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**ORIGINAL ARTICLE**

# Relative bioavailability of 13C5-folic acid in pectin-coated folate fortified rice in humans using stable isotope techniques

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**BACKGROUND/OBJECTIVES:** The aim of the study was to measure the relative bioavailability of labeled pteroylglutamic acid (13C5-PteGlu) from a pectin-coated fortified rice *in vivo* to measure any effect of the edible coating on folic acid bioavailability.
**SUBJECTS/METHODS:** Healthy volunteers ($N = 26$) aged 18–39 years received three test meals in three randomized short-term cross-over trials: Trial 1: aqueous 400 µg 13C5-PteGlu, Trial 2: 200 g cooked white rice+400 µg 13C5-PteGlu,Trial 3: 200 g fortified cooked white rice with pectin-coated premix containing 400 µg 13C5-PteGlu. Blood samples were drawn at 0,1,2,5 and 8 h postprandial. The concentration of 13C5-5 methyl-tetrahydrofolate appearing in plasma was quantified using high performance liquid chromatography–mass spectrometry (MS)/MS. For 24 h before baseline estimation and during the area under the curve (AUC) study, the subjects were placed on a low folate diet (~100 µg/day). The relative bioavailability of the folic acid following Trial 3 was measured by comparing the 13C5-5 methyl-tetrahydrofuran (THF) AUC with Trials 1 and 2.
**RESULTS:** The bioavailability of folic acid in a pectin-coated rice premix was 68.7% (range 47–105) and 86.5% (range 65–115) in uncoated fortified rice relative to aqueous folic acid.
**CONCLUSION:** This study is the first demonstration of the bioavailability of folate in pectin-coated fortified rice in humans.

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## INTRODUCTION

Australia implemented mandatory fortification of bread flour in September 2009 in order to increase the folate status of childbearing women and reduce the risk of neural tube defect affected pregnancy.[1] Other cereal foods namely pasta, savoury biscuits, ready-to-eat breakfast cereals, rice as well as fruits/vegetable juices are voluntarily fortified. Rice, however, is difficult to fortify as it is prepared and eaten as an intact grain. Additionally, grain washing and drainage can significantly reduce the folic acid content of fortified rice (>90% losses have been shown).[2] It has been demonstrated that pectin coating protects the folic acid content in fortified rice (~10 and 70% washing/cooking losses, respectively) compared with non-coated rice (~50 and 90% washing/cooking losses, respectively).[2] Pectin is suitable as it is water-soluble, inexpensive and common in fruits and vegetables. However, it is not known whether the pectin coat significantly affects the bioavailability of the folic acid.

Stable isotope techniques make it possible to detect and quantify low concentrations of specifically labeled folate in human plasma derived from physiological doses of aqueous folate (200–400 µg).[3–6] Equivalence has been demonstrated between pteroylglutamic acid (PteGlu), $^{13}C_5$-PteGlu, $^{2}H_2$-PteGlu and $^{2}H_4$-PteGlu[7–11] provided the test and reference dose use the same route of administration.[7,12–15]

This paper outlines a bioavailability study designed to investigate short-term absorption of $^{13}C_5$-PteGlu from fortified pectin-coated rice, using the area under the time—plasma concentration curve (area under the curve (AUC)) relative to an equivalent $^{13}C_5$-PteGlu dose in water and in uncoated fortified rice. The levels of plasma $^{13}C_5$-5-methyl-THF were quantified using liquid chromatography-tandem mass spectrometry (liquid chromatography (LC)–MS/MS).

## SUBJECTS AND METHODS

### Subjects

The appropriate sample size, calculated from the statistic *n*, was obtained using resolution $= X_1 - X_2 = \sigma_p/\sqrt{n}.Ttest$ where resolution = the difference in the means of the urinary excretion of folate in the subjects of the fortified ($X_1$) and unfortified ($X_2$) foods and $\sigma_p$ is the standard deviation of the pooled population.

Healthy volunteers (21 women; 5 men) aged 18–39 years were recruited from UNSW Australia in 2004. Subjects were excluded under the following criteria: if serum folate, red cell folate, B12, hemoglobin, mean cell volume, leucocytes or platelets were outside the accepted range for their specific gender and age groups; tobacco use; excessive alcohol consumption as defined by the Australian Alcohol Use Disorders Identification Test;[16] history of clinical folate, zinc, iron or B12 deficiency; recent pregnancy/miscarriage or neural tube defect affected pregnancy. Prescription and non-prescription drug use during the trials was noted (including oral contraceptives). This study was conducted according to the guidelines laid down in the Declaration of Helsinki and the UNSW Ethics Committee approved all procedures involving the human subjects. Written informed consent was obtained from all the subjects. Each subject used their own code name disclosed only to the pathologist to facilitate the identification of blood samples and survey responses.

### Design

The subjects took part in three short-term crossover trials with a two-week wash out period as follows: Trial 1 (positive control) was an aqueous oral dose of 400 µg $^{13}C_5$- PteGlu, Trial 2 (matrix control) was 200 g cooked white rice along with an aqueous dose of 400 µg $^{13}C_5$- PteGlu and Trial 3 (test food) was 200 g of cooked fortified white rice with a

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low-methoxy-pectin-coated premix containing 400 μg $^{13}C_5$- PteGlu. Only the fortified (200 g) and non-fortified rice (200 g) were given to the subjects double blinded.

At each trial, meals were prepared immediately before consumption following an overnight fast. Blood samples were drawn at 0,1,2,5 and 8 h postprandial. Blood (8 ml) was collected from an antecubital vein into K2-EDTA Vacutainers, centrifuged (1500 *g*, 10 min) and the plasma placed into 10 ml K2-EDTA sterile plastic tubes (Sarstedt, Nümbrecht, Germany), immediately cooled on solid $CO_2$, flushed with nitrogen and stored at –80 °C. The concentration of plasma $^{13}C_5$-5 methyl-THF and $^{13}C_5$-PteGlu were quantified using LC–MS/MS with $^2H_2$-PteGlu (synthesized by School of Chemistry, UNSW, Australia) as an internal standard[17] with the $^2H$ atom occupying the atoms at the 3′, 5′ positions of the 4-aminobenzoyl moiety.

### Diet

During the trials, the subjects were placed on a low-folate basal diet (designed using AUSNUT, professional edition on SERVE 95 software, Sydney, NSW, Australia) to standardize any whole-food matrix effects on folic acid absorption. The total folate content was verified using direct microbiological analysis with cryoprotected *Lactobacillus casei* and tri-enzyme extraction (α-amylase, protease and chicken pancreas conjugase).[18] The diet supplied total folate (112 ± 12 μg/day) and 9500 kJ (2270 kcal).

### Preparation of pectin-coated fortified rice premix

Preparation was based on the method adopted by[2] with modification. Raw long-grain rice (250 g) was placed in a tablet coating pan (ErwekaAR401, Serial No, 71378:ab53/D63150, Germany) rotating at 16 r.p.m. and blown with cool air during the coating process. Folic acid suspension (4 g/l) was sprayed onto the rice. Once dry, the rice was sprayed with pectin coating solution (5% w/w) of low-methoxy citrus fruit pectin (polygalacturonic acid methyl ester powder, 86.3% galacturonic acid as is, 8.9% methoxyl as is, 7% moisture). The rice premix was dried in a cabinet dryer at 55 °C for 1 h, vacuum packed and stored in a cool, dark area. The mean $^{13}C_5$-PteGlu enrichment of the fortified rice premix was analyzed using a validated LC–MS/MS technique[17] and used to determine the weight required to provide a 400 μg $^{13}C_5$-folic acid dose.

### Extraction and purification

Extraction of the $^{13}C_5$-PteGlu in the fortified rice premix was according to[19] with modification. Rice sample (1 g) was added to 0.05 N CHES-HEPES, pH 7.85 extraction buffer (50 ml) and stomached for 10 min. The contents were flushed with nitrogen, stoppered and autoclaved for 10 min at 15 psi (121 °C). The cooled flask contents were centrifuged (15 min, 10 000 r.p.m., 4 °C) and filtered through a Minisart CE syringe filter (0.4 μm pore size). Supernatants were flushed with nitrogen and stored at − 18 °C. Immediately before purification, the extract was diluted (1:50) and 50 ng $^2H_2$-PteGlu internal standard was added.

Plasma extraction was based on Rychlik *et al.*[5] Plasma samples (1 ml) were spiked with $^2H_2$-PteGlu internal standard (50 ng), centrifuged (10 min, 2500 *g*, 4 °C) and the supernatants diluted with 2 ml extraction buffer (0.05 M CHES/HEPES, 2% sodium ascorbate, 0.2 M 2-mercaptoethanol, pH 7.85).

The plasma and rice extracts were purified using solid-phase extraction with a strong anion exchange cartridge (3 ml/500 mg quaternary amine) as given by Freisleben *et al.*[17]

### LC–MS/MS

Isotopic enrichment of the plasma with $^{13}C_5$-5-methyl-THF and $^{13}C_5$-PteGlu was analyzed using an LC–MS/MS technique based on Freisleben *et al.*[17] $^{13}C_5$-PteGlu and $^{13}C_5$(6 R,S)-5-methyl-5,6,7,8- tetrahydrofolic acid, calcium salt (99% purity) were purchased from Eprova AG, (Schaffhausen, Switzerland). The $^{13}C$ atoms occupied each of the carbons on the glutamic acid portion of the molecule. Mass spectra were obtained using a LCQ Deca XP Plus ion trap mass spectrometer (Thermo Finnigan, Somerset, NJ, USA), coupled to a surveyor MS pump high performance liquid chromatography system with cooled autoinjector (4 °C) (Thermo Finnigan). The liquid chromatography separation was performed on an Eclipse XDB C-18 column (2.1 × 150 mm, 4 μm) with guard column by eluting the samples (50 μl) in variable mixtures of 0.1% formic acid and 0.1% acetonitrile at a flow rate of 180 μl/min. The mass spectrometer was operated in positive electrospray mode using selected-reaction monitoring.

### Bioavailability calculation

The positive area under the concentration time curve from time 0–8 h ($AUC_{0–8}$) was calculated from the combined area of trapezoid portions between the collected data points: the linear trapezoidal rule.[20] The labeled plasma 5-methyl THF concentrations were calculated using baseline subtraction of the labeled plasma folate at time zero. Data were statistically analyzed using one-way analysis of variance models on SPSS software (SPSS incorporated, 1998, Chicago, IL, USA).

## RESULTS

### Total AUC

The relative absorption calculations were based on the positive $AUC_{0–8}$ areas taken from the labeled 5-methyl-THF concentration time curves. The AUC areas were calculated from the combined area of trapezoid portions between the collected data points, that is, the linear trapezoidal rule.[20] The mean population AUC profiles (labeled plasma folate) from the three test foods are shown in Figure 1. Greater variation (relative standard deviation %) was observed between the subjects' AUC values for the test dose in the pectin-coated fortified rice premix (25.5%) than either the aqueous folic acid dose (15.4%) or the plain uncoated folic acid fortified rice (17.5%) because of the larger variability in the labeled folic acid dose within the rice premix. The mean folic acid content of eight separately extracted portions of pectin-coated fortified rice premix was 48.95 ± 3.13 μg per gram as is basis, giving a 25.6 μg error associated with the 400 μg dose (standard deviation × grams of rice premix required per test dose portion).

Mean AUC and relative absorption values were compared between men ($n = 5$) and women ($n = 21$), and between women taking the contraceptive pill ($n = 7$) and those not taking the pill ($n = 14$). No significant differences ($P > 0.05$) were found, thus the percentage absorption for each food was calculated using the entire population. Mean relative absorption was calculated using the subjects' individual AUC comparisons. For the 400 μg folic acid embedded within the pectin rice premix, relative absorption was 68.7 ± 4.5% (range 47–105%) relative to 400 μg standard aqueous folic acid. Without the pectin coat, the relative absorption was significantly higher ($P < 0.05$), 86.5 ± 3.8% (range 64–115%) relative to 400 μg standard aqueous folic acid. Figure 2 shows each subject's mean percentage absorption of 400 μg $^{13}C_5$-folic acid in two rice formulations relative to the aqueous standard. The

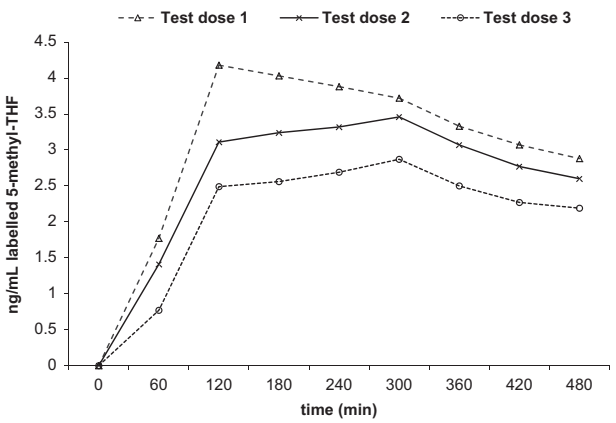


**Figure 1.** Mean labeled plasma 5-methyl-THF $AUC_{0–8\,h}$ of subject group ($n = 26$) for three bioavailability trial test doses: (1) 400 μg $^{13}C_5$-PteGlu, (2) 400 μg $^{13}C_5$-PteGlu plus 200 g cooked white rice and (3) 200 g cooked fortified rice with a pectin-coated premix containing 400 μg $^{13}C_5$-PteGlu.

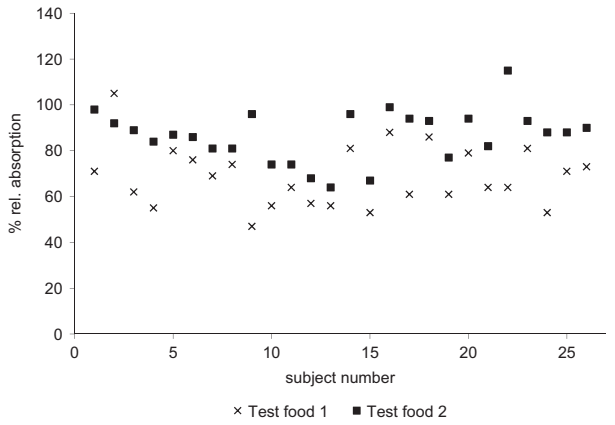


**Figure 2.** The percentage absorption of 400 μg $^{13}C_5$-PteGlu in two test foods relative to 400 μg aqueous $^{13}C_5$-PteGlu, in 26 subjects (5 men, 21 women). Test food 1: 400 μg $^{13}C_5$-PteGlu embedded within a pectin-coated rice premix and Test food 2: 400 μg $^{13}C_5$-PteGlu in 200 g plain cooked rice.

relative absorption of the folic acid within the pectin rice premix did not display a high level of bioequivalence (90% confidence interval entirely within the 80–125% boundary)[21] to either aqueous folic acid (68 ± 4.5%) or the folic acid in plain uncoated rice (79.9 ± 4.6%). Moreover, the folic acid absorption in the pectin rice premix displayed an 80% probability of being below the 80% absorption reference, relative to the aqueous standard dose ($z$ score = 0.2005), indicating that the bioavailability of folic acid in the pectin rice premix was significantly ($P < 0.05$) lower than that of an equivalent aqueous dose.

### Unmetabolized plasma folic acid

The concentration of unmetabolized plasma $^{13}C_5$-folic acid was monitored as it was expected to contribute to the total labeled serum folate value.[22] A small concentration of $^{13}C_5$-folic acid was found in the plasma of less than half of the subjects (seven women, three men) with the folic acid peak occurring and then decreasing below the detectable limit. Mean $^{13}C_5$-folic acid plasma peak concentrations of 0.85 ± 0.29 ng/ml (1–2 h post dose), 1.12 ± 0.35 ng/ml (2–5 h post dose) and 0.64 ± 0.26 ng/ml (2–5 h post dose) were found during trials 1, 2 and 3, respectively.

## DISCUSSION

Designation of the AUC sampling times to accurately capture the plasma peaks and elimination rates was difficult. Although pilot studies were carried out in order to obtain reasonably precise estimates of the plasma AUCs from the three separate food formulations, it was apparent that the plasma peak times, absorption rates and elimination rates differed between formulations and between subjects, possibly due to the differences in intestinal transit times and gastric motility.[4–6] Differing plasma peaks between subjects has also been observed for labeled 5-methyl-THF arising from single doses of 13C5-PteGlu in fortified bread ($T_{max}$ 90–240 min).[23] A later plasma peak (5 h) observed in 34% of the subjects' plasma profiles meant that elimination constants and logarithmic linear regression could not be used to obtain an accurate extrapolation to infinity, even though this had been successfully achieved in the pilot studies.

The single-dose AUC model does not allow for the assessment of the slow-turnover folate pools or long-term adaptation by the body. Instead it is limited to the fast metabolized, absorbed 5-methyl-THF portion in the plasma pool.[24] Future studies could address long-term bioavailability (under steady-state conditions) and whether these particular matrices show a slower rate of absorption over a longer period than simply a lower short-term bioavailability.[25]

No studies can be found that have investigated folic acid bioavailability in rice coated with edible coatings. Another study[14] found that 76 μg of aqueous $^{13}C_5$-folic acid taken with 185 g of white rice showed an 83% (range 57–123) absorption relative to standard aqueous folic acid in non-saturated subjects on the basis of urinary excretion ratios, which is similar to the absorption level found in this study (86.5 ± 3.8%, range 64–115). One milligram of unlabeled folic acid in fortified rice showed 58% absorption (range 42–84%) from the day-4 increase in serum folate.[26] The saturation scheme and high test dose (1 mg), however, may have caused larger excretion levels.[27]

As pectin is a non-digestible fibre, it may be able to decrease the bioavailability of folic acid in the pectin-coated rice by means of folate-pectin binding and physical encapsulation.[28,29] Although the lower digestive tract of humans contain the pectate lysases and hydrolases of *Bacteroides thetaiotaomicron, Clostridium beijerinckii* and *C. butyricum*,[30] these faecal microbes will use other carbon sources before they begin degrading pectin.[31] If the cellular fibrous structures in foods remain intact in the intestine, they can create barriers to diffusion and inhibit the access of the digestive enzymes.[32] Entrapment may also be possible through the binding of the starch hydroxyl and pectin methoxy components. Low-methoxyl pectin has more free-carboxyl groups than high-methoxyl pectin and is favored for coating use due to the greater number of binding sites available to form the polymer network (especially with the hydroxyl group in rice starch).[33]

An important question is whether the pectin coat affects the folic acid bioavailability to the point where it outstrips the edible coatings' advantage of increased protection against washing and cooking losses. Washing and cooking losses of folic acid in uncoated rice were 49% and 86%, respectively, compared with 9% and 69%, respectively, in pectin-coated rice (fortified at the 40 mg PGA per 100 g raw rice level).[2] As such, using the present study's bioavailability values, from a 400 μg folic acid dose per serve of enriched rice (washed and cooked in excess water), 29 μg is bioavailable in uncoated rice compared with 90 μg in a pectin-coated premix. Assuming negligible losses under absorption cooking, a 400 μg folic acid dose per serve of enriched rice (washed and then cooked under absorption conditions) gives a bioavailable portion estimate of 204 μg in uncoated rice compared with 291 μg in a pectin-coated premix. It is important to note, however, that the thickness of the pectin coat and the manner of washing and cooking rice (for example, rice to water ratio, time, wash repetitions, level of abrasion and agitation) largely determine the amount of folic acid lost during preparation. This study highlights the importance of educating the consumer as washing rice and cooking in excess water both significantly affect the amount of folic acid retained on the fortified grains and should, where appropriate, be discouraged.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

Dr Alison de Ambrosis was responsible for conducting the study as part of her PhD program. Dr Shyamala Vishnumohan assisted with the writing of the manuscript. Dr Paterson, Dr Arcot and Dr Haber contributed to the knowledge and design of the experimentation.

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