193	202
Ileostomal effluent									
0–10 h postdose (μg)	5	2	150	78	62	3	69	39	88
0–10 h excreted as % of intake	–	–	38	40	31	–	19	20	44
0–12 h postdose (μg)	^b	3	151	80	64	5	80	42	91
0–24 h postdose (μg)	49	7	161	85	82	26	108	54	102
urinary folate									
0–12 h postdose (μg)	0.9	10.4	2.0	1.2	1.1	3.7	2.9	2.1	2.2
12–24 h postdose (μg)	1.2	1.1	1.3	1.3	1.3	2.8	2.9	2.3	2.1
0–24 h postdose (μg)	2.2	11.5	3.4	2.5	2.5	6.5	5.7	4.3	4.3
Total excreted as% of intake	–	3	1	1	1	2	2	2	2

Random order of individual study days for each subject at intervals of 2–4 weeks: N: no folate application; O: oral application of (6S)-5-CH₃-H₄folate with 100 ml tap water; I: intramuscular injection of (6S)-5-CH₃-H₄folate; B: ingestion of 300 g steamed broccoli; S: ingestion of 400 g thawed strawberries.

^aCalculated as free (6S)-5-CH₃-H₄folic acid.

^b12 h sample accidentally not collected, sample included in pooled night sample.

affected by the folate status of body stores, which could result in variable plasma responses. In the present study, volunteers' folate status was standardized and hepatic first-pass folate uptake was minimized by saturating the body stores with a daily dose of a commercial folic acid supplement according to procedures described by Gregory's group (14, 19). Folic acid presaturation was completed 2 days before each study day to avoid interference from bile containing folate derived from the saturation dose.

The enterohepatic circulation results in overestimation of absorbed folate using plasma AUC when biliary folate is completely reabsorbed, or results in overestimation of non-absorbed folate in stomal effluent when reabsorption is incomplete. Information on biliary folate excretion is inconsistent. This study standardized for possible effects from biliary folate excretion by strictly standardizing ingestion of test foods, collection of samples and consumption of a low-fat/low-folate snack at defined times. Volunteers were not allowed to consume coffee or tea until 10 h postdose. Data from study days N and I, when no oral folate dose was administered, could be carefully interpreted as baseline data regarding effects from the enterohepatic circulation. The minimal AUC on day N after no folate administration (<5% of the AUC from day I, volunteer 1) indicates that neither bile folate nor the low-folate/low-fat snack at lunch influenced postdose plasma folate concentrations remarkably. It is recom-

mended that an N day is included for each volunteer to account for possible interindividual variation in bile excretion.

The effects of the size of the test dose on plasma response were assessed, considering 200 and 400 μg (435 and 870 nmol) folate as moderate to large physiological doses. Both doses resulted in increased folate concentrations in plasma, urine and ileostomal effluent (Tables 1 and 2). Postdose plasma AUCs increased 6-fold to more than 20-fold compared with the small AUC after no folate administration (day N, volunteer 1).

The chosen number and time-points of blood collection allowed kinetic interpretation of plasma curves, indicating a similar pattern for both volunteers regarding gut passage and folate absorption. Gut passage time increased for pharmaceutical 5-CH₃-H₄folate < strawberries < broccoli, as interpreted from increasing *t*_{max} (Fig. 1). Pharmaceutical preparations of 5-CH₃-H₄folate administered by intramuscular injection resulted in greater AUCs for both volunteers than all oral doses, which could be accounted for by incomplete intestinal absorption and/or possibly folate destruction during gut passage. When estimating relative folate absorption from test meals by AUC, it is important to refer to the AUC of a controlled (known) amount of ingested pharmaceutical. This is guaranteed by the application of a reference dose by injection, where no confounding influences can reduce the resulting AUC size. Konings et al. (18) and others (11) used

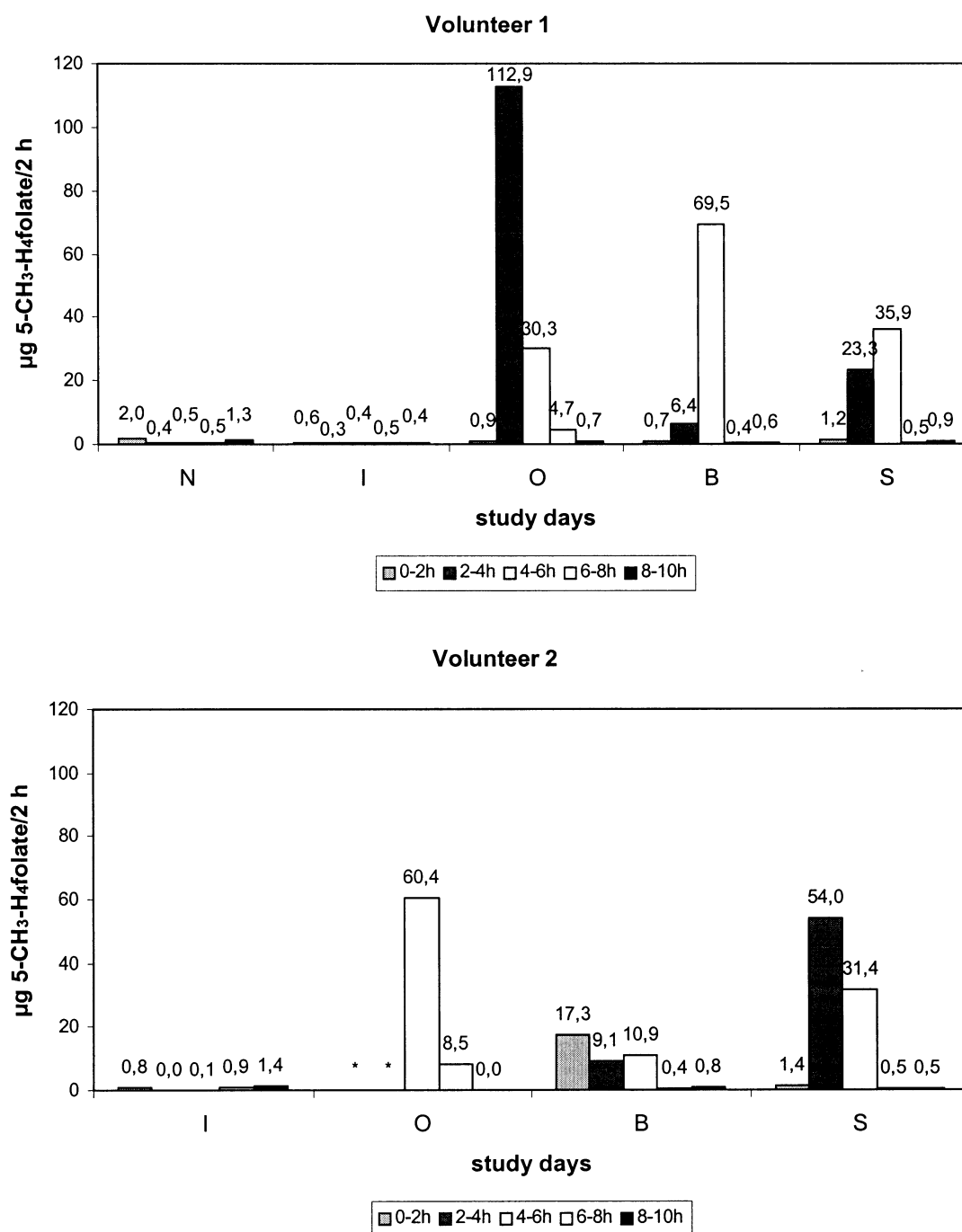


Fig. 2. 5-CH₃-H₄folate concentrations into stomal effluent over time after single doses of test food or pharmaceutical 5-CH₃-H₄folate preparations. Random order of individual study days for each subject at intervals of 2–4 weeks; N: no folate application; O: oral application of (6S)-5-CH₃-H₄folate with 100 ml tap water; I: intramuscular injection of (6S)-5-CH₃-H₄folate; B: ingestion of 300 g steamed broccoli; S: ingestion of 400 g thawed strawberries. * Volunteer 2/day O: three samples collected at 2 h intervals but pooled for analysis owing to low content of stomal effluent in bag.

an oral pharmaceutical dose of folic acid as a reference dose. That might result in overestimation of relative folate absorption from food samples, as it is not possible to account for underestimation of the

reference AUC caused by destruction of the pharmaceutical folate dose during gut passage.

In dual-label stable isotope trials, usually a bolus intravenous injection of folic acid is usually pre-

ferred as reference dose (7, 14, 17). In this study the reference dose was administered by intramuscular injection, because with a given number and time-points of blood sampling the assessment of the plasma concentration curve is more accurate after an intramuscular than an intravenous injection owing to a slower folate invasion into the plasma compartment. Furthermore, this study used a new approach, using as a reference substance the biological folate form (6S)-5-CH₃-H₄folate, which is dominantly present in food, to ensure similar handling by the body.

Volunteers' absolute and relative plasma AUCs were different in size; this may be carefully interpreted as interindividual variation. High interindividual variation in AUCs was observed by Konings et al. (18) (variation of individual AUCs on 3 test days resulted in CVs between 75 and 90%, $n = 11$) and Prinz-Langenohl et al. (11) (variation in individual AUCs on 3 test days resulted in CVs between 34 and 47%, $n = 20$). Because only two volunteers completed the current study, it is not possible to analyse the results statistically.

Usually, especially after administration of the smaller folate dose (200 µg, 435 nmol), plasma folate concentrations returned to baseline levels 8–10 h postdose, indicating the completeness of plasma collection. However, plasma folate curves can be extrapolated to estimate the total AUC if initial concentrations are not quite reached. It is desirable to keep the period of postdose blood collection and fasting as short as possible, to increase compliance by the volunteers.

Using dual-label stable isotope protocols, urinary isotope excretion ratios are often chosen as criteria of absorption, requiring either large folate doses exceeding the threshold of renal absorption or saturation of body tissues (7, 15) to improve uniformity among subjects. After application of labelled folic acid from fortified foods and intravenous injections, only 1–3% of the dose was found as intact folate in 24 h urine (14), while major excretion appears via metabolites. According to the present results (Table 2), intact 5-CH₃-H₄folate amounting to 1–3% of the administered doses was excreted into 24 h urine. Pfeiffer et al. (14) reported that after ingestion of folic acid 4–13% of the urinary folate was excreted in the form of folic acid and not as 5-CH₃-H₄folate, the only folate form present in predose urine. Folic acid was not given in the present pilot trial. The parameter urinary folate

excretion was considered to be limited using this study design (without isotopic labelling), possibly providing qualitative rather than quantitative information.

The ileostomy model to study nutrient digestion and absorption was introduced by Sandberg et al. (25). It offers a method for direct and quantitative determination of small bowel excretion, providing that the influence of bacteria on the effluent can be minimized. In the current study, both volunteers had a very low intestinal bacterial activity according to an earlier study (data not shown). Since this study used a strictly standardized protocol for effluent collection, in which the bag was changed every 2 h with immediate freezing on dry ice, it may be assumed that no interference occurred from minor bacterial activity.

So far, only one folate ileostomy trial has been published (18), indicating a high folate absorption between 73 and 91% from two spinach meals and an oral dose of folic acid based on 24 h postdose stomal folate excretion. Volunteers showed a remarkably high interindividual variation, as expressed by CVs of 60–90% ($n = 11$) for individual ileostomal folate excretion on the same test days. Variable folate excretion was observed for both volunteers (Table 2), and folate absorption from the test foods estimated from 10 h postdose ileostomal effluent excretion was between 55 and 80%. In agreement with Konings et al. (18), the main folate form detected in stomal effluent samples was 5-CH₃-H₄folate after ingestion of test foods containing this vitamer as the dominant folate form.

Collection of individual 2 h effluent samples, a procedure newly introduced by this model, demonstrates that a 10–12 h effluent collection under standardized conditions may be sufficient to estimate non-absorbed folate (Fig. 2). Furthermore, matrix-dependent differences in gut passage time are indicated for pharmaceutical preparations and test foods, as seen by delayed maximal folate excretion.

Based on the results of this pilot study, a 10–12 h collection period of individual ileostomy samples is recommended, including for each volunteer a baseline day with no folate application (N day) to account for individual basal folate excretion. Effluent samples from the N and I days, both after no oral folate application, demonstrated that neither the intake of the low-folate snack nor periods of fasting remarkably increased stomal folate excretion

during the standardized 10–12 h postdose period (Table 2), as supposed by Konings et al. (18).

Reports regarding age-related effects on folate absorption are inconsistent, partly owing to trials using inadequate test doses exceeding physiological test doses, as reviewed by Gregory (26). However, using an intestinal perfusion technique to study luminal disappearance and subsequent urinary recovery of radiolabelled folate compounds, Bailey et al. (27) showed that the absorption of monoglutamyl and polyglutamyl folates was equivalent in young and elderly volunteers. Owing to both a lack of recent studies showing altered absorption with age, and the good health status of the volunteers, there was no apparent reason to exclude one volunteer, aged 77 years, as no age range for exclusion could be defined.

In summary, the human model presented is a suitable tool to study folate absorption from foods, using stomal folate excretion and plasma AUC to determine absolute and relative folate absorption. The new study design allows correction for inter-individual variation by inclusion of a day (N day) accounting for basic plasma and stomal effluent data. A new approach is the use of the biological form of (6S)-5-CH₃-H₄folate as a reference dose, given by intramuscular injection. Folate determination in individual 2 h effluent samples postdose gives information on the gut passage times of test foods. The volunteers complied well with the strictly standardized study design. The intervals chosen for taking samples, as well as the portion sizes of the test foods, aiming for a folate dose of approximately 200 µg (435 nmol), seemed appropriate. This model will be used to determine folate absorption from various processed foods and from pharmaceutical folate preparations using a greater number of volunteers to enable statistical and biokinetic interpretation of the data.

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