Use of Isotopic Labels and Mathematical Modelling to Investigate Mineral and Vitamin Bioavailability in Humans

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Abstract

This thesis describes the application of compartmental modelling techniques to data from human intervention studies with the objective of studying the absorption and metabolism of minerals (iron, copper, calcium) and vitamins (folate, carotenoids, riboflavin) labelled with stable isotopes. Compartmental modelling is a mathematical tool that uses differential equations to describe a dynamic process, such as nutrient metabolism, by adjusting the parameters of the equations until the model is a good fit to the real experimental data.

A single compartment model was developed that can be used to estimate the quantity of absorbed (unlabelled) iron from a test drink containing a minimum of 10mg iron. Studies on copper demonstrated that approximately 74% of the absorbed dose is sequestered by the liver on “first-pass” and that large quantities of copper (2.4mg/d) are lost via the bile. Data from a calcium intervention trial was evaluated using a multi-compartment model and indicated that a moderately high salt intake (11.2g/d) was associated with a significantly negative bone calcium balance with a high calcium (1284mg/d) compared to a low calcium (518mg/d) diet (P=0.024). Modelling the initial metabolism of an absorbed dose of $^{13}$C-labelled folic acid resulted in the finding that it is not metabolised in the mucosal cells but (probably) in the liver. The absorptive efficiency of carotenoids was investigated by isolating the triglyceride rich lipoprotein fraction in plasma and a simple model was used to estimate $\beta$-carotene and lutein bioavailability. Results from the first stable isotope labelled riboflavin study indicated that the bioavailability of riboflavin from spinach (60±30%) was not significantly different to the bioavailability of riboflavin derived from milk (67±21%).

The thesis has shown how compartmental modelling, in conjunction with stable isotope labelling, can reveal new insights into human mineral and vitamin metabolism, especially in the study of nutrient bioavailability.
Acknowledgements

Human studies are a team effort and require multidisciplinary collaboration to make them successful. I would like to express my thanks to all the co-authors on the papers that are submitted in this thesis. In particular, Sue Fairweather-Tait (iron), Linda Harvey (copper), Birgit Teucher (calcium), Paul Finglas (folate), Tony Wright (folate), Richard Faulks (beta-carotene), Kees de Meer (folate) and Hilary Powers (riboflavin). Most of this work took place between 1996 and 2006 when I was part of the Nutrition Programme at IFR. Although I was based within the Minerals Group, I collaborated with other scientists who were interested in vitamin metabolism and this thesis reflects the work I did on both mineral and vitamin studies.

Thank you to Pippa for her love and support during the past 15 years. Finally, I would like to dedicate this PhD to the memory of my father who passed away last year.
Contents

Abstract 2

Acknowledgements 3

Contents 4

1. Introduction 5

2. Critical Analysis 11
   2.1 Minerals 11
      2.1.1 Iron 11
      2.1.2 Copper 20
      2.1.3 Calcium 27
   2.2 Vitamins 32
      2.2.1 Folate 32
      2.2.2 Carotenoids 44
      2.2.3 Riboflavin 49

3. Conclusions 53

4. References 54

Appendix A: List of submitted papers 72

Appendix B: Copies of submitted papers 75

Appendix C: Letters from co-authors 76

Appendix D: Full list of publications 77
1. Introduction

Organisation and Aim of the Thesis
The thesis begins with an introduction of the definition and historical use of stable isotopes and compartmental modelling. This is followed by a critical analysis of the development of the submitted publications (see Appendix B) and their impact on the field of human nutrition. It is divided into six sections that describe the work undertaken on a particular mineral or vitamin and demonstrates the author’s unique contribution, to each area. The aim of the thesis is to show how compartmental modelling, in conjunction with stable isotope labelling, can reveal new insights into human mineral and vitamin metabolism, especially in the study of nutrient bioavailability.

Stable Isotopes
Most minerals have several, naturally occurring stable isotopes, all with a fixed abundance in nature. Vitamins are compounds that are made from several elements but they also have a fixed molecular mass (abundance). To study the in vivo metabolism of a mineral or vitamin, it is necessary to ingest it in an altered form so that it can be distinguished from the naturally occurring nutrient. The form of a mineral can be altered by enriching one of its low abundance isotopes. The alteration in the mass of a vitamin is more complex and involves changing one or more of its naturally occurring elements, from the higher to the lower abundance isotope (e.g. $^{12}\text{C}$ to $^{13}\text{C}$). Whilst carbon is a popular choice for alteration, hydrogen ($^{1}\text{H}$ to $^{2}\text{H}$), nitrogen ($^{14}\text{N}$ to $^{15}\text{N}$) and oxygen ($^{16}\text{O}$ to $^{18}\text{O}$) are also good candidates. The “new” form of the mineral or vitamin is known as a “tracer” or “label” and can be injected and/or taken orally. The concept of a tracer is important in isotope work. A true tracer is one that does not perturb the system it is investigating. Only radioisotopes can be called true tracers since they are administered in extremely small quantities. The term “label” is more appropriate for stable isotopes because they are usually given in quantities equivalent to the amount received in the normal diet. The word “tracer” is, however, still used in some stable isotope studies with the implicit understanding that it will almost certainly perturb the system under investigation. The underlying assumption in
this type of work is that the label (or tracer) behaves in the same kinetic way as the naturally abundant nutrient (sometimes called the tracee), otherwise the use of labels to mimic the behaviour of the native mineral or vitamin is invalid. There must be no discrimination by the body between the two forms of the nutrient.

The use of tracers (labels) allows the nutrient to be monitored in the plasma, urine and faeces over several days or weeks depending on the “enrichment” in vivo and the precision of the mass spectrometer that is measuring the study samples. Enrichment is another important concept in stable isotope experiments. There is a large, endogenous background concentration of most elements in plasma, urine and faeces and the dose of tracer needs to be of a sufficient quantity that the mass spectrometer can detect it with good precision against the “natural” background. Enrichment can be defined as the ratio of the quantity of tracer (or label) to quantity of naturally abundant nutrient, in the measured sample. Good mass spectrometer measurement quality is vital for the accuracy and precision of the resultant model and the measurement precision needs to be known before the experiment is designed so that an effective dose size can be ascertained. Depending on the nutritional hypothesis that is being investigated, designing an experiment that takes samples for days, rather than hours, usually reveals more features of the underlying metabolism. It is, therefore, very important to simulate the combination of dose size, mass spectrometer precision and different sampling times to achieve the optimal (theoretical), experimental design. In designing a study, it is important to avoid administering a nutrient dose that would exceed the reference nutrient intake (RNI), as the subsequent metabolism of the absorbed nutrient might be different from that of a dose in the “normal” range and the results of the study could not be generalised to the wider population. Simulated mass spectrometer data can also be generated from a compartmental model. Random variation (sometimes called, “noise”) can be added to this data from knowledge of the precision (RSD) of the instrument that will measure the real samples. This allows investigators to simulate when the potential limit of detection (LOD), and limit of quantification (LOQ), may occur, in the sampling procedure. Knowledge of the time required to reach the LOQ and LOD should prevent unnecessary extra plasma, faecal or urine collection, which would be unethical, as well as a waste of resources.
Although stable isotopes were the first tracers to be used in nutritional science (Schoenheimer & Rittenburg, 1935), radioisotopes quickly became the standard amongst researchers after the Second World War. Cyanocobalamin (vitamin B12) was one of the first vitamins to be labelled with a radioisotope (Chaiet et al, 1950) and a radioisotope of calcium was used at a similar time (Harrison & Harrison, 1950). Mineral and vitamin stable isotope studies started to be published in the early 1960’s (iron - Lowman & Krivit, 1963; B12 - Ekins et al, 1967). The first studies using calcium (McPherson, 1965) and chromium (Donaldson et al, 1968) followed shortly afterwards and the late 1970’s saw the first zinc and copper stable isotope studies performed (King et al, 1978). Human studies using stable isotope labelled folic acid (Gregory & Toth, 1988) and beta-carotene (Dueker et al, 1994) appeared more than ten years later. Stable isotope labelled riboflavin was not used in a human study until 2007 (Dainty et al, paper [13]). A combination of advances in mass spectrometry, and concern about exposure to ionising radiation, led to a growing number of researchers switching to stable isotope tracers in the 1980’s. Several reviews and guides on the use of stable isotopes in nutrition have been written (Buckley, 1988; Turnlund, 1989; Turnlund, 1991; Turnlund, 1994; Mellon & Sandstrom, 1996; Lowe & Jackson, 2001). The isotopic labels are normally incorporated in food, either intrinsically (Janghorbani et al, 1981; Weaver, 1985; Fox et al, 1991; Hart et al, 2006), or extrinsically (Janghorbani et al, 1982; Christensen et al, 1983; Johnson & Lykken, 1988; Weaver, 1988). An intrinsic label is one in which the stable isotope is incorporated biosynthetically into a plant or animal, whereas an extrinsic label is simply added to the food, prior to ingestion.

**Compartmental Modelling**

The advances in mathematical modelling in nutrition, since the 1980’s, have been described in conferences and workshops (Canolty & Cain, 1985; Hoover-Plow & Chandra, 1988; Abumrad, 1991; Siva Subramanian & Wastney, 1995; Coburn & Townsend, 1996; Clifford & Muller, 1998; Novotny et al, 2003). The vast majority of the work published from those meetings was concerned with compartmental and empirical modelling. A comprehensive literature exists on this type of modelling and it is well grounded, theoretically (Atkins, 1969; Shipley & Clark, 1972; Anderson, 1983; Carson et al, 1983; Green & Green, 1990; Wolfe, 1992; Jacquez, 1996; Wastney et al, 1999). Dedicated software tools such as SAAMII (1997) allow non-
mathematicians to set up compartmental and empirical models, without the need to define the differential equations that underpin them.

Before describing why models are useful tools for gaining insight into human nutrition, some definitions must be given to clarify the terminology. A compartment is a theoretical construct that may combine material from several different physical spaces. It is defined as an amount of material that acts as though it is well mixed and kinetically homogeneous and a compartmental model consists of a finite number of compartments with specified interconnections between them. The interconnections are a representation of the flux of material that is transported from one location to another. By defining a compartmental model in this way, the researcher can reduce a complex metabolic system into a small number of pathways and compartments. The outputs from such a model include the quantity of mineral or vitamin stored in certain body pools, the rate and extent to which the nutrient moves from one pool to another and how long it will remain in the body before being excreted. This can inform the approach of the nutritionist about status, daily requirements and retention and, only through modelling, can this type of information be obtained from humans in a non-invasive way. As an example, consider the 2-compartment model shown in the figure below.

The circles represent compartments and the arrows are pathways showing the direction of transfer of material. Associated with each pathway is a rate constant, which is conventionally written as \( k_{(i,j)} \) that denotes transfer of material, from compartment “\( j \)”, to compartment “\( i \)”, per unit time. The type of compartment models which are usually found in nutrition are described by constant coefficient, ordinary, first-order differential equations. The basis of these equations is that the rate constants do not alter over the course of the experiment and, by applying The Conservation Of
Mass to the system under study, it is possible to solve the resultant series of equations. If the mass of material in compartment 1 is denoted as $P$ (for plasma) and that in compartment 2 as $E$ (the “rest” of the body), the system can be described by:

$$\frac{dP}{dt} = k_{1,2} \cdot E \cdot (k_{2,1} + k_{0,1}) \cdot P$$

$$\frac{dE}{dt} = k_{2,1} \cdot P \cdot k_{1,2} \cdot E$$

To solve these equations, one of the two compartments needs to be sampled (measured) so that the parameters ($k_{0,1}$, $k_{1,2}$ and $k_{2,1}$) can be fitted to the resultant, measurement data. It is often the case that the only compartment that can be sampled is the plasma. This limits the number of “other” compartments that can be used in the model, if the parameters are to be identified, uniquely. This is called a priori identifiability and is used to identify, given noise-free data, the number of compartments a model may have in order that all its parameters will have a single solution. Often investigators have an in-depth knowledge of the metabolism of a particular nutrient, and want to include many compartments, but this can lead to uncertainty in the value of the subsequent parameters. Any model that is not a priori identifiable must be carefully evaluated and the results from it used cautiously. The fitted parameters from a model should always be quoted with their calculated precision so that an assessment of the quality of the model can be made. This is called a posteriori identifiability and plays an important part in drawing nutritional conclusions from the modelling of real data. The a posteriori identifiability is dependent on good quality data and the correct choice of model structure for the system under investigation. The 2-compartment model is a priori identifiable. If the data from the plasma samples are of sufficient quality and they support a 2-compartment rather than, say, a one or three compartment structure then the model should be a posteriori identifiable. A compartment model is, therefore, constrained by the type of data collected, the location it is collected from and the statistical considerations involved in fitting experimental data to models.

Closely linked with compartmental modelling is empirical modelling. This is usually applied to data generated by multiple sampling of the plasma, over a few hours, after an intravenous (IV) label has been administered. The disadvantage of empirical models is that they cannot inform the researcher about mineral or vitamin absorption,
endogenous losses or bioavailability. This type of modelling also makes the assumption that the nutrient introduced into the plasma, via an IV injection, behaves in the same kinetic way as the nutrient that appears in the plasma from the oral route. It cannot be assumed that a mineral or vitamin introduced into the body by different methods will be transported in the plasma in the same way. This can lead to the subsequent kinetics being “route dependent”. In modelling a nutritional system, the oral route of label administration is preferable because this is how minerals and vitamins enter the body from food. Due to these considerations, compartmental approaches are more widely used than empirical and this is reflected in the models described in the Critical Analysis section of this thesis.

The remaining text focuses on the human metabolism of copper, calcium, iron, folates, carotenoids and riboflavin. All the work is “whole body” in the sense that plasma, urine or faecal samples are taken from volunteers after the ingestion of a stable isotope labelled test meal or drink.
2. Critical Analysis

2.1 Minerals

2.1.1 Iron

Compartmental modelling has been applied extensively to a number of studies on iron metabolism (Franzone et al, 1982; Nathanson et al, 1985; McLaren et al, 1995; Lao & Kamei, 2006). All of these previous papers attempt very detailed analysis of the kinetics of iron and require several compartments and rate constants to describe the system under study. The models developed in papers [2] and [10] were simpler because only one aspect of iron metabolism was being investigated, namely, bioavailability. The original motivation to use compartmental modelling to estimate bioavailability was to avoid the problems associated with existing methods that measured iron absorption.

Methods to estimate iron absorption, using isotopic labels

Since the 1980’s, safety concerns have prevented the use of radioisotopes and whole body counting in the UK and researchers have switched to stable isotope labels to measure iron absorption using one of three main methods:

(1) faecal monitoring (Janghorbani et al, 1980)
(2) dual isotope (plasma) method (Werner et al, 1983a)
(3) erythrocyte incorporation (Dyer & Brill, 1972)

The first of these (faecal monitoring) is no longer a method of choice as it was recognised that it overestimated absorption, probably due to losses of isotope during collection and processing (Van den Heuvel et al, 1997). The dual isotope method is considered to be very accurate (it has been validated against the “gold standard” technique of whole body counting (Werner et al, 1983b)) but an intravenous (IV) dose of labelled iron has to be administered at the same time as an oral dose. Erythrocyte incorporation is the third method that is widely used and usually requires only a single (oral) stable isotope label to estimate iron absorption. It is assumed that a large fraction (usually, 80%) of newly absorbed iron is eventually incorporated into
erythrocytes, reaching a “peak” of enrichment approximately 14 days (post-dose) after which a single blood sample can be taken to determine absorption.

**Problems with existing, stable isotope methods to measure iron bioavailability**

The assumption that 80% of newly absorbed iron is taken up by red cells is based on studies that measured erythrocyte incorporation of labelled iron from intravenous doses (e.g. Hosain et al, 1967; Barrett et al, 1992; Van den Heuvel et al, 1998; Roe et al, 2007). This has led to most, subsequent iron bioavailability studies administering an oral label to volunteers and assuming a fixed value of 80% red cell incorporation of the newly absorbed iron. However, on closer inspection, the papers mentioned above also showed that individuals varied markedly (62% - 99%) in their incorporation of iron into red cells. This would suggest that an intravenous dose of labelled iron should be given with the oral dose if an accurate determination of iron absorption is required for an individual, or a small group comparison study.

The need for an intravenous dose with the dual isotope, or erythrocyte incorporation methods, is inconvenient but not insurmountable. The real motivation for the development of compartmental models were the other, more intractable problems associated with using these methods to measure iron absorption in mixed meals (meat and vegetables) and in food fortified with iron (powder).

First, it is often assumed by researchers that the iron found naturally in food is absorbed to the same extent as an added, extrinsic stable isotope label. Extrinsically labelled non-haem iron is thought to enter a “common pool” which means that native iron in a food will be absorbed to the same extent as extrinsically labelled iron (Bothwell, 1979). However, there is evidence that this is not always true as at least one, intrinsic/extrinsic label comparison study has shown that retention of an intrinsic label was significantly lower in rats compared to extrinsic labels (Boza et al, 1995). Also, a review listed several publications that highlighted problems with extrinsically labelled iron not fully exchanging with native iron (Van Campen & Glahn, 1999).

The second problem is that iron is found in two main forms in foods, haem and non-haem, which are absorbed by independent pathways (Sharp, 2004). Extrinsic labels cannot be used to measure haem iron absorption and intrinsically labelling meat is
expensive (Weaver, 1985). Also, iron absorption can vary from day-to-day; any study hoping to estimate absorption from a multitude of iron sources in a meal (e.g. meat, vegetables and drinks) should ideally be performed on the same day. Another limitation is that there are only three, relatively low abundance stable isotopes of iron, so only three iron sources could be quantified in the same experiment.

Third, to combat iron deficiency in developing countries, different iron fortification methods have been trialled to assess the extent of the iron absorption of the various compounds, but the evidence of their efficacy is inconsistent (Hurrell, 2002). This paper acknowledges that the physicochemical characteristics of the commercial iron powders that influence bioavailability (e.g. particle size, porosity, solubility and chemical form) cannot easily be reproduced with an extrinsic or intrinsic label of iron and, therefore, the absorption results from stable isotope labelled fortificant studies may not be accurate, especially if they involve an iron powder. A possible resolution to these problems is to use intrinsically labelled iron products but the cost is prohibitive (Weaver, 1985) and reproducing commercial iron fortificants of the right particle size and porosity is extremely difficult.

**Compartmental modelling**
An alterative, novel solution is to measure the absorption of the iron that is naturally present in the food or fortificant without recourse to adding stable isotope labels. The measurement of this “natural”, unlabelled iron in a biological sample (e.g. plasma) is straightforward but to distinguish the iron in a sample whose origin is exogenous (e.g. from the last meal) compared to iron whose origin is endogenous is more of a challenge as they cannot be differentiated, analytically.

The following section will outline how compartmental modelling can be used to estimate iron absorption from the plasma appearance of newly absorbed iron. It will provide more detail of the modelling process than the critical analysis of the other minerals and vitamins. This will avoid repetition of modelling description in other sections where similar analysis was undertaken but will serve as an in-depth illustration of the process of modelling changes in the plasma concentration over time.
It is known from pharmacokinetic studies, that the bioavailability of certain drugs can be estimated from their appearance in the plasma, over the first few hours post-ingestion (Gabrielsson & Weiner, 2000). In an analogous way, the study described in paper [2] was designed to test the hypothesis that a compartmental model could accurately predict the absorption of a dose of unlabelled iron, by assuming that certain key features of iron and drug absorption are shared and, that the plasma appearance of newly absorbed iron, would be measurable.

The initial problem in this type of study is the measurement of new, “dietary” iron, against the natural background concentration of endogenous iron, already in the plasma. The plasma needs to be perturbed, sufficiently, to enable accurate quantification of absorption, by the model. Simulations, performed prior to the study design, indicated that a dose of 10mg of iron (as ferrous sulphate) would be sufficient to ensure a large perturbation in the plasma iron concentration. The simulation, with a 10mg dose, suggested that the change in concentration (from baseline) would be large enough for good measurement precision for up to eight hours (post-dose), using a Ferrozine kit (total iron diagnostic kit; Sigma, Poole, Dorset, UK). This simulation also had to take account of the diurnal variation in serum iron concentration, which is known to increase from early morning (8am), when the volunteers would start the study (Statland et al, 1976; Dale et al, 2002).

This introduced two other issues that are very important for modelling of this type: (i) the duration of the experiment and (ii) the number of blood samples required to ensure that all the relevant iron kinetics are captured. The work performed by Gonzalez et al (2001) had indicated that 360 minutes (post-dose) was an optimal time to allow an estimate of the rate of disappearance of iron from plasma. This is a vital parameter for the model because the underlying mathematics (differential equations) depend on the principle of mass balance – the rate of change in the plasma iron concentration is equal to the rate of iron absorption minus the rate of removal of iron from the plasma for utilisation and storage. So, the estimation of rate of iron removal is very important if the absorption is to be estimated correctly. Intensive blood sampling was carried out in this first study (paper [2]) to ensure that the peak in plasma concentration was not missed and the changes in iron concentration were fully characterised. Simulation of ten sampling times, equally spaced over 360 minutes, indicated that there would be
enough degrees of freedom to estimate all the model parameters “a priori” from a one or two compartment model, given noise free data. This is an important part of the modelling process and must be performed to ensure that a model is theoretically possible, given the experimental design. The number of parameters that need to be estimated in the one compartment model are the quantity of iron absorbed, the rate of iron loss from the plasma and the time period during which absorption is taking place. The same parameters would be estimated from a two compartment model, as well as the rate of re-appearance of iron from the second compartment (rest of body).

Modelling work from other researchers had suggested the need for more complicated models (i.e. more than two compartments) for iron metabolism but they had sampled the plasma for days, rather than hours, which allows far more details of the kinetics to be estimated (e.g. McLaren et al, 1995). In developing a model whose only function is the estimation of iron absorption, sampling for days is unnecessary because the absorptive process takes place over a few hours.

It became possible to decide which of the two competing models (one or two compartment) was the most appropriate after the human study was performed and the data were collected. This was carried out in SAAMII (SAAM Institute Inc, Seattle, WA) by looking at the residual plots, examining the uncertainty on the parameters and using the model comparison parameters called Akaike's information criterion, AIC (Akaike, 1974) and Bayesian information criterion, BIC (Schwarz, 1978). When comparing two or more model structures, the one with the lowest AIC (or BIC) is preferred, because these parameters measure the goodness of fit of the model and also take into account the number of parameters that are used in fitting it. If two models have a comparable goodness of fit to the data, the simplest model (one with the least parameters) is preferred.

Figure 1 in paper [2] illustrates the final model which is a single compartment. It is assumed that mass, \( M \), of the iron dose, is absorbed from the small intestine, into the plasma, at a constant rate, \( R \), for a time, \( T \). This is known as “zero-order” absorption and equates to a constant infusion of iron from the gut into the plasma, which is, of course, a simplification, as the true process is more likely to be first-order (time-varying) in nature (Schumann et al, 1999). A first-order absorptive process had been
examined as part of the model development but it did not improve the absorption predictions and also required the estimation of an additional parameter. Therefore, by applying the Principle of Parsimony, the simpler process of zero-order absorption was preferred. Had the labelled iron been mixed with solid food instead of a liquid test drink, a first-order absorptive process would almost certainly have been preferable.

By fitting equations 2 and 3 in paper [2] to the plasma concentration data (C) for either labelled or unlabelled iron, it is possible to estimate the mass of iron absorbed (M), the period of time when absorption has taken place (T) and the rate constant of elimination from the plasma (k). All fitting can be performed in Excel (Microsoft Corporation, 2002) or SAAMII but the latter is preferable as it gives statistics that indicate how good the fit is. Initially, the volume of distribution (V) was estimated from the model and was found to be, approximately, equal to the plasma volume. It was decided to use plasma volume estimates rather than model fits in the published work because it meant that fewer parameters had to be estimated in the model. Plasma volume was calculated from nomograms by knowledge of the volunteer’s age and weight (Lentner, 1984). The fractional iron absorption can then be calculated by knowledge of the mass of iron (M) appearing in plasma, divided by the mass of the dose of iron.

When the plasma data were fitted to the model, it was necessary to baseline subtract the measured values of iron concentration so that the model examined the change in plasma iron concentration due solely to the unlabelled iron absorbed from the test dose. There is some uncertainty associated with this baseline correction, because the value that is subtracted is the measurement at time=0 minutes (just before the dose is given). It is known that unlabelled plasma iron concentration is subject to a diurnal variation, which means that the “true” baseline plasma iron concentration at 360 minutes post-dose is likely to be higher than that at time=0 (Dale et al, 2002). Some account was taken of this variation by adding more uncertainty to the plasma iron concentration at the later time points, compared to those at the beginning. Modellers refer to this as “weighting” the data. The literature suggests that the diurnal variation causes a maximum 10% change in iron concentration, in the period from 8am to 2pm (Statland et al, 1976; Dale et al, 2002). Simulations indicate that not taking account of
the diurnal variation will lead to a 2% (approximate) overestimation of the quantity of iron absorbed.

The experimental design of paper [2] consisted of five volunteers receiving 10mg of iron (as ferrous sulphate) in a drink of water, followed by regular (25mL) blood sampling at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300 and 360 minutes. In order to validate the results from the unlabelled iron study, a labelled iron experiment was performed at the same time. Hence, a dual isotope study was carried out with the same volunteers and 1mg of highly enriched Fe-57 (as ferrous sulphate) was given orally, in a drink, and 0.225mg of Fe-58 (as ferrous citrate) was given intravenously, in order to estimate absorption using the well validated, area under the (plasma) curve (AUC) method (Barrett et al, 1994). When the results of the model (unlabelled iron geometric mean absorption = 19%, -1SD=12%, +1SD=28%) were compared to those using the AUC dual isotope method (labelled iron geometric mean absorption = 17%, -1SD=10%, +1SD=29%), there was no significant difference in the results. In addition, the model estimated the quantity of iron absorbed from the labelled iron’s plasma appearance and found it to be 16% (geometric mean). This demonstrated that there was no significant difference in the estimates of unlabelled iron absorption from the model, compared to the AUC method for the labelled appearance of iron.

Another study examined some of the modelling assumptions in more detail (paper [10]). IV doses of 0.2mg of highly enriched Fe-58 were infused slowly into nine, female volunteers and the resultant plasma appearance was modelled using a single compartment model. If the model is accurate, it should predict the quantity of the known IV dose (approx. 0.20mg). The model estimated the IV dose to be 95% of its true value (0.19mg) but this was found to be a non-significant difference.

There is, therefore, independent evidence from two data sets (papers [2, 10]) that the model accurately estimates the quantity of iron that has been absorbed or infused despite initial concerns that the single compartment structure might be too simple, or that the diurnal variation in serum iron concentration might cause the method to be inaccurate.
Other Methods to Estimate Iron absorption from unlabelled test meals

Previous authors have used the plasma appearance of newly absorbed iron to assess bioavailability. This work can be broadly categorised as either, (a) relating the (post test meal) peak change in serum iron concentration to iron absorption (Ekenved et al, 1976; Heinrich & Fischer, 1982; Werner et al, 1983b; Gonzalez et al, 2001; Hoppe & Hulthen, 2006; Conway et al, 2006) or, (b) relating the (post-test meal) area under the serum iron concentration (versus time) curve (AUC) to iron absorption (Ekenved et al, 1976; Werner et al, 1983b; Gonzalez et al, 2001; Hoppe et al, 2004; Conway et al, 2006). Method (b) is sometimes called the iron tolerance test (ITT). Both of these methods lead to regression equations of the form \( y = mx + c \), where “\( y \)” is the fraction or percent of iron absorbed and “\( x \)” is the peak increase in serum iron or the AUC (“\( m \)” and “\( c \)” are estimated constants). The drawback to this type of analysis is that it is only valid for the range of iron absorption that led to the development of the equation (i.e. it should not be used for extrapolation) and it can only be used for the particular iron compound that the original data/equation was constructed for. It is, therefore, a very restrictive method and probably explains why it has not become popular as a technique for measuring iron absorption. Only the work by Gonzalez et al (2001) attempted something more flexible but this required the administration of a labelled IV dose as well as measuring the AUC (or peak change in serum iron). This paper does not fully describe the method that was used to quantify absorption and, therefore, it is impossible to reproduce. The work described in papers [2, 10] has an obvious advantage over these “regression” methods, in that it will measure the absorption of any form of iron. Whether it can measure low quantities of absorbed, unlabelled iron remains to be demonstrated but that is a problem with any serum-based method, as the change in iron concentration (post-test meal) must be large enough to allow accurate quantification, against the background of endogenous diurnal changes in plasma concentration.

Conclusions

The impact of the work in papers [2, 10] is, firstly, that there is no need to give an intravenous dose of iron in future, labelled studies that estimate absorption from plasma appearance. This is an improvement because it reduces the invasiveness for the volunteers. It also reduces the cost of the study and improves the overall safety as, injecting any substance, carries a slight risk of an adverse event. Secondly (and more
importantly), it allows the possibility to estimate absorption of unlabelled iron, which was successfully demonstrated in paper [2] with 10mg ferrous sulphate in a liquid carrier. If it is to become a really valuable tool for future bioavailability work, the model needs to be tested with different forms of unlabelled iron at lower doses in real food systems. If substantiated, it does hold out the promise to be the first technique that will accurately measure the total iron absorption in a meal containing both haem and non-haem iron. It also has the potential to measure the unlabelled iron absorption from commercial iron powders which is something that no method, to date, has been able to achieve. This should help policy makers to decide which iron fortification product is the most bioavailable.

Limitations and future work
The drawbacks to the technique are threefold, but not insurmountable. First, using a Ferrozine kit to analyse unlabelled iron, requires quite a large overall blood volume to be taken; this makes it unlikely to be used in cross-over studies in the same 6 month period, as the limit on blood volume withdrawal is 450ml. This problem would be overcome if the samples were analysed using a mass spectrometer (e.g. ICP-MS) as the method would then require a much smaller blood volume, for each sample. Second, the experimental protocol is quite invasive and inconvenient for volunteers. However, unpublished results from our work (J Dainty, unpublished) show that it is possible to accurately estimate iron absorption, from the model, using a single blood sample at 120 minutes. Further work is necessary before we could validate this. If that could be accomplished, it raises the possibility of a cheap and fast clinical technique for assessing iron absorption. The third and final drawback is the diurnal variation in plasma iron concentration which could make estimates of unlabelled iron concentration less accurate, the longer blood is sampled for. If the model could be reduced to one blood sample at 120 minutes, this problem should be reduced. More work is needed to assess the implications of diurnal changes in the serum iron concentration on the accuracy of the model estimates of iron absorption.
2.1.2 Copper

Copper is an essential element in the human diet and is involved as a cofactor in a number of enzymes (Linder, 1991). There is homeostatic control over copper absorption and endogenous excretion and this requires human bioavailability trials to measure both absorption and retention (Turnlund, 1998). Copper bioavailability studies are problematic because the element only has two isotopes ($^{63}\text{Cu}$, $^{65}\text{Cu}$). Consequently, dual tracer experiments cannot be performed and only, either, absorption (using oral isotope administration and faecal monitoring) or, retention (using intravenous isotope administration and faecal monitoring) can be investigated in any one study. This has led researchers to design expensive, separate trials (an oral study and an IV study) on the same volunteers to quantify both absorption and endogenous losses.

New Method to Estimate Endogenous loss of Copper

Reducing the time and cost of this type of experiment was the motivation for the first human trial that is outlined here (paper [1]). This work presents a technique that allows an estimate of the absorption and endogenous loss of copper, from a single, stable isotope-labelled, study. Ten volunteers were given a variety of test meals (on separate study days) containing between 1-3mg of highly enriched Cu-65 (for details, see Table 2 in paper [1]) and faeces were collected for 10 days, post-dose. This gives sufficient time for the last few faecal samples to contain only labelled copper that has been absorbed and then excreted (henceforth, called “labelled, endogenous copper”) (see Figures 1 and 2 in paper [1]). There is a linear relationship between the mole fraction of the labelled, endogenous copper and (post-dose) time (Figure 3 in paper [1]). From the linear equation that describes this relationship, an estimate can be made of the mole fraction (and quantity) of the endogenous, labelled copper in the earlier stools that also contain unabsorbed, labelled copper. Therefore, a mathematical correction can be made in these earlier stools and this allows the calculation of the endogenous loss as well as the true absorption of copper from the oral dose. This is validated by examining Figures 5 and 6 in paper [1] (wrongly labelled as the data for volunteer 230 when, in fact, it is the entire volunteer set). Figure 5 illustrates the relationship between labelled, faecal copper and the faecal appearance of the rare earth element, holmium, which is assumed to have the same excretory pattern as
copper. Theoretically, the data should fall on the y=x line (slope=1.00) but the slope is 0.84, indicating that the two elements do not have the same excretory pattern and that copper travels through the GI tract faster than holmium. However, if the labelled faecal copper is corrected for endogenous losses and the data are plotted again, the slope is estimated to be 0.99 and the data does lie, approximately, on the y=x line (Figure 6).

Further validation of this method was carried out in paper [8] using a cross-over design. Volunteers were given an oral dose of Cu-65 on one study day and an intravenous dose of the same isotope, some weeks later. Faecal samples were collected for 14 days (post-dose) on both occasions. The results from six volunteers indicated that there was no significant difference between the two methods (endogenous loss from IV label=32% of dose; from oral label=35% of dose). A final validation is the finding that this quantity of endogenous loss is similar to that reported previously, using radioisotope labels (Bush et al, 1955; Weber et al, 1969).

This new method for estimating endogenous loss was used in another study (paper [4]) that examined the effect on absorption and retention of consuming a high, medium and low copper diet. Twelve male volunteers completed a longitudinal crossover trial in which they were assigned to consume either 6.0mg/d Cu (high), 1.6mg/d Cu (medium) or 0.7mg/d Cu (low) for six weeks each, with a minimum four-week washout period after each intervention. Before and after each intervention, the volunteers were given a stable isotope labelled copper dose of Cu-65 (3mg), and complete faecal collections were carried out for 14 days, to allow an estimation of the copper absorption and endogenous loss. The results indicated that absorption of copper did not change between diets, but there were differences in endogenous loss, with significantly higher excretion on the high, compared to the medium (P=0.001) and low copper diet (P<0.001). A similar study had been performed in the US (Turnlund et al, 1989), but the strength of the work in paper [4] was that both absorption and endogenous loss were estimated from the faecal appearance of copper from the oral dose, whereas the study by Turnlund et al had used an oral and IV dose in separate experiments.
In paper [1], a linear equation (\(y=mx+c\)) was used to describe the temporal appearance of the labelled, endogenous copper but there was no attempt to examine whether there was a better, mathematical description. In paper [4] there was an opportunity to examine different equations to describe the pattern of endogenous loss. The effect on the estimation of endogenous loss was compared using linear, mono-exponential and bi-exponential formulae (see Figure 2 and Table 7 in paper [4]). The figure in the paper is slightly misleading as it shows that the bi-exponential model is the best fit to the data for this particular example, but was found to be incorrect for the whole data set, for which the linear expression was the only model that could be justified from the data. Other researchers (Owen, 1982; Linder et al, 1986) have shown, in rats, that the endogenous loss of copper from the body follows a biphasic pattern.

The underlying pattern of endogenous excretion, in humans, is probably more complex than has been proposed in papers [1, 4] but only a simple linear model could be justified from the data generated by these studies. More precise measurement of the quantity of labelled copper in these final faecal samples is required before a complicated excretion model could be proposed. It can be seen from Table 7 (paper [4]) that the effect of a bi-exponential fit to the data is to increase the estimates of copper absorption and excretion. The isotope enrichment in these samples was close to the limit of detection of the mass spectrometer. A larger isotopic dose could increase the enrichment but the quantity of copper used in paper [4] was above the UK RNI (1.2mg). Increasing it further could prevent the study results being generalised to the wider population.

**New Method to Estimate Absorption of Copper**

Prior to 2005, faecal monitoring was the only method available to calculate copper absorption. Paper [8] describes a study that estimates copper absorption from plasma appearance in an analogous way to the iron studies contained in papers [2, 10]. A single compartment model was deemed to be the most appropriate choice after assessing the goodness of fit and suitability (via AIC and BIC) of two and three compartment alternatives. Again, the movement of copper, from the GI tract into the plasma compartment, was modelled as a constant rate infusion (zero order process). The volume of distribution of copper was more difficult to estimate, compared to that
of iron, and some unpublished data was used (N Lowe, personal communication) to calculate a value. This volume is the “apparent” space that the mineral occupies in the sampled compartment (blood). For iron, it had been found that this is equal to the plasma volume which is dependent on a subject’s age and weight (Lentner, 1984). The estimate for the copper volume of distribution (5 litres) was higher than the plasma volume (less than three litres for a typical subject). The model estimated that copper absorption from a 3mg labelled dose was 8±2%, but the faecal monitoring method found the absorption to be 48±5%. Even allowing for the fact that faecal monitoring can sometimes overestimate absorption, the model is clearly not fit for purpose.

The reason for this became apparent when a full compartmental model of copper metabolism was analysed. The copper that is absorbed across the mucosal cells, is carried, mainly, by albumin (Venelinov et al, 2007) in the portal vein to the liver, where a large proportion of it is removed. This is known as the first-pass effect (Gabrielsson & Weiner, 2000). It is particularly marked in copper metabolism because most newly absorbed copper, although initially transported on albumin, has to be repackaged into ceruloplasmin by the liver. This “new” form of copper is slowly released into the general circulation over a period of 4-5 days (Figure 2A, paper [8]). This means that only a small fraction of the absorbed copper appears, immediately, in the plasma and explains why the simple, one compartment model grossly underestimated the absorption of copper from the test dose. Whilst this invalidates the use of the model for predicting the quantity of copper absorbed from a meal/dose, it does allow the estimation of relative bioavailability. To date, no such studies have taken place. It would be useful if the bioavailability of unlabelled copper could be assessed using the model, with the proviso that it would only be possible to measure relative bioavailability.

**Detailed compartmental model**

Whilst parts of the work from paper [8] have already been mentioned, additional insights into copper metabolism were obtained from the full compartmental model (Figure 1 in paper [8]). Before describing our model, it is important to highlight previous work in this area so that the novel aspects of paper [8] can be understood in the right context.
The first compartmental model of human copper metabolism was developed by Cartwright & Wintrobe (1964), but was unconventional because no experiment was performed to gather data for the model. Instead, literature values were used for serum and urinary copper values and autopsy data were utilised for total copper in specific body parts. These data, together with the current knowledge of copper metabolism, allowed a simple compartmental model to be built, which had albumin as the main copper transporter to other tissues. It is now known that this is incorrect because ceruloplasmin is the main copper transporter in plasma, but the model stimulated debate and led to a study, in rats, that was designed to generate data for compartmental modelling (Hazelrig et al, 1966). For this trial, the radioisotope $^{64}\text{Cu}$ was injected into ten rats, and bile, blood and liver was sampled over the next five hours. This resulted in a detailed model of copper liver metabolism and reinforced the view that biliary excretion was important in copper homeostasis. This model was viewed as definitive over the next 25 years and was extended to allow for simulations of copper metabolism in man (Blincoe, 1992; 1993) although, again, no human experimental data were generated.

Dunn et al (1991) performed a detailed experiment that injected doses of $^{64}\text{Cu}$ in 33 rats and sampled various tissue sites over three days to generate data for compartmental modelling. This study used the Hazelrig et al (1966) model as a basis, but included many other tissue compartments, for the first time. Like the Cartwright & Wintrobe (1964) model, it concluded that the tissues are not supplied with copper transported by ceruloplasmin but by another (unknown) carrier. As mentioned previously, this is not correct in the human but is, perhaps, a good example of why the results from animal studies must be viewed with caution when trying to extrapolate their findings to human metabolism. One of the interesting points to note in the development of these copper metabolism models is that, until the early 1990’s, there were no papers published describing a compartmental model that used data generated from a human study. This is probably due to ethical reasons, as there are dangers associated with the ionising radiation from a $^{64}\text{Cu}$ dose.

It was not until 1994 when a study was finally published on human copper metabolism, using stable isotope data (Scott & Turnlund, 1994). The study was originally designed to examine the absorption and retention of labelled copper, during
and after three periods of (unlabelled) copper intervention using low, medium and high copper diets (Turnlund et al, 1989). As such, the data were not ideal for developing a compartmental model, as the blood was not sampled regularly enough after oral dose administration to permit an accurate model fit to the plasma appearance of labelled copper. Nevertheless, this was the first model to correctly identify that ceruloplasmin is the protein that transports copper to body tissues, but it underestimated the percentage of copper in the plasma that is bound to ceruloplasmin (62-72%) in comparison to the model in paper [8] (mean=99%).

The detail of our model (paper [8]) was such that it gave a good representation of most of the copper storage sites and estimated the quantity of liver copper more accurately (2.4mg) than Scott & Turnlund’s work (88.8mg) when compared to the reference value (9.9mg) from autopsy work (Linder, 1991). This autopsy data also suggest that the average human has 110mg of copper stored in the body, but at least 60mg of this is in the skeleton. The study in paper [8] was too short in duration to estimate the size of the skeletal pool, but this could be possible in a longer study, assuming that the enrichment of labelled copper in plasma was measurable for more than the estimated 30 days that would be needed in such an experiment. An interesting finding was the large quantity of copper that is lost through the bile. Previous work had suggested that this is the primary mechanism of copper homeostasis, since loss through the urine is negligible (Cartwright & Wintrobe, 1964). Post-mortem examinations have indicated that large quantities of copper are stored in the bile duct (Cartwright & Wintrobe, 1964) and evidence for copper excretion via bile has been demonstrated with radio and stable isotopes (Bush et al, 1955; Turnlund et al, 1998).

In paper [8], a large, first-pass effect for copper was quantified as 74% of the absorbed dose. This is the first report of a copper first-pass effect in humans; the Scott & Turnlund (1994) paper failed to recognise that newly absorbed copper enters the liver before being circulated to other tissues. The quantity of copper removed on first-pass is, unexpectedly large, but the reasons for this are unclear. We speculate that it could be connected with the repackaging of newly absorbed copper, from albumin, into ceruloplasmin but there might also be a need to store newly absorbed copper for subsequent release to maintain fasting plasma levels, which are quite tightly
controlled and generally not thought to be linked with status (Medeiros et al, 1991; Milne, 1994) although a recent systematic review concluded that plasma copper concentrations may reflect dietary intake under some circumstances (Harvey et al, 2009). Another explanation for the first-pass effect could be the liver’s role in maintaining homeostatic control over retention, by excreting excess copper via the bile. The liver is clearly at the centre of copper metabolism, and this is reflected in its predominant role in the model (Figure 1, paper [8]).

**Conclusion and future work**

The model developed in paper [8] is only the second of its kind to be published, and is the most detailed and accurate representation of copper metabolism, in humans. Extra detail would be forthcoming if an experiment could be designed that took samples over a longer period, but this might not be possible with a physiological dose of Cu-65, since the determination of accurate copper ratios, in plasma, are currently not feasible with ICP-MS after about 14 days (post-dose), due to insufficient enrichment.
2.1.3 Calcium

The structure of bone is highly dynamic and about 10% of the skeleton is undergoing constant “remodelling”. Osteoporosis is a bone disease that can lead to increased risk of fracture and the underlying cause of this is more bone being broken down (bone resorption), than is made (bone formation). Bone resorption releases calcium, whereas formation requires it, but although skeletal integrity is important, the main homeostatic driver for calcium is the maintenance of the calcium plasma concentration, within very tight tolerances. This is because calcium has an important role in intracellular signalling, neurotransmission, muscle contraction and many other functions. These vital processes take precedent over maintaining a healthy skeleton and therefore calcium will be released from bone to ensure that they are preserved. Therefore, there is greater risk of developing osteoporosis when (over a prolonged period) the daily quantity of absorbed (dietary) calcium is less than the daily endogenous calcium excretion because more bone will be resorbed in order to maintain the “correct” plasma concentration (Heaney et al, 1977).

Objective

High salt intake is considered to be a risk factor for osteoporosis because urinary calcium excretion (calciuria) is increased by diets high in sodium (Teucher & Fairweather-Tait, 2003). However, many previous studies that investigated this hypothesis used intervention diets that contained salt levels far in excess of those commonly consumed. The objective of the study presented in paper [14] was to clarify the effects of salt (and calcium) on calcium metabolism at normal dietary levels.

Calcium Balance

Calculations from earlier studies have indicated that the average additional body loss of calcium is 40mg for every 2290mg of sodium consumed (Zarkadas et al, 1989). The increased urinary loss of calcium must be compensated for by increased absorption, or increased calcium intake, otherwise there is the potential for the skeleton to lose 10% of its calcium within a decade. Measurements of calcium “balance” are crucial in assessing the loss of calcium from the skeleton (so-called negative balance), and at what rate. This measure of balance can be achieved by the
use of compartmental modelling because, in conjunction with stable isotope labelling, the major calcium fluxes can be quantified either directly (calcium used in bone formation, calcium excretion), or indirectly (calcium lost in bone resorption). This is particularly useful when studying calcium metabolism as the vast majority (>99%) is stored in bone, which turns over too slowly to allow its size to be measured.

The study that is described here (paper [14]) is a 4-way cross-over that attempts to examine the effect of salt and calcium on overall calcium balance. Eleven postmenopausal women completed a randomised cross-over trial consisting of four successive, five week periods of controlled dietary intervention, each separated by a minimum 4 week washout. Moderately low and high calcium (518mg versus 1284mg) and salt (3.9g versus 11.2g) diets, reflecting lower and upper intakes in postmenopausal women consuming a Western-style diet, were provided. On the morning of day 30 (at the end of each intervention period), subjects consumed a drink of water containing an extrinsically labelled dose (20mg) of Ca-42 (as CaCl$_2$) with a standard breakfast that contained 183mg of unlabelled calcium. Approximately 30 minutes later, a solution of Ca-43 (4.5mg as CaCl$_2$) was infused over 20 minutes through a cannula. Blood was sampled intensively over the first six hours and then at days 1, 4, 8 and 10 post-dosing. In addition, 24-hour urine and faecal samples were collected for 10 days post-dose. Compartmental modelling was used to estimate calcium absorption, excretion and bone calcium balance. Biochemical markers of bone formation and resorption were also measured in blood and urine.

**Compartmental Model**

Arriving at the final two-compartment model (Figure 1 in paper [14]) was a process of trial and error. Prior to data collection, it was thought that a three-compartment model would be optimal as several other studies had used this approach (Neer et al, 1967; O’Brien et al, 1998; Wastney et al, 1996, 2000). The model should attempt to reflect the known physiology but must also be the simplest that fits the data (Principle of Parsimony). After extensive model comparison and goodness of fit tests, it was found that a two-compartment model best represented the data. The uncertainty estimates for the rate constants were quite low for modelling of this type with a typical coefficient of variation being less than 10%. This was due to the large data set that was collected and the unique way it was used by the model fitting program (SAAMII). The program
has a facility that allows both the oral and the IV data to be fitted simultaneously to the same model, thereby doubling the degrees of freedom that are used. Typically, the model was attempting to estimate seven parameters, but there were approximately sixty data points with which to do so (thirty from each isotope “source”), and this meant that the parameters could be estimated with good precision.

Compartment 1 represents the blood and compartment 2 is probably a combination of soft tissue pools (see Figure1, paper [14]). The rate of whole body calcium loss or gain (balance, $V_{\text{bal}}$) was estimated from the principles of mass balance (= rate of calcium into system – rate of calcium out of system, i.e. $V_{\text{bal}} = V_a - V_u - V_f$). This requires a knowledge of the rate of calcium absorbed ($V_a$), and the rate of calcium excreted via urine ($V_u$) and into faeces (endogenous Ca loss, $V_f$). It is also useful to estimate the rate of calcium loss ($V_{0-}$) and gain ($V_{0+}$) to bone. The gain to bone ($V_{0+}$) can be measured directly from the compartmental model but the loss ($V_{0-}$) cannot, and has to be estimated by difference ($V_{0-} = V_{0+} - V_a + V_u + V_f$). The reason that $V_{0-}$ cannot be estimated directly is that labelled calcium, taken up into bone from the oral dose, will not be broken down and returned to the plasma within the timeframe (10 days) of the experiment.

The results of the study demonstrated that a moderately high salt intake (11.2g/d) elicited a significant increase in urinary calcium excretion ($p < 0.001$) and significantly affected bone calcium balance with the high calcium diet ($p = 0.024$). Efficiency of calcium absorption was higher following a period of moderately low calcium intake ($p < 0.05$) but was unaffected by salt intake. Salt was responsible for a significant change in bone calcium balance, from positive to negative, when consumed as part of a high calcium diet. With a low calcium intake, the bone calcium balance was negative with both high and low salt diets (see Table 2 in paper [14]).

The strengths of this study were that the volunteers acted as their own controls which eliminated the confounding effects of genotype that are known to be strongly related to the probability of developing osteoporosis (Parfitt, 1997). An additional strength of the design was the use of a range of salt levels that are commonly consumed in the UK. This allows the conclusions of the study to be applied more widely than if higher (or lower) levels had been used. A potential weakness was that the study gave the
same calcium load for the test breakfast on day 30, irrespective of which calcium diet (high or low) the volunteers were on. It is known that absorption is influenced by calcium load (Heaney et al, 1989) as well as by physiological requirements and the test breakfast should have been tailored to the level of calcium that the volunteers were consuming at breakfast over the 30 days of the intervention diet. The 183mg of calcium that was fed would have been appropriate for the low calcium diet but it would have been preferable to have given about 450mg of calcium at breakfast on the test days for the high calcium diet.

The standard deviations for the estimated, mean calcium balances are worth examining in more detail. For each individual set of data, the compartmental modelling estimated the model parameters (rate constants) with good precision. However, the means of the two model parameters for calcium excretion ($V_f$ and $V_u$) had large uncertainties associated with them (see Table 2 in paper [14]). This is due to the large, inter-individual variation in excretion and is explained by certain volunteers being “non-responders”, described as the fact that some people do not alter calcium excretion in response to salt challenges (Shortt et al, 1988; Leitz et al, 1998). $V_f$ and $V_u$ were used in the derivation of calcium balance ($V_{bal}$) and this partly explains why the parameter has a large uncertainty associated with its mean. Another reason is that, on adding or subtracting one quantity from another, the errors in these quantities propagate to form larger uncertainties in the derived parameter ($V_{bal}$). The fact that the calcium balance was estimated with such large uncertainty explains why some of the findings did not reach statistical significance, even though a trend was evident. Other studies that derive calcium balance in this way have suffered from similarly large uncertainties (e.g Wastney et al, 2000; Bryant et al, 2003). This highlights the need for a larger study with possibly even longer adaptation to the intervention diets before clear conclusions can be reached.

**Comparison of findings to other studies**

On comparing the work carried out in paper [14] to other studies, several points emerge. First, as has been mentioned earlier, other authors have used an extra compartment to model their data (e.g. Wastney et al, 1996). This was found unnecessary with the data generated in paper [14] and the resulting model parameters were estimated with similar precision to other work. The model parameter values
were also similar, so there was no perceived loss of accuracy by having one less compartment.

Second, some previous studies did not collect faeces but still managed to estimate endogenous loss in the faeces, with reasonable precision (O’Brien et al, 1998; Kerstetter et al, 2005). This is somewhat surprising given that others estimate this parameter with less precision, having collected faecal samples. Some preliminary work was performed with the data in Paper [14] to examine the effect of not fitting the faecal data, but the parameter estimates were badly affected and the faecal data was subsequently included in all analysis. The other advantage of including faecal data is its use in absorption estimates.

A third point that arose in comparing paper [14] with other work, was the lack of controlled diets and small sample sizes in previous papers (e.g. Smith et al, 1996). Even though the study in paper [14] had controlled diets and eleven volunteers it was still under-powered to detect significant changes between some of the dietary interventions. Power calculations performed prior to the study design had indicated that 16 volunteers would be needed but, due to the invasive and time consuming nature of the study, five volunteers dropped out.

**Conclusions**

Paper [14] presents a model that estimates the calcium flux from bone and therefore allows an estimation of bone calcium balance after four intervention diets in the same subjects. Although the parameters (rate constants) of the model are estimated with good precision, the calcium balance has a large uncertainty associated with it because of the combined effect of error propagation, and its derivation from the calcium excretion parameters ($V_u$ and $V_f$), which are subject to large inter-individual variation. There is a significant difference ($P=0.024$) of bone calcium balance between the low salt ($V_{bal}=90±46$ mg/d) and high salt ($V_{bal}=-12±84$ mg/d) regimen on the high calcium diet.

**Future work**

A larger study is warranted that has a longer adaptation period to each diet.
2.2 **Vitamins**

### 2.2.1 Folate

Folate is the generic name for the members of a family of water soluble B-group vitamins. They are primarily comprised of reduced methyl and formyl folypolyglutamates that have similar chemical structures to folic acid (a monoglutamate), the synthetic form of the vitamin. There is considerable interest in folates because of their involvement in 1-carbon metabolism which is crucial for nucleotide biosynthesis (Stover, 2009). In addition, low folate status has been implicated in neural tube defects and higher risks of cardiovascular disease and stroke (McNulty et al, 2007).

**Plasma Appearance of Labelled Folate**

The rationale for the initial work (paper [3]), was to compare the bioavailability of three forms of folate by observing their appearance, in plasma, as 5-methyltetrahydrofolate (5-methylTHF) in human volunteers. At the time of publication (2003), other researchers were using folic acid as a reference dose because $^{14}$C labelled folate studies had shown that about 90% of a dose of folic acid was absorbed (Clifford et al, 1998). Stable isotope labelled folates were generally not available so folate bioavailability studies were designed with unlabelled folate doses in conjunction with sampling of the plasma for a few hours to quantify the area under the (plasma concentration) curve (AUC). These were “relative absorption” studies as an unlabelled dose of folic acid was usually given and its AUC compared to that of the other folates, with the assumption being that folic acid absorption was of the order of 90-100% (Pietrzik et al, 1990; Prinz-Langenohl et al, 1999).

Paper [3] is an account of one of the first experiments to use stable isotope labelled folates and the study it describes was designed to replicate the protocol of previous, unlabelled folate studies, in order to test the validity of the labelled “tracer” approach. The experimental protocol employed two groups of volunteers receiving either 285µg of $^{13}$C-labelled folic acid (n=12) or 200-230µg of $^{13}$C-labelled 5-formyltetrahydrofolate (n=12). The study was performed after an overnight fast and serial blood samples were taken regularly for 8 hours post-dose. It is well known that the main form of folate in the plasma is 5-methylTHF and the first task, after
measuring the plasma samples by LC-MS, was to quantify the labelled plasma portion of the 5-methylTHF (from the doses) from the total 5-methylTHF. The calculations for this are shown in Appendix 1 of paper [3].

The results indicated that the plasma profile had perturbations of labelled and unlabelled folate (Figure 1 in paper [3]). This was unexpected because only a labelled dose had been given to the volunteers and it was concluded that the origin of the unlabelled folate was endogenous. A similar finding has subsequently been found for vitamin C (Bluck et al, 2005) which could indicate that all water-soluble vitamins have a similar mechanism. Paper [3] speculated that the original site or store of this displaced, unlabelled folate was the liver. The amounts of displaced folate varied widely between volunteers as evidenced by the far bigger error bars on the “total” 5-methylTHF compared to the “labelled” 5-methylTHF (Figure 1, paper [3]) but there was no significant difference in the AUC of the displaced, unlabelled 5-methylTHF generated after the folic acid dose compared to that after the 5-formyltetrahydrofolate (5-formylTHF) dose.

**Unexpected Findings**

The reason why the unexpected displacement of endogenous folates is an important finding, is that previous researchers had given unlabelled folate doses and measured the appearance of folate in the plasma to ascertain the “relative” bioavailability of the dose (Konings et al, 2002; Pentieva et al, 2004). This assumed that the change in plasma folate was all due to the dose, an assumption which our results indicate is not true. This finding invalidates these earlier, unlabelled folate studies, and leads to the conclusion that only labelled-tracer studies can be used to assess folate bioavailability.

A second, unexpected finding was the difference in plasma profile of the labelled 5-methylTHF from the folic acid dose compared to profile from the 5-formylTHF dose. There is a long delay in the peak appearance of 5-methylTHF derived from folic acid ($T_{\text{max}}=173$ minutes) compared to that derived from the 5-formylTHF dose ($T_{\text{max}}=41$ minutes). This was the first time such a discrepancy in plasma profiles had been observed, and appeared to suggest that folic acid and 5-formylTHF are handled very differently by the body. The folic acid had been given as a capsule, and an adjustment in the $T_{\text{max}}$ was required to reflect the delayed breakdown of the capsule. This delay was taken into account in a later paper (paper [7]) and reduced the “absorption time”
of folic acid by approximately 35 minutes so the “true” $T_{\text{max}}$ is probably about 140 minutes.

The third unexpected finding from paper [3] was that the (dose corrected) AUC of labelled 5-methylTHF was significantly smaller ($P<0.001$) after the folic acid dose compared to the 5-formylTHF dose. This meant that the relative absorption of the dose of 5-formylTHF was 221% compared to that of folic acid (assumed to be 100%) which led to two possible conclusions with regard to folic acid metabolism:

1) Previous papers are incorrect when they suggest that a dose of folic acid is almost completely (90-100%) absorbed, or,
2) Folic acid is metabolised in a different way to 5-formylTHF.

It is safe to dismiss conclusion (1) as the previous papers that found ~90% absorption, had used state-of-the-art measurement techniques with $^{14}$C-labelled folic acid doses.

**Initial Site of Metabolism of Folic Acid**

Conclusion (2) deserves more scrutiny, especially when the evidence of the apparent low absorption of folic acid is added to the finding of a delay in the peak appearance of labelled 5-methylTHF after a labelled folic acid dose. It had been argued for some time in reviews of folate metabolism, that all forms of folate were metabolised by the mucosal cells before being transported in the portal vein as 5-methylTHF to the liver (e.g. Gregory & Quinlivan, 2002). The problem with this theory, for folic acid, is that it originates from studies performed on rats which were then extrapolated to humans (Tani & Iwai, 1983). In fact, there was evidence before 1983 to show that folic acid entered the portal vein intact (Whitehead & Cooper, 1967; Melikian et al, 1971) but these papers were dismissed because the folic acid dose was deemed to be too large (non-physiological) even though the Melikian study had given just 500µg of folic acid. This earlier work by Whitehead & Cooper (1967) and Melikian et al (1971) and the results of paper [3] began to lead us to speculate that the site of reduction of folic acid had to be the liver, not the mucosal cells.

Whilst the site of initial metabolism may be different for folic acid compared to other folates, this hypothesis isn’t enough to explain the findings of paper [3]. It wasn’t until the publication of paper [7] that the reasons for the apparent, low folic acid absorption were revealed. Paper [7] presents the modelling approach (the Critical
Analysis of paper [5] will provide the overall modelling detail) and uses the data set from paper [3], plus additional data from that study which was not analysed at the time paper [3] was in press. It includes the data from an intrinsically labelled ($^{15}$N) spinach meal (n=14) as well as the full data set from the ($^{13}$C) 5-formylTHF dose (n=16) and ($^{13}$C) folic acid dose (n=14). The folate in the spinach was predominantly 5-methylTHF and the labelled dose was approximately 264µg of folate. This experiment was originally designed as a cross-over but very few of the volunteers managed to complete more than one of the interventions, so the data are presented and analysed as independent observations. The intrinsically labelled spinach has a 5-methylTHF plasma appearance similar to that seen after the 5-formyl labelled drink but quite different to the labelled folic acid (Figure 2, paper [7]). The labelled 5-methylTHF from the folic acid dose had a significantly (P<0.001) delayed appearance in the plasma ($T_{\text{max}} = 171\pm9$ minutes) in comparison to that from the 5-formylTHF ($T_{\text{max}} = 54\pm10$ minutes) and the spinach derived 5-methylTHF ($T_{\text{max}} = 60\pm13$ minutes). Here were further results that the folic acid was being handled differently by the body, contrary to what had been reported consistently since 1983. Additional evidence was apparent when the results from the modelling were examined. The model predicted that the apparent absorption of the folic acid dose (24±2%) was significantly lower (P<0.05) than that from the 5-formyl (38±6%) and the spinach (44±6%). Taking the apparent absorption of folic acid as the reference dose (i.e. absorbed at 100%, as many papers have done by using AUC (e.g. Konings et al, 2002)), it was concluded that 5-formyl is absorbed at 158% and the spinach folate at 183% of the quantity in the dose. This is clearly incorrect but was the first time that anomalous absorption results had been published using folic acid as a reference dose.

**First-pass effect**

It is speculated that the reason for the low, apparent absorption of folic acid is that, all newly absorbed folates are subject to a first-pass effect of the liver (Steinberg et al, 1979) and, that the liver has a higher affinity for folic acid than other folate forms (Steinberg et al, 1979; Selhub, 1989; Zhao et al, 2009). When these two vital pieces of information are added to the hypothesis that folic acid, uniquely amongst the folates, is transported intact to the liver, the reason for the relatively, low apparent absorption of folic acid becomes clear. The Steinberg et al (1979) paper used rats to demonstrate the first-pass effect and the work in paper [7] is the first to confirm it in humans. The
liver must be “removing” a larger fraction of the absorbed folic acid dose in comparison to other folates (on first-pass) if the results from papers [3, 7] are to be reconciled with previous work that reports high absorption (~90%) of folic acid. By assuming that the absorption of all three folate doses was 90%, an estimate can be made of the first-pass effect for each folate. The folic acid dose (73±2%) had a significantly higher (P<0.05) fraction of the absorbed dose removed by the liver on first-pass than did 5-formyl (58±6%) and spinach folate (52±6%). Interestingly, Table 2 of paper [7] contains data on the correlation coefficients of percentage first-pass effect versus fasting plasma folate concentrations which shows that there is a significant inverse (wrongly assigned in paper as being positive) correlation for folic acid (r=-0.703, P=0.005) and 5-formyl (r=-0.770, P<0.001) and a borderline inverse correlation with spinach folate (r=-0.507, P=0.054).

**Homeostatic control**

This suggests that the quantity of folate the liver removes on first-pass is under homeostatic control to ensure that the fasting plasma concentration of folate is maintained. Folate is a water soluble vitamin and cannot, therefore, be stored in lipid tissue. However, the finding from paper [3] that a large quantity of endogenous folate appears in the plasma after a meal does suggest that the liver has a short-term storage capacity. This observation, in conjunction with the inverse correlations seen in paper [7], lead to the conclusion that the liver sequestrates a certain fraction of newly absorbed folate to maintain fasting levels of 5-methylTHF. The biological mechanisms for this homeostatic control are unknown but warrant further investigation. It was originally speculated in paper [3] that the mechanism was the enterohepatic recirculation of folate. In hindsight, this overstated the role of enterohepatic recirculation in maintaining a fasting level of 5-methylTHF, as it is a process that happens infrequently, namely when the gall bladder is emptied following a meal, and therefore cannot be expected to contribute significantly to the overall plasma concentration.

**Explanation for delay in plasma appearance of folic acid derived folate**

Paper [3] also suggested that enterohepatic recirculation could explain the delay in appearance of labelled 5-methylTHF from a folic acid dose in comparison to that from other folate forms. A much more plausible theory for this phenomenon was put
forward in a recent paper by Bailey & Ayling (2009). All forms of dietary folate are metabolised to 5-methylTHF by an enzyme called dihydrofolate reductase (DHFR). The Bailey & Ayling paper has shown that humans have a reduced capacity (more than 50-fold) to metabolise folic acid by DHFR compared to rats. That paper predicts that the absorption of a folic acid dose of 400µg would be metabolised by DHFR (in humans) in about an hour after peak absorption which would explain the delayed peak appearance of labelled 5-methylTHF, after the folic acid dose in papers [3, 7].

Paper [7] adds evidence to support the proposal that folic acid is handled differently to other folates by the human body. Until its publication, researchers had used the plasma appearance of unlabelled folic acid as a “reference” dose (usually presented as an AUC) as a standard against which to compare the relative bioavailability of other forms of folate. The work in papers [3, 7] has shown that this is invalid for two reasons. Firstly, large quantities of endogenous 5-methylTHF appear in the plasma after an oral dose of folates and there is no method of distinguishing this from the newly absorbed folate (paper [3]). Secondly, folic acid appears to be handled in a different way, compared to other forms of folate (paper [7]), which makes it unsuitable to use as a reference.

The results from papers [3, 7], in conjunction with the work by Whitehead & Cooper (1967), Melikian et al (1971), Steinberg et al (1979) and Bailey & Ayling (2009) have explained why folic acid is handled differently, by humans, in comparison to other folates. The conclusion from all these studies is that human folate bioavailability can only be assessed properly by the use of labelled folates in combination with mathematical modelling.

Collaborative work (I)

Paper [5] provided details of a novel modelling approach to estimating bioavailability using labelled folate doses. Five volunteers were given two simultaneous oral doses of folic acid (\(^{13}\)C and \(^2\)H labelled) and serial blood samples were collected for 8 hours (post-dose). The \(^{13}\)C-folic acid (dose=224µg) was in the form of a capsule and \(^2\)H-folic acid (dose=207µg) was a drink. We took a similar modelling approach to the one that had been taken with iron (see papers [2, 10] and earlier discussion in the Iron section of the Critical Analysis). A priori identifiability suggested that a one or two
compartment model would be feasible to estimate the absorbed mass of folate, from the plasma appearance of 5-methylTHF, over a minimum of 6 hours post-dose, using the same assumptions of zero-order absorption. It was thought to be more difficult to estimate folate bioavailability as compared to iron, since it was reported (in rats) that a significant fraction of newly absorbed folate did not appear in the plasma due to some of the folate being sequestered by the liver as a first-pass effect (Steinberg et al, 1979; Steinberg, 1984). No attempt had ever been made to quantify this in humans. In addition, the volume of distribution of folate was not the plasma volume but was, in fact, a far larger space which Loew et al (1987) had estimated to be 387ml/kg body weight. After much preliminary work on model comparison (using AIC and BIC parameters), with the data generated from the study, it was decided that the one-compartment model was preferable in terms of goodness of fit and simplicity.

For paper [5], we focussed on estimating the first-pass effect and just assumed the fractional absorption of folic acid to be 90%, based on previous findings (89% - Clifford et al, 1998; 90% - Butterworth et al, 1969; 92% - Krumdieck et al, 1978). A large fraction of the absorbed folate was removed on first-pass (72±10%) which compared favourably to an estimate we made of work by Clifford et al (1998) that used accelerator mass spectrometry to measure the plasma appearance of a 14C-labelled dose of folic acid. That paper had reported the most detailed study to date on human folic acid metabolism and, although it hadn’t estimated the liver first-pass effect, it was possible to quantify it from the data in the paper, to be about 65% (Finglas et al, 2002). The equivalent value of 72% in paper [5] is almost certainly an overestimate since non-metabolised folic acid was detected in the plasma (see Figure 3a in paper [5]) but this wasn’t corrected for in the model (the model assumed that the entire labelled dose of folic acid had been converted to labelled 5-methylTHF). This “free” folic acid was a manifestation of the drawback in the design of this study (paper [5]) because the combined dose of folic acid from the capsule and the drink was 431µg which is above the known folic acid dose threshold (~300µg) when intact folic acid will appear in the plasma (Kelly et al, 1997). More reliability can be placed on the other data presented in the paper such as the absorption time (T), which is defined as:

\[ T = T_{\text{max}} - T_{\text{lag}} \]

where:

- \( T_{\text{max}} \): time for labelled folate plasma concentration to reach a maximum after test dose
- \( T_{\text{lag}} \): time for labelled folate concentration to be detectable after test dose
The absorption time (T) for folic acid from the capsule (T=146±38 minutes, T_{max}=182 mins, T_{lag}=36 mins) was larger than that from the drink (T=122±21 minutes, T_{max}=122 mins, T_{lag}=0 mins). This compares with a T_{max} of 173 minutes for the peak appearance of labelled 5-methylTHF from the folic acid capsules reported in paper [3].

**Collaborative work (II)**
The combined methodology of stable isotope labelled folate and compartmental modelling was used successfully in two more studies [9, 11]. In paper [9] an attempt was made to estimate the “true” absorption of folic acid by taking account of the first-pass effect. The study comprised 12 young (<30y) and 12 middle-aged (>50y) volunteers who were randomized into two groups to receive daily supplementation with 400µg of folic acid or 5-methylTHF for five weeks. Before and after supplementation, an oral test dose of 202µg \(^{2}\)H-folic acid was given to the volunteers to estimate their absorption and metabolism of folate. Leaving aside the post-supplementation results, it is worth examining the data from the two age groups before supplementation (n=12 in each group). Folic acid apparent absorption was significantly higher (P=0.031) in the young (39±12%) compared to the middle aged adults (29±7%). This was an unexpected finding, and hadn’t been reported before. After supplementation with 5-methylTHF, the apparent absorption was lower and the differences between age groups less marked (33±10%; <30y and 28±11%; >50y). After supplementation with folic acid, the apparent absorption was higher for the young adults but about the same for the middle aged adults (43±8%; <30y and 28±14%; >50y). It is difficult to interpret the “after supplementation” results as the group sizes were small (n=6) and a larger study would be needed to assess any differences in folate absorption after different supplements, but there is clearly a trend for younger adults to absorb more folate than middle aged adults.

The first-pass effect was set to be a constant fraction (fraction of absorbed dose that is removed by liver = 0.65) of the absorption for all volunteers in both supplementation regimen based on previous work (Finglas et al, 2002). This figure would appear to be too high for the young volunteers (<30 y) as the estimated true absorption values are, on average, above 100%. The absorption values for the older volunteers appear to be
Collaborative work (III)

Paper [11] uses compartmental modelling to estimate the apparent absorption of folate from unlabelled test foods and capsules containing either 5-methylTHF or folic acid. This paper also used ileostomists as subjects so that the predicted bioavailability from the modelling could be verified. Faecal balance in normal subjects cannot be used to assess bioavailability because the gut microflora can degrade folate. Nine ileostomy volunteers were recruited to the study; they each consumed the same seven test foods on different days and had serial blood samples taken for 10 hours (post-dose) on each occasion. The bioavailability of folic acid and 5-methylTHF were estimated from both the AUC and a model developed from previous work (papers [5, 7]). The volunteers also collected all their ileostomy effluent over the same 10 hour period, enabling an assessment of the quantity of non-absorbed folate to be calculated. To try and ensure that all volunteers had the same folate status prior to the test days, each consumed a daily capsule of 960µg folic acid. This is the so-called “presaturation” protocol that other unlabelled folate studies have used (Tamura & Stokstad, 1973).

A single compartment model was sufficient to fit the plasma appearance data from all the test foods except for the bread, where two compartments were required. The second compartment was equivalent to modelling the absorption as a first order process. This results in a model that equates to the Bateman function (Equation 3, paper [11]). The apparent absorptions (median) of folate from the test doses were as follows: fermented milk 86%, fermented milk with folate binding protein (FBP) 62%, fermented milk pasteurised with FBP 55%, bread 74%, yeast crème 80%. Previous research (paper [3]) had found that the plasma response would consist of newly absorbed as well as endogenous folates. However, it was reasoned that the comparison of the results for each test meal would be easily interpretable because the
nine volunteers had each of the test meals (randomised) in a cross-over design. Having established that endogenous folates would form part of the plasma response, one of the aims of pre-saturation was to minimise this and, also, to minimise any first-pass effect. This probably explains why the apparent absorption values were higher than those seen in previous work (papers [7, 9]) using the same method. The excretion of 5-methylTHF as measured from the effluent samples is shown in Table 4 of paper [11].

Paper [13] contains a summation of our work on modelling folate metabolism. The two main findings from papers [3, 5, 7, 9, 11] are, firstly, that folic acid appears to be absorbed to a lesser extent than other folate forms. This was surprising because many previous studies had demonstrated that about 90% of the dose is absorbed. The observation can be explained by assuming that folic acid is metabolised in the liver and all other folate forms are metabolised in the mucosal cells. The liver has a higher affinity for folic acid than other forms of folate and therefore it sequesters a higher fraction of absorbed folic acid, on first-pass, than it would if it were 5-methylTHF.

The second important finding is that the 5-methylTHF peak appearance in plasma (after a folic acid dose) is about 80 minutes later than other forms of folate. This delay in plasma appearance after a folic acid dose is important because it had not been observed before with unlabelled folate doses and confirms the finding that the human, almost uniquely, has very low capacity to metabolise a folic acid dose to 5-methylTHF. A recent paper in PNAS (Bailey & Ayling, 2009) has demonstrated that the human DHFR activity is 50 fold lower than that of the rat. Their data suggest that a 400µg dose of folic acid will take about 60 minutes to be metabolised to 5-methylTHF after peak absorption has occurred, which, for a liquid test dose, would happen about 40 minutes after ingestion of the dose. This suggests that the plasma peak in 5-methylTHF appearance, after a folic acid dose of 400µg, should be approximately 100 minutes. Paper [5] reported the time of maximum 5-methylTHF concentration (T\text{max}) to be 122±21 minutes, for a 207µg dose. Although paper [9] did not report the T\text{max} for the 202µg dose of folic acid, it was 119±39 minutes (J Dainty, unpublished). After correcting for the time taken for capsule dissolution (approximately 30 minutes), paper [7] reported a T\text{max} of about 140 minutes for a 285µg dose of folic acid. These peak times to convert folic acid to 5-methylTHF are all longer than predicted by Bailey & Ayling (2009) but of the right order of
magnitude. This suggests that the *in vitro* data overestimate the activity of *in vivo* DHFR by about 50% but further work is needed to clarify this.

Paper [13] hypothesises that the reason many people living in the US have folic acid circulating in their plasma is that this form of folate is metabolised in the liver, not in the mucosal cells. The constant exposure of the liver to folic acid fortified products, coupled with its low DHFR activity, should lead to some folic acid appearing in the plasma. Evidence to corroborate this hypothesis has come from a large study in the US (Troen et al, 2006) that found 78% of 105 postmenopausal women had measurable fasting concentrations of folic acid in their plasma. This paper also showed a significant relationship between reduced natural killer (NK) cell toxicity and elevated levels of folic acid in plasma, which suggests that “free” folic acid compromises the immune function in some way. Other papers have suggested a link between high intake of folic acid and risk of colorectal neoplasia (Van Guelpen, 2005; Cole et al, 2007) and breast cancer (Stolzenberg-Solomon, 2006). These findings are particularly pertinent for the US which has had a mandatory folic acid fortification policy since 1998 and intakes of more than 1.0mg/d are not uncommon (Choumenkovitch et al, 2002; Quinlivan & Gregory, 2003). As the threshold dose for folic acid appearing in the plasma is about 0.3mg, it would not be surprising if a significant proportion of the US population have detectable concentrations of folic acid in their plasma.

**Conclusions**

Studies using labelled folates have led to the observation that (1) large quantities of endogenous folate appear in the plasma after a labelled folate dose, (2) folic acid is metabolised more slowly to 5-methylTHF and, is absorbed to a lesser extent, in comparison to test doses of 5-formylTHF and spinach folate. Mathematical modelling has helped to explain finding (2) by showing that more folic acid is removed on first-pass through the liver than other folates. This indicates that folic acid is metabolised by the liver and other folates are metabolised by the mucosal cells.

**Future work**

The portal vein connects the GI tract to the liver and all newly absorbed folate must pass through it. By sampling this vein after a folic acid test meal, it would be possible
to identify the folate form that enters the liver. In addition, *in vitro* studies predict an earlier plasma peak of 5-methylTHF, after a folic acid dose, than is observed from *in vivo* work. Modelling the enzyme kinetics of the conversion (by DHFR) of folic acid to 5-methylTHF could help to pinpoint why these differences arise.
2.2.2 Carotenoids

Epidemiological studies indicate that the consumption of foods rich in carotenoids is associated with a reduced risk for certain types of cancer and cardiovascular disease (e.g. Gey et al, 1993; van Poppel, 1993). There is controversy over the effectiveness of carotenoids to prevent disease because several studies have concluded that they are poorly absorbed (O’Neill & Thurnham, 1998; Castenmiller et al, 1999). These studies examined the plasma response after a test meal and found little or no change in the carotenoid concentration. However, another study hypothesised that, in fact, the absorption was quite high (greater than 50%) but the clearance from the plasma was so rapid that very little of the absorbed carotenoid could be observed (Faulks et al, 1997). Paper [6] describes an experiment that tests this hypothesis.

Methods used to assess carotenoid absorption

There are four techniques that have been commonly used to assess bioavailability:

1) Appearance of unlabelled carotenoids in plasma after a test meal (Brown et al, 1989).

2) Appearance of unlabelled carotenoids in the triglyceride rich lipoprotein (TRL) fraction in plasma after a test meal (van Vliet et al, 1995).

3) Appearance of stable isotope labelled carotenoids in plasma after a test meal (Novotny et al, 1995).

4) Appearance of radio isotope labelled carotenoids in faeces after a test meal (Dueker et al, 2000).

Approach (1) has been discredited because it is not sensitive enough and requires pharmacological doses before any plasma response is observed (van den Berg, 2000). Techniques (3) and (4) are sensitive and selective for the absorbed dose but they are expensive and may require intrinsic labelling of the food under study for valid results. Approach (2) is relatively inexpensive and can be used with any food containing a relatively high concentration of carotenoid. At the time of publication of paper [6], approach (2) had been used by other groups but they had concluded that carotenoid absorption was quite low (van Vliet et al, 1995 - absorption=11%; O’Neill & Thurnham, 1998 – absorption=4%). The models used in these papers appear to
calculate a low absorption because of an underestimation in the rate of clearance of carotenoid from the plasma.

**Novelty of study design**

Newly absorbed carotenoids are found in the triglyceride rich lipoprotein (TRL) fraction and these can be isolated from blood samples (Goodman et al, 1966). The TRL fraction consists of chylomicrons and chylomicron remnants (e.g. VLDL). The papers of van Vliet et al (1995) and O’Neill & Thurnham (1998) had measured carotenoid from the TRL and then estimated absorption with the assumption that the half-life of newly absorbed carotenoids in plasma was 11.5 minutes (mean of reported half-lives for chylomicron remnants). The study in paper [6] hypothesised that the true half-life of newly absorbed carotenoids is less than 11.5 minutes as the TRL contains a mixture of chylomicrons (Grundy & Mok, 1976: $T_{1/2}=2-5$ minutes) and chylomicron remnants. To allow for individual variation, paper [6] estimates the half-life of newly absorbed carotenoids for each subject in the study.

To validate the modelling approach in paper [6], we recruited ileostomy subjects in order to collect their non-absorbed carotenoid. The carotenoid had not been degraded by colonic bacteria as it had only passed through part of the small intestine. This allowed a comparison of estimated absorption (by modelling plasma appearance of carotenoid) with actual absorption (measurement of unabsorbed carotenoid in the volunteers’ effluent, collected via the stoma).

Some previous studies had used β-carotene capsules to test bioavailability (e.g. Traber et al, 1994), but the study reported in paper [6] examined the plasma response using a real food. Spinach was selected as the test meal as it was known to contain two types of carotenoid (lutein and β-carotene) in relatively large amounts. Nutrition advice is based on the consumption of food and it is important to clarify the bioavailability of carotenoids from commonly consumed meals, rather than supplements.

**Initial metabolism of carotenoids**

During the absorptive process, a variable fraction of the β-carotene is metabolised to retinyl ester in the enterocyte (Goodman et al, 1966; Parker et al, 1996) followed by both species being packaged into chylomicrons and transported out of the enterocyte.
At the time of publication of paper [6], very little was known about the initial metabolism of absorbed lutein and it was assumed that it was carried in the chylomicrons, in an identical way to that of β-carotene.

Modelling approach
Carotenoids are absorbed into the enterocyte, incorporated into chylomicrons and secreted into lymph before appearing in the plasma. This will lead to a delay in their peak plasma appearance and, coupled with an expected slower absorption linked to the need to break down the food structures in the gut, it was decided to sample the plasma every two hours over a 12-hour period. Due to the expected variation in the measurements and possibility that the appearance of carotenoid would be limited, a very simplistic view of the TRL kinetics was taken and it was assumed that the plasma clearance would be acceptably modelled as a single compartment. The modelling strategy was to measure the area under the curve (AUC) of the carotenoid plasma TRL response over time, and then calculate a clearance rate constant (k), so that the model estimated fractional absorption was similar to that based on the recovery of carotenoid in the ileostomy effluent. The carotenoid half-life (T\(_{1/2}\)) in plasma is related to the clearance rate constant (k) by the simple expression, 
\[ T_{1/2} = \log_2 \frac{2}{k} \].
The requirements for this approach to work are: (1) the carotenoid plasma concentration (in the TRL fraction) must return to baseline within the timeframe of the experiment, (2) that a single compartment is adequate (mathematically) to model the change in plasma appearance of carotenoids, (3) all of the absorbed carotenoid (β-carotene or lutein) is contained in the TRL and, (4) no significant cleavage of the absorbed β-carotene and lutein takes place in the enterocyte prior to them being packaged into chylomicron (we only measured the quantity of β-carotene and lutein in the TRL, not the retinyl ester).

Summary of results
Whole or chopped-leaf cooked spinach (in 150g portions) were fed to volunteers (n=7, paired), with vegetable oil in yoghurt. The quantity of lutein was approximately 15mg in each spinach portion and there was approximately 10mg of β-carotene, in the same portion. The results of the study were slightly disappointing. As other studies had reported, none of the volunteers had measurable carotenoid plasma concentrations (e.g. van Vliet, 1995). The isolated TRL fraction did have measurable carotenoid
concentrations for three (out of six) volunteers but only in two of these did the concentration return to baseline by the end of the 12 hour study period. Volunteers with the highest absorption (C and F) did not show any plasma TRL response.

Table 2 (in paper [6]) details the results for the two volunteers whose temporal plasma concentration data was applicable for modelling. The Table shows the estimated absorption of carotenoid for six different half lives of TRL clearance. We expected there to be a “common” half-live, for each volunteer, that would allow the model to correctly estimate both lutein and β-carotene absorption in whole and chopped leaf spinach. Comparison of Tables 1 and 2 (paper [6]) suggest that the model underestimated the lutein and β-carotene absorption from whole leaf spinach in three out of the four instances where data was obtained. Lutein and β-carotene absorption from chopped leaf spinach was more successfully estimated by the model, with the same plasma TRL half-life for volunteer B ($T_{1/2} = 8$ minutes for lutein and β-carotene clearance) and approximately the same for volunteer G ($T_{1/2} = 3$ minutes for lutein and β-carotene clearance).

**Limitations of the study**

There are several limitations to the study design that affect the ability of the model to accurately predict the quantity of β-carotene and lutein that has been absorbed from the spinach.

First, previous studies of initial lutein (post-absorption) kinetics, have found that it peaks in plasma later (Kostic et al, 1995 – 16 hours; Yao et al, 2000 – 16 hours; Kurilich et al, 2003 – 11 hours; Burri & Clifford, 2004 – 11 hours) than an equivalent β-carotene dose (6-8 hours). This suggests that β-carotene and lutein are transported and cleared from the plasma in different ways. Therefore, some of the absorbed lutein may not have appeared in the plasma TRL fraction but could have been transported in plasma by another, unmeasured carrier. This will lead to an underestimation of lutein absorption and could explain why half the volunteers had no measurable lutein in their TRL.

Second, a large fraction of the absorbed β-carotene will, potentially, be converted to retinyl esters in the enterocytes. This fraction has been estimated to be more than 50% of the total $^{14}$C-label from a β-carotene test meal (Lemke et al, 2003; Burri & Clifford, 2004; Ho et al, 2009). Interestingly, Burri & Clifford (2004) also show data to suggest
that only 70% of the total $^{14}$C plasma concentration peak at 6-hours (post $^{14}$C-labelled β-carotene dose) is accounted for by the sum of the contribution from labelled β-carotene and retinyl esters. The consequence of this for the study reported in paper [6] would be that the majority of the absorbed spinach β-carotene was not measured. Again, this will lead to an underestimation of β-carotene absorption and could explain why half the volunteers had no measurable β-carotene in their TRL.

Third, the isolated TRL fraction contains two entities with different half-lives in plasma (chylomicrons, $T_{1/2} \sim 2-5$ minutes; chylomicron remnants (VLDL), $T_{1/2} \sim 11$ minutes). There is no way of distinguishing the two different components and, therefore, no mechanism of modelling their different kinetics. Without the validation from the measurement of non-absorbed carotenoid in the ileostomy effluent, it would have been impossible to choose the “correct” half-life for carotenoid clearance.

Conclusions
When the half-life of the TRL in the plasma is set in a range between 2-11 minutes, the predicted model absorption for lutein and β-carotene from chopped leaf spinach is similar to that estimated by analysis of ileostomy effluent. Only three of the six volunteers had measurable carotenoid concentrations in their plasma TRL fraction even though “true” absorption was reasonably high. The model is not a reliable method for the estimation of carotenoid absorption.

Future work
The use of labelled carotenoid studies appears to be the only reliable way to assess absorption, other than using ileostomists. Stable isotope labelled carotenoids can be measured accurately in plasma and urine, but not in faeces. This makes absorption estimates difficult but stable isotope labels are useful for tracing the metabolic fate of ingested carotenoids. Extremely small doses of $^{14}$C labelled carotenoid can be traced in plasma and faeces for over 100 days using AMS. Future bioavailability work should focus on $^{14}$C labelled studies.
2.2.3 Riboflavin

Riboflavin is a B vitamin that is the central component in the co-factors flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) and is, therefore, vital in a wide variety of cell processes. Green leafy vegetables, meat and milk are all good sources of riboflavin but very little is known about the bioavailability from food. The study in paper [12] was designed to measure the absorption of riboflavin from commonly consumed foods.

Requirement for bioavailability studies

At the time of publication of paper [12], the scientific literature did not contain any studies that had investigated the human bioavailability of riboflavin from food. Although this was unexpected, it was probably related to the apparent consensus that riboflavin deficiency was not a significant problem. Influential studies by Sauberlich et al (1972) and Boisvert et al (1993) had demonstrated that the measurement of the erythrocyte glutathione reductase activity coefficient (EGRAC) was a reliable indicator of riboflavin deficiency. From these studies, it appeared that a dietary intake of 1.2mg/d was adequate for most people to remain below the EGRAC cut-off for deficiency (=1.4). However, recently, the National Diet and Nutrition Survey of British Adults (Henderson et al, 2003) reported biochemical riboflavin deficiency in between 54% and 80% of the population, and the National Diet and Nutrition Survey of Young People aged 4–18 y (Gregory & Lowe, 2000) reported biochemical riboflavin deficiency in 95% of adolescent girls. On examining the riboflavin intakes from these studies, it became clear that the populations with riboflavin deficiency were consuming the recommended daily amounts of riboflavin. This led to our hypothesis that the foods being consumed by the deficient individuals, had low riboflavin bioavailability. To test this, we designed a study that fed two stable isotope labelled foods (milk and spinach) to female volunteers and collected blood and urine to estimate absorption by mathematical modelling (paper [12]).

Novelty of study design

There are a number of original features of the experimental design in study [12]. It was the first riboflavin study to administer stable isotope labels to volunteers and it was the first to estimate the quantity of riboflavin absorbed from food. Riboflavin was
synthesised with different labels (milk: $^{13}$C-riboflavin as an extrinsic label; spinach: $^{15}$N-riboflavin as an intrinsic label). A $^{13}$C labelled intravenous dose of riboflavin was also synthesised, which was administered with the test meals. It is well known in trace element work that this type of “dual” stable isotope method can estimate the absorption of a labelled dose from plasma and/or urine samples (Turnlund, 1991). The labelling of the spinach and milk meant that the labelled riboflavin from these meals would be handled identically, \textit{in vivo}, to the unlabelled vitamin. The two foodstuffs were chosen because they have completely different food matrices and this made it possible to investigate whether riboflavin absorption is matrix dependent. In addition, milk contains ‘free’ riboflavin bound to specific binding proteins, in contrast to most foodstuffs (e.g. spinach) that contain predominantly FAD and riboflavin phosphate, tightly bound to enzymes. These different “forms” of riboflavin would be labelled within the foods and their metabolic fate could be traced in plasma and urine to extract more detailed kinetic information.

\textbf{Compartment model}

The kinetics of riboflavin had been investigated before (Zempleni et al, 1996), but that study used very high, non-physiological doses of riboflavin, up to 60 times higher in quantity than the RNI. It also used unlabelled doses which made it difficult to distinguish between newly absorbed and endogenous riboflavin. The study in paper [12] required extensive mathematical modelling to estimate a number of important kinetic parameters including the riboflavin pool size, volume of distribution and rate of elimination from the plasma. A two compartment model was deemed most appropriate to represent the plasma appearance of riboflavin from a labelled meal. This decision was taken after extensive “goodness of fit” tests, both before and after data collection. The movement of absorbed, labelled riboflavin into the plasma volume is modelled as a zero order process as was the case with previous work on iron (papers [2, 10]) and folate (papers [5, 7, 9]).

\textbf{Study design}

The study used a cross-over design, with a sample comprising 20 female volunteers randomly assigned to consume either 734µg riboflavin (334µg unlabelled, 400µg labelled) in whole milk or 642µg riboflavin (334µg unlabelled, 308µg labelled) in spinach soup. An intravenous dose of 200µg of free riboflavin (labelled) was
administered as a bolus whilst the volunteers consumed their test meals. Four weeks later, the study was repeated with the other test meal. Serial blood samples were collected for up to 7 hours on the test day. 24h urine samples were collected for 3 days over the study period, starting at 24h before the test meals.

**Novel findings**

Results in Figures 2 and 4 of paper [12] indicate that the riboflavin in the milk and spinach appeared as “free” riboflavin in the plasma, not FMN or FAD (no labelled FMN or FAD was detected in the plasma for any volunteer). The peak in plasma appearance for riboflavin from the milk (24±13 minutes) was slightly earlier than that from the spinach (35±12 minutes) which may reflect the fact that riboflavin from milk is in the free form and did not have to undergo any conversion before appearing in the plasma. The riboflavin from spinach is largely FMN and had to undergo conversion to free riboflavin. This may also account for the differences in the rate constants from the model (see Table 4 in paper [12]). The apparent absorption of riboflavin from milk (23±21%) was not significantly different from that of spinach (20±11%). These estimates were obtained using the plasma appearance of absorbed, labelled riboflavin in conjunction with the 2-compartment model. The true absorption of riboflavin from milk (67±21%) was not significantly different from that of spinach (60±30%). These estimates were obtained using the dose corrected ratio of urinary appearance of labelled riboflavin from the oral and intravenous doses, over the 48h period post dosing. The intravenous dose was also used to quantify the apparent volume of distribution of riboflavin in the sampled compartment, and it was found to be 549±71ml/kg body weight.

It is speculated that the large discrepancy between true and apparent absorption is due to the liver sequestering a large fraction of the absorbed riboflavin from the portal vein before its appearance in the general circulation. We estimate this first-pass effect to be something of the order of 60% of the absorbed dose. This is similar to the findings with folate (papers [3, 5, 7, 9]) and is probably common to all water soluble vitamins in order for the body to maintain stores and to prevent the fasting concentration becoming too low. It is also interesting to note that there appears to be no difference in absorption between riboflavin from milk and that from spinach. Both
are quite well absorbed and validate the analysis used to set the dietary reference values for riboflavin (Department of Health, 1991).

Conclusions
The model predicted a large first-pass effect in line with previous work on other water soluble vitamins (folate - paper [7]; vitamin C - Bates et al, 2004; Bluck et al, 2005). This means that the compartmental model cannot be used to assess true absorption by plasma appearance but could be useful in measuring relative absorption of several sources of riboflavin. True absorption of riboflavin can be estimated by urinary monitoring of the appearance of oral and IV labelled riboflavin.

The study makes a useful contribution to the knowledge of riboflavin bioavailability and leads towards a conclusion that the perceived problem of riboflavin deficiency, as indicated by biochemical indices, may need to be reassessed. The threshold for deficiency (EGRAC > 1.4) could be changed depending on the outcome of future work (see below).

Future work
It would be interesting to repeat the study with a larger sample size and different foods. The volunteers would need to be better characterised in terms of biochemical indices of riboflavin deficiency (e.g. EGRAC) and food diaries kept for a month to determine dietary intake. This would enable a clear link to be made between daily quantity of riboflavin absorbed and riboflavin status.
3. Conclusions

It has been demonstrated that stable isotope labels, in conjunction with compartmental modelling, can make useful contributions to the study of human mineral and vitamin metabolism. In particular, significant progress has been made in studying the bioavailability of iron and folate. It is now possible to quantify the absorption of iron from an unlabelled dose which should benefit those areas of research where iron particle size or chemical species is a factor in determining what quantity of iron can pass across the mucosa. Labelled folate studies have demonstrated for the first time that folic acid is handled very differently from other forms of folate, in vivo. This has major implications for countries where folic acid is used as a food fortificant, since we hypothesise that it is metabolised in the liver, not in the mucosa. The biological implications of this finding are beyond the critical review but it warrants further investigation, especially as many people living in the US have detectable levels of folic acid circulating in their plasma. The modelling of the plasma appearance of newly absorbed, labelled folate has also allowed an estimate of its bioavailability after assumptions are made regarding the volume of distribution, and fraction removed on first-pass through the liver. This technique has been successfully applied in many other folate studies and also in the first stable isotope labelled riboflavin study. An unlabelled carotenoid study used modelling to estimate the absorption of β-carotene and lutein from whole and chopped leaf spinach. It was concluded that the assessment of carotenoid bioavailability was more accurately estimated using isotopic labels.

New insights into the kinetics of calcium and copper have been obtained from the papers outlined in this thesis. A moderately high salt intake significantly affects bone calcium balance with a high calcium diet in comparison to a low calcium diet. Finally, the copper compartmental model is the most detailed of its kind to be developed. The higher than expected endogenous losses and large liver first-pass effect are new insights into the way this mineral behaves in vivo.
4. References


Christensen MJ, Janghorbani M, Steinke FH, Istfan N & Young VR (1983) Simultaneous determination of absorption of selenium from poultry meat and selenite


Dyer NC & Brill AB (1972) Use of the stable tracers \(^{58}\)Fe and \(^{50}\)Cr for the study of iron utilisation in pregnant women. In: *Nuclear activation in the life sciences*, pp 469-477. Vienna: IAEA.


Lowe N (personal communication)


Appendix A: List of submitted papers


Appendix B: Submitted papers
Holmium as a faecal marker for copper absorption studies in adults

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The objective of the present study was to investigate the validity of using holmium as a faecal marker in a copper absorption study using a highly enriched $^{65}$Cu stable isotope label. Ten volunteers (nine female, one male) aged 24–55 years were recruited from the Norwich area to take part in a free-living study. The study was conducted in the Human Nutrition Unit at the Institute of Food Research, Norwich, U.K., and involved feeding breakfast test meals containing foods labelled intrinsically or extrinsically with a highly enriched $^{65}$Cu stable isotope label. A 1 mg oral dose of the rare earth element holmium was administered to volunteers simultaneously with an oral dose of highly enriched $^{65}$Cu stable isotope as a label in the breakfast test meal. Complete faecal collections were made for 10 days after dosing, and the mean percentage holmium recovery ($\pm$ S.D.) was 105 ± 15%. After correcting for re-excreted copper label, the labelled copper and holmium had similar excretory profiles. It was concluded that holmium is a valid faecal marker in adult human copper absorption studies using stable isotopes.

INTRODUCTION

Copper homoeostasis in the human body is maintained by changes in both the absorptive efficiency in the gut and biliary copper excretion. Therefore an estimate of endogenous copper losses is essential in order to measure true copper absorption. Assessments of copper absorption in human studies have traditionally relied solely on the use of the faecal monitoring technique using stable isotopes. In order to accurately determine true copper absorption using the faecal monitoring technique, it is essential that complete faecal collections are made for at least 10 days and that the quantity of re-excreted copper label is estimated [1]. Faecal monitoring is time-consuming and unpleasant, which may result in volunteer non-compliance. Therefore it is important to have an accurate method for determining completeness of faecal collection. An ideal faecal marker would have an identical excretory profile to that of copper, be completely non-absorbable and allow the delineation of non-absorbed copper stable isotope excretion from that which is due purely to re-excretion. In addition, any such marker must be of naturally low abundance in the diet and easily quantifiable.

The rare earth elements are a group of 14 inert elements (lanthanides) that are found in trace amounts in the diet, are absorbed in negligible quantities by the mammalian gastrointestinal tract [2–4] and are non-toxic [5]. Hutcheson et al. [3] suggested that rare earth elements could be used to monitor various nutrients without monitoring food intake directly, as the rare earth element content of food was found to be negligible. Several workers have suggested the use of rare earth elements as faecal markers in mineral absorption studies involving

Key words: adults, copper absorption, holmium, rare earth element, stable isotope.

Abbreviations: AAS, atomic absorption spectrometry; ICP-MS, inductively coupled plasma MS.

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stable isotopes, but published data are somewhat limited. In particular, there is a lack of satisfactory studies on the validity of using holmium for this purpose in copper absorption studies.

In the present paper we report data from a study undertaken to evaluate the usefulness of the rare earth element holmium as a non-absorbable faecal marker in copper isotope absorption studies. The holmium data also allow the re-excretion of the label to be accurately quantified. All data presented here formed part of a larger study investigating copper absorption from a variety of foods labelled either intrinsically or extrinsically with a highly enriched ⁶⁵Cu label. The absorption data will be published separately.

**METHODS**

**Subjects**

Volunteers were recruited through advertisements placed in the Norwich area. Ten healthy adult volunteers (nine female, one male) were recruited to take part in the study; subject characteristics are given in Table 1. A 10 ml blood sample was taken from each volunteer for clinical screening to ensure that biochemical indices and haematological measurements (MD 8 Coulter Counter), including haemoglobin, haematocrit and mean red cell volume, were within the normal range. Other exclusion criteria included chronic illness, the taking of medication or nutritional supplements, and smoking. The study protocols were explained to the volunteers and written, informed consent was obtained. The research was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and the protocol was approved by the Norwich District Ethics Committee.

**Study design**

The study involved the measurement of copper absorption from a range of test meals consisting of foods labelled either intrinsically or extrinsically with a maximum of 3 mg of highly enriched (99.6%) ⁶⁵Cu stable isotope or from a 3 mg reference dose in a diet cola drink (data to be published elsewhere). Details of the composition of the test meals are given in Table 2. Following an overnight fast, test meals were administered in the Human Nutrition Unit, with a minimum interval of 4 weeks between meals. A 1 mg oral dose of holmium was given with the test meal, either incorporated into the test food or in a diet cola drink with the meal. Volunteers refrained from eating for a period of 4 h after dosing. Radio-opaque faecal markers were given with each test meal and also with breakfast on the subsequent three consecutive mornings (a total of 40 markers). Complete faecal collections were made for 10 days following the test meal plus a baseline sample. Complete recovery of all radio-opaque markers in the faecal samples confirmed volunteer compliance. All subjects consumed their habitual diet during the course of the study.

**Dose preparation**

Isotopically enriched copper chloride (⁶⁵CuCl₂) was prepared from elemental ⁶⁵Cu (Europa Scientific Ltd, Crewe, U.K.) by dissolving the metal in 10 ml of concentrated HNO₃ (BDH, Poole, Dorset, U.K.; Aristar grade) and evaporating to virtual dryness. The sample was then taken up in 25 ml of 0.1 M HCl (BDH; Aristar grade) and again evaporated almost to dryness; this stage was then repeated twice more, and finally the sample was taken up in an appropriate volume of 0.1 M HCl. The solution was divided into individual doses and stored in plastic vials at −20 °C until required. The concentration was determined accurately by atomic absorption spectrometry (AAS).

The holmium oral doses were prepared by dissolving holmium chloride (Avocado Research Chemicals Ltd, Heysham, Lancs., U.K.) in demineralized, purified water (Elga, Cambridge, U.K.) to a concentration of approx. 0.5 mg/ml. The solution was divided into individual 1 mg doses and stored in plastic vials at −20 °C until required. The concentration was determined accurately by inductively coupled plasma MS (ICP-MS).

**Production of intrinsically labelled food**

Sunflower (Helianthus annuus) seeds and soya beans (Glycine max) were intrinsically labelled with the highly enriched ⁶⁵Cu stable isotope by cultivating the plants using hydroponic techniques [6]. Copper concentrations and isotope enrichments were determined by AAS and ICP-MS respectively.

**Sample preparation and analysis**

Faecal samples were autoclaved, freeze-dried, ground to a fine powder in a mortar and pestle and sub-sampled. The number of radio-opaque markers in each sample was recorded. Two 0.5 g replicates of each powdered faecal sample were microwave-digested until a clear solution was obtained. For quantitative analysis of copper and holmium, 1 ml of the acid digest was diluted to 10 ml

---

**Table 1  Subject characteristics**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean (S.D.)</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37 (9)</td>
<td>24–55</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.71 (0.06)</td>
<td>1.63–1.83</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>75.4 (17.8)</td>
<td>60.0–120.6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>25.6 (4.4)</td>
<td>20.8–36.0</td>
</tr>
</tbody>
</table>

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Holmium as a faecal marker

Table 2 Details of test meals administered

<table>
<thead>
<tr>
<th>Test meal</th>
<th>No. of subjects</th>
<th>Meal details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reference dose</td>
<td>10</td>
<td>3 mg of $^{65}\text{Cu}$ and 1 mg of Ho in a 125 ml diet cola drink</td>
</tr>
<tr>
<td>Red wine</td>
<td>10</td>
<td>3 mg of $^{65}\text{Cu}$ and 1 mg of Ho in 125 ml of red wine (1996 Côtes du Rhône)</td>
</tr>
<tr>
<td>Extrinsically labelled mushrooms</td>
<td>10</td>
<td>3 mg of $^{65}\text{Cu}$ and 1 mg of Ho added to a mushroom soup (350 g of mushrooms blended with 6.5 g of butter, 143 g of demineralized water and seasoning)</td>
</tr>
<tr>
<td>Extrinsically labelled sunflower seeds</td>
<td>8</td>
<td>1 mg of $^{65}\text{Cu}$ in 37.5 g of sunflower seed butter (25 g of finely chopped sunflower seeds with 12.5 g of butter) served on 35 g of toasted white bread; 1 mg of Ho was given simultaneously in 125 ml of diet cola</td>
</tr>
<tr>
<td>Intrinsically labelled sunflower seeds</td>
<td>8</td>
<td>1 mg of $^{65}\text{Cu}$ in 75 g of sunflower seed butter (50 g of finely chopped intrinsically labelled sunflower seeds with 25 g of butter) served on 70 g of toasted white bread; 1 mg of Ho was given simultaneously in 125 ml of diet cola</td>
</tr>
<tr>
<td>Extrinsically labelled soya beans</td>
<td>5</td>
<td>3 mg of $^{65}\text{Cu}$ and 1 mg of Ho in 450 g of cooked and homogenized soya beans</td>
</tr>
<tr>
<td>Intrinsically labelled soya beans</td>
<td>9</td>
<td>0.3 mg of $^{65}\text{Cu}$ and 1 mg of Ho in 450 g of cooked and homogenized soya beans</td>
</tr>
</tbody>
</table>

Table 3 Typical ICP-MS operating conditions

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RF power (W)</td>
<td>1350</td>
<td></td>
</tr>
<tr>
<td>Gas flow (litres/min)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coolant</td>
<td>14.0</td>
<td></td>
</tr>
<tr>
<td>Auxiliary</td>
<td>0.7</td>
<td></td>
</tr>
<tr>
<td>Nebulizer</td>
<td>0.80</td>
<td></td>
</tr>
<tr>
<td>Spray chamber temperature (°C)</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Dwelling time (µs)</td>
<td>320</td>
<td></td>
</tr>
<tr>
<td>Mass range for scan (AMU)</td>
<td>110–239</td>
<td></td>
</tr>
<tr>
<td>No. of channels per AMU</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>No. of runs per sample</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Holmium internal standard</td>
<td>Bismuth</td>
<td></td>
</tr>
</tbody>
</table>

Mathematical analysis

Some calculations are necessary in order to estimate the quantity of recovered copper label that has been re-excreted. Firstly, the mole fraction of labelled copper in a stool is defined as the quantity of recovered copper label divided by the quantity of recovered total copper (all corrected for differences in molecular mass). The calculation of this is shown in the Appendix. Assuming that all subsequent stools after the complete collection of holmium only contain re-excreted labelled copper, it is possible to plot the mole fraction of labelled copper against time for these latter stools, fit a straight line through the points (in the form $y = mx + c$) and extrapolate back to the time of dose administration. This allows the calculation of the mole fraction of re-excreted labelled copper in the earlier stools. Knowledge of this, along with the measured total copper content in those stools, permits calculation of the quantity of re-excreted copper label in each stool, which can be applied as a correction to estimate the true absorption of the labelled copper dose.

All data are expressed as means (± S.D.). Correlation analysis was used to compare the excretory patterns of labelled copper and holmium.

RESULTS

A mean percentage faecal recovery (± S.D.) of 105 ± 15% was obtained for the holmium dose; the baseline faecal sample for each volunteer contained only negligible amounts of holmium. In all cases, the unabsorbed copper label and the holmium had very similar excretory patterns, and there was also no difference in holmium recoveries between the different test meals. Individual transit times of the volunteers varied; however, the majority of the holmium was generally recovered in the faeces within 6 days after dosing. There was no difference in the excretory profile of either copper or holmium when the copper dose was given as an intrinsic or an extrinsic label.

using purified water. The remaining digest solution was used for isotope ratio analysis by ICP-MS after separation of bivalent cations, including copper, from univalent cations and other contaminants, using ion-exchange chromatography. The precision of the $^{63}\text{Cu}/^{65}\text{Cu}$ ratios was 0.46%. Analysis of GBW 07605 certified reference material tea (Cu 17.3 ± 3 µg/g) for copper gave a mean value of 16.2 ± 1.0 µg/g ($n = 4$). Samples were analysed quantitatively for copper by AAS using a Thermo-Unicam Solaar 969 atomic absorption spectrophotometer, which had been calibrated with standards prepared from 1000 mg/l SpectrosoL (BDH) stock solution. Measurements were made as described previously [7]. Holmium was quantified by ICP-MS using a VG PQ2+ instrument (VG Elemental, Winsford, Cheshire, U.K.). Typical operating conditions are given in Table 3.

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Figure 1 Examples of excretory patterns of the rare earth element holmium (points) and a highly enriched $^{65}$Cu stable isotope label (bars)

Excretory patterns were recorded following a reference dose test meal (top panel; volunteer 230), (b) a mushroom test meal (middle panel; volunteer 540) and (c) a red wine test meal (bottom panel; volunteer 507)

Figure 1 (top panel) and Figures 2–4 represent data from one subject following a single test meal, and are shown to illustrate the general trend of results among the group of volunteers. Examples of excretory patterns of holmium and copper label following different test meals are illustrated in Figure 1. The holmium was completely recovered within 3–6 days after dosing, and at this point it was assumed that all subsequent stools contained re-excreted labelled copper. Figure 2 displays the recovered re-excreted label against time for the same volunteer as in Figure 1 (top panel). The excretory pattern appears to be a random series of recoveries. By converting the same data into the mole fraction of recovered, re-excreted label against time (Figure 3), a trend emerges. A straight line of the form $y = mx + c$ was fitted through these points, which allowed an extrapolation back to the time of dose administration, thus giving an estimate of the mole fraction of re-excreted label in the earlier stools. For each stool, the quantity of labelled, re-excreted copper can be estimated from the mole fraction and the quantity of total copper in the stool (see Appendix for details). Figure 4 shows how the estimated quantity of labelled, re-excreted (endogenous) copper varies with time. The quantity of re-excreted label in each stool is used to correct the amount of recovered labelled copper. An estimate can then be made of the unabsorbed copper from the administered dose. The total recovery of rare earth and label (corrected and uncorrected) can be calculated by...
Holmium as a faecal marker

Figure 5 Relationship between copper label (uncorrected for endogenous loss) and holmium in individual stools (volunteer 230)
The broken line represents the line $y = x$. The solid line represents the linear best fit through the data points.

Figure 6 Relationship between copper label (corrected for endogenous loss) and holmium in individual stools (volunteer 230)
The broken line (overlaid by the solid line) represents the line $y = x$. The solid line represents the linear best fit through the data points.

summing all the holmium and labelled copper (corrected and uncorrected for re-excreted label) in the volunteer’s stools. If the individual stool recoveries are then divided by total recoveries, the fractions of holmium and label in each stool can be calculated. These are plotted against each other in Figure 5 (uncorrected label recovery) and Figure 6 (corrected label recovery). The slope ($\pm$ S.E.M.) of the line in Figure 5 is $0.84 \pm 0.01$, and that in Figure 6 is $0.99 \pm 0.01$. A slope equal to 1 would indicate that the copper and holmium share identical excretory patterns.

DISCUSSION

Copper absorption can be described as a three-stage process, consisting of uptake from the gastrointestinal lumen by the mucosal cells, intra-enterocyte transfer and serosal transport into the systemic circulation. Copper that has not been transported into the body, but is lost through mucosal cell exfoliation, is usually not classified as absorbed. When copper is absorbed into the circulation it is transported rapidly to the liver as a complex bound to serum albumin, amino acids and possibly transcuprein [8,9], where it is incorporated into the long-term transport protein caeruloplasmin. Copper excretion from the body is achieved via the secretion of bile and other intestinal secretions such as pancreatic juice [8]. Copper homoeostasis in the human body is maintained by changes in both the absorptive efficiency in the gut and biliary copper excretion. When measuring absorption, the difference between labelled copper intake and excretion in faeces (i.e. luminal disappearance) is defined as apparent absorption. However, when an allowance is made for the re-excreted copper, the term true absorption is used. Previous studies have used rare earth elements as non-absorbable faecal markers to delineate the excretion of unabsorbed isotope from endogenous secretion, and also to correct for completeness of collection [10,11]. To date, however, there has been no validation of the reliability of using the rare earth element holmium as a faecal marker in $^{64}$Cu stable isotope studies.

In order for rare earth elements to be valid markers in mineral absorption studies, it is essential that they are completely non-absorbed and have similar temporal patterns of excretion. If both criteria are met, it is then possible to use rare earth element methodology to reduce the period of faecal collection to a few days if only apparent absorption data are required. For the estimation of endogenous losses of copper, it is still essential to collect individual stools for about 10 days. It should be noted that a complete recovery coupled with dissimilar excretory patterns only permits the use of rare earth elements as markers of complete faecal collection by the subject.

Previous studies have evaluated the use of various rare earth elements as faecal markers in human stable isotope studies. Dysprosium has been used as a quantitative faecal marker in human mineral absorption studies, where the excretion kinetics were found to parallel those of zinc and magnesium, but not copper [10]. However, in all cases dysprosium recovery from the faeces was complete. A more recent study has supported these earlier findings, concluding that dysprosium does not share the same excretory pattern as copper and therefore is not a valid faecal marker [12]. However, in that study, faecal samples were collected for only 4 days post-dosing in three volunteers and for 14 days in a fourth volunteer, which meant that complete recovery of the dysprosium dose was only achieved in the last volunteer. In order to verify the validity of rare earth elements it is essential to attempt complete recovery of the dose and, therefore, only limited conclusions can be drawn from these data. The complete recovery of the rare earth elements samarium and
 ytterbium has been reported previously in human studies [11,13,14]. In one of the studies, samarium was administered with iron as a highly enriched $^{59}$Fe label, ytterbium with a highly enriched $^{58}$Fe label and dysprosium with a highly enriched $^{64}$Fe label [11]. In each case there was complete recovery of the rare earth element, and the excretory patterns of the iron isotopes and the rare earth elements were very similar. As a result, predicted values of iron absorption from the first 4 days of pooled faeces using rare earth element recovery data were in close agreement with the measured values.

The holmium used in the present study was found to be virtually unabsorbed in the gastrointestinal tract, as demonstrated by its complete recovery. In addition, the negligible holmium content of the baseline faecal samples demonstrated that the habitual diets of the volunteers contained only trace amounts of holmium. Any significant contribution of holmium from either the test meals or the habitual diets of the volunteers would have caused an increase in faecal holmium recovery. Therefore all holmium recovered in the faeces originated from the holmium dose. The strong correlation between the excretory patterns of holmium and unabsorbed copper label demonstrates the validity of using holmium as a predictor of apparent copper absorption. This could be achieved by measuring the holmium content of all the stools and the copper label content of any of the first three. All the other stools could then have their copper content estimated from the pattern of holmium recovery. In order to calculate true copper absorption, it is necessary to quantify the endogenous copper losses. This can only be achieved by collecting individual stools for approx. 10 days and analysing each sample for copper and holmium. The first recovered stool that does not contain holmium indicates that any copper label found in that, and subsequent, stools has been absorbed previously (Figure 1). Figure 2 shows how the quantity of this re-excreted labelled copper varies with time.

In order to correct content in preceding stools for endogenous losses, an estimate needs to be made of re-excreted copper isotope. There are several potential ways to do this. One method is to fit a line to the re-excreted labelled copper against time data (Figure 2) and extrapolate it back to the point of label administration. It can be seen from Figure 2 that this approach is not valid, because the quantity of copper in these stools is dependent not just on the amount of re-excretion but also on the quantity of the stool material collected. This will vary in a random manner, as can be seen clearly in Figure 2. The variation with time of the mole fraction of re-excreted labelled copper (Figure 3) appears to avoid these random fluctuations. This was consistent for the majority of the volunteers. By fitting a line to these data and extrapolating back to the times of previous collections, the mole fraction of the labelled re-excreted copper was calculated for the earlier stools. The Methods section describes how the quantity of labelled endogenous copper loss over the time course of the experiment was calculated, and an example is shown in Figure 4. When this correction was applied to the labelled copper in each appropriate stool, the quantity of unabsorbed labelled copper could be calculated, which provides an estimate of the true copper absorption of the volunteer. Examination of Figures 5 and 6 provides compelling evidence that holmium and copper share a common excretory pattern once endogenous losses have been taken into account. A slope equal to 1.0 would indicate identical patterns. It also gives a strong validation of the technique to estimate endogenous losses by fitting an equation to the copper data from the last few stool samples.

It can be concluded that the rare earth element holmium used in the present study was found to be virtually unabsorbed in the gastrointestinal tract, as demonstrated by its complete recovery. The strong correlation between the excretory patterns of holmium and the copper label demonstrates the validity of using holmium as a predictor of copper absorption.

**ACKNOWLEDGMENTS**

The research was supported by the Biotechnology and Biological Sciences Research Council, the European Commission (grant CT95-0813 FOODCUE), and the Ministry of Agriculture Fisheries and Food (grant ANO 509).

**APPENDIX**

There are two possible sources of copper in our biological samples: the tracer (the oral dose of copper that was highly enriched in $^{65}$Cu) and the tracee (naturally abundant copper). The mass spectrometer response for any particular isotope, say $^{65}$Cu, will depend on the number of $^{65}$Cu atoms present in the sample that have come from the two sources. Examining each source separately, the number of $^{65}$Cu atoms in the sample that have come from the tracer (defined as $^{65}$Cu$_{\text{tracer}}$) is proportional to the number of moles of the tracer present in the sample (defined as mol$_{\text{tracer}}$) and the percentage of the tracer that is actually made up from $^{65}$Cu (defined as $^{66}$Cu$_{\text{tracer}}$). Therefore:

$$^{65}\text{Cu}_{\text{sample}} \propto ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}}$$

In a similar fashion, the number of atoms of $^{66}$Cu in the sample that have been derived from the tracee (naturally abundant) source is:

$$^{65}\text{Cu}_{\text{sample}} \propto ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}}$$

The overall contribution from the two sources to the total number of $^{65}$Cu atoms will be a linear combination.
Therefore the number of atoms of $^{65}\text{Cu}_{\text{sample}}$ is:

$$\frac{^{65}\text{Cu}_{\text{sample}}}{^{65}\text{Cu}_{\text{tracer}}} \cdot \text{mol}_{\text{tracer}} + \frac{^{65}\text{Cu}_{\text{tracee}}}{^{65}\text{Cu}_{\text{tracer}}} \cdot \text{mol}_{\text{tracee}}$$

(1)

Similarly for $^{63}\text{Cu}_{\text{sample}}$:

$$\frac{^{63}\text{Cu}_{\text{sample}}}{^{63}\text{Cu}_{\text{tracer}}} \cdot \text{mol}_{\text{tracer}} + \frac{^{63}\text{Cu}_{\text{tracee}}}{^{63}\text{Cu}_{\text{tracer}}} \cdot \text{mol}_{\text{tracee}}$$

(2)

On dividing eqn (2) by eqn (1):

$$\frac{^{65}\text{Cu}_{\text{sample}}}{^{65}\text{Cu}_{\text{tracer}}} = \frac{^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}}}{\left( ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracee}} \right)} \cdot \left( ^{63}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{63}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}} \right)$$

(3)

The ratio $^{65}\text{Cu}_{\text{sample}}/^{65}\text{Cu}_{\text{tracer}}$ is equal to the mass spectrometer ratio of the sample, $^{53/65}R$ (once any corrections for mass discrimination have been accounted for). Therefore:

$$^{53/65}R = \frac{^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}}}{\left( ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}} \right)}$$

(4)

Similarly for the $^{53/65}$Cu sample ratio:

$$^{53/65}R = \frac{^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}}}{\left( ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}} \right)}$$

(5)

If we set the denominator in eqns (4) and (5) to be equal to $1/k$, then:

$$1/k = ^{65}\text{Cu}_{\text{tracer}} \cdot \text{mol}_{\text{tracer}} + ^{63}\text{Cu}_{\text{tracee}} \cdot \text{mol}_{\text{tracee}}$$

(6)

Eqns (4) and (5) can now be rewritten:

$$^{53/65}R = ^{65}\text{Cu}_{\text{tracer}} \cdot k \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot k \cdot \text{mol}_{\text{tracee}}$$

(7)

$$^{53/65}R = ^{65}\text{Cu}_{\text{tracer}} \cdot k \cdot \text{mol}_{\text{tracer}} + ^{65}\text{Cu}_{\text{tracee}} \cdot k \cdot \text{mol}_{\text{tracee}}$$

(8)

Eqns (7) and (8) can be represented in matrix notation:

$$\begin{bmatrix} ^{53/65}R \\ ^{53/65}R \end{bmatrix} = \begin{bmatrix} ^{65}\text{Cu}_{\text{tracer}} & ^{65}\text{Cu}_{\text{tracee}} \\ ^{65}\text{Cu}_{\text{tracer}} & ^{65}\text{Cu}_{\text{tracer}} \end{bmatrix} \cdot \begin{bmatrix} k \cdot \text{mol}_{\text{tracer}} \\ k \cdot \text{mol}_{\text{tracee}} \end{bmatrix}$$

(9)

or:

$$R = A \cdot x$$

(10)

Solving for $x$:

$$x = A^{-1} \cdot R$$

(11)

where:

$$x = \begin{bmatrix} k \cdot \text{mol}_{\text{tracer}} \\ k \cdot \text{mol}_{\text{tracee}} \end{bmatrix}$$

In terms of mole fractions:

Mole fraction of tracer = Cu $\cdot$ fractiontracer

$$= k \cdot \text{mol}_{\text{tracer}} / (k \cdot \text{mol}_{\text{tracer}} + k \cdot \text{mol}_{\text{tracee}})$$

(12)

Mole fraction of tracee = Cu $\cdot$ fractiontracee

$$= k \cdot \text{mol}_{\text{tracee}} / (k \cdot \text{mol}_{\text{tracer}} + k \cdot \text{mol}_{\text{tracee}})$$

(13)

It is now a simple task to calculate the quantity of tracer or tracee in any biological sample, because the total copper (defined as Cu-total) in the sample is known from an AAS measurement. Care must be taken to take account of the molecular masses of the tracer (defined as MW-tracer) and tracee (defined as MW-tracee).

$$\text{Quantity of tracer} = \frac{\text{Cu-tracer (sample)} \cdot \text{Cu-total} \cdot \text{MW-tracer}}{\text{Cu-tracer (sample)} \cdot \text{MW-tracee} + \text{Cu-tracer (sample)} \cdot \text{MW-tracer}}$$

(14)

$$\text{Quantity of tracee} = \frac{\text{Cu-tracee (sample)} \cdot \text{Cu-total} \cdot \text{MW-tracee}}{\text{Cu-tracee (sample)} \cdot \text{MW-tracer} + \text{Cu-tracee (sample)} \cdot \text{MW-tracer}}$$

(15)

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Quantification of unlabelled non-haem iron absorption in human subjects: a pilot study

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A method for measuring unlabelled Fe absorption has been investigated in a pilot study using a simple mathematical model. The metabolism of newly absorbed Fe can be approximated as a single-compartment model with the sampled compartment being the plasma pool. Five female volunteers (aged 30–55 years) were recruited to participate in the pilot study. After a 10 mg oral dose of unlabelled ferrous sulfate, the change in plasma Fe concentration over the following 6 h was used to estimate the quantity of absorbed Fe from the mathematical model. To assess the accuracy of the new technique, a 1 mg oral dose of 57Fe-labelled iron sulfate was given simultaneously with a 225 μg intravenous dose of 58Fe as iron citrate. The plasma appearance of the labelled Fe was used to estimate the absorption of the oral label from the traditional area under the curve method. There was no significant difference (P = 0.61) between the geometric mean absorption of the unlabelled (19 (±1 SD 12, +1 SD 28) %) and the labelled Fe (17 (±1 SD 10, +1 SD 29) %). These initial results are encouraging, but further work needs to be undertaken with smaller doses, as typically found in meals. The effect of diurnal variation in serum Fe concentration on the estimation of unlabelled Fe absorption needs further assessment.

Iron: Mathematical models: Kinetics

Fe absorption has traditionally been measured from the appearance of radio- or stable-isotope-labelled Fe doses in either blood or faecal samples. Newly absorbed labelled Fe is incorporated into erythrocytes and a single blood sample taken 14 d after the oral dose is sufficient to enable an estimate of Fe absorption to be made. Alternatively, a complete faecal collection can be undertaken for approximately 10 d and the unabsorbed label measured directly. Both of these techniques require radio- or stable-isotope-labelled Fe in the test meal because the unlabelled Fe that naturally occurs in foods cannot be distinguished from endogenous Fe once it is inside the body or in faeces. Almost all non-haem Fe bioavailability studies have used extrinsic labels, based on the assumption that the Fe from the label will form a common pool in the gut with non-haem Fe from unlabelled dietary sources and be absorbed identically. Generally, the UK diet contains a mixture of haem (10 to 15 %) and non-haem Fe (85 to 90 %) and to quantify dietary Fe absorption, a method is required that can measure the absorption of both forms simultaneously. If this method can be applied to single meals with an Fe content of about 5 mg then the total quantity of unlabelled Fe absorbed from a meal can be determined without the need to use extrinsic or intrinsic isotope labels.

Previous authors (Gonzalez et al. 2001) have attempted to use the increase in serum Fe concentration to estimate unlabelled Fe absorption. The present paper is the first, however, to describe the development of a method for estimating unlabelled non-haem Fe absorption with the use of a simple compartmental model. Compartmental modelling is a well-established technique for estimating fluxes and pool sizes without the need for invasive sampling of body tissues (Jacquez, 1996). Its use in Fe metabolism is based on radioisotope work, published mainly in the 1960s, which resulted in the development of complicated multicompartmental models (for example, Pollycove & Mortimer, 1961). In the present study a simpler compartmental approach has been taken that attempts to model the short-term metabolism of Fe following a single unlabelled dose of ferrous sulfate. The techniques employed are similar to pharmacokinetic drug trials.

Abbreviations: AUC, area under the curve; iv, intravenous.
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where single-compartment models are often the best method for the approximate estimation of parameters such as drug half-life, turnover time and fractional absorption (Fox et al. 1993). A technique for measuring labelled Fe absorption, the plasma area under the curve (AUC) method (Barrett et al. 1994), is used to evaluate the potential of the compartmental approach.

Materials and methods

Subjects

Five female volunteers aged 30–55 years took part in the study. All were in apparent good health as assessed by biochemical screening (full blood count, urea, electrolytes, liver enzymes, glucose and cholesterol) and were Fe replete (haemoglobin >120 g/l). All subjects gave written informed consent and the Norwich District Ethics Committee approved the study.

Preparation and administration of iron solutions for oral and intravenous use

Labelled ferrous sulfate for oral use was prepared from elemental $^{57}$Fe (95 % $^{57}$Fe; AEA Technology, Didcot, Oxon, UK) using a previously developed method (Fox et al. 1998). Iron citrate for intravenous (iv) use was prepared by dissolving 22 mg $^{58}$Fe (92 % $^{58}$Fe; Isotec, Saint-Quentin Fallavier, France) in 5 ml concentrated HCl (12 M) in a silica crucible and heating until almost dry. Sodium citrate (20 ml of 0.5 M) was added to the iron chloride, giving a citrate:Fe molar ratio in excess of 20:1, which is required to convert iron chloride to iron citrate. The pH was adjusted to 7.0 with 0.1 M-sodium carbonate and the solution was diluted to 250 ml with water for injection. Iron citrate portions for iv use were checked for sterility by the Norfolk and Norwich Hospital Pharmacy.

Experimental protocol

The five subjects were each given 10 mg Fe as sulfate and 20 mg ascorbic acid. In all subjects, following a 12 h overnight fast, a dose of 225 $\mu$g $^{58}$Fe as citrate was infused and 5 min later the subject was given 300 ml cola drink containing the 1 mg oral dose of $^{57}$Fe and 10 mg unlabelled Fe, both as sulfate. For the duration of the study the subjects were only allowed drinking water. Blood samples (25 ml) were taken at 0, 20, 40, 60, 80, 100, 120, 150, 180, 240, 300 and 360 min after the ingestion of the oral doses and collected into trace element-free vacutainers.

Sample analysis

Total serum Fe was measured in duplicate using Ferrozine (total iron diagnostic kit; Sigma, Poole, Dorset, UK). The manufacturer’s stated single measurement precision (CV) is approximately 7 %, which was calculated from a group of four serum pool preparations (range of serum Fe concentration 510–2220 $\mu$g/l) assayed on eight occasions. According to our calculations, duplicate analysis of a sample should provide a standard error of the mean of about 40 $\mu$g/l for a mean of 1000 $\mu$g/l. Recovery of a set of Fe standards (Fe concentrations of 2000, 3000 and 4000 $\mu$g/l) were 100, 98 and 101 % respectively. Isotope ratios of $^{57}$Fe:$^{56}$Fe and $^{58}$Fe:$^{56}$Fe were measured by thermal ionisation quadrupole MS (Finnegan MAT, GmbH, Bremen, Germany). The measured precision (% relative sd) for these ratios is approximately 1 % and the method has been described previously (Fairweather-Tait et al. 1995). A baseline blood sample was also analysed for plasma ferritin using an in-house ELISA with WHO-certified reference standards.

Calculations

Plasma concentrations of the $^{57}$Fe oral dose and the $^{58}$Fe iv dose were calculated by solving a set of simultaneous equations generated from the mass spectrometer ratios. The AUC was calculated for appearance of the oral and iv doses in plasma using the trapezoidal method.

With reference to Fig. 1, changes in plasma concentration of Fe after an oral dose (doseoral) can be approximated as a single-compartment model provided the measurement period is between 3 and 6 h. This length of time allows for the completion of the absorptive process and a good characterisation of the decay of the plasma concentration. The plasma appearance of Fe from the oral dose is assumed to approximate that of an infusion of rate R over time T. The definition of R is:

$$ R = \frac{\text{mass of dose that is absorbed}}{\text{time period for absorption}} = \frac{M}{T} \quad (1) $$

If V is the apparent volume of distribution and k is the rate constant of elimination from the compartment, then the Fe concentration in the compartment (C) can be approximated as:

$$ C = \frac{M}{V.T.k} \cdot (1 - e^{-kT}) \quad (0 < t < T) \quad (2) $$

$$ C = \frac{M}{V.T.k} \cdot (1 - e^{-k(T-t)}) \cdot e^{-k(T-t)} \quad (T < t) \quad (3) $$

By fitting equations 2 and 3 to the plasma concentration data, M, T and k can be calculated. All fitting was performed in Excel (Microsoft Corporation, 2002) using a standard desktop personal computer. Plasma volume, V,

Fig. 1. Schematic overview of the compartmental model.
is estimated from nomograms by knowledge of the volunteer's age and weight (Lentner, 1984). The fractional absorption can then be calculated according to the equation:

\[
\text{Fractional absorption} = \frac{M}{\text{dose}_{\text{oral}}}. \tag{4}
\]

Results and discussion

Table 1 compares calculated percentage Fe absorption, from measurements of plasma appearance of isotopically enriched oral and iv doses (AUC), with serum appearance of orally administered unlabelled Fe, as calculated by the model. Geometric mean absorption measured by plasma appearance of \(^{57}\text{Fe}\) using the AUC method (17 (−1 SD 10, +1 SD 29)%) was not significantly different (P=0.61, as calculated by two-tailed, paired, Student's \(t\) test) from unlabelled Fe absorption calculated using the model (19 (−1 SD 12, +1 SD 28)%).

There are several criticisms of this new method. The first is the potential error introduced by reliance on an estimate of the plasma volume. The plasma volume estimates were calculated according to the nomograms published in the Geigy tables (Lentner, 1984), and depended on weight, age and sex of the volunteer. An underestimation of plasma volume would lead to an underestimation of the Fe absorption as calculated by the model. For comparison, a different plasma volume method was tried, which was based on body surface area (Hurley, 1975). There was no significant difference between the estimates, and whilst this does not imply that the estimation of plasma volume is accurate, it gives no grounds on which to say that it is not. This remains as a possible small source of error in the model estimate of Fe absorption.

A second source of error is encountered in assuming that a single-compartment model adequately describes the kinetics of newly absorbed Fe. The modelling process can be broken down into two distinct parts: absorption of Fe; plasma Fe kinetics. The absorption process is modelled by assuming that the plasma appearance of newly absorbed Fe has come from the gut via a constant infusion, with a sharply defined start and endpoint. This is a simplification of how Fe is transported across the enterocytes, since the rate of absorption will depend on the quantity of Fe presented to the absorptive cells and the rate of systemic delivery. It would probably be better modelled as a first-order process and this will be investigated in future work. However, as a first approximation, the flux of Fe entering the blood appears to be adequately described as a zero-order process. Any remaining modelling error will be due to the assumptions made concerning Fe kinetics in the plasma. Previous studies have shown that a two- or three-compartment model best describes plasma Fe kinetics in ‘normal’ subjects studied over a few days (Sharney et al. 1965). By simplifying this to a single compartment, it was predicted that the calculation of the elimination rate constant, \(k\), would potentially underestimate the true value. This is due to the Fe ‘reflux’ from extravascular tissues, which causes Fe, once removed from the plasma, to be returned, thereby increasing the plasma Fe concentration. Since the present study was performed over 6 h, it was thought that the inherent error of assuming a single compartment would be quite small.

A simulation was performed in order to assess whether this technique is sensitive enough to measure small differences in Fe absorption. The modelling program SAAMII (version 1.1.1; SAAM Institute Inc., Seattle, WA, USA) was used to generate a set of simulated Fe serum concentration data over 360 min. The input conditions for the simulation were that 200% of a 10 mg oral dose of Fe was absorbed into a single compartment of 3000 ml volume (\(V\)) over 100 min (\(T\)) and the rate of elimination (\(k\)) was 0.002/min. These input variables are approximately what were estimated from our volunteers. The simulated output data (serum Fe concentration over 360 min) were then ‘overlaid’ with an approximate diurnal variation in measured baseline serum Fe concentration estimated from the data of twenty volunteers (Dale et al. 2002) by the use of a polynomial function. These output data were processed through our model and it was found that the absorption was now 18.9%, so the diurnal variation affected the accuracy and led to a systematic error (underestimate) in the ‘real’ absorption by 5% of its value (18.9% compared with 20.0%). No attempt has been made to quantify how random error in the diurnal variation would affect the precision of the absorption estimate but crossover studies are designed to test the same volunteer on more than 1 d and, if the diurnal variation did not remain constant, the precision as well as the accuracy of the estimated absorption would be altered. This has implications for resolving differences in absorption between two or more test meals and will have to be dealt with in a future, more in-depth study.

Finally, using Monte Carlo techniques, random measurement error was added to the data to account for the theoretical variability in the Sigma kit used to measure total serum Fe. The resulting data with randomised error were then generated several times (\(n\) 25) and the simulated absorption calculated for each data set using the model described earlier. The average absorption was found to be 19.0 (SD 1.8)% compared with the noise-free simulation of 18.9% absorption for the simulated data overlaid with a diurnal variation. Two conclusions can be drawn from this

<table>
<thead>
<tr>
<th>Volunteer no.</th>
<th>Labelled (AUC)</th>
<th>Unlabelled (Model)</th>
<th>Serum ferritin ((\mu g/l))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>21</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>17</td>
<td>19</td>
<td>23</td>
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</tr>
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<td>5</td>
<td>20</td>
<td>23</td>
<td>39</td>
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<tr>
<td>Geometric mean</td>
<td>17</td>
<td>19</td>
<td>25</td>
</tr>
<tr>
<td>−1 SD</td>
<td>10</td>
<td>12</td>
<td>17</td>
</tr>
<tr>
<td>+1 SD</td>
<td>29</td>
<td>28</td>
<td>37</td>
</tr>
</tbody>
</table>

AUC, area under the curve.
simulation with regard to the use of the single-compartment model for estimating Fe absorption. First, there is a potential loss of accuracy due to the underestimation of the true Fe absorption because of the decrease in the ‘real’ baseline serum Fe concentration (diurnal effect). In using the model for the pilot study, it was assumed that the baseline serum Fe concentration was the same throughout the experiment (i.e. the $t = 0$ value). This underestimation will become proportionately larger the lower the dose of Fe and this has implications for extending the use of the model in situations where the Fe dose is less than 10 mg.

Second, the effect of the measurement variation in the Sigma Ferrozine kit leads to an uncertainty of approximately 1.8% in a simulated absorption of 20% from a 10 mg dose. For the purposes of the following statistical work, it has to be assumed that the diurnal variation in serum Fe concentration does not vary significantly between any 2 d. Power calculations estimate that at a 0.05 level of significance with a power of 80%, a two-tailed test for an experiment would require eight volunteers in a crossover design to guarantee being able to resolve a difference in absorption of 3% between two test meals. This rises to sixteen and fifty-one volunteers for a 2 and 1% difference in absorption respectively. The Ferrozine kit therefore appears to be inadequate for situations where an attempt is being made to resolve a small difference (<2%) in Fe absorption and in these cases the use of inductively coupled plasma MS or thermal ionisation MS would be expected to measure total Fe in serum with a precision (% relative SD) of less than 1%. This would lead to an uncertainty in model absorption estimation of approximately 0.5% and only six volunteers would be required to resolve a difference of 1% in Fe absorption between two test meals assuming, again, that the diurnal variation in serum Fe concentration is the same on the two test meal days. Also, the Ferrozine kit requires 5 ml whole blood for triplicate analysis but less than 1 ml would be required for inductively coupled plasma MS or thermal ionisation MS thereby reducing the total blood volume taken in a study. This would have obvious benefits in studies where the same volunteer is required to participate in more than one intervention.

The use of a single-compartment model to estimate unlabelled Fe absorption requires a larger study, with smaller doses of Fe, both haem and non-haem, to be undertaken. The 10 mg dose is higher than the quantity consumed in most meals, thus the technique must be shown to work with doses of 5 mg or less. However, the results of the present pilot study demonstrate that the estimation of the absorption of total Fe from an unlabelled source has promise and highlight the potential of mathematical modelling in nutritional studies.

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References


Single oral doses of $^{13}$C forms of pteroylmonoglutamic acid and 5-formyltetrahydrofolic acid elicit differences in short-term kinetics of labelled and unlabelled folates in plasma: potential problems in interpretation of folate bioavailability studies

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Single $^{13}$C$_6$-labelled doses of pteroylmonoglutamic acid (PteGlu; 634 nmol) or 5-formyltetrahydrofolic acid (431–569 nmol) were given to fasted adult volunteers, and the rise in total and $^{13}$C-labelled plasma 5-methyltetrahydrofolic acid metabolite monitored over 8 h by HPLC and liquid chromatography–MS. The dose-adjusted area under the curve (AUC) for total (labelled plus unlabelled) plasma 5-methyltetrahydrofolic acid following a 5-formyltetrahydrofolic acid test dose was 155 % that obtained following a PteGlu test dose. Surprisingly, an average 60 and 40 % of the total plasma 5-methyltetrahydrofolic acid response to $[^{13}$C$_6]$PteGlu and $[^{13}$C$_6]$5-formyltetrahydrofolic acid, respectively, was unlabelled; an observation never before reported. Short-term kinetics of plasma $[^{13}$C$_6]$5-methyltetrahydrofolic acid showed a slower initial rate of increase in plasma concentration and longer time to peak following an oral dose of $[^{13}$C$_6]$PteGlu compared with that for an oral dose of $[^{13}$C$_6]$5-formyltetrahydrofolic acid, while the $[^{13}$C$_6]$5-methyltetrahydrofolic acid AUC for $[^{13}$C$_6]$5-formyltetrahydrofolic acid was 221 % that for $[^{13}$C$_6]$PteGlu. These data indicate that PteGlu and 5-formyltetrahydrofolic acid, which are thought to be well absorbed (about 90 %) at physiological doses, exhibit dramatically different rates and patterns of plasma response. A limitation in the rate of reduction of PteGlu before methylation could result in slower mucosal transfer of $[^{13}$C$_6]$5-methyltetrahydrofolic acid derived from $[^{13}$C$_6]$PteGlu into the plasma. This, when coupled with an observed similar plasma clearance rate for $[^{13}$C$_6]$5-methyltetrahydrofolic acid metabolite derived from either folate test dose, would yield a comparatively smaller AUC. These findings suggest potential problems in interpretation of absorption studies using unlabelled or labelled folates where the rate of increase, the maximum increase, or the AUC, of plasma folate is employed for test foods (mainly reduced folates) vs. a ‘reference dose’ of PteGlu.

**Pteroylmonoglutamic acid: Folate: Absorption: Plasma: Stable isotopes**

Folate is a generic term for B-group vitamins found widely in foodstuffs, mainly reduced methyl and formyl folates (Perry, 1971), that have nutritional properties and chemical structures similar to those of pteroylmonoglutamic acid (PteGlu) (Scott & Weir, 1976). PteGlu is the synthetic form of the vitamin that is used extensively for food fortification purposes and supplements, but which does not occur naturally in significant amounts. Folates are crucial for methionine and nucleotide biosynthesis (Shane, 1995). Periconceptual supplementation of women with PteGlu has been shown to significantly reduce the incidence and recurrence of neural tube defects, such as spina bifida (Anonymous, 1991; Czeizel & Dudas, 1992). Marginal folate deficiency is also associated with elevated plasma homocysteine, an emerging risk factor for vascular diseases (Bousey et al. 1995), epigenetic factors such as cytosine-phosphate-guanosine dinucleotide methylation-associated transcription factor-binding (Nephew & Huang, 2003) and uracil-induced genomic instability (Blount et al. 1997). Either manipulation of the diet or fortification of food with physiological levels of PteGlu may be expected to reduce the prevalence of these folate-related diseases. However, the bioavailability of different folate vitamers is not well understood. Is folate status increased best by the intake of folate supplements, fortified foods or natural food folates? Is optimal folate status easily achievable in countries that do not permit the PteGlu fortification of foodstuffs?

Methods for the assessment of folate bioavailability in human subjects include the comparison of the serum or

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**Abbreviations:** AUC, area under the curve; 5-CH$_3$H$_4$PteGlu, 5-methyltetrahydrofolic acid; 5-CHOH$_4$PteGlu, 5-formyltetrahydrofolic acid; 5MTHF, 5-methyltetrahydrofolic acid; PteGlu, pteroylmonoglutamic acid.

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plasma response to a single oral dose relative to that of a PteGlu reference dose (Gregory, 1997, 2001). This may entail either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over 2 to 3 h (Perry & Chanarin, 1970, 1972; Brown et al. 1973; Lucock et al. 1989; Bower et al. 1993; Kelly et al. 1997), or measurement of the rise in plasma folate concentration (the area under the curve (AUC) of the increase in plasma folate concentration above fasting baseline level) over 6 h or more (Markkanen, 1968, 6 h AUC; Bailey et al. 1988, 8 h AUC; Fenech et al. 1999, 7 h AUC; Prinz-Langenohl et al. 1999, 10 h AUC). In respect of methodological aspects in vitamin bioavailability testing, the AUC is accepted as a valuable indicator of bioavailability provided the post-dosing plasma measurement test period is long enough to capture ~80% of the whole AUC (Pietrzik et al. 1990).

Because of the potential benefits of sensitivity and specificity, it is increasingly common to study the absorption and metabolism of folates in human subjects by using stable-isotope-labelled folates (Wolfe et al. 2001). Our group has used such an approach to assess the relative absorption of two different folate vitamers; PteGlu and 5-formyltetrahydrofolic acid (5-CHOH 4PteGlu). This way, folate can be followed and differentiated from the natural store of folate already present in the body. Thus, more accurate estimates of ‘relative absorption’ should be able to be calculated as a result.

During the course of these studies it became apparent that there are a number of problems in interpreting comparative folate absorption based on short-term plasma folate responses, even when folate test doses are labelled. The present paper presents the evidence for this statement, which emerged in a human volunteer study. Human plasma 5-methyltetrahydrofolic acid (5-CH3H4PteGlu) AUC excursion profiles were followed over a period of 8 h following ingestion of single oral physiological doses of 13C6-labelled PteGlu or (6S)- 13C6-labelled 5-CHOH 4PteGlu. Folate-binding affinity columns were used to isolate extracted plasma folate and, following HPLC analysis of folate concentrations, a newly developed liquid chromatography–MS analytical method was used to determine the proportions of 13C-labelled and unlabelled 5-CH3H4PteGlu (Hart et al. 2002).

Materials and methods

Materials

Purified [2H2]PteGlu was synthesised using a previously published procedure (Gregory, 1990), stored frozen at −18°C, and used as the internal standard. Proton NMR, HPLC and mass spectrometric results gave a purity of >99% and an isotopic enrichment of >95%. Purified [13C6]PteGlu with an isotope enrichment of 96% was synthesised by the method of Mauder et al. (1999), and then encapsulated in gelatin capsules (‘softgels’) and stored at +4°C (Finglas et al. 2002b). These capsules release their contents rapidly once in contact with acid stomach contents and the bioavailability of encapsulated water- and acid-soluble compounds (such as folates), as measured by plasma appearance, has been shown to be indistinguishable from oral aqueous solutions (Seager et al. 1988). Purified (6S)- [13C6]5-CHOH 4PteGlu was synthesised using a previously published enzymic–chemical procedure (Moran et al. 1986), and stored frozen, as a solid, at −18°C. The purified [13C6]5-CHOH 4PteGlu was checked by HPLC analysis, u.v. spectrum and MS. The mass spectrum gave an isotopic enrichment of >94%. There were no detectable impurities and contaminants from the HPLC chromagram, and u.v. spectra gave an estimated purity >98%. Intermittently, as required, a batch of oral doses of [13C6]5-CHOH 4PteGlu were prepared. Approximately 10 µmol solid was weighed out and dissolved in 0.5 ml NaOH solution (0.1 mol NaOH/L). High-grade water was then added to obtain a solution of about 1.2 µmol/ml. This was then dispersed, in doses of 0.5 ml (about 600 nmol), into 2.0 ml plastic screw-capped microfuge tubes. One dose was retained for spectroscopic analysis, with folate concentration being calculated after further dilution in PBS (0.1 mol/l, pH 7.0) using a molar extinction coefficient (E(mol, 10 mm)) of 37200 (Blakkey, 1969). The residual doses were temporarily stored frozen at −18°C until required, and used within 2 weeks.

Human study design

The present work forms part of an ongoing crossover, within-subject, study comparing the absorption of 13C6-labelled folate isolates or 15N-intrinsically labelled spinach folates in a group of healthy male and female adults. The study was approved by the Norwich and District Ethics Committee (Norfolk & Norwich Area Health Care Trust). After giving written consent, a 12 h fasting blood sample was taken and analysed at the haematology department of a local hospital for full-blood-count, blood glucose, erythrocyte-folate, serum B12, urea and electrolytes, and liver function tests. If all results were inside normal assay ranges, volunteers then attended test days where, following an overnight fast, a baseline (time zero) blood sample (10 ml) was taken and where they were then given a single oral dose of [13C6]PteGlu (634 nmol) or (6S)- [13C6]5-CHOH 4PteGlu (431–569 nmol). The dose of [13C6]PteGlu was administered in gelatin capsules washed down with a glass of bottled ‘still’ mineral water. The thawed dose of (6S)- [13C6]5-CHOH 4PteGlu was added to a glass of 25 ml bottled ‘still’ mineral water and then drunk by the volunteer. The glass was quickly rinsed out twice with further water, which was also drunk. A timer was started after the test doses had been completely swallowed and rinsed down. Volunteers were always allowed access to water, and were given a light lunch only after a 4 h post-dosing venous blood sample had been obtained. With few exceptions, the sub-set of volunteers reported in the present paper did not complete both 13C6-labelled PteGlu and 5-CHOH 4PteGlu test days. The present paper therefore reports essentially independent results from ten volunteers (age 30·9 (SE 1.3) years; BMI 25·7 (SE 1.3) kg/m2) who had (6S)- [13C6]5-CHOH 4PteGlu as the test dose and twelve volunteers (age 31·1 (SE 1.2) years; BMI 25·4 (SE 1.3) kg/m2) who had [13C6]PteGlu as the test dose.
Blood sampling and storage

Venous blood samples (10 ml) were taken by cannula at time zero and at eleven further time points over an 8 h period following each test dose; 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6 and 8 h. Blood samples were transferred immediately into tubes containing K2-EDTA (Sarstedt Ltd, Beaumont Leys, Leics., UK) and mixed gently. Samples were centrifuged (1500 g, 10 min), plasma removed and frozen immediately over solid CO2, and then stored at −30°C until analysis.

Sample preparation, folate extraction and analysis

Following storage, plasma samples were mixed thoroughly and 5 ml transferred into 50 ml screw-top glass centrifuge tubes. Extraction buffer (20 ml, 0.1 m-K2HPO4, pH 7.0; containing 0.05 m-sodium ascorbate and 0.01 m-mercaptoethanol as antioxidants) and an internal standard (0.1 ml [2H2]PteGlu; 2 µg/ml) were added. The tubes were then flushed with N2, capped, and the sample mixed thoroughly before being placed into a boiling water bath for 10 min for proteins to precipitate. The samples were cooled over ice, precipitates agitated, then centrifuged for 10 min (1000 g). The supernatant fraction was removed and stored in a clean tube. Extraction buffer (15 ml) was added to the pellet and thoroughly mixed. The sample was re-centrifuged and the wash supernatant fraction removed and combined with the first supernatant fraction. All folate from each extracted plasma sample was purified on an affinity column using folate-binding protein and eluted with HCl (0.1 mol/l) into a 5 ml volumetric flask containing ascorbic acid solid (10 mg), mixed thoroughly, and transferred in three roughly equal portions to microfuge tubes (Hart et al. 2002). These folate isolates were temporarily stored in liquid N2 (with no loss of potentially labile 5-CH3H4PteGlu, even after several months) and subsequently analysed, utilising a cooled (4°C) autoinjector, in negative ion mode by liquid chromatography–MS as previously described (Hart et al. 2002). Selected ion monitoring was conducted on the [M-H]− ion: m/z 458 for analysing 5-CHOH4PteGlu derived from the label dose; m/z 444 for analysis of 5-CH3H4PteGlu, even after 30 h). Chromatographic separation was on a 250 mm × 4.6 mm internal diameter column packed with LiChrospher 100 RP C18 (Merck Ltd, Poole, Dorset, UK), performed isocratically at 1.0 ml/min with a mobile phase consisting of phosphoric acid (0.05 mol/l)–acetonitrile (9:1, v/v) with fluorescence detection for 5-CH3H4PteGlu (excitation at 292 nm and emission at 356 nm) and u.v. absorbance (288 nm) for both PteGlu and 5-CHOH4PteGlu. HPLC quantification was against external standards. Stock folate solutions were made up in borate buffer (0.1 mol/l, pH 7.0; van den Berg et al. 1994) before true folate concentrations were established using appropriate molar extinction coefficients (Blakley, 1969). When required, sub-samples of stock solutions (that had been stored in liquid N2) were thawed and diluted to suitable concentrations with HCl (0.1 mol HCl/l containing 2 mg/ml ascorbic acid as an antioxidant); the same matrix as used for isolated plasma samples. Plasma concentrations of unlabelled and 13C6-labelled 5-CH3H4PteGlu were calculated using selected ion monitoring ratios (Hart et al. 2002) (see Appendix 1). Step 1 involves calculating the fraction of each source in the sample. Step 2 involves multiplying the measured total 5-CH3H4PteGlu concentration by the derived fraction of each source to give the actual plasma concentrations of unlabelled and 13C6-labelled 5-CH3H4PteGlu.

Area under the curve calculations of the rise in plasma folate above initial fasting baseline concentration

The AUC was calculated using the trapezoidal method, and then adjusted to an oral dose equivalent to 600 nmol folate. For each test dose, in each volunteer, both the ‘total’ and the ‘unlabelled’ plasma 5-CH3H4PteGlu AUC (the AUC of the rise in plasma folate concentration) were calculated after deduction of each individual’s baseline (time zero) concentration from the concentration values obtained for each of their post-absorption time points. The 13C6-labelled 5-CH3H4PteGlu AUC was calculated without adjustment, since there was no excess label at baseline (time zero).

Statistics

Originally, our ongoing study (of which the volunteers reported on in the present paper form a part) had a crossover within-subject design. However, partly because of the intrusive nature of 8 h cannulations, most subjects reported here did not complete both 13C6-labelled PteGlu and 5-CHOH4PteGlu test days. Hence, for statistical purposes, the data that are reported in the present paper are treated as independent observations. For PteGlu and 5-CHOH4PteGlu test meals, plasma total, labelled and unlabelled AUC, unlabelled:labelled ratio and total:labelled ratio were examined by the Kolmogorov–Smirnov one-sample continuous distribution test. As distributions were not significantly different from normal, each parameter for the two test doses was compared by parametric Student’s unpaired t test for: (i) total plasma 5-CH3H4PteGlu AUC; (ii) plasma 13C6-labelled 5-CH3H4PteGlu AUC; (iii) plasma unlabelled 5-CH3H4PteGlu AUC; (iv) plasma unlabelled:labelled 5-CH3H4PteGlu AUC ratio; (v) plasma total:labelled 5-CH3H4PteGlu AUC ratio. Linear regression analysis was used to assess associations (r2) with either fasting plasma baseline folate concentration, or erythrocyte-folate concentration. It was also used, within subject, to assess association between labelled and unlabelled plasma 5-CH3H4PteGlu AUC response.
Results
Selected ion monitoring indicated that neither of the test doses appeared unmetabolised in the plasma as PteGlu or 5-CHOH4PteGlu. The total plasma response to both labelled PteGlu and 5-CHOH4PteGlu test doses was significantly greater than their labelled plasma response. This indicates that there was a significant appearance of unlabelled folate in the plasma following both test folates (Fig. 1).

The labelled plasma response to PteGlu was slower, and peaked much later than the response to the 5-CHOH4PteGlu test dose. \( T_{\text{max}} \) is the time between the administration of the dose and the occurrence of the peak plasma concentration of labelled 5MTHF.

Table 1 shows (i) 'total', (ii) 'labelled', and (iii) 'unlabelled' plasma AUC responses, together with the (iv) unlabelled:labelled and total:labelled AUC ratios to the PteGlu and 5-CHOH4PteGlu test doses.

![Plasma folate concentration](image)

**Fig. 1.** (a) Plasma response to (6S-) \(^{13}C_6\)5-formyltetrahydrofolic acid (5-CHOH4PteGlu). Mean values with their standard errors of total (●) and \(^{13}C_6\)-labelled (○) plasma 5-methyltetrahydrofolinic acid (5-CHOH4PteGlu) areas under the curve (AUC) of the rise in plasma folate concentration in response to an oral test dose (\( n = 10 \)) of (6S-) \(^{13}C_6\)CHOH4PteGlu. AUC responses are adjusted to an oral dose of 600 nmol. The unlabelled response, not of test-dose origin, is the difference between the two responses shown. (b) Plasma response to \(^{12}C_6\)_PteGlu or 5-CH$_2$H$_4$PteGlu AUC of the rise in plasma folate concentration in response to an oral test dose (\( n = 12 \)) of \(^{12}C_6\)PteGlu. AUC responses are adjusted to an oral dose of 600 nmol. The unlabelled response, not of test-dose origin, is the difference between the two responses shown.

On average, the 'total' plasma AUC response to 5-CHOH4PteGlu was 155% of the response to PteGlu; a significant trend for difference (\( P = 0.062 \)).

In comparison, the average plasma 'labelled' AUC response to 5-CHOH4PteGlu was 221% of the response to PteGlu; a significant difference (\( P < 0.001 \)).

In contrast to the 'total' and 'labelled' response, the average plasma 'unlabelled' AUC response to 5-CHOH4PteGlu was the same as the response to PteGlu; no significant difference (\( P = 0.864 \)).

Plasma 'unlabelled' and 'labelled' AUC varied greatly between volunteers but an 'unlabelled' plasma response was always present. Notably, there was no significant association, within subject, between 'unlabelled' and 'labelled' plasma AUC responses to either PteGlu (\( r^2 0.039 \); \( P = 0.541 \)) or 5-CHOH4PteGlu (\( r^2 0.166 \); \( P = 0.243 \)) test doses.

A comparison of the plasma 'unlabelled':'labelled' AUC ratios for the two oral folate test doses indicated that the mean ratio for PteGlu (1.43) was more than double, and significantly different (\( P = 0.012 \)) from the mean ratio for 5-CHOH4PteGlu (0.61).

A comparison of the plasma 'total':'labelled' AUC ratios for the two oral folate test doses indicated that the mean ratio for PteGlu (2.27) was significantly different (\( P = 0.027 \)) from the mean ratio for 5-CHOH4PteGlu (1.47).

Table 2 shows the results of linear regression analysis between both labelled and unlabelled plasma AUC responses to PteGlu and 5-CHOH4PteGlu test doses with (a) fasting plasma and (b) erythrocyte-folate concentrations. It also shows that there was no relationship between fasting plasma and erythrocyte-folate concentrations. Neither 'unlabelled' nor 'labelled' plasma AUC responses were significantly associated (\( r^2 \)) with volunteer fasting baseline erythrocyte-folate concentrations. Furthermore, neither the 'unlabelled' plasma AUC response to both test folates, nor the 'labelled' plasma AUC response to the 5-CHOH4PteGlu test dose, were significantly associated (\( r^2 \)) with volunteer fasting baseline plasma concentrations. However, interestingly, there was an extremely high degree of association between the 'labelled' plasma AUC response to the PteGlu test dose, and volunteers' fasting baseline plasma folate concentration (\( r^2 0.880 \); \( P < 0.001 \)).

Discussion
The size of the oral folate test doses used in the present study did not exceed any threshold for the reported direct appearance of unmetabolised PteGlu in the plasma (Kelly et al. 1997); consequently no evidence of unmetabolised PteGlu or 5-CHOH4PteGlu appearing in any of the analysed samples was found.

Results indicated that a significant proportion of the total plasma AUC response to an oral load of labelled PteGlu or labelled 5-CHOH4PteGlu did not originate directly from the oral test doses because it was not labelled (Fig. 1(a) and (b)). Approximately 40% (range 7 to 64%) of the plasma response to the dose of labelled 5-CHOH4PteGlu, and approximately 60% (range 38 to 75%) of the plasma response to the dose of labelled PteGlu did not
originate from the labelled oral test doses (Table 1). This result has not been demonstrated previously.

The ‘unlabelled’ plasma AUC response cannot be as a result of metabolic cleavage and loss of label from the test folates during subsequent metabolism. The underlying parent structure of all monoglutamate folate compounds that exhibit a common vitamin activity is based on the parent structure of PteGlu (pteroyl-L-monoglutamic acid). The pteroylmonoglutamate molecule consists of an aromatic pteridine ring linked through a methylene bridge to para-amino-benzoic acid, and then to one L-glutamic acid residue. Cleavage between the pteridine ring and benzoic-acid ring (generating a pteridine residue + para-aminobenzoylglutamic acid), between the benzoic-acid ring and glutamic acid (generating pteroic acid + glutamic acid), or between both (generating a pteridine residue + para-aminobenzyol residue + glutamic acid), will result in molecules with no vitamin activity that human metabolism cannot reassemble. Hence, the classification of folates as ‘vitamins’. It is thus self-evident that the benzene ring of our labelled test folates that carries the six 13C-carbons cannot be removed and substituted with a non-labelled ring without irreversible destruction of the vitamin folate molecule.

The large, and highly variable, displacement of endogenous folate is problematic in interpreting absorption studies using ‘unlabelled’ folates because the portion of plasma response due to oral v. endogenous folate cannot be determined. There was no relationship between this ‘unlabelled’ folate response and folate status. Furthermore, there does not appear to be a simple relationship between oral and endogenous response as there was no significant association, within subject, between ‘unlabelled’ and ‘labelled’ plasma AUC response to either ‘labelled’ PteGlu or ‘labelled’ 5-CHOH4PteGlu test doses. It could be speculated that the larger variation in unlabelled plasma folate response may be due to variable absorptive epithelial cell exposure to folate intake in the day-or-so immediately preceding the test days, and a knock-on perturbation effect. The latter effect would result in the previously absorbed ‘cold’ folate being pushed out of the epithelial cells into the body-proper to generate an unlabelled folate plasma response that is completely unpredictable, and unrelated to the absorption of any labelled test dose. If unlabelled

Table 1. Plasma 5-methyltetrahydrofolic acid (5-CH₃H₄PteGlu) area under the curve (AUC) responses and unlabelled:labelled and total:labelled ratios in fasting healthy adult volunteers following an oral dose of either [¹³C₆]pteroylmonoglutamic acid (PteGlu) or (6S-) [¹³C₆]5-formyltetrahydrofolic acid (5-CHOH₄PteGlu) (Mean values with their standard errors)

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Total AUC</td>
<td>2437 300</td>
<td>3771 646</td>
</tr>
<tr>
<td>¹³C₆-labelled AUC</td>
<td>1149 128</td>
<td>2542 187</td>
</tr>
<tr>
<td>Unlabelled AUC</td>
<td>1497 257</td>
<td>1589 503</td>
</tr>
<tr>
<td>Ratio of unlabelled:labelled</td>
<td>1-43 0.23</td>
<td>0.61 0.17</td>
</tr>
<tr>
<td>Ratio of total:labelled</td>
<td>2.27 0.26</td>
<td>1.47 0.20</td>
</tr>
</tbody>
</table>

*All AUC responses are adjusted to an oral dose of 600 nmol, and are over the post-dosing period from 0 to 480 min. † Student’s unpaired t-test.

Table 2. Linear regression analysis of the association (r²) between unlabelled or [¹³C₆]-labelled plasma 5-methyltetrahydrofolic acid (5-CH₃H₄PteGlu) area under the curve (AUC) response in fasting healthy adult volunteers, following an oral dose of either [¹³C₆]pteroylmonoglutamic acid (PteGlu) or (6S-) [¹³C₆]5-formyltetrahydrofolic acid (5-CHOH₄PteGlu), and fasting plasma folate concentration or fasting erythrocyte-folate concentration

<table>
<thead>
<tr>
<th>Test meal Fasting plasma folate</th>
<th>Fasting erythrocyte folate</th>
<th>Oral dose [¹³C₆]PteGlu (n 12)</th>
<th>Oral dose (6S-) [¹³C₆]5-CHOH₄PteGlu (n 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabelled AUC per 600 nmol dose</td>
<td>0.000</td>
<td>0.007</td>
<td>0.066</td>
</tr>
<tr>
<td>r²</td>
<td>0.000</td>
<td>0.805 (NS)</td>
<td>0.505 (NS)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>0.990 (NS)</td>
<td>0.878 (NS)</td>
</tr>
<tr>
<td>[¹³C₆]-labelled AUC per 600 nmol dose</td>
<td>0.880</td>
<td>0.222</td>
<td>0.226</td>
</tr>
<tr>
<td>r²</td>
<td>0.880</td>
<td>0.022</td>
<td>0.174</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>0.990 (NS)</td>
<td>0.265 (NS)</td>
</tr>
<tr>
<td>Fasting plasma (oral dose PteGlu)</td>
<td>0.032</td>
<td>0.600 (NS)</td>
<td>0.102</td>
</tr>
<tr>
<td>r²</td>
<td>0.032</td>
<td>0.600 (NS)</td>
<td>0.442 (NS)</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.001</td>
<td>0.990 (NS)</td>
<td>0.887 (NS)</td>
</tr>
</tbody>
</table>
folate was mainly a result of hepatic biliary excretion then one may have expected the ‘unlabelled’ AUC to be related to the initial fasting plasma folate concentration of volunteers, because it too is a function of enterohepatic recirculation (Steinberg et al. 1979). Such a relationship was not observed.

The oral dose of 5-CHOH4PteGlu induced larger ‘total’ plasma AUC responses than equivalent oral doses of PteGlu (Table 1). If the AUC of an unlabelled oral test folate is compared with the AUC of an equivalent unlabelled dose of PteGlu (i.e. using PteGlu as the ‘reference’ dose) then this would have indicated that the ‘relative bioavailability’ of 5-CHOH4PteGlu was 155% that of PteGlu. Similarly, the ‘relative bioavailability’ of 5-CHOH4PteGlu calculated when using only the plasma ‘labelled’ AUC responses was 221% that of PteGlu. Under the conditions of the present trial in which relatively small folate test doses (within the physiological range) were given to fasting volunteers, one would expect that a high proportion (approximately 90%) of the dose would be absorbed (Bhandari & Gregory, 1992; Clifford et al. 1998). Thus, differences in plasma kinetics and AUC as seen in the present study are almost assuredly due to differences in rate or extent of conversion to 5-CH3H4PteGlu, displacement of tissue folates, and release into the circulation. These data suggest that a reduced folate such as (6S-)-5-CH3H4PteGlu or (6S-) 5-CHOH4PteGlu would be a more appropriate ‘reference’ dose in bioavailability studies of natural folates, especially comparisons carried out ‘within volunteer’.

A review by Selhub et al. (1983) concluded that the intestine is capable of both reduction and one-carbon substitution (methylation) of physiological doses of folate before absorbed folate is transported from the mucosal to the serosal side. It is therefore expected that physiological oral doses of both PteGlu and 5-CHOH4PteGlu will only result in the transfer of 5-CH3H4PteGlu into the hepatic portal vein. Though an extensive hepatic uptake of reduced folates and calculation of ‘relative absorption’ should still be valid. However, a comparison of labelled plasma AUC responses indicated that the ‘initial rate’ and ‘time to peak’ response to PteGlu test doses was slower and longer, respectively, than that to 5-CHOH4PteGlu test doses (Fig. 1(a) and (b)). Arguably, a slower mucosal transfer of labelled 5-methyltetrahydrofolic metabolite, derived from labelled PteGlu, to the plasma (possibly due to a limitation in the rate of initial reduction of PteGlu to H2-PteGlu, before further reduction to H4-PteGlu, subsequent 1-carbon addition and re-arrangement to 5-CH3H4-PteGlu; Mathews & Huennekens, 1963) when coupled with a similar plasma clearance rate to 5-methyltetrahydrofolic metabolite derived from 5-CHOH4PteGlu would result in a comparatively smaller ‘labelled’ plasma AUC.

The lack of association (Table 2), with one exception, between labelled or unlabelled plasma AUC responses to PteGlu or 5-CHOH4PteGlu test doses, and either volunteer fasting baseline plasma or erythrocyte-folate concentrations, indicates that plasma AUC responses cannot be predicted using either of these criteria. The exceptional observation of a high degree of association ($r^2$ = 0.980; $P < 0.001$) between the labelled plasma AUC response to a labelled PteGlu oral test dose and volunteers’ fasting baseline plasma folate concentration could be real, and could eventually provide an insight into the specific metabolism of PteGlu. Alternatively, the association could be an artifact of having undertaken a total of ten statistical comparisons (i.e. including testing of associations between ‘unlabelled’ and ‘labelled’ plasma responses to both test folates). So, without an experimental ‘a priori’ intention to make such comparisons, the associations should, strictly speaking, be left to future examination. However, since fasting baseline plasma folate concentration is maintained by enterohepatic recirculation (Steinberg et al. 1979), it is too tempting to avoid the speculation that this strong relationship suggests that absorbed PteGlu is reduced and methylated in the liver, and not the mucosa, as has been the accepted wisdom for at least the past two decades (Selhub et al. 1983).

In summary, the present paper reports that after an oral folate test dose there is a large and unexpected concurrent displacement of tissue 5-CH3H4PteGlu into the plasma. This makes comparisons of the relative bioavailability of unlabelled reduced folates (i.e. from test food doses), labelled PteGlu reference doses, ambiguous. It is also reported that there is a large difference in plasma ‘labelled’ response to a ‘labelled’ naturally occurring reduced folate compared with ‘labelled’ unlabelled PteGlu. This difference would lead to spurious conclusions in bioavailability studies based on comparisons of oral doses of reduced ‘labelled’ folate (for example, labelled food folate) v. unlabelled PteGlu reference doses, ambiguous. It is also reported that there is a large difference in plasma ‘labelled’ response to a ‘labelled’ naturally occurring reduced folate compared with ‘labelled’ unlabelled PteGlu. This difference would lead to spurious conclusions in bioavailability studies based on comparisons of oral doses of reduced ‘labelled’ folate (for example, labelled food folate) v. a ‘labelled’ PteGlu reference dose. It could be speculated that this phenomenon may be due to a slower rate of mucosal processing of PteGlu compared with 5-CHOH4PteGlu. This may result in a slower transfer of 5-CH3H4PteGlu metabolite to the plasma, which will, when coupled with a similar clearance rate to that of 5-CH3H4PteGlu metabolite derived from oral 5-CHOH4PteGlu, result in comparatively smaller labelled plasma AUC. Contrary to current theory, it is suggested that absorbed physiological doses of PteGlu mainly enter the hepatic portal vein unmodified with immediate removal to the liver for biotransformation, with subsequent plasma 5-CH3H4PteGlu AUC response being entirely a function of enterohepatic recirculation.

Thus, neither comparison of ‘total’ or ‘labelled’ plasma AUC responses appears to give a reliable index of ‘relative bioavailability’. It is concluded that currently held views on the absorption, metabolism and subsequent tissue distribution of folates (which suggest that the bioavailability of oral test doses can be estimated by contrasting the plasma response with that from an equivalent ‘reference dose’ of PteGlu), need re-evaluation.

Two potential solutions to this problem are suggested. First, an adaptation could be made to the oral and intravenous dual-label stable-isotope ‘urinary excretion ratio’ protocol of Gregory et al. (1992), using isotopically labelled (6S-) 5-CH3H4PteGlu as the intravenous dose, rather than isotopically labelled PteGlu. This adaptation to the method may overcome differences in the kinetics
of urinary excretion of oral and intravenous doses that have been observed when using labelled PteGlu as the intravenous dose (Gregory et al. 1992; Rogers et al. 1997; Finglas et al. 2002b). However, this manoeuvre may eventually prove to be only a partial solution and have to be restricted to the estimation of the ‘relative absorption’ of oral doses of ‘natural’ reduced folates and not PteGlu (as either supplement or fortificant). Second, one could mathematically model plasma-labelled response data (entrance and clearance rates) using a single-compartment model with estimates for the size of pool mass and volume, and (perhaps) first-pass liver effect. The advantages of this approach may be at least to obtain an accurate estimate of ‘relative bioavailability’, if not absolute absorption. Metabolic models are currently being investigated (Finglas et al. 2002a).

Acknowledgements

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References

Rogers LM, Pfeiffer CM, Bailey LB & Gregory JF (1997) A dual-label stable-isotopic protocol is suitable for determination of


Appendix 1

There are two possible sources of $^{13}$C in the 5-CH$_3$H$_4$PteGlu in our plasma samples: the tracer (the $^{13}$C$_6$-labelled 5-CH$_3$H$_4$PteGlu; 5MTHF) and the tracee (unlabelled 5-CH$_3$H$_4$PteGlu). The mass spectrometer is set up to measure the largest fraction of the 5-CH$_3$H$_4$PteGlu coming from the tracer (mass 464; M + 6) and the largest fraction coming from the tracee (mass 458; M + 0). The response for either M + 0 or M + 6 will depend on the number of 5-CH$_3$H$_4$PteGlu molecules present in the sample that have come from the two sources. Examining each source separately, the number of 5-CH$_3$H$_4$PteGlu molecules in the sample, measured in the M + 6 channel, that have come from the tracer (defined as M + 6$_{\text{sample tracer}}$) is proportional to the number of mol of the tracer present in the sample (defined as mol$_{\text{tracer}}$) and the fraction of the tracer that is actually present at mass 464 (defined as M + 6$_{\text{tracer}}$). Therefore:

$$M + 6_{\text{sample tracer}} \propto M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}}.$$  

and, similarly for M + 0$_{\text{sample}}$,

$$M + 0_{\text{sample}} \propto M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}.$$  

On dividing (2) by (1):

$$\frac{M + 6_{\text{sample}}}{M + 0_{\text{sample}}} =$$

$$= \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(3)

The ratio $M + 6_{\text{sample}}/M + 0_{\text{sample}}$ is defined as $M + 6/M + 0R$. Therefore:

$$M + 6/M + 0R =$$

$$= \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(4)

Similarly for the $M + 0_{\text{sample}}/M + 0_{\text{sample}}$ ratio:

$$M + 0/M + 0R =$$

$$= \frac{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(5)

If the denominator in equations 4 and 5 is set to be a constant, k, then:

$$1/k = \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(6)

Equations 4 and 5 can now be rewritten:

$$M + 6/M + 0R =$$

$$= \frac{M + 6_{\text{tracer}} \times k \times \text{mol}_{\text{tracer}} + M + 6_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(7)

$$M + 0/M + 0R =$$

$$= \frac{M + 0_{\text{tracer}} \times k \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}{M + 0_{\text{tracer}} \times \text{mol}_{\text{tracer}} + M + 0_{\text{tracee}} \times \text{mol}_{\text{tracee}}}. $$  

(8)

Equations 7 and 8 can be represented in matrix notation:

$$\begin{bmatrix} M + 6/M + 0R \\ M + 0/M + 0R \end{bmatrix} = \begin{bmatrix} M + 6_{\text{tracer}} & M + 6_{\text{tracee}} \\ M + 0_{\text{tracer}} & M + 0_{\text{tracee}} \end{bmatrix} \begin{bmatrix} k \times \text{mol}_{\text{tracer}} \\ k \times \text{mol}_{\text{tracee}} \end{bmatrix}. $$  

(9)

or,

$$R = A \times x. $$  

(10)

Solving for x:

$$x = A \times R. $$  

(11)

where

$$x = \frac{1}{k} \times \frac{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}}}{M + 6_{\text{tracer}} \times \text{mol}_{\text{tracer}}}. $$  


In terms of mol fractions:

mole fraction of tracer (i.e. \( 5\text{MTHF}_{\text{fractiontracer}} \))
\[
= \frac{k \times \text{mol}_{\text{tracer}}}{k \times \text{mol}_{\text{tracer}} + k \times \text{mol}_{\text{tracee}}},
\]  

(12)

mole fraction of tracee (i.e. \( 5\text{MTHF}_{\text{fractiontracee}} \))
\[
= \frac{k \times \text{mol}_{\text{tracee}}}{k \times \text{mol}_{\text{tracer}} + k \times \text{mol}_{\text{tracee}}},
\]  

(13)

It is now a simple task to calculate the quantity of the 5-\( \text{CH}_3\text{H}_4\text{PteGlu} \) that has come from the tracer or tracee in any plasma sample because the total 5-\( \text{CH}_3\text{H}_4\text{PteGlu} \) (defined as \( 5\text{MTHF}_{\text{total}} \)) in the sample is known from an HPLC measurement.

Quantity of tracer = \( \frac{5\text{MTHF}_{\text{fractiontracer}} \times 5\text{MTHF}_{\text{total}}}{5\text{MTHF}_{\text{fractiontracer}} + 5\text{MTHF}_{\text{fractiontracee}}} \);  

(14)

Quantity of tracee = \( \frac{5\text{MTHF}_{\text{fractiontracee}} \times 5\text{MTHF}_{\text{total}}}{5\text{MTHF}_{\text{fractiontracer}} + 5\text{MTHF}_{\text{fractiontracee}}} \).  

(15)
Adaptive responses in men fed low- and high-copper diets

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The study of Cu metabolism is hampered by a lack of sensitive and specific biomarkers of status and suitable isotopic labels, but limited information suggests that Cu homeostasis is maintained through changes in absorption and endogenous loss. The aim of the present study was to employ stable-isotope techniques to measure Cu absorption and endogenous losses in adult men adapted to low, moderate and high Cu-supplemented diets. Twelve healthy men, aged 20–59 years, were given diets containing 0·7, 1·6 and 6·0 mg Cu/d for 8 weeks, with at least 4 weeks intervening washout periods. After 6 weeks adaptation, apparent and true absorption of Cu were determined by measuring luminal loss and endogenous excretion of Cu following oral administration of 3 mg highly enriched 65Cu stable-isotope label. Apparent and true absorption (41 and 48 % respectively) on the low-Cu diet were not significantly different from the high-Cu diet (45 and 48 % respectively). Endogenous losses were significantly reduced on the low- (0·45 mg/d; \( P = 0·001 \)) and medium- (0·81 mg/d; \( P = 0·001 \)) compared with the high-Cu diet (2·46 mg/d). No biochemical changes resulting from the dietary intervention were observed. Cu homeostasis was maintained over a wide range of intake and more rapidly at the lower intake, mainly through changes in endogenous excretion.

Copper: Fructose: Stable isotopes: Copper absorption: Copper endogenous losses

Whole-body Cu balance (homeostasis) is maintained by a combination of changes in absorptive efficiency of the intestinal mucosal cells and quantity of endogenous Cu excreted via the gastrointestinal tract. At low and high intakes the efficiency of absorption is up and down regulated respectively, but adaptation is more rapid with a low Cu intake (Turnlund et al. 1989). The extent to which this endogenously excreted Cu is reabsorbed from the gut is not known (Linder, 1991).

Cu absorption decreases with malnutrition and in various disease states with malabsorption syndromes, for example, coeliac disease (Cordano et al. 1964; Goyens et al. 1985), and several dietary factors reduce absorption, for example, antacids, infant cows’ milk formulas (Dorner et al. 1989), Zn (Turnlund et al. 1988; August et al. 1989) and fructose. The Cu–fructose interaction is potentially the most important due to the increasing consumption of fructose in the Western diet. However, the mechanisms are uncertain because the results of human studies have proved difficult to interpret. Fructose has been found to impair Cu status, whilst Cu balance appears to be increased (Reiser et al. 1985).

Cu absorption has been measured in human volunteers using a highly enriched 65Cu stable isotope, with doses corresponding to 50–200 % of typical daily intakes (August et al. 1989; Turnlund et al. 1991). In these studies, the unabsorbed Cu isotope is excreted in the faeces within 5–7 d, followed by smaller amounts of isotope that have been absorbed and then re-excreted (endogenous loss). Thus the period of time over which the faecal collections are made is critical; if it is too short then the apparent absorption (intake minus luminal loss) will be overestimated, as not all of the unabsorbed Cu will have been excreted. In addition, it is only possible to calculate true absorption if endogenous excretion has been estimated from the appearance of absorbed isotope in the later faecal collections.

The aim of the present study was to determine Cu absorption, retention and endogenous losses following equilibration at different levels of intake of Cu, from that found in diets low in Cu to a level generally only attainable with Cu supplements. In the absence of a sensitive and specific index of Cu status, this was believed to be the best approach for generating information needed to assess the public health significance of low and high Cu intakes. A range of biochemical assays was undertaken at the beginning and end of each dietary intervention in order to underpin the metabolic measurements.

Abbreviations: GPx, glutathione peroxidase; ICP–MS, inductively coupled plasma–mass spectrometry; IFR, Institute of Food Research; SOD, Cu,Zn-superoxide dismutase.

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Methods

Subjects

Twelve healthy men were recruited to a residential, longitudinal intervention study (subject characteristics are given in Table 1). A screening blood sample (10 ml) was taken to exclude volunteers whose biochemical and haematological indices lay outside the normal range. Other exclusion criteria included chronic illness, taking medication or nutritional supplements, and smoking. The aims and procedures of the study were explained to the volunteers during a visit to the Human Nutrition Unit at the Institute of Food Research (IFR) and written informed consent was obtained. The IFR Human Research Ethics Committee approved the protocol and the study was conducted in accord with the Helsinki Declaration of 1975 as revised in 1983.

Study design

The subjects were resident in the Human Nutrition Unit for three periods of 8 weeks with a minimum washout of 4 weeks between study periods. A diet low in Cu was fed throughout the study using a 7 d rotating menu consisting of three meals per day and snacks. Additional Cu was added to the diets in the form of copper sulfate with each meal, such that the total daily intake was 1.6 and 6.0 mg during the first and third dietary periods respectively. The diets were regularly analysed to ensure that the Cu content remained constant from batch to batch. During each study period, the energy intakes of the subjects were adjusted as necessary in order to maintain body weight by the addition of either a commercially available dextrose drink or yoghurt. The mean Cu content of the diet analysed by atomic absorption spectroscopy was 0.69 (SD 0.02) mg/d. All other dietary variables were within customary UK limits.

Dose preparation

Isotopically enriched copper chloride (\(^{65}\text{CuCl}_2\)) was prepared from elemental \(^{65}\text{Cu}\) (Europa Scientific Ltd., Crewe, UK) by dissolving the metal in 10 ml concentrated HNO\(_3\) (BDH, Poole, UK) (Aristar grade) and evaporating to nearly dryness. The sample was then taken up in 0.1 M HCl (25 ml; BDH, Aristar grade) and again evaporated to almost dryness; this stage was then repeated twice more and finally the sample was taken up in an appropriate volume of 0.1 M HCl to give an approximate concentration of 1 mg/ml. The concentration was accurately determined by ICP–MS. The solution was divided into individual doses and stored in plastic vials at \(-20°C\) until required.

The Ho oral doses were prepared by dissolvingholmium chloride (Avocado Research Chemicals Ltd., Heysham, UK) in demineralised, purified water (Elga, Cambridge) to concentrations of approximately 0.5 mg/ml. The solution was divided into individual 1 mg doses and stored in plastic vials at \(-20°C\) until required and the concentration accurately determined by ICP–MS.

Sample preparation and analysis

Faecal samples were autoclaved, freeze-dried, ground to a fine powder using a mortar and pestle, and sub-sampled. Before analysis by ICP–MS, portions of faecal samples (0.5 g) were digested in concentrated nitric acid (5 ml) using a high temperature–pressure microwave digestion system and the digest fluid diluted 50-fold with distilled water containing Ga (50 ng/ml) as an internal standard. The total levels of Cu and the \(^{65}\text{Cu}:^{63}\text{Cu}\) isotope value in the solutions were measured using a VG Plasmaquad Turbo II Plus ICP–MS (VG Elemental, Winsford, UK) (Baxter et al. 1997). The quality of the analytical data was assessed in two ways; first, by the use of a certified reference material (BCR 422 cod muscle), and second, by the recovery of a known amount of added analyte. Ho concentrations were also quantified by ICP–MS (Baxter et al. 1997).

Biochemical assays

Blood samples collected on days 1 and 42 were prepared for various analyses performed either at the IFR or at the Chemical Pathology Department of the Norfolk and
Norwich Hospital (Norwich, UK). Analyses performed by the Chemical Pathology Department included serum Cu, caeruloplasmin, C-reactive protein and a full lipid screen. Serum Cu was measured by atomic absorption spectrophotometry (Phillips model no. PU9200; Phillips, Cambridge, UK) and total caeruloplasmin by an immuno-turbidimetry assay (Dako, High Wycombe, UK). The inter-assay CV were 6.4 and 10 % for the Cu and caeruloplasmin respectively. Serum samples were also analysed for triacylglycerols and total, LDL- and HDL-cholesterol; LDL-cholesterol was calculated using the Friedewald equation (Friedewald et al. 1972). Apolipoprotein A1 and B concentrations were determined using immunoturbidimetry assays (IMMUNO, Ltd., Sevenoaks, Kent, UK) automated on a Cobas Fara centrifugal analyser (Roche, Welwyn Garden City, UK). In order to eliminate raised caeruloplasmin or ferritin concentrations that resulted from an inflammatory response or infection, C-reactive protein concentrations were determined using an immunoturbidimetry assay (Dako). Caeruloplasmin activity was determined at the Northern Ireland Centre for Diet and Health using a modified method of Henry (1960) on an autoanlyser (Cobas Fara; Roche).

The remaining assays were performed at the IFR using an autoanlyser (Cobas Mira; Roche). Control serum (Serum N; Roche) and quality control samples prepared in-house were used for each assay as appropriate:

(a) Cu,Zn-superoxide dismutase (SOD) was measured in erythrocytes using a commercial kit (Ransod kit; Randox Laboratories, Crumlin, UK) based on a modified method of Jones & Suttle (1981). SOD activity was expressed in terms of SOD units/g haemoglobin measured by Drabkin’s method (Drabkins & Austin, 1932) standardised with cyanomethaemoglobin (BDH standard).

(b) Glutathione peroxidase (GPx) activity was determined in plasma, platelets and erythrocytes using a commercially available kit (Ransel test kit; Randox Laboratories) based on a modified method of Paglia & Valentine (1967). Gpx activity was expressed in terms of protein concentration (Unimate 7 Total protein kit; Roche) in plasma and platelets (GPx activity/g protein) and haemoglobin concentration in erythrocytes (Gpx activity/g haemoglobin).

(c) Plasma ferritin was determined by an in-house ELISA assay using A133-rabbit anti-human ferritin coating anti-body and P145-rabbit anti-human ferritin detector antibody, peroxidase conjugated (Dako). The assay was standardised using the 3rd International Standard for Ferritin (National Institute for Biological Standards and Control, Potters Bar, UK).

(d) Platelet aggregation was determined as a response to ADP at a final concentration of $2 \times 10^{-5}$ mol/l in citrated blood using a platelet aggregation profiler (model PAP-4; Bio/Data Corporation, Horsham, PA) (Williams et al. 1977).

(e) Other haematological indices including haemoglobin, packed cell volume and mean cell haemoglobin concentration were measured using an MD8 Coulter Counter (Coulter Electronics Ltd, Hialeah, FL, USA).

Mathematical analysis

Quantities of labelled and unlabelled Cu were calculated according to the method described in Harvey et al. (2002); this paper also details the calculation of endogenous losses from the labelled Cu dose. Fig. 1 shows an overview of the labelled and unlabelled Cu absorption and excretion that was measured and calculated in the present study. The following equations were used to generate the results shown in Tables 2 and 6.

**Labelled copper absorption and excretion.**

$$\text{Apparent absorption} = \frac{(D_L - R_L)}{D_L},$$

$$\text{True absorption} = \frac{(D_L - R_L + E_L)}{D_L} = \frac{A_L}{D_L} = \frac{A_U}{D_U},$$

where $D_L$ is the labelled Cu dose, $R_L$ is the labelled Cu recovered in faeces, $A_L$ is the absorbed labelled Cu, $A_U$ is the absorbed unlabelled Cu and $D_U$ is the unlabelled Cu in the diet. Endogenous loss ($E_L$, mg) = $F_L + S_L$ where $F_L$ (mg) is the loss of labelled Cu from a ‘fast’ pool and $S_L$ (mg) is the loss of labelled Cu from a ‘slow’ pool. Labelled Cu from the fast pool is defined to be Cu that was absorbed from the dose and then excreted within 14 d. Labelled Cu from the slow pool is defined to be Cu that was absorbed from the dose and excreted more than 14 d after the dose. Since the study period is only 14 d, labelled Cu from the slow pool is not measured and $S_L = 0$. Therefore all the endogenous losses of labelled Cu are from the fast pool, i.e. $E_L = F_L$.

Endogenous loss (as a fraction of dose)

$$= \frac{E_L}{D_L} = \frac{F_L}{D_L} = \frac{F_U}{D_U},$$

where $F_U$ is the rate of loss of unlabelled Cu from the ‘fast’ pool in mg/d.

**Fig. 1.** Overview of labelled and unlabelled copper absorption and excretion.
Unlabelled copper balance and excretion. In an analogous way to the labelled Cu, unlabelled Cu from the fast pool is defined as Cu that was absorbed from a meal and then excreted within 14 d. Unlabelled Cu from the slow pool is defined to be Cu that was absorbed from a meal and excreted more than 14 d after the meal.

\[
\text{Balance (mg/d)} = DU - RU,
\]

where \(RU\) is the unlabelled Cu recovered in faeces.

Endogenous loss \((EU; \text{mg/d}) = RU - DU + AU = RU - DU + (DU \times AL)/DL\) (from equation (1)).

Unlabelled Cu from the ‘fast’ pool \((FU; \text{mg/d}) = EL \times DU/ DL\) (from equation (2)).

Since \(EL, DU\) and \(DL\) are all known or measured, \(FU\) can be calculated. These results are shown in Table 2 in the ‘fast pool’ section.

Unlabelled Cu from the ‘slow’ pool \((SU; \text{mg/d}) = EL \times DU + (DU \times AL)/DL\) (from equation (3)).

Since \(RU, RL, DU\) and \(DL\) are all known or measured, \(SU\) can be calculated. These results are shown in Table 2 in the ‘slow pool’ section.

Statistical analysis

All data are expressed as means and standard deviations. ANOVA with repeated measures was used to determine the effect of dietary Cu intake on the efficiency of Cu absorption and excretion. If a significant difference was found, the least squares difference test was used to determine which treatment means differed. Student’s \(t\) test was used to determine the difference between measured and calculated habitual dietary Cu intakes. A significance level of \(P < 0.05\) was used for all statistical tests.

Results

The biochemical data are shown in Table 3. In every case, none of the parameters measured on day 42 was significantly different from those measured on day 1 and there was also no significant difference between the values measured on day 1 of each dietary period; thus only the day 42 results are presented. All erythrocyte SOD, serum Cu, serum total caeruloplasmin and plasma caeruloplasmin activity measurements fell within normal ranges; statistical evaluation of the data demonstrated that none of the parameters was affected by dietary Cu intake. Equilibration for 6 weeks at each Cu intake level was also found to have no significant effect on various risk factors related to cardiovascular disease, including ADP-stimulated platelet aggregation, plasma lipoproteins (total, HDL-, LDL- cholesterol), triacylglycerols, apolipoproteins A1 and B and GPx (Table 4). Other haematological factors were also unaffected by dietary Cu intake including haemoglobin, mean cell haemoglobin concentration and packed cell volume (Table 5).

All data for efficiency of Cu absorption for the low (0.7 mg/d), medium (1.6 mg/d) and high (6.0 mg/d) Cu intake levels are presented in Table 6. Apparent absorption data for eleven of the twelve volunteers are presented due

---

### Table 2. Unlabelled copper endogenous losses and balance data†

<table>
<thead>
<tr>
<th>Dietary Cu intake (mg/d) . . .</th>
<th>6.0</th>
<th>1.6</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu recovered in faeces‡ (mg/d)</td>
<td>12</td>
<td>5.25</td>
<td>1.05</td>
</tr>
<tr>
<td>Balance (mg/d)</td>
<td>6</td>
<td>0.75</td>
<td>1.05</td>
</tr>
<tr>
<td>Slow pool§ (mg/d)</td>
<td>6</td>
<td>1.33</td>
<td>1.15</td>
</tr>
<tr>
<td>Fast pool¶ (mg/d)</td>
<td>6</td>
<td>1.14</td>
<td>0.29</td>
</tr>
<tr>
<td>Total (slow pool + fast pool) (mg/d)</td>
<td>6</td>
<td>2.46</td>
<td>1.11</td>
</tr>
</tbody>
</table>

* Mean value was significantly different (\(P < 0.05\)) from the value on the high Cu diet.
† For details of subjects and procedures, see Table 1 and p. 162.
‡ Based on the unlabelled Cu recovered 14 d after label administration.
§ Cu absorbed from a meal and excreted at least 14 d after the meal.
¶ Cu absorbed from the meal and excreted within 14 d of the meal.

### Table 3. Putative indices of copper status after 6 weeks of each dietary period*

<table>
<thead>
<tr>
<th>Dietary Cu intake (mg/d) . . .</th>
<th>6.0</th>
<th>1.6</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erythrocyte superoxide dismutase activity (U/g haemoglobin)</td>
<td>1016</td>
<td>79</td>
<td>1070</td>
</tr>
<tr>
<td>Serum Cu ((\mu)mol/l)</td>
<td>15.5</td>
<td>2.2</td>
<td>13.9</td>
</tr>
<tr>
<td>Serum caeruloplasmin (g/l)</td>
<td>0.28</td>
<td>0.14</td>
<td>0.23</td>
</tr>
<tr>
<td>Plasma caeruloplasmin activity (U/mg)</td>
<td>2.62</td>
<td>0.48</td>
<td>2.29</td>
</tr>
</tbody>
</table>

* For details of subjects and procedures, see Table 1 and p. 162.
data for true absorption are reduced to six data sets because the method used to calculate true absorption failed on five other volunteers due to slow transit times of these subjects. This resulted in an inadequate number of stools being available in the later stages of the faecal collection period containing only $^{65}$Cu stable isotope dose resulting from endogenous losses. The mean apparent absorption of the 3 mg label for eleven of the volunteers on the low Cu intake was not significantly different from the apparent absorption of the dose at the high dietary intake level. There was no significant difference in apparent absorption between the medium and high or medium and low Cu intakes.

### Table 4.
Biochemical indices denoting increased risk of cardiovascular disease measured after 6 weeks at each level of copper intake

<table>
<thead>
<tr>
<th>Dietary Cu intake (mg/d)</th>
<th>6.0</th>
<th>1.6</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet aggregation (%)</td>
<td>80</td>
<td>80</td>
<td>82</td>
</tr>
<tr>
<td>Plasma lipoproteins (mmol/l)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>5.0</td>
<td>5.2</td>
<td>5.1</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>3.3</td>
<td>3.4</td>
<td>3.5</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>1.1</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.24</td>
<td>1.51</td>
<td>1.19</td>
</tr>
<tr>
<td>Apolipoprotein A1 (g/l)</td>
<td>1.14</td>
<td>1.10</td>
<td>1.19</td>
</tr>
<tr>
<td>Apolipoprotein B (g/l)</td>
<td>1.08</td>
<td>0.96</td>
<td>0.93</td>
</tr>
<tr>
<td>Ferritin‡ (ng/ml)</td>
<td>45</td>
<td>47</td>
<td>47</td>
</tr>
<tr>
<td>MCHC (g/l)</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Platelets (× 10^12/μl)</td>
<td>265</td>
<td>225</td>
<td>347</td>
</tr>
</tbody>
</table>

*MCHC, mean cell haemoglobin concentration.
†Values for eleven subjects.
‡Values are geometric means ($-\overline{1}$ SD, $+\overline{1}$ SD).

### Table 5.
Haematological and haemostatic measurements after 6 weeks at each level of copper intake

<table>
<thead>
<tr>
<th>Dietary Cu intake (mg/d)</th>
<th>6.0</th>
<th>1.6</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Packed cell volume (%)</td>
<td>45.7</td>
<td>44.5</td>
<td>44.1</td>
</tr>
<tr>
<td>Haemoglobin (g/l)</td>
<td>151</td>
<td>150</td>
<td>148</td>
</tr>
<tr>
<td>MCHC (g/l)</td>
<td>331</td>
<td>336</td>
<td>335</td>
</tr>
<tr>
<td>Ferritin‡ (ng/ml)</td>
<td>29.3</td>
<td>336</td>
<td>29.3</td>
</tr>
</tbody>
</table>

*For details of subjects and procedures, see Table 1 and p. 162.
†Values are geometric means ($-\overline{1}$ SD, $+\overline{1}$ SD).

### Table 6.
Copper absorption and endogenous losses (from an oral dose of 3 mg copper label) measured after 6 weeks of each dietary period

<table>
<thead>
<tr>
<th>Dietary Cu intake (mg/d)</th>
<th>6.0</th>
<th>1.6</th>
<th>0.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apparent absorption (%)</td>
<td>11</td>
<td>45</td>
<td>42</td>
</tr>
<tr>
<td>mg</td>
<td>1.34</td>
<td>1.27</td>
<td>1.27</td>
</tr>
<tr>
<td>True absorption (%)</td>
<td>6</td>
<td>48</td>
<td>45</td>
</tr>
<tr>
<td>mg</td>
<td>1.45</td>
<td>1.36</td>
<td>1.45</td>
</tr>
<tr>
<td>Endogenous loss of label†</td>
<td>6</td>
<td>40</td>
<td>38</td>
</tr>
<tr>
<td>mg</td>
<td>0.58</td>
<td>0.48</td>
<td>0.48</td>
</tr>
</tbody>
</table>

*For details of subjects and procedures, see Table 1 and p. 162.
†Absorbed label excreted within 14 d of dose administration.
Generally, the non-absorbed label was excreted in the faeces within 6 d of administration of the dose. This was validated by the simultaneous and complete recovery of the Ho dose (mean recovery was 98 (SEM 5) %). Labelled Cu appearing in the faeces after this time was attributed to endogenous losses. Labelled endogenous losses were calculated using the method of Harvey et al. (2002) and were significantly lower on the low- and medium-Cu diets compared with the high-Cu diet (Table 6).

True absorption was estimated by adjusting the apparent absorption, as determined from faecal monitoring, with the endogenous loss measurements of the recovered label in these subjects (Table 6). True absorption was found to follow the same pattern as apparent absorption; absorption on the low-Cu diet was not significantly different from the high-Cu diet. There was no significant difference in the absorption between the medium and either the high or low Cu intakes.

Balance is measured as the difference between dietary intake of unlabelled Cu and unlabelled Cu recovered in the faeces. It can be seen from Table 2 that, on average, the volunteers are in negative balance on the low-Cu diet and positive balance on the medium- and high-Cu diets. Balance on the high-Cu diet was significantly greater than for both the medium- and low-Cu diets. However, the difference between the medium- and low-Cu diets was not significant. For the labelled Cu, volunteers on the low-Cu diet retained 1·05 (SD 0·40) mg of the label 14 d after administration compared with 0·88 (SD 0·44) mg on the medium-Cu diet and 0·87 (SD 0·26) mg on the high-Cu diet. The difference between the low- and high-Cu diet values was not significant.

In the present experiment, total endogenous losses can be divided into two parts: fast and slow loss. The ‘fast’ loss is Cu that has been absorbed from a meal and then excreted within 14 d. The ‘slow’ loss is absorbed Cu that is excreted in the faeces more than 14 d after the meal was consumed. The total daily endogenous loss is calculated from the quantity of absorbed Cu per d plus the difference between dietary intake and recovered faecal Cu. This assumes that the quantity of Cu lost via other routes (for example, urine) is small. These data are presented in Table 2 for the unlabelled Cu. The total endogenous loss on the high-Cu diet was significantly different from both the medium- (P<0·001) and low-Cu diets (P<0·001). A similar significant difference was observed for the rate of endogenous loss from the fast pool on the high-Cu diet compared with the medium- (P<0·001) and low-Cu diets (P<0·001). The rate of endogenous loss from the slow pool was calculated as the difference between the total and the fast rates of loss (Table 2). The rate of loss was significantly greater on the high- than on the low-Cu diet (P=0·023). No significant difference was observed between the high- and medium-Cu diets.

The habitual dietary Cu intake of each of the volunteers was determined from both calculated and direct measurements. The mean analysed daily intake was 1·8 (SD 0·5) mg with a range of 0·8–2·8 mg/d and was significantly higher (P=0·021) than the calculated value of 1·4 (SD 0·5) mg with a range of 0·7–2·5 mg/d.

Discussion

From the results presented it appears that Cu homeostasis is maintained through control of only endogenous excretion and not absorption. Although the present study does not include data on urinary output, it has been reported that this form of excretion is minimal (Ishihara & Matsushiro, 1986) and is not dependent on dietary Cu intake. Turnlund et al. (1989, 1998) have performed two dietary intervention studies with male volunteers, feeding Cu levels similar to those used in the present study. Stable-isotope methodology was used to determine apparent absorptions of 56 and 54 % following equilibration at Cu intake levels of 0·785 and 0·66 mg/d respectively, higher than the mean apparent absorption of 41 % found on the low-Cu diet (0·7 mg/d) in the present study. Daily intakes of 7·53 and 1·68 mg Cu were found to result in apparent absorptions of 12 and 36 % respectively (Turnlund et al. 1989). These values are, however, lower than the values found in the present study, which may be the result of differences in experimental protocol. First, the present study measured absorption from a single test meal, which has been reported to over-estimate absorption compared with measurement over several meals as used by Turnlund et al. (1989). Second, in the present study, in order to be able to calculate endogenous losses, 3 mg labelled Cu was given in a single test meal on each of the high-, medium- and low-Cu diets, whereas in the study by Turnlund et al. (1998) Cu in the study meals was replaced with the same quantity of Cu label.

The study by Turnlund et al. (1998) estimated endogenous losses by measuring the faecal appearance of intravenous doses of highly enriched 65Cu stable isotope. In agreement with our data, endogenous losses were found to be reduced with low dietary Cu intake when compared with higher intake levels. A Cu metabolism model based on the disappearance of both infused and oral 65Cu dose has suggested that tissue uptake of oral and intravenous Cu is different, with flow between the plasma and liver compartment from the intravenous dose only being 80 % of the oral dose (Scott & Turnlund, 1994). The intravenous technique gives a general indication of the relative changes in endogenous losses resulting from different dietary Cu intakes, whereas the technique used in the present study has the advantage of reflecting the metabolism of the absorbed oral dose in the same volunteer during the same time period as the absorption efficiency is assessed.

Within 14 d, 38 (SD 20) % of the absorbed oral dose had been excreted when the volunteers were on the medium-Cu diet. From the retention data of the unlabelled Cu it is known that the volunteers were in balance (Table 2). This indicates that the other 76 % of the absorbed label will be excreted at a future indeterminate time from a slowly exchangeable pool in the body. When the volunteers were on the high-Cu diet, 40 (SD 11) % of the absorbed dose was excreted 14 d after the dose was given. The volunteers were not, however, in balance (0·75 mg/d was retained). It is not clear from our study or others (Turnlund et al. 1989; 1998) how long, if at all, it would take to restore Cu balance on such a high-Cu
Adaptation to low and high copper intakes

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References


5-Methyltetrahydrofolic acid and folic acid measured in plasma with liquid chromatography tandem mass spectrometry: applications to folate absorption and metabolism

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Abstract

We describe a liquid chromatography (LC) tandem mass spectrometry (MS-MS) method for the determination of 5-methyltetrahydrofolic acid (5-methylTHF) and folic acid concentrations and enrichments in human plasma. It was used to study absorption and initial metabolism in five volunteers with two simultaneously administered oral test doses ([13C6]folic acid in capsules and [2H2]folic acid in a drink). [13C5]5-methylTHF and [2H4]folic acid were used as internal standards. Plasma samples (2 ml) were purified using folate binding protein affinity columns, followed by a concentration step. After LC separation, folates were detected using positive electrospray ionization MS-MS under multiple reaction monitoring conditions. Calibrations were linear for 5-methylTHF over the range 1.2 \times 10^{-10} \text{ mol/L} to 3.2 \times 10^{-7} \text{ mol/L} and for folic acid over the range 5 \times 10^{-10} \text{ mol/L} to 4.5 \times 10^{-8} \text{ mol/L}. For 5-methylTHF concentration in plasma, intraassay coefficient of variation was within 8.6% (and for unlabeled 5-methylTHF it was within 2.8%) and interassay coefficient of variation was within 9.0%. For folic acid concentrations these coefficient of variations were within 7.5% and within 6.5%, respectively. The [13C6] and [2H2] isotopomers of folic acid and 5-methylTHF were measured in the plasma of each volunteer for 8 h. After accounting for the time delay due to capsule opening, the modeling results showed no significant differences in absorption time, first pass effect, and elimination rate in the folic acid test doses in capsule or drink. We conclude that LC-MS-MS offers increased sensitivity for quantification of plasma concentrations and enrichments of 5-methylTHF and folic acid and is applicable to stable-isotope studies in humans.

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Keywords: Folate; Liquid chromatography tandem mass spectrometry; Bioavailability

Folates are a group of compounds derived from food stuffs. They belong to the B vitamin group and are an essential element in the diet. In human pathology, folate deficiency causes anemia and neurological disturbances. Diminished folate status is associated with hyperhomocysteinemia, an independent risk factor for vascular disease [1]. Folic acid, a synthetic product not appearing in nature, is used as an oral supplement by patients with these disorders and is recommended to women of childbearing age to reduce the risk of neural tube defects in offspring. In some countries, folic acid is also used as a food fortificant in cereals and dairy products.

Folic acid is absorbed in the small intestine and is converted in the body through several steps into the methylated derivative of tetrahydrofolic acid, 5-methyltetrahydrofolic acid (5-methylTHF). 1 5-MethylTHF either is then stored in tissues or appears in plasma. If folic acid is consumed in amounts exceeding

1 Abbreviations used: 5-methylTHF, 5-methyltetrahydrofolate glutamate; LC, liquid chromatography; MS, mass spectrometry; MS-MS, tandem mass spectrometry; tlag, time that 5-methylTHF enrichment remains at baseline; TTR, tracer to tracee ratio; CV, coefficient of variation.
5.7 × 10⁻⁷ mol (250 µg) in a single dose or as fortificant in a meal, its free form can also appear in plasma [2]. To study the in vivo absorption and metabolism of these folate forms, several stable isotopes are available. In stable isotope studies, mass spectrometry (MS) is used for the measurement of enrichment in folic acid derivatives, in particular 5-methylTHF in plasma. With the use of isotope dilution techniques, MS can also be used to measure the plasma 5-methylTHF and folic acid concentrations. For that purpose, blood samples of 5-methylTHF must be protected against degradation because the compound is sensitive to heat, UV light, and oxidation.

For assessment of 5-methylTHF isotopic enrichment, several methods using LC-MS have been described. These methods used folate binding protein affinity columns to purify the folate from the plasma samples. The time-consuming steps required in extraction and derivatization inherent in previous approaches using gas chromatography-mass spectrometry [3,4] can thus be overcome. In recent years, LC-MS has been applied to quantify folate monoglutamates [5-8]. In a study applied to human folate bioavailability, Hart et al. [9] described good linearity for 5-methylTHF using LC-MS with “negative ion” electrospray ionization; using [2H₂]folic acid as internal standard, limits of detection were 2-5.5 × 10⁻¹⁰ mol/L (coefficient of variation (CV) 7.4%). After ingestion of [1³C₆]folic acid and [1³C₅]5-HCOH₄folic acid by volunteers, this method allowed the measurement of tracer (mass+6) enrichment in plasma 5-methylTHF samples.

In the present study, we developed a LC tandem MS (LC-MS-MS) technique to measure 5-methylTHF and folic acid enrichments in human plasma. The method is applied in a human stable isotope study in which folic acid was administered orally in two forms, i.e., [2H₂]folic acid dissolved in water and [1³C₆]folic acid in capsules. Both forms were administered in a single bolus, and this was followed by measurements of plasma 5-methylTHF isotopomer enrichments. Absorption and initial metabolism of both folate tracers was studied with short-term kinetic modeling, and characteristics for both administration forms were compared.

**Methods**

**Materials**

Folic acid (purity 98%) and 5-methylTHF (6R/S; purity 90%) were obtained (Sigma, Deisenhofen, Germany), and aqueous stock solutions were prepared (1.6 × 10⁻⁴ mol/L folic acid and 5-methylTHF, with 0.1 w/v% ascorbic acid added as antioxidant). The labeled folates [2H₂]folic acid, [3H₂]folic acid, and gelatin capsules containing [1³C₅]folic acid were obtained from the Institute of Food Research (Norwich, UK), and [1³C₅]5-methylTHF was obtained from Eprova AG. Aqueous solutions were prepared (3.6 × 10⁻⁶ mol/L [2H₂]folic acid and [1³C₅] 5-methylTHF, with 0.1 w/v% ascorbic acid added as antioxidant) for use as internal standards. One dose of the stock solutions and internal standard solutions was diluted (with 0.1 M K₂HPO₄ buffer, pH 7.0) and folate concentration measured with spectroscopic analysis using molar extinction coefficients (E [mmol⁻¹, cm⁻¹]) of 31,700 for 5-methylTHF and 27,600 for folic acid [10]. The residual doses were stored at −80°C in the dark.

For the in vivo experiments, [1³C₆]folic acid in gelatin capsules and [2H₂]folic acid in water were used as test doses. The capsules (containing 1.27 × 10⁻⁷ mol [1³C₆]folic acid, with isotopic purity 96% [11,12], and glass vials with [2H₂]folic acid in pyrogen-free water (concentration 1.18 × 10⁻⁴ mol/L with isotopic purity 99%, determined using LC-MS-MS as described below) were stored in the dark at 4°C.

**Human study protocol**

Five nonsmoking healthy volunteers (one female and four males, mean age 27 years, mean body weight 72 kg) were studied. The volunteers did not use medication or vitamin supplements and were asked to avoid excessive consumption of folate-rich food during the week preceding the experiment (systematic food fortification with folic acid is not practiced in The Netherlands). The study protocol was approved by the ethics committee of the VU University Medical Center (Amsterdam, The Netherlands), and informed consent was obtained from each subject.

The subjects reported to the VU University Medical Center at 0800 h, following an overnight fast. An intravenous catheter was placed in a dorsal hand vein for blood sampling. After baseline blood sampling, the volunteers were given simultaneously two kinds of labeled folic acid (i.e., [1³C₅]folic acid in capsules and [2H₂]folic acid as a drink). First, four gelatin capsules (total tracer dose 5.1 × 10⁻⁷ mol (=224 µg) [1³C₅]folic acid) were swallowed, followed immediately by a 4-ml solution (total tracer dose 4.7 × 10⁻⁷ mol (=207 µg) [2H₂]folic acid) dissolved in water. Volunteers were given only a light lunch after a 4-h postdosing blood sample had been obtained. Venous blood was drawn at t = 0 (baseline), 20, 40, 60, 90, 120, 150, 180, 240, 300, 360, and 480 min, collected in EDTA vacutainers (Becton-Dickinson, Plymouth, UK), and immediately centrifuged (at 4°C; 2000g, 10 min); the plasma was stored at −80°C.
Sample preparation and folate extraction from blood

Folate affinity columns were prepared overnight by binding folate binding protein (Scripps Laboratories, San Diego, USA) to Affigel 10 (Bio-Rad, Hemel Hempstead, UK) in 0.1 M sodium bicarbonate at 4°C, stored in 0.1 M K2HPO4, buffer pH 7 (containing 0.2 mM vitamin B6). Both supernatant fractions were conditioned by rinsing three times with 5 ml of 0.1 M K2HPO4, 1 M NaCl buffer (pH 7), and stored at 4°C. The affinity columns were checked regularly and found to be stable over a period of 6 months.

5-MethylTHF extraction from plasma was based on the method described by Hart et al. [9], with minor modifications. Plasma (2 ml) was transferred into 50-ml propylene tubes and 20 µl of an internal standard solution (containing 3.6 × 10⁻⁶ mol/L [13C5]5-methylTHF and [2H4]folic acid) was added. Protein was precipitated and folates were protected against oxidation by adding 10 ml extraction buffer (0.1 M K2HPO4, pH 7), containing 50 mM ascorbic acid and 10 mM mercaptoethanol). To further protect the folates against oxidation the tubes were flushed with nitrogen prior to placing them in a boiling waterbath for 20 min. Samples were placed on ice and centrifuged (2000 × g, 10 min). The supernatant was collected and the pellet was extracted with an additional 10 ml extraction buffer. Folate affinity columns were used for sample cleanup; they were preconditioned by rinsing three times with 5 ml of 0.1 M K2HPO4 (pH 7). Both supernatant fractions were introduced onto the column and the columns were washed with 5 ml of 0.1 M K2HPO4, 1 M NaCl buffer (pH 7), 5 ml of 0.05 M K2HPO4 (pH 7), and 5 ml of 0.025 M K2HPO4 (pH 7). The eluate was collected in tubes (containing 5 mg ascorbic acid), and 5.5 ml of 0.1 M HCl (containing 3.6 × 10⁻⁶ mol/L [13C5]5-methylTHF and [2H4]folic acid) was added. Protein was precipitated and folates were protected against oxidation by adding 10 ml extraction buffer (0.1 M K2HPO4, pH 7), containing 50 mM ascorbic acid and 10 mM mercaptoethanol). To further protect the folates against oxidation the tubes were flushed with nitrogen prior to placing them in a boiling waterbath for 20 min. Samples were placed on ice and centrifuged (2000 × g, 10 min). The supernatant was collected and the pellet was extracted with an additional 10 ml extraction buffer. 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The 0.1 M HCl fraction was then introduced onto the column. The columns were washed with 2 ml of water and eluted with 500 µl of methanol into vials (containing 2 mg ascorbic acid). Subsequently, the methanol fraction was evaporated under nitrogen at 40°C and redissolved in 500 µl of eluent (10 mM formic acid, 10% acetonitrile). The samples were stored at −20°C. A volume of 50 µl was used for LC-MS-MS analysis.

Liquid chromatography tandem mass spectrometry

All analyses were performed on an API 3000 triple quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, USA). Side instrumentation consisted of a Perkin–Elmer Series 200 HPLC pump, a Perkin–Elmer Series 200 auto sampler (operated at 4°C), and a Harvard Apparatus Pump 11 infusion pump. Liquid chromatography was performed on a Xterra MS C18 analytical column (4.6 × 250 mm; 5-µm beadsize) (Waters) using 10 mM formic acid:acetonitrile (90:10), pH 2.3, as mobile phase at a flow rate of 1 ml/min. The column was connected to the turbo ion electrospray source by a splitter (1:20), MS was performed in the “positive ion” mode, with temperature of the turbo ion electrospray set to 450°C, and nitrogen was used as turbo ion gas (flow rate 8 L/min, ion spray 5000 V). The mass spectrometer was optimized under computer control by constant infusion of the target analytes. For each precursor fragment transition a dwell time of 0.150 s was applied. The optimization included collision-induced dissociation, which was performed in the second quadrupole using nitrogen as the collision gas (at 0.06 kPa).

The mass spectrometer was operated under unit resolution. The selected precursor and fragment ions used for the measurement of the labeled and unlabeled folate vitamers are summarized in Table 1. The LC-MS-MS data were acquired and processed using Analyst for Windows NT software (Applied Biosystems). Diluted folate standards within the linear range of the calibration curves (see Results) were measured before and after human plasma samples in all measurement series for calibration verification. Duplicate injections were performed for plasma samples and standards.

Calculations and statistics

Calibration curves were constructed and concentrations of sample enrichments and concentrations were calculated using linear regression analyses (least

Table 1

<table>
<thead>
<tr>
<th>Folic acid</th>
<th>Precursor ion (m/z)</th>
<th>Fragment ion (m/z)</th>
<th>5-MethylTHF</th>
<th>Precursor ion (m/z)</th>
<th>Fragment ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlabeled compound</td>
<td>442.2</td>
<td>295.1</td>
<td></td>
<td>460.2</td>
<td>313.1</td>
</tr>
<tr>
<td>Labeled site: glutamic acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1H3]folic acid</td>
<td>446.2</td>
<td>295.1</td>
<td>[13C5]5-methylTHF</td>
<td>465.2</td>
<td>313.1</td>
</tr>
<tr>
<td>Labeled site: benzoyl ring</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>[1H3]folic acid</td>
<td>444.2</td>
<td>297.1</td>
<td>[2H2]5-methylTHF</td>
<td>462.2</td>
<td>315.1</td>
</tr>
<tr>
<td>[13C6]folic acid</td>
<td>448.2</td>
<td>301.1</td>
<td>[13C6]5-methylTHF</td>
<td>466.2</td>
<td>319.1</td>
</tr>
</tbody>
</table>
The isotopic enrichment was expressed as tracer to tracee ratio,
\[ \text{TTR} \% = \left( \frac{R_b - R_a}{} \right) \times 100\% \],
where \( R_a \) and \( R_b \) are the ratios of the areas at the \( m/z \) values of the labeled and unlabeled compounds for the enriched sample and a sample of natural abundance, respectively.

In vivo folate absorption was estimated from the data sets of the labeled and unlabeled 5-methylTHF concentrations. Multiple folate pools exist in the human body and can be characterized as either fast or slow turnover pools. Prediction of whole-body folate turnover and metabolism require characterization of the slow-turnover pools in long-term studies [13]. Short-term protocols do not allow assessment of slow-turnover pools which include intracellular folate polyglutamates but can be applied to study the short-term distribution of absorbed (monoglutamate) folate. During a measurement period of >6 h after an orally administered labeled folic acid dose, changes in plasma concentration of 5-methylTHF isotopomers can be approximated as a single-compartment model of the fast monoglutamate pools [14,15]. In this model, the time after ingestion during which the plasma 5-methylTHF isotopomer enrichment remains at baseline \((t_{lag})\), the absorption time (i.e., the time between the first rise in enrichment and the peak \((t_{max})\)), the first pass effect (i.e., the removal of part of the absorbed dose, presumably by the liver), and the rate of elimination \((k)\) can be estimated. The model, assumptions, and calculations are described in the Appendix.

Data from each in vivo experiment were analyzed in the single-pool model with SAAM II software (version 1.1.1, Saam Inc., Seattle, USA), and parameters for first pass effect and rate of elimination were estimated with SAAM II using standard goodness of fit criteria.

Student’s \( t \) tests for paired samples were performed to compare test folates (SPSS 9.0, SPSS Inc., Chicago, USA). Values are presented as means ± SD, unless otherwise stated. Differences were considered significant if \( p < 0.05 \) (two-tailed).

Results

**LC tandem mass spectrometry of folic acid and 5-methylTHF in plasma**

The LC-MS-MS spectra with collision-induced decomposition of folic acid and 5-methylTHF isotopomers (depicted in Fig. 1) showed intense fragment ions. Due to the loss of the glutamic acid residue from the protonated molecular ion, fragments \( m/z \) 295 and 313 (and additional isotopomers for benzoyl-ring-labeled compounds) were found for folic acid and 5-methylTHF, respectively. These fragments were used for multiple reaction monitoring (Fig. 2).

The calibration curves for 5-methylTHF were linear over a concentration range of \( 4.5 \times 10^{-11} \) to \( 3.2 \times 10^{-7} \) mol/L (correlation coefficients for intraassay and interassay analyses: 0.9976 ± 0.0016 \((n = 6)\) and 0.9993 ± 0.0016 \((n = 14)\), respectively). Linearity for calibration curves of folic acid also existed over a concentration range of \( 1.5 \times 10^{-9} \) to \( 4.5 \times 10^{-8} \) mol/L (correlation coefficients for intraassay and interassay analyses: 0.9942 ± 0.0043 \((n = 6)\) and 0.9971 ± 0.0017 \((n = 5)\), respectively).

Limits of detection were derived by analyzing diluted folate standards for 5-methylTHF and diluted plasma for folic acid. The limit of detection for 5-methylTHF was \( 1.2 \times 10^{-11} \) mol/L (=6.1 \times 10^{-18} mol on column) and that for folic acid was \( 5.0 \times 10^{-10} \) mol/L (=2.5 \times 10^{-15} mol on column). The limits of quantification (with signal-to-noise ratio >10) were \( 4.5 \times 10^{-11} \) mol/L for 5-methylTHF and \( 1.5 \times 10^{-9} \) mol/L for folic acid. Results of reproducibility studies are shown in Table 2. The mean intraassay and interassay CVs for plasma folate acid concentration measurements were ≤7.5 and ≤6.5, respectively. For plasma 5-methylTHF the mean intraassay and interassay CVs were, respectively, ≤8.6% (and for unlabeled 5-methylTHF ≤2.8%) and ≤9.0% for concentration values ≤6.5 and ≤1.3% for enrichment values.

The method was further tested by adding known amounts of (6S)5-methylTHF and folic acid (and the labeled internal standards) to human plasma samples, which then went through the folate extraction and LC-MS-MS procedure. After addition of 40, 80, and \( 120 \times 10^{-9} \) mol/L 5-methylTHF and 6, 11, and \( 17 \times 10^{-9} \) mol/L folic acid to human plasma, the increase in concentration over baseline values ranged between 98.7 and 102.5% of the added amount for 5-methylTHF and between 96.6 and 104.7% of the added amount for folic acid.

**In vivo folate absorption and comparison of test doses**

Plasma concentrations of \(^{[2H_2]}\) and \(^{[13C_6]}\)folic acid and \(^{[2H_2]}\) and \(^{[13C_6]}\)5-methylTHF were simultaneously quantified for each volunteer experiment. The data set from one experiment is shown in Fig. 3. Table 3 shows the results of the absorption modeling. The \( t_{lag} \) for the capsule opening was approximately 40 min; there was no delay for the drink. Absorption time for \(^{[2H_2]}\)folic acid from the drink was 122 ± 21 min and that for \(^{[13C_6]}\)folic acid from the capsules was 146 ± 38 min \((p = 0.11)\). For unmetabolized \(^{[2H_2]}\) and \(^{[13C_6]}\)folic acid, peak plasma concentrations were reached within 60 min after ingestion of the test doses. Thereafter, the plasma concentration of the folic acid isotopomers rapidly diminished to values below the detection limit. For \(^{[2H_2]}\) and \(^{[13C_6]}\)5-methylTHF, plasma peak concentrations were reached later than for the folic acid isotopomers, and concentrations declined slowly and remained markedly higher than the baseline values during the 8-h
experiment. Compartmental analysis showed similar means for first pass effect and rate of elimination in the study volunteers. Parameter estimation for the elimination rate constant was less accurate than for first pass effect (as shown by the individual CV values in Table 3).

Discussion

We have presented a LC-MS-MS method for the measurement of 5-methylTHF and folic acid in plasma and showed its applicability to the study of initial metabolism of folic acid in humans.

Our LC-MS-MS method includes a concentration step of the folate binding protein column eluent and combines the use of an acidic mobile phase with a reverse-phase column for separation with “positive ion” electrospray MS-MS. We modified the LC-MS method described by Hart et al. [9], with the aim to reduce plasma volume (at present 2 ml) and to improve sensitivity for the measurement of concentrations and enrichments of 5-methylTHF and folic acid. The use of an

Fig. 1. Tandem mass spectrum with collision-induced dissociation of the protonated molecular ion for (A) folic acid (m/z 442) and (B) 5-methylTHF (m/z 460). Results were obtained with “positive ion” electrospray.
acidic mobile phase offers the advantage of yielding highly polar folates (especially for 5-methylTHF), with longer retention on reversed-phase-type columns, facilitating their separation from signal-suppressing impurities eluting at or near the void volume of the LC column. For MS determination of folates, “negative ion” elec-

![Graph](image-url)
trospray is often used to prevent formation of satellite ions [8,9]. Satellite ions (mainly sodium and potassium adducts) are observed when “positive ion” electrospray MS-MS is used and lower the protonated molecular ion. However, in contrast to its use in combination with “positive ion” electrospray, acidic mobile phases compromise the signal in “negative ion” electrospray MS-MS, requiring in the latter case the use of post-column addition of organic bases (such as triethylamine [8]). The combination of “positive ion” electrospray with an acidic mobile phase precludes such complications. Therefore we applied positive electrospray despite the drawback of this technique with respect to the formation of satellite ions. Recently, “positive ion”

Table 2
Intraassay and interassay precision of LC/MS/MS for folic acid and 5-methylTHF measurements in human plasma

<table>
<thead>
<tr>
<th>Concentration</th>
<th>Unlabeled</th>
<th>[2H2]-labeled</th>
<th>[13C6]-labeled</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>intraassay</td>
<td>interassay</td>
<td>intraassay</td>
</tr>
<tr>
<td>Folic acid</td>
<td><em>a</em></td>
<td><em>a</em></td>
<td>4.95 ± 0.57</td>
</tr>
<tr>
<td></td>
<td>(11.5%)</td>
<td>(6.5%)</td>
<td>(7.7%)</td>
</tr>
<tr>
<td></td>
<td>4.66 ± 0.16</td>
<td>(3.4%)</td>
<td></td>
</tr>
<tr>
<td>Mean CV(%)<em>b</em></td>
<td>7.5%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-MethylTHF</td>
<td>12.62 ± 0.48</td>
<td>10.16 ± 0.02</td>
<td>0.035 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>(3.8%)</td>
<td>(0.2%)</td>
<td>(8.6%)</td>
</tr>
<tr>
<td></td>
<td>16.57 ± 0.15</td>
<td>16.84 ± 0.29</td>
<td>1.55 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(0.9%)</td>
<td>(1.7%)</td>
<td>(5.9%)</td>
</tr>
<tr>
<td></td>
<td>45.26 ± 1.63</td>
<td>47.07 ± 0.27</td>
<td>3.53 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>(3.6%)</td>
<td>(0.6%)</td>
<td>(6.5%)</td>
</tr>
<tr>
<td>Mean CV(%)<em>b</em></td>
<td>2.8%</td>
<td>0.8%</td>
<td>7.0%</td>
</tr>
<tr>
<td>Enrichment</td>
<td>7.09 ± 0.52</td>
<td>8.77 ± 0.09</td>
<td>9.77 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>(7.3%)</td>
<td>(1.1%)</td>
<td>(4.2%)</td>
</tr>
<tr>
<td></td>
<td>10.74 ± 0.79</td>
<td>11.24 ± 0.18</td>
<td>21.56 ± 0.77</td>
</tr>
<tr>
<td></td>
<td>(7.4%)</td>
<td>(1.6%)</td>
<td>(3.6%)</td>
</tr>
<tr>
<td></td>
<td>18.24 ± 0.89</td>
<td>14.74 ± 0.17</td>
<td>46.14 ± 1.34</td>
</tr>
<tr>
<td></td>
<td>(4.9%)</td>
<td>(1.1%)</td>
<td>(2.9%)</td>
</tr>
<tr>
<td></td>
<td>6.5%</td>
<td>1.3%</td>
<td>3.6%</td>
</tr>
<tr>
<td>Mean CV(%)<em>b</em></td>
<td>6.5%</td>
<td>1.3%</td>
<td>7.0%</td>
</tr>
</tbody>
</table>

Samples were selected from human volunteers to obtain values for unlabeled and labeled vitamers. TTR, tracer-to-tracee ratio.

"Not available.

"CV denotes coefficient of variation (n = 6; CV between parentheses).

Fig. 3. Sample set of data from a human volunteer for plasma concentrations of (A) [2H2] and [13C6]folic acid isotopomers and (B) [2H2] and [13C6]5-methylTHF isotopomers. Two oral test doses ([13C6]folic acid in capsules and [2H2]folic acid in a drink) were administered at t = 0. A. [2H2] Isotopomer; B. [13C6] Isotopomer. Peak plasma concentration for folic acid ranged 1.8–2.9 × 10⁻⁹ mol/L for [2H2]folic acid and 3.1–5.0 × 10⁻⁹ mol/L for [13C6]folic acid (n = 5 volunteers). Note that folic acid levels were below the limit of quantification at t = 20 min and t ≥ 120 min.
With the use of absorption time (which corrects for study but no differences in absorption time and kinetics capsule opening in the human volunteer bioavailability ma-enrichment data sets. The data showed a delay in folates and a single-pool model for 5-methylTHF plas-
vivo absorption and bioavailability using two test the full 8-h time course of the study. quantification folic acid could not be followed during plasma peak concentration, but due to the limit of concentrations could be accurately quantified near the human volunteers. The unmetabolized folic acid con-
tations, validating its use in human (patho)physiology. turnover pools in the body and is limited to the short-term protocol does not allow assessment of slow-
turnover pools in the body and is limited to the absorbed and metabolized folic acid as 5-methylTHF monoglutamate in the plasma pool. Second, folate ab-
absorption is assumed to be 90%. This value for absor-
pation was obtained in 1 healthy human volunteer after an oral dose of 0.8 × 10⁻⁷ mol (=35 μg) monoglutamate-
t[¹⁴C]folic acid [19]. A similar absorption (92%) was found in a patient in remission with Hodgkin’s disease after ingestion of 7.25 × 10⁻⁷ mol [14C]folic acid [20], which is close to the dose ingested by our study subjects (total for [²H₂] and [¹³C₆]folic acid: 9.8 × 10⁻⁷ mol). Absorption characteristics of both folic acid isotopo-
mers were also very similar to those measured with the same model in 27 human volunteers who received an oral dose of 4.5 × 10⁻¹⁰ mol (200 μg) [²H₂]folic acid [13]. Third, the model does not allow the simultaneous estimation of both absorption fraction and first pass effect. Assumption of one of these parameters is needed to estimate the other. In the present study, we focused on first pass effect of the labeled folic acid doses. Fourth, using the enrichment in plasma 5-methylTHF over an 8-h time course, the model estimates the rate constant of elimination in each individual with considerable error. A longer sampling protocol (e.g., 10 or 12 h) would reduce this.

The rate constants of elimination (k) from the single pool in the present study were very similar for both test folates (k = 0.105 h⁻¹). These results can be compared with the literature, reporting results of a radioactive folic acid experiment in one volunteer using 48-h collection of urine samples to follow elimination [20]. Biphasic elimination kinetics, with an initial rapid decline (k = 0.022h⁻¹) followed by a long decline (k = 0.0069h⁻¹), were found [20]. Our study results indicate an even higher initial elimination from the fast pool in humans. A possible explanation for the difference in initial k-value between our study and the work of Krumdieck et al. [20] is the difference in sampling method (plasma versus urine) and sampling frequency. Whether the
higher initial elimination rate of labeled 5-methylTHF found in our study reflects a higher biological catabolism and metabolism from the central compartment than previously thought requires further study, preferably also in a larger group of subjects.

In conclusion, the study with dual orally administered folic acid stable isotopes demonstrates the potential of LC-MS-MS for application in human bioavailability studies of folate vitamers. High sensitivity of LC-MS-MS for measuring 5-methylTHF enrichments might be useful for research in the in vivo absorption and kinetics of folates and interactions with one-carbon metabolism. Also, experiments to further develop the single-pool model for folate absorption into two-compound, multiple-pool models including unmetabolized folic acid can be designed. The accurate and simultaneous measurement of concentrations and enrichments of 5-methylTHF and folic acid using 2-ml plasma samples can now be achieved with LC tandem MS.

Acknowledgments

The work was funded by the European Union under the 5th Framework Programme (Grant QLRT-1999-00576). Y.M. Smulders is supported by a fellowship from the Netherlands Heart Foundation (2001D044). We thank A.J.A. Wright (Institute of Food Research, Norwich, UK) for comments.

Appendix. Estimation of in vivo folate absorption and elimination

Changes in 5-methylTHF isotopomer plasma concentrations are approximated in a single-compartment model (see Fig. 4). Following an oral dose of $[^{13}C_{6}]$folic acid or $[^{2}H_{2}]$folic acid in a measurement protocol of $>6$ h, which allows for the completion of the absorptive process and characterization of the decay of the plasma concentration, the plasma appearance of labeled folic acid is assumed to approximate that of an infusion of rate ($R$), over the absorption time period. The absorption time period ($T$) is defined as the time from the first rise ($t_{lag}$) of 5-methylTHF isotopomer enrichment in the plasma to the peak ($t_{max}$).

The definition of $R$ is

$$R = \frac{\text{mass of dose that is absorbed}}{\text{time period for absorption}} = \frac{M}{T}. \quad (A.1)$$

If $V$ is the apparent volume of distribution for folate, $k$ is the rate constant of elimination from the compartment (the decay slope of $\log_e$ plasma labeled folate when $t > t_{max}$), and $t$ is time after ingestion of the test dose (doseoral), then the labeled 5-methylTHF concentration in the compartment ($C$) can be approximated as

$$C = \frac{M}{V.T.k} \cdot (1 - e^{-k(t-t_{lag})}) \quad (0 < t < t_{max}) \quad \text{and} \quad (A.2)$$

$$C = \frac{M}{V.T.k} \cdot (1 - e^{-k.T}), e^{-k((t-t_{lag})-T)} \quad (t > t_{max}). \quad (A.3)$$

The time during which the plasma enrichment remains at baseline ($t_{lag}$) and the value of $T$ (i.e., $t_{max}$ minus $t_{lag}$) are established from the plasma 5-methylTHF enrichment curve, and $V$ is estimated as 389 ml/kg according to Loew et al. [21]. By fitting Eqs. (A.2) and (A.3) to the labeled 5-methylTHF plasma concentration data over time, $M$ can be calculated. The apparent absorption can then be calculated according to the equation

$$\text{Apparent absorption} = \frac{M}{\text{dose}_{oral}}. \quad (A.4)$$

A first pass effect is present when folate is absorbed from a meal [22]. By assuming that the absorption fraction of a folic acid test dose in humans is approximately 0.90 [19], the first pass effect (as % of doseoral) can be estimated:

$$\text{First pass effect} = \left(1 - \frac{0.9 \cdot \text{dose}_{oral}}{M}\right) \cdot 100\%. \quad (A.5)$$

References


Richard M. Faulks
David J. Hart
Gary M. Brett
Jack R. Dainty
Susan Southon

Kinetics of gastro-intestinal transit and carotenoid absorption and disposal in ileostomy volunteers fed spinach meals

Summary  Background  Reports of low carotenoid absorption from food sources has undermined their postulated ‘protective’ role as one of the active agents in diets rich in vegetable matter. Aims of the study  This study quantified β-carotene and lutein absorption from a representative green vegetable with different degrees of processing, using both mass balance and metabolic modelling of triglyceride-rich lipoprotein plasma fraction (TRL) response. Method  Whole or chopped-leaf cooked spinach was fed to volunteers (n = 7, paired) with vegetable oil (40 g) in yoghurt. Blood and ileal effluent samples were collected for up to 24 h. Effluent and TRL samples were analysed for lutein and β-carotene by HPLC. A digesta transit model was used to describe meal transit and a single compartment model used to predict percentage absorption from the plasma TRL response. Results  Mass balance showed 25 % of lutein and β-carotene were absorbed from chopped spinach, compared with 25 % β-carotene and 40 % lutein from whole-leaf spinach. Increased lutein absorption correlated to slower gastrointestinal (GI) transit for the whole-leaf meal. An area under the curve (AUC) response for the TRL fraction, found in 50 % of cases, was not confined to those with the greatest percentage absorption. Absorption by mass balance and TRL AUC indicate a half-life of newly absorbed carotenoid around 11 min. Conclusion  GI residence time appears to have an effect on the absorption of lutein but not β-carotene. Rapid clearance is probably the main reason for absence of measurable plasma concentration excursions. Lack of plasma response cannot be interpreted as lack of carotenoid absorption without knowledge of the absorption and disposal kinetics.

Key words  Lutein – β-carotene – absorption – ileostomy – model

Introduction  The absorption of carotenoids from test meals is reported to range between 2–90 % depending upon the test material, the model used to assess absorption and interpretation of data obtained [1]. Reports of low absorption values [2, 3], obtained using changes in plasma concentration have undermined the hypothesis that the carotenoids are one of the active protective constituents of diets high in vegetables and fruits, and that supplemental green vegetable feeding may be ineffective in relieving retinol deficiency [4]. Plasma concentrations of the carotenoids may be elevated significantly in chronic dosing studies [5, 6] but the absolute amount absorbed cannot be quantified by this approach.

Abbreviations

AUC  Area under the curve
BMI  Body mass index
GI  Gastro-intestinal
HDL  High-density lipoproteins
HPLC  High performance liquid chromatography
LDL  Low-density lipoproteins
SD  Standard deviation
SI  Small intestine
TGWM  Total gastrointestinal washout method
TRL  Triglyceride rich lipoproteins
VLDL  Very low density lipoproteins
IV  Intravenous
The absorption of carotenoids has been attempted in human volunteers by a number of methods. Oral-faecal mass balance [7], whole plasma response after chronic [3, 8–10,] or acute [11, 12] dosing, triglyceride-rich lipoprotein (TRL) response after an acute dose [2, 13], radioactive tracers [14, 15], stable isotope tracers [16–18], total gastrointestinal washout, mass balance method (TGWM) [19] and the ileostomy mass balance model [20].

Of the mass balance methods, the oral-faecal approach has been the most commonly used. However, the method is prone to losses of carotenoid through their exposure to the microflora of the large intestine and precise faecal collection may be a problem. Estimates of absorption by this method are therefore predicted to be high and variable.

A second, frequently used approach is measuring carotenoid concentration in whole plasma following an acute (single meal), or chronic, dose (multiple meals over several days/weeks). Plasma response after a single meal has identified many individuals who do not appear 'to respond', i.e. no measurable change in plasma concentration; sometimes interpreted as no, or low, carotenoid absorption. This interpretation has been challenged following consideration of plasma clearance kinetics and the likelihood, or not, of observing a perturbation in the plasma pool after a single meal [20]. In chronic feeding studies, plasma concentrations of carotenoids are more likely to be significantly elevated, but the amount absorbed still cannot be quantified without a knowledge of the clearance kinetics from all appropriate plasma pools and re-exportation kinetics from liver and other tissues. Furthermore, changes in plasma concentration after feeding a 'standard' isolated compound vs. a food are usually assumed to follow a linear dose response. At best, absorption from the food can only be expressed as a percentage of the absorption from the 'standard' dose.

In the present study the ileostomy approach has been selected as the preferred mass balance method for obtaining quantitative data on the absorption of carotenoids from an important food source (green leafy vegetable). Whilst there may be some microbial activity in the SI through colonisation of the terminal ileum, appropriate collection and storage of effluent minimises the potential confounding influence of microbial action, and variable residence time in the large bowel.

As an alternative to mass balance measurements, the study described here also included carotenoid analysis of whole plasma and the triglyceride-rich lipoproteins to produce comparative data between loss of carotenoid from ileal effluent and appearance of carotenoids in blood. Newly absorbed carotenoids, from a meal given after an overnight fast, appear in the TRL. The AUC for the TRL-carotenoid response is measured, clearance rate assumed to be the same as for chylomicrons and carotenoid absorption quantified [13]. However, the actual clearance kinetics for chylomicron remnant half life [21–23], or chylomicron triglyceride half life are not known for the individual volunteers, and have to be assumed from limited published information [24], gives rise to large differences in carotenoid absorption values, depending on the half life value selected. By quantifying the amount of carotenoid lost in ileal effluent, together with the size of the TRL excursion, calculation of the carotenoid half-life was possible and assumptions relating to chylomicron remnant half-life tested.

In summary, the objectives of the study were to quantify β-carotene and lutein absorption from a representative green vegetable with different degrees of processing; using both mass balance and metabolic modelling of TRL response. This dual approach allowed comparison of the data sets obtained and testing of assumptions relating to clearance kinetics of carotenoids from the TRL fraction.

### Experimental

A group of seven ileostomy volunteers who had minimal ileal resection (< 15 cm) for ulcerative colitis (5 women and 2 men), mean (SD) age 51 (7.6) y, weight 80.1 (16.4) kg gave informed written consent to the study which was approved by the Norwich District Ethics Committee. Volunteers had BMI values of 19–27, fasting plasma cholesterol ≤ 6.5 mmol/l, fasting plasma triglycerides ≤ 2.3 mmol/l, plasma β-carotene 0.1–1.0 µmol/l and plasma retinol ≥ 0.1 µmol/l, were non-smokers, not taking medication or dietary supplements. All but one consented to provide serial blood samples over the duration of the 2 study periods. Volunteers attended the Human Nutrition Unit on two occasions, at least 6 weeks apart, having avoided excessive carotenoid intake (a list of foods was provided) for 24 h before the study day. They arrived at 08.00 h having fasted from 19.00 h the previous evening and having performed their usual morning routines.

Volunteers were cannulated (antecubital/cephalic), provided a baseline fasting blood sample (20 ml), emptied their appliances (baseline effluent collection) and were then given approximately 150 g of either cooked whole, or cooked finely chopped, leaf spinach prepared from the same harvest. The spinach meal contained approximately 15 mg lutein and 10 mg β-carotene. The spinach, which had been blanched, drained, frozen and stored at −40 °C in heat sealed foil laminate pouches, was reheated in boiling water to a core temperature in excess of 72 °C for 3 min. A sub-sample of the spinach meal was retained at −70 °C for analysis. The spinach meal was followed by 400 g of skimmed milk yoghurt containing 40 g of low vitamin E sunflower oil, 20 g of sucrose and chocolate flavouring. The study was timed from when...
the spinach was consumed (t = 0). Defined carotenoid-free midday (t = 4.5h) and evening (t = 10h) meals were provided and carotenoid free drinks were freely available at all times. The midday meal provided 20 g of fat (25% of energy) and the evening meal 42 g of fat (28% of energy).

Volunteers remained seated in an armchair for the duration of the study except for toilet visits. This procedure was adopted to harmonise physical activity in all volunteers although it is recognised that it may have had an impact on gut motility. Blood samples (20 ml) were drawn every 2h from t = 0 up to 12h, into lithium heparin blood tubes, centrifuged, the plasma separated and frozen at −70 °C before further treatment. Total ileal effluent was collected every 2h up to 12h and then as discrete timed samples up to 24h at the volunteers’ own convenience. All effluent samples and retained food samples, collected into polythene bags, were spread into thin sheets within the bag, frozen on solid CO2, weighed and stored at −70 °C. Plasma (5 ml) was density adjusted with potassium bromide, layered into saline (1.006 sg) and ultracentrifuged for 4h at 64,000 g [25] to separate the lipoproteins into TRL, LDL, and HDL fractions. The fractions were then aspirated from the centrifuge tube and stored at −70 °C.

Plasma and plasma fractions were extracted using hexane and the carotenoid content assayed by HPLC [6] with a limit of quantification < 1 ng. Effluent and spinach samples (ca. 8 g) were broken from the frozen sheets, placed in 50 ml screw top glass centrifuge tubes and 20 ml of acetone added. The effluent was thoroughly dispersed using a small homogeniser (Ultra Turrax), the mixture centrifuged for 10min at 2000 g to pellet the solids and the supernatant transferred to a 100 ml volumetric flask. The pellet was re-suspended in 20 ml acetone and the process repeated 3 more times. The acetone extract was made up to 100 ml, thoroughly mixed and a filtered (No.1 paper. Whatman) sub-sample (20 ml) stored at −20 °C. A sub-sample (1 ml) of the filtered acetone extract was dried under N2, made up in HPLC mobile phase, diluted if needed, and assayed by HPLC as above.

Statistics

One tailed Student’s paired t-test was used to determine whether carotenoid absorption (within subject) was significantly different (p < 0.10, because of the low value of n) between the whole leaf and finely chopped leaf spinach test meals. A one-tailed test was used because it was believed that absorption from the chopped spinach would be greater than from whole leaf spinach because of the more extensive tissue disruption. A ‘within subject’ correlation of the absorption of β-carotene from whole leaf and chopped leaf spinach was undertaken to test if the magnitude of absorption was volunteer consistent, i.e. whether a higher absorption from whole leaf spinach predicted a higher absorption from chopped leaf spinach, and vice versa. Likewise, a correlation for lutein absorption was also undertaken. Fasting plasma concentrations of β-carotene and lutein were correlated with percentage absorption to test if there was a relationship between habitual plasma concentration and absorption from the test meal. Regression analysis was used to assess if physical performance of the meal in the GI tract (lag phase, rate, t1/2) was related to absorption.

Data treatment

To characterise the appearance of the spinach meal in the ileal effluent, lutein was used as a marker. The percentage of the original spinach meal collected at each time point was calculated from the total recovered lutein and the amount of lutein found in each collection. Normalised cumulative collection (percentage) of the spinach meals were plotted against time to provide a transit profile of the spinach and the data fitted using a logistic model to calculate the t1/2 of the gastrointestinal residence time. The portion of the curve that appeared to be steeply increasing with time was fitted with a regression model. The slope is equivalent to the effluent production rate, the intercept (x at y = 0%) the lag time and time for complete passage of the meal (x at y = 100%).

The total loss of lutein and β-carotene to the effluent and the amount in the spinach meal were calculated and expressed as percentage absorption of the carotenoid content of the meal.

Digesta transit model

The normalised cumulative effluent loss (y) vs. Time (t) data was fitted using the following 2-parameter equation:

\[
y = 100 \cdot \frac{e^{ax+b \cdot t}}{1+e^{ax+b \cdot t}}
\]

The time taken to reach 50% loss is given by:

\[t_{1/2} = a/b\]

The slope and y-intercept were found from the linear regression of the data points in the central (linear) section of the normalised cumulative effluent loss vs. time plot. The regression model can be summarised as:

\[y = mt + c\]
where \((m)\) is the slope and is equivalent to the effluent production rate and \((c)\) is the y-intercept. The x-intercept of this line is the lag time and is calculated from:

\[
lag \text{ time} = -\frac{c}{m}
\]

100% transit time \((y = 100)\) can be calculated from:

\[
t = \frac{100 - c}{m}
\]

**TRL area under the curve model.**

The areas under the curve (AUCoral) of these plots were calculated using the Altman trapezoidal approximation method [26]. The integrated area under the curves for the TRL response in two of the volunteers who gave complete TRL curves within the 12 h blood sampling period were calculated and compared to the theoretical TRL AUC that would have been obtained if the carotenoids had been given as an intravenous bolus. The half life of the carotenoids in the TRL is not known, thus the theoretical TRL AUC from the bolus was calculated at a number of time points between 2 and 11 min to embrace the chylomicron triacylglycerol \(t_{1/2}\) of 2–5 min, up to the chylomicron remnant \(t_{1/2}\) of 11 min.

After correcting for the background TRL concentration of carotenoid (lutein and \(\beta\)-carotene), a plot of TRL concentration against time was constructed which represents the TRL response to the oral dose. A single compartment model (Fig. 1) can be assumed with only disposal \((k_1)\) from the TRL fraction, because TRL was the only pool to have an input exclusively (theoretical or real) of newly absorbed carotenoid.

By assuming that a single compartment model (Fig. 1) will approximate chylomicron clearance from the plasma, various plasma half-lives \((t_{1/2})\) were simulated using the SAAM II modelling package [27] to investigate the absorption of an oral dose of carotenoid.

For each \(t_{1/2}\), the plasma response to an intravenous (IV) dose was simulated using the above model. The plasma volume of each volunteer was estimated [28] and given as an input parameter in the model. The IV dose was adjusted until the simulated area under the curve (AUCiv) was the same as that found experimentally from the oral dose (AUCoral). Under these conditions, the simulated IV dose was the same as the amount of carotenoid actually absorbed from the oral test meal.

### Results

**Meal behaviour in the GI tract**

Figs. 2a and 2b show the normalised appearance of spinach meal in the ileal effluent.

The calculated mean lag phase of the initial appearance of the effluent from the chopped leaf spinach meal (2.6 h, range 0.1–4.5 h) was shorter \((p = 0.078, n = 7)\) than that from the whole leaf spinach meal (3.6 h, range 0–6 h). The mean half-life of the chopped spinach meal in the stomach and small intestinal (SI) tract (6.5 h, range 3.4–10 h) was shorter \((p = 0.08, n = 7)\) than that for the whole leaf meal (7.4 h, range 4–11.3 h). The mean rate of mass transit through the SI tract as estimated from the slope of the cumulative collection of spinach in the ileal effluent was the same with both meals. The chopped spinach meal passed the whole SI (12.6 h, range 8–16 h) more rapidly \((p = 0.099, n = 7)\) than the whole leaf spinach meal (13.4 h, range 10–18 h). The transit...
time of the whole leaf spinach meal at all time points was about 1 h longer than that of the chopped leaf meal.

Absorption of lutein and β-carotene by mass balance

All the meals contained approximately 15 mg lutein and 10 mg β-carotene, the exact amount being measured by weighed intake and assay of a retained portion of the meal. The mean ratio of lutein:β-carotene was 3:2 in the whole leaf and chopped spinach. Chopping and drip loss from the prepared spinach meals did not affect this ratio.

The percentage absorption of lutein and β-carotene from leaf and chopped spinach is shown in Table 1.

Lutein absorption from whole leaf spinach (mean 44 %, range 28–58 %) was greater (p = 0.01, n = 7) than from chopped leaf spinach (mean 26 %, range 8–58 %) whereas the mean absorption of β-carotene from both meals was similar (chopped 23 %, range 4–41 %, whole leaf 26 %, range 9–58 %). Percentage lutein absorption from all meals was weakly correlated (R² = 0.27, p = 0.05, n = 13) with lag phase of initial appearance of spinach in ileal effluent by the equation: percentage absorption = 4.6 x lag phase (h) + 20.4. This indicated about 20 % lutein absorption with the shortest (> 2 h) lag phase and up to 55 % absorption with the longest (6 h) lag phase. Residence time in the SI tract altered the ratio of β-carotene: lutein absorbed. There was equal mass absorption of both carotenoids for short lag phase rising to 3:2 lutein:β-carotene at longer residence times. Absorption of β-carotene and lutein were also weakly correlated (R² = 0.43, p = 0.01, n = 14); percentage β-carotene absorbed = 0.49 x percentage lutein absorbed + 7.7.

There was no relationship between fasting plasma concentration of lutein or β-carotene and the amount absorbed measured by mass balance.

Plasma and lipoprotein β-carotene and lutein response

Fasting plasma β-carotene (mean ± SD) was 216 ±171 ng/ml and lutein 135 ±72 ng/ml.

None of the volunteers (n = 6) showed a plasma response for β-carotene or lutein with either chopped or whole leaf spinach, probably because the amounts absorbed over the time taken for the spinach to pass through the SI tract were too low (β-carotene, 2.5 mg; lutein 3.75–7.5 mg). There was also no measurable carotenoid response in either LDL or HDL fractions. However, there was a measurable TRL fraction response in 50 % of the volunteers (n = 3) for both β-carotene and lutein from chopped and whole leaf spinach, but complete curves were obtained for only 2 volunteers within the 12 h period of blood sampling. The absorption, measured by mass balance in these 2 volunteers, was compared to that predicted at various half life (t₁/₂) values (Table 2).

Table 1 Percentage absorption lutein and β-carotene from spinach (whole leaf and chopped leaf) meals by mass balance in ileostomy volunteers

<table>
<thead>
<tr>
<th>Volunteer</th>
<th>Lutein % absorbed</th>
<th>β-Carotene % absorbed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole</td>
<td>Chopped</td>
</tr>
<tr>
<td>A</td>
<td>33.6</td>
<td>28.0</td>
</tr>
<tr>
<td>B</td>
<td>28.0</td>
<td>16.0</td>
</tr>
<tr>
<td>C</td>
<td>51.0</td>
<td>26.7</td>
</tr>
<tr>
<td>D</td>
<td>52.7</td>
<td>7.3</td>
</tr>
<tr>
<td>E</td>
<td>29.6</td>
<td>14.9</td>
</tr>
<tr>
<td>F</td>
<td>57.0</td>
<td>57.9</td>
</tr>
<tr>
<td>G</td>
<td>58.5</td>
<td>33.2</td>
</tr>
<tr>
<td>Mean</td>
<td>44.3*</td>
<td>(13.4)</td>
</tr>
</tbody>
</table>

* Whole leaf lutein is significantly greater (p < 0.05) than chopped leaf lutein

Table 2 Predicted and measured absorption of carotenoids from spinach meals by TRL response. Lutein and β-carotene TRL absorption kinetics from spinach meals: Predicted percentage absorption from a single compartment model with various values of t₁/₂ and absorption measured by mass balance in two volunteers

<table>
<thead>
<tr>
<th>Volunteer Meal Type</th>
<th>Lutein</th>
<th>β-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>B</td>
</tr>
<tr>
<td></td>
<td>WL</td>
<td>CL</td>
</tr>
<tr>
<td>t₁/₂(min)</td>
<td>Predicted Absorption %</td>
<td>Predicted Absorption %</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>3</td>
</tr>
<tr>
<td>Measured Absorption%</td>
<td>28</td>
<td>16</td>
</tr>
</tbody>
</table>

WL Whole leaf spinach. CL Chopped leaf spinach
In most cases, the measured absorption falls within the range predicted from \( t_{1/2} \) values in the range 2–11 min. The exception was the measured absorption of lutein from whole leaf spinach, which was greater than could be accounted for by even the most rapid plasma clearance.

**Discussion**

Understanding the concept of bioavailability is essential to all involved in food production, nutritional assessment and determination of diet:health relationships. However, the absorption and post-absorptive metabolism of many of the bioactive organic components of foods is complex and not fully understood. The carotenoids provide an excellent example of where too little understanding of the complexity of the behaviour of a food component within the food matrix, during digestion, absorption and clearance and within human tissues, can lead to naïve interpretation of study results.

Faecal mass balance studies are constrained by (a) dietary modification, (b) prolonged sample collection, and (c) the assumption that loss is the same as absorption. Some of these criticisms can be overcome by using modified mass balance methods such as the ileostomy model [20] and TGWM [19].

Interpretation of plasma or plasma fraction carotenoid excursions can only be undertaken with a clear knowledge of the absorption and clearance mechanisms and by sampling the most appropriate ‘pool’. Even so, there are inevitably assumptions that need to be invoked and justified when using plasma response models.

Particular care must be taken with chronic dosing when comparing ‘relative bioavailability’ for four main reasons, (a) the absence of a knowledge of dose response, (b) change in plasma concentration induced by a test food relative to the free compound does not provide an absolute absorption, (c) change or rate of change of plasma carotenoid concentration can be constrained if the doses exceed the capacity of the gut to absorb or the plasma to carry, thus all excessive doses will provoke the same plasma response, (d) at what point during the supplementation period are the relative plasma responses to be measured?

Acute studies also present specific problems. These relate to (a) observing small changes in plasma concentration against a high endogenous background, (b) avoiding the confounding influence of sequestration from and re-export to the plasma pool, (c) lack of knowledge of absorption and clearance kinetics, (d) lack of knowledge of dose response relationship curves.

Newly absorbed carotenoids appear in the plasma chylomicron fraction (TRL), which is virtually free of carotenoids in the fasting state; thus measurement of the TRL response avoids both the problems of quantifying small changes against a high endogenous background and the problems arising from carotenoid trading between other body pools.

The present study sought to quantify \( \beta \)-carotene and lutein absorption from a representative green vegetable, using two experimental systems (together with metabolic modelling) to allow comparison, and more rigorous examination and interpretation, of the data sets obtained.

- **Mass balance: effects of food structure**

Most nutrients have specific absorption mechanisms but many minor lipophilic components are passively absorbed from the gut as an integral part of lipid absorption. Such components, if present in foods of plant origin, must be extracted from their native environment and dissolved in appropriate lipid carriers. Intuitively, breaking up the cellular structure of the food, the presence of lipid, bile salts, lipases and the correct pH should increase the probability of achieving maximum absorption. Absorption from cooked processed foods may be very different by comparison with that from raw; however, disruption of plant cell architecture, beyond that occurring during the relatively mild processing used here, did not influence the proportion of \( \beta \)-carotene absorbed from spinach in vivo (Table 1).

The increased absorption of lutein from the whole leaf spinach was unexpected, since it was assumed that the larger particle size would slow down mass transfer to absorbable lipid structures in the stomach and ileum. However, recent studies of carotenoid mass transfer in an *in vitro* gastric and duodenal environment (using samples taken from spinach as fed to human volunteers) demonstrate that whilst the rate and limit of mass transfer are the major controlling factors of transfer to the lipid phase, time is much more crucial for the transfer of lutein [polar] than \( \beta \)-carotene (apolar) (Fillery-Travis, A. Personal Communication). This would explain the enhanced absorption of lutein from the whole leaf spinach and the lack of self-consistency of lutein absorption (Table 1), which might be confounded by unquantified transit rate fluctuations or changes in luminal conditions which affect lutein, but not \( \beta \)-carotene, absorption.

- **Mass balance: inter- and intra-individual response**

Inter-individual \( \beta \)-carotene absorption response to the same food was highly variable but intra-individual response was consistent (\( R = 0.887, n = 7, p = 0.01 \)) between different forms of the same food (Table 1). A self-consistency of plasma response has also been seen in...
chronic supplementation studies [6]. This could be due to two mechanisms: those individuals who exhibit low plasma concentrations of β-carotene (a) absorb less β-carotene, or (b) the β-carotene is absorbed to the same extent but is not retained in the plasma pool. The converse would be true for those showing high plasma β-carotene concentrations.

The data show wide variation between individuals with regard to lutein absorption but, unlike β-carotene, there was no self-consistency between the different forms of spinach meal despite there being a weak correlation between lag phase and amount absorbed ($R^2 = 0.27$, $p = 0.05$, $n = 13$), with the longer residence time doubling the absorption.

### Modelling of TRL response

Those individuals that showed a TRL response to β-carotene and lutein, from both chopped and whole leaf spinach, were the same in both cases but were not the individuals that gave the greatest absorption. The fact that there is a measurable TRL response at all levels of absorption (depending on the individual) is a reflection of clearance from the plasma rather than absorption (confirmed by mass balance). Although all the volunteers had ‘normal’ plasma lipids, turnover was not measured. It would be expected that individuals with rapid clearance of chylomicrons would show the smallest change in TRL concentration of carotenoids because they would not accumulate in the plasma to any measurable extent. Those volunteers with slower lipid turnover may be those that exhibit measurable TRL carotenoid excursions. This might be the reason why the volunteers fell into consistent groups of TRL ‘responders’ and ‘non-responders’ despite the fact that carotenoid absorption occurred in all cases.

Two individuals gave complete TRL AUCs for both lutein and β-carotene, over the 12 h of blood sampling, for both whole and chopped spinach meals (Table 2). Using the kinetic model described in this paper, the measured absorption of lutein from the whole leaf meal exceeded that which is predicted from the model, whereas for the chopped leaf meal the measured absorption falls within the range of acceptable $t_{1/2}$ (2–11 min). This indicates that GI transit rate of whole leaf spinach is positively associated with loss of lutein in the GI tract, which contributes to an increased loss (elevated measured absorption) but which is not seen in the TRL response. Alternatively, TRL is not an appropriate measure for newly absorbed lutein, which, because of its more polar nature, may be partially transported in portal blood by a mechanism un-associated with the chylomicrons and thus it was not detected. For the TRL lutein response to chopped spinach the percentage absorption can be predicted if the TRL $t_{1/2}$ is in the range 2–11 min. The range of $t_{1/2}$ does not allow any decision as to whether the lutein is cleared from the TRL in the extrahepatic capillary bed along with the triacylglycerols ($t_{1/2} = 2–5$ min), or remains with the chylomicron remnants (mean $t_{1/2} = 11.5$ min).

For β-carotene the measured absorption exceeds that which would be predicted from a TRL $t_{1/2}$ of 11 min (Table 2). This is not surprising because some of the absorbed β-carotene will be converted to retinol in the enterocytes and it will therefore not appear in the plasma as β-carotene. However, if conversion is low ($\approx 10\%$) it will not significantly reduce the TRL β-carotene concentration and could still indicate that some of the β-carotene is absorbed in the extrahepatic capillary bed and the remainder being cleared by the liver along with the chylomicron remnants indicating a shorter $t_{1/2}$. Carotenoid losses, here interpreted as absorption because it is assumed that there are no microbial or oxidative losses, may in fact have occurred. If this is the case then absorption by mass balance in the ileostomy model (and oral-faecal model) is an overestimate and $t_{1/2}$ would be longer than that indicated.

### Conclusion

Whole leaf spinach has approximately a 1 h longer GI transit time than chopped leaf spinach and this doubles the loss of lutein (25% → 40%) but has no effect on β-carotene (25%). The attenuated delivery of the small amounts of both lutein and β-carotene from the spinach meals did not cause a measurable plasma AUC or responses in LDL and HDL fractions. TRL carotenoid responses were seen in 50% of the volunteers but in only 2 cases were the whole curves within the 12 h blood sampling window. Future studies with foods should be run for at least 16 h to ensure the complete curves needed for modelling. In both volunteers, the measured absorption of lutein and β-carotene exceeds that predicted from a theoretical TRL $t_{1/2}$ of 11.5 min and this could be a result of luminal losses that are taken as absorption in the mass balance model but which do not appear in the TRL fraction. In the specific case of β-carotene some of the ‘loss’ will be accounted for by conversion to retinol.

### Acknowledgements

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Differential Kinetic Behavior and Distribution for Pteroylglutamic Acid and Reduced Folates: a Revised Hypothesis of the Primary Site of PteGlu Metabolism in Humans

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Nutrition Department, Institute of Food Research, Norwich NR4 7UA, United Kingdom; and *Food Science and Human Nutrition Department, University of Florida, Gainesville, FL 32611-0370

ABSTRACT Single 13C6-labeled doses of pteroylmonoglutamic acid (PteGlu: 634 nmol; n = 14), (65)-5-formyltetrahydrofolic acid (431–569 nmol; n = 16), or [15N1–7]-intrinsically labeled spinach (mainly 5-methyltetrahydrofolate) (588 nmol; n = 14) were fed to fasting adult volunteers. Plasma-labeled 5-methyltetrahydrofolic acid responses were monitored for 8 h. There was a slower rate of increase in plasma-labeled 5-methyltetrahydrofolic acid and longer time to peak (171 ± 9 min; mean ± SEM) following an oral dose of [13C6]PteGlu than either [13C6]5-formyltetrahydrofolic acid (54 ± 10 min) or [15N1–7]spinach folate (60 ± 13 min) suggesting saturated metabolic capacity for the biotransformation of PteGlu. Mathematical modeling generated a significantly higher mean “apparent absorption” for 5-formyltetrahydrofolic acid (38%) and spinach folate (44%) than for PteGlu (24%). The high “relative absorption” of reduced folates to PteGlu was unexpected given that PteGlu itself, from 14C-tracer mass balance experiments, is almost completely absorbed. Although it is ubiquitously accepted that a physiological dose of PteGlu is reduced and methylated in the epithelial cells of the small intestine, and that essentially only 5-methyltetrahydrofolic acid is exported into the hepatic portal vein (HPV), as is the case for absorbed reduced 1-carbon-substituted folates, modeling indicated greater liver sequestration when PteGlu was used as the test dose, suggesting that PteGlu enters the HPV unaltered and that the liver is the primary site of initial metabolism. Because of the observed differential plasma response and the hypothesized difference in the site of initial metabolism, the historical use of PteGlu as a “reference folate” in studies of folate bioavailability is seriously questioned. J. Nutr. 135: 619–623, 2005.

KEY WORDS: • pteroylmonoglutamic acid • folate • absorption • isotopes • modeling

Because there is an extensive hepatic uptake (liver “first-pass”) of newly absorbed folate (1–4), which prevents direct estimation of the degree of folate absorption from any plasma response, the vast majority of work over the past 35 or more years attempting to assess folate absorption in humans has centered on methods comparing the serum/plasma response to a single unlabeled oral test-dose relative to that of an equal “reference” dose of pteroylmonoglutamic acid (PteGlu)1 (4,5) and the subsequent computation of “relative absorption.” This may entail either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over 2 to 3 h (6–11) or measurement of the dose-normalized rise in plasma folate concentration area under the curve (AUC) over 6 h or more (12–19).

Comparison of AUC between test and reference folate has been accepted as a valuable indicator of absorption, provided that the postdosing plasma measurement test period is long enough to capture ≥80% of the whole AUC (20). This approach also requires experimental conditions that satisfy the following 3 principles: Assumption 1 states that PteGlu is absorbed by the same mechanism as reduced folates and in a similar manner. PteGlu and reduced folates are absorbed mainly in the proximal small intestine by a saturable, carrier-mediated, pH- and energy-dependent transport mechanism, which, unlike other epithelial tissues, appears to be unique in its lack of hierarchy of transport, having a similar affinity for both PteGlu and reduced folate forms (21,22). Assumption 2 states that physiological doses of PteGlu are initially reduced and then methylated in the epithelial cells of the small intestine and that only 5-methyltetrahydrofolic acid is exported from the mucosa to the hepatic portal vein. Without exception, review articles from 1983 onward agree that the small intestine efficiently reduces and methylates physiological doses of PteGlu and, as with absorbed naturally occurring reduced and 1-carbon-substituted folates, subsequently transfers only 5-methyltetrahydrofolic acid (5-CH3H4PteGlu) to the hepatic portal vein (e.g., 22,23,23). Thus, logically, one would expect

1 Supported by UK Food Standards Agency, the UK Biotechnology & Biological Sciences Research Council, and EU project “Folate: From Food to Functionality and Optimal Health,” QLK1-CT-1999–00576.
2 To whom correspondence should be addressed.
3 Abbreviations used: 5-CH3H4PteGlu, 5-methyltetrahydrofolic acid; 5-HCOH,PteGlu, 5-formyltetrahydrofolic acid; AUC, area under the curve; PteGlu, pteroylmonoglutamic acid; m/z, mass-to-charge ratio; spinach folate, mainly 5-methyltetrahydrofolate polyglutamate.

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no differential in hepatic uptake in response to oral doses of PteGlu compared to other test folates, and the calculation of relative absorption from the comparative plasma AUC response of a test-dose to that for PteGlu is a valid “pragmatic” maneuver. Assumption 3 states that the kinetics of the plasma response to PteGlu is the same as that for reduced 1-carbon-substituted naturally occurring folates. Until recently, no systematic difference has been reported between the plasma 5-methyltetrahydrofolic acid response to test folates and PteGlu.

Controversy has arisen recently because it has been shown that much of the plasma AUC response to oral folate doses is induced by, but is not actually derived from, the dose itself (18), a phenomenon recently reported to also affect vitamin C (24). We interpreted our reported large, variable, and unpredictable displacement of tissue 5-methyltetrahydrofolic acid into the plasma pool as making a comparison of the relative absorption of folate from an unlabeled test dose (food or supplement) versus an unlabeled PteGlu reference dose untenable. Also, concurrent examination of the plasma 13C-labeled 5-methyltetrahydrofolic acid response to 13C-labeled [6S]-formyltetrahydrofolic acid (5-HCOH4PteGlu) and a “reference dose” of 13C-labeled PteGlu unmasked an underlying serious discrepancy in plasma response that completely violated Assumption 3, rendering direct estimates of relative absorption using even labeled AUCs potentially invalid.

Fortunately, the violation of Assumption 3 need not necessarily be fatal to the overall methodologic approach. The application of suitable mathematical modeling (25), which makes allowance for any differences in the kinetics of plasma-labeled 5-methyltetrahydrofolic acid response, can be used to estimate important parameters. We have applied such an approach to the current study where, in fasting human volunteers, plasma response profiles for labeled 5-CH2H3PteGlu are followed over a period of 8 h after ingestion of single oral doses of [13C6]PteGlu, (6S-)[13C6]5-HCOH4PteGlu, or intrinsically labeled [13N1–7]spinach folate. Folate binding affinity columns were used to isolate extracted plasma folate and, following HPLC analysis of folate concentrations, a recently developed LC-MS analytical method (26) was used to determine the proportions of labeled and unlabeled 5-CH2H3PteGlu in the plasma samples.

**MATERIALS AND METHODS**

**Materials.** With the exception of intrinsically labeled spinach folate, a description of materials was given previously (18). A hydroponics system was used for the production of [13N]-intrinsically labeled spinach, which leads to intrinsic labeling of some or all of the 7 nitrogen atoms in the folate molecule. In brief, spinach seeds were grown under glass and, once seed leaves opened, were watered daily with a growing medium containing 50% of its nitrogen as [13N]-nitrates. Plants were harvested after 8 wk. Woody stems and outer leaves were removed and the remaining leaves were washed in lightly salted cold water, rinsed in cold water, shaken dry, and shredded and a composite was made. Portions (200 g) were then weighed into bowls and blanched in a microwave oven (750 W) for 3 min. After the bowls were cooled in cold water, the contents (leaf and liquid) were transferred to sealable bags, frozen over solid CO2, and stored at −20°C. Total spinach folate, 2.94 ± 0.38 μmol/kg, predominantly 5-CH2H3PteGlu but with some 5-HCOH4PteGlu, was determined using liquid chromatography-microbiologic assay (27) after polyglutamates were deconjugated to the monoglutamate form with hog kidney deconjugase (28). Isotopic enrichment of spinach [13N5]-CH2H3PteGlu isolated on folate binding affinity columns, compared to natural abundance 5-CH2H3PteGlu, was measured using negative ion [M−H] electrospray LC-MS (m/z 458–465) (26). Isotopic spectra revealed that spinach [13N5]-CH2H3PteGlu had measurable ions at m/z 458–465 (i.e., M to M + 7), with 18% of all the naturally occurring 15N atoms being replaced by 15N atoms.

**Human study design.** The study was approved by the Norwich Local Research Ethics Committee (Norfolk & Norwich University Hospital NHS Trust). After written consent was obtained, a blood sample was taken from healthy adult volunteers who had fasted for 12 h and analyzed at the hematology department of the Norfolk & Norwich University Hospital for full blood count, blood glucose, erythrocyte folate, serum vitamin B-12, urea and electrolytes, and liver function tests. If all results were within normal ranges, volunteers were then invited to attend 3 test days. Following an overnight fast and the measurement of blood pressure, a baseline blood sample (10 mL) was taken via cannula. Volunteers were then given a single oral dose of [13C6]PteGlu (634 nmol), (6S-)[13C6]5-HCOH4PteGlu (431–569 nmol), or a portion of [13N1–7]-labeled spinach (588 nmol folate). The dose of [13C6]PteGlu or (6S-)[13C6]5-HCOH4PteGlu was administered as previously described (18). Spinach portions were thawed, homogenized, reheated by microwave, and then served and consumed within 10 min. A timer was started only after the spinach had been completely consumed. Volunteers were always allowed access to water, and were given a light lunch (a sandwich with a only slice of either processed chicken breast or processed cream cheese spread and cucumber) only after the 4 h postdosing venous blood sample had been obtained. Few volunteers completed all 3 test days.

**Blood sampling and storage.** Venous blood samples (10 mL) were taken by cannula: a baseline “time zero” prior to test dose and 11 time points over an 8-h period following each test dose; 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, and 8 h. Blood samples were transferred immediately into tubes containing K2-EDTA and mixed gently. Samples were centrifuged (1500 × g, 10 min), and plasma was removed and frozen immediately over solid CO2 and then stored at −30°C until analysis.

**Sample preparation, folate extraction, and analysis.** The extraction of folate from plasma samples, purification on affinity columns using folate binding protein, and subsequent analysis by HPLC (total plasma 5-CH2H3PteGlu) or LC-MS (labeled and unlabeled plasma 5-CH2H3PteGlu) was conducted as described previously (18) with the exception that selected ion monitoring was conducted additionally on the [M−H] ion over the range of m/z 458–465 to determine 5-CH2H3PteGlu derived from the [13N1–7]-labeled spinach.

**Mathematical modeling of the labeled plasma response.** We started with the application of a simple one-compartment model (Fig. 1), which is designed specifically to elicit an understanding of the kinetics of initial absorption, metabolism, and transport of absorbed folates. Mathematical modeling was derived from pharmacokinetic principles (29); all formulas were executable within cells of standard operating Microsoft Excel spreadsheet software. Following an oral dose of labeled folate, the plasma appearance of labeled 5-CH2H3PteGlu is assumed to approximate that of an infusion of rate R over an absorption time period. The absorption time period (T) is defined as the time to peak labeled 5-CH2H3PteGlu concentration (tmax) minus the time during which the plasma-labeled enrichment initially remains at baseline (t0).

**FIGURE 1** Simple 1-compartment model for folate absorption.
The definition of $R$ is

$$R = \frac{\text{mass of dose that is absorbed}}{\text{time period for absorption}} = \frac{M}{T}. \quad (1)$$

If $t$ is time after ingestion of the test dose ($\text{dose}_{oral}$), $k$ is the rate constant of elimination from the plasma compartment to body tissues and/or excretion, and $V$ is the apparent volume of distribution for folate in the sampled plasma compartment, then plasma concentration ($C$) of labeled 5-CH$_3$H$_4$PteGlu can be approximated as

$$C = \frac{M}{V \cdot T \cdot k} \left(1 - e^{-k \cdot (t - t_{\text{lag}})}\right) \quad (0 < t < t_{\text{max}}). \quad (2)$$

$$C = \frac{M}{V \cdot T \cdot k} \left(1 - e^{-k \cdot t}\right) \cdot e^{-k \cdot (t - t_{\text{lag}})} \quad (t > t_{\text{max}}). \quad (3)$$

The time during which the plasma enrichment remains at baseline ($t_{\text{lag}}$), the peak time to plasma concentration ($t_{\text{max}}$), the value of $T$ ($t_{\text{max}} - t_{\text{lag}}$), and the rate constant of elimination ($k$) are established from the plasma-labeled 5-CH$_3$H$_4$PteGlu enrichment curve. The volume ($V$) of distribution in the sampled compartment is large and estimated to be 387 mL/kg body wt in humans (30).

By fitting the pair of simultaneous equations (Eqs. [2] and [3]) to the plasma concentration of labeled 5-CH$_3$H$_4$PteGlu over time ($t$), $M$ can be calculated. The apparent absorption can then be calculated according to

$$\text{Apparent absorption} = \frac{M}{\text{dose}_{oral}} \quad (4)$$

A liver first-pass effect occurs when folate is absorbed from a meal (12–15). By assuming that the absorption, at least of PteGlu, in the present study is ~90% (31–34), the first pass effect can be estimated:

$$\text{First pass effect} = \left(1 - \frac{M}{0.9 \cdot \text{dose}_{oral}}\right) \times 100\% \quad (5)$$

**Statistics.** Originally, our study had a crossover within-subject design. However, because of the intrusive nature of 8-h cannulations, most subjects did not complete all 3 test days and data are treated as independent observations. Because distributions were not significantly different from normal (Kolmogorov-Shapiro test), data (means ± SEM) were compared using ANOVA. When the ANOVA was significant ($P < 0.05$), means for reduced-folate test doses were compared to that for the PteGlu reference folate test dose by $t$ test. Linear regression analysis was used to assess the correlation ($r$) of mathematically modeled “apparent absorption,” “first-pass effect,” and “plasma 5-CH$_3$H$_4$PteGlu elimination rate constant” ($k$) to fasting baseline plasma or erythrocyte folate concentration.

**RESULTS**

Age, BMI, erythrocyte, and plasma (at $t = 0$ h) folate concentrations of volunteers did not differ in each of the 3 folate test groups (Table 1).

Selected ion monitoring indicated that neither PteGlu nor 5-formyltetrahydrofolic acid from the respective test doses appeared in the plasma in its unmetabolized form.

Plasma-labeled 5-methyltetrahydrofolic acid response to the 3 test folates is depicted in Figure 2.

The labeled plasma response to PteGlu was slower and peaked much later ($t_{\text{max}} = 171 ± 9$ min) than the response to either of the reduced-folate test doses; $t_{\text{max}} = 54 ± 10$ min in response to 5-formyltetrahydrofolic acid test dose and $t_{\text{max}} = 60 ± 13$ min in response to spinach-folate test dose (Table 1). The mean modeled apparent absorption (an estimate of folate absorption that takes no account of the fact that a fraction of newly absorbed folate may be sequestered by the liver) for reduced folates (38% for 5-formyltetrahydrofolic acid and 44% for spinach-folate) was significantly higher than for the PteGlu (24%) reference dose (Table 1). Calculation of relative absorptions (i.e., response for test dose divided by response for PteGlu reference dose) yields 158 and 183% for 5-formyltetrahydrofolic acid and spinach-folate, respectively.

By assuming that the actual absorption of the test folate dose is 90%, the mean calculated first-pass-effect, where a percentage of absorbed labeled folate is sequestered (presumably to the liver), was significantly greater for PteGlu (73%) than for either 5-formyltetrahydrofolic acid (58%) or spinach-folate (52%) (Table 1).

The rate constant of elimination ($k$) of plasma-labeled 5-CH$_3$H$_4$PteGlu response was not associated with fasting baseline plasma or erythrocyte folate concentration (Table 2).

Apparent absorption and the first-pass effect were not correlated to fasting erythrocyte folate concentration, but they did significantly correlate with fasting baseline plasma folate concentration (Table 2).

**DISCUSSION**

Most previous work over the past 4 decades attempting to assess relative folate absorption in humans in studies of folate bioavailability has centered on methods comparing the serum/plasma AUC response toward a single oral test dose (food or supplement) to that of an equal reference dose of PteGlu. This

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**TABLE 1**

<table>
<thead>
<tr>
<th></th>
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<tbody>
<tr>
<td></td>
<td>PteGlu</td>
<td>5-HCOH$_2$PteGlu</td>
<td>Spinach</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td>$31.4 ± 1.2$</td>
<td>$30.3 ± 0.9$</td>
<td>$33 ± 2$</td>
</tr>
<tr>
<td><strong>BMI, kg/m$^2$</strong></td>
<td>$25.2 ± 1.1$</td>
<td>$24.9 ± 0.9$</td>
<td>$25 ± 1$</td>
</tr>
<tr>
<td><strong>Erythrocyte folate, nmol/L</strong></td>
<td>$958 ± 70$</td>
<td>$976 ± 70$</td>
<td>$972 ± 70$</td>
</tr>
<tr>
<td><strong>Plasma (at $t = 0$) folate, nmol/L</strong></td>
<td>$18.1 ± 3.2$</td>
<td>$15.4 ± 2.7$</td>
<td>$12.9 ± 3.4$</td>
</tr>
<tr>
<td><strong>$t_{\text{max}}$ min</strong></td>
<td>$171 ± 9$</td>
<td>$54* ± 10$</td>
<td>$60* ± 13$</td>
</tr>
<tr>
<td><strong>Apparent absorption, % of dose</strong></td>
<td>$24 ± 2$</td>
<td>$38* ± 6$</td>
<td>$44* ± 6$</td>
</tr>
<tr>
<td><strong>First-pass effect, % of absorbed dose sequestered to liver</strong></td>
<td>$73 ± 2$</td>
<td>$58* ± 6$</td>
<td>$52* ± 6$</td>
</tr>
</tbody>
</table>

$^1$ Data are means ± SEM, $n = 14$ (PteGlu and spinach) or 16 (5-HCOH$_2$PteGlu). * Different from mean for PteGlu reference folate test dose, $P < 0.05$.  

$^2$ Time to maximum plasma-labeled 5-methyltetrahydrofolinic acid response.  

$^3$ An estimate of folate absorption that takes no account of the fact that a fraction of newly absorbed folate may be sequestered by the liver.  

$^4$ An estimate of the percentage of newly absorbed folate that is sequestered by the liver (assuming true absorption to be 90%).
spinach) or 16 (5-HCOH 4PteGlu). Neither labeled PteGlu nor 5-HCOH4PteGlu is detected in plasma, only their labeled 5-methyltetrahydrofolic acid response. PteGlu and spinach folate (35). A delay in the rate of initial reduction of PteGlu to H2-PteGlu (35). A limit due to a limitation in the capacity for the biotransformation of PteGlu was saturated. We interpret this delay to indicate that the metabolic response to labeled folate test doses. PteGlu (41) and spinach folate (14, PteGlu or spinach) or 16 (5-HCOH4PteGlu). Neither labeled PteGlu nor 5-HCOH4PteGlu is detected in plasma, only their labeled 5-methyltetrahydrofolic acid metabolite. To convert 5-MTFH to nmol/L, multiply ng/mL by 2.17.

approach has been undertaken universally using unlabeled test and reference folates. We have already demonstrated that a substantial portion of any total plasma response is elicited by, but does not originate directly from, an oral test dose itself (18). Arguably, in all previous studies utilizing unlabeled test doses, calculations of relative absorption to PteGlu have been ambiguous and potentially erroneous.

When restricting our current analysis to plasma-labeled 5-methyltetrahydrofolic acid response, we note that the time to maximum response to a dose of PteGlu is ~3 h in comparison to 1 h for reduced folates. This large difference in the kinetics of plasma response violates Assumption 3 and thus disqualifies the idea of comparing the plasma AUC response to an oral test dose of folate with that of an equal reference dose of PteGlu and the subsequent computation of relative absorption. We interpret this delay to indicate that the metabolic capacity for the biotransformation of PteGlu was saturated when using our dose of 634 nmol, possibly due to a limitation in the rate of initial reduction of PteGlu to H2-PteGlu (35). A study published recently (36), using accelerator mass spectrometry to quantify the plasma [14C]5-methyltetrahydrofolic acid response to a small 80 nmol dose of [14C]PteGlu, reports a similar delayed peak plasma response of 3 h. This evidence suggests that the metabolic capacity for the biotransformation of PteGlu in humans may be rate limiting; a conclusion that is consistent with the lower dihydrofolate reductase activity detected in human tissue compared to corresponding animal tissue (37–39) and a report that concludes that low levels of dihydrofolate reductase activity are a feature peculiar to humans (40).

Fortunately, the violation of Assumption 3 is not fatal because mathematical modeling of plasma response circumvents the issue. Modeling indicates an apparent absorption of reduced folates that is significantly higher than for the reference dose of PteGlu. These generate relative absorptions significantly in excess of 100%. This is unexpected and biologically impossible, because the “true absorption” of doses of [14C]PteGlu in 2 human volunteers by mass balance has been reported to approximate 90% or more (33,34), and tritiated forms of PteGlu and reduced folates are almost fully absorbed in rats (31). It is only when the first-pass effect is estimated from the current data (Table 1) that it becomes apparent that more absorbed folate may be sequestered by the liver when PteGlu is the test dose than when reduced-folates are the test dose. However, such a differential in distribution to body tissues should not be so if, as generally assumed, absorbed folate forms undergo mucosal biotransformation and successive transfer to the hepatic portal vein primarily as 5-methyltetrahydrofolic acid. Therefore, contrary to current theory, it appears that a substantial fraction of absorbed [13C]PteGlu may be entering the hepatic portal vein unchanged, to be more effectively removed by the liver than would 5-methyltetrahydrofolic acid, prior to subsequent biotransformation and partial enterohepatic recirculation. Although we agree that physiologic doses of PteGlu are indeed extensively reduced and methylated in the mucosal epithelial cells of the small intestine in rats (the historical experimental animal model used in formulating much of our current understanding of folate absorption and metabolism), we are not the first to suggest that this may not take place, or not so extensively, in humans. High concentrations of untransformed PteGlu were reported to appear in the hepatic portal vein of humans following oral administration of both 1.0 mg (2264 nmol) PteGlu (41) and

![FIGURE 2](Image 56x572 to 272x735) Labeled plasma 5-methyltetrahydrofolic acid (5-MTHF) response to labeled folate test doses. PteGlu ([ ), 5-HCOH4PteGlu ( ), and spinach folate ( ). Values are means ± SEM, n = 14 (PteGlu or spinach) or 16 (5-HCOH4PteGlu). Neither labeled PteGlu nor 5-HCOH4PteGlu is detected in plasma, only their labeled 5-methyltetrahydrofolic acid metabolite. To convert 5-MTFH to nmol/L, multiply ng/mL by 2.17.

| TABLE 2 | Correlation among apparent absorption, first-pass effect, or labeled plasma 5-CH3H4PteGlu clearance rate in fasting healthy adult volunteers following oral labeled-folate test doses and fasting baseline plasma or erythrocyte folate concentration1 |
|---|---|---|---|---|---|---|
| | PteGlu | 5-HCOH4PteGlu | Spinach |
| | Plasma folate | Erythrocyte folate | Plasma folate | Erythrocyte folate | Plasma folate | Erythrocyte folate |
| Apparent absorption, % of dose | 0.702 | 0.115 | 0.735 | 0.336 | 0.522 | 0.258 |
| r | 0.005 | 0.709 | <0.001 | 0.221 | 0.056 | 0.373 |
| First-pass effect, % of absorbed dose sequestered to liver | 0.005 | 0.676 | <0.001 | 0.257 | 0.054 | 0.359 |
| r | 0.703 | 0.128 | 0.770 | 0.312 | 0.507 | 0.265 |
| P | 0.051 | 0.058 | 0.406 | 0.103 | 0.328 | 0.370 |
| Labeled plasma 5-CH3H4PteGlu clearance rate, k | 0.862 | 0.851 | 0.119 | 0.715 | 0.252 | 0.193 |

1 Data are for n = 14 (PteGlu and spinach) or 16 (5-HCOH4PteGlu).
0.5 mg (1132 nmol) PteGlu (42). Despite criticisms of these studies, e.g., the use of relatively high doses of PteGlu and the analysis of folate concentrations by possibly problematic differential microbiological assay, we think that reports of the almost complete absence of assayable 5-methyltetrahydrofolic acid in the hepatic portal vein are remarkable—particularly when a dose of 0.5 mg (1132 nmol) was used (42). This is only twice the dose of 260 μg (589 nmol) reported much later (11) to be the threshold at which PteGlu may start to appear in the plasma.

It is noteworthy that apparent absorption correlates positively with fasting baseline plasma folate concentration (Table 2). Because a large fraction of newly absorbed labeled folate undergoes immediate removal to the liver, irrespective of the form appearing in the hepatic portal vein, much of any subsequent plasma-labeled response may actually be derived from enterohepatic recirculation. Greater enterohepatic folate recirculation would not only result in a higher fasting baseline plasma folate concentration but also then automatically assist in generating a greater labeled plasma response to any test folate by recycling a larger proportion of newly sequestered folate, mainly as 5-methyltetrahydrofolic acid.

We have serious doubts as to the ubiquitous use of PteGlu as the reference folate in most nutritional studies conducted in humans and conclude that currently held views on the absorption, metabolism, and subsequent tissue distribution of folates need careful re-evaluation, as does the methodology in current use for estimating the relative absorption of single folate test doses. We think it is essential, using labeled folates and sensitive HPLC/MS techniques, to establish whether a substantial fraction of absorbed folate undergoes immediate removal to the liver, irrespective of the form appearing in the hepatic portal vein, much of any subsequent plasma-labeled response may actually be derived from enterohepatic recirculation. Greater enterohepatic folate recirculation would not only result in a higher fasting baseline plasma folate concentration but also then automatically assist in generating a greater labeled plasma response to any test folate by recycling a larger proportion of newly sequestered folate, mainly as 5-methyltetrahydrofolic acid.

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Use of mathematical modeling to study copper metabolism in humans


ABSTRACT

Background: An improved understanding of copper metabolism is needed to derive more precise estimates of dietary requirements.

Objectives: The objectives were to validate a method for estimating endogenous losses of copper, test whether a simple model can predict true absorption from the plasma appearance of labeled copper, and develop a compartmental model for copper metabolism by using stable isotopes.

Design: A stable isotope of copper was intravenously administered to 6 men, and fecal samples were collected for 14 d. Four weeks later the study was repeated, but with an oral dose, and blood samples were collected for 7 d and fecal samples for 14 d.

Results: There was no significant difference ($P = 0.48$) in the estimated endogenous loss of copper calculated by using either the excreted intravenous dose ($\bar{x} \pm SD: 32 \pm 5\%$) or the absorbed and excreted oral dose ($35 \pm 2\%$). A simple mathematical model fitted to plasma isotope appearance data estimated true absorption to be $8 \pm 2\%$ compared with $48-49\%$ measured by fecal monitoring. A more complicated compartmental model predicted that, when newly absorbed copper first enters the blood, $74\%$ is removed by the liver and $99\%$ is bound to ceruloplasmin in the plasma. The exchangeable pool of copper was estimated to be $43 \pm 30\%$. Daily endogenous losses were predicted to be $2.4\%$.

Conclusions: The results showed that fecal monitoring is the only method that can reliably measure labeled copper absorption, and it is not necessary to administer an intravenous dose of copper to estimate endogenous losses. The compartmental model provides new insights into human copper metabolism. Am J Clin Nutr 2005;81:807–13.

KEY WORDS Copper, stable isotopes, men, compartmental modeling, endogenous losses, true absorption

INTRODUCTION

Copper is an essential nutrient for humans, and stable isotopes of copper have been used for many years to investigate its metabolism (1). However, because copper has only 2 stable isotopes, it is not possible to administer an oral and intravenous copper label simultaneously. Thus, fecal monitoring has been used to estimate copper absorption from an oral isotope dose. The drawback to this technique is that a large unknown quantity of copper—excreted endogenously via the bile and salivary, gastric, pancreatic, and duodenal routes—a Appears in the feces, which will result in an error in the calculation of unabsorbed oral isotope. Previously, the endogenous loss was quantified by administering an intravenous dose of labeled copper either some weeks before or some weeks after the oral dose and by quantifying its appearance in the feces (2). Recently, Harvey et al (3, 4) described a new method that only requires an oral isotope dose to quantify the endogenous loss. The first objective of the present work was to validate this method by comparing estimates of endogenous loss from oral and intravenous doses of labeled copper.

It is generally agreed that the fecal monitoring method is not ideal in terms of volunteer compliance, ease of sample preparation, analysis, or cost. An alternative method used for iron absorption studies (5, 6) involves giving an oral dose and taking serial blood samples over 6 h. The plasma appearance of iron is modeled by a simple technique, from which the quantity of iron absorbed from the oral label can be calculated. The second aim of the present study was to construct a similar model by measuring the appearance of labeled copper in the plasma and to compare the results with fecal monitoring data to see whether the iron technique could be used for copper.

Only one compartmental model of copper metabolism has been developed for humans (7). Although this model incorporates the main physiologic features, it has some experimental drawbacks, such as limited sampling of blood, which means that there is incomplete characterization of the initial metabolism of absorbed copper. Also, much of the earlier work on copper metabolism has focused on animal models, the results of which are assumed to mimic human copper metabolism. Some of the assumptions of the animal models have yet to be validated in humans. Therefore, the third aim of our study was to develop a comprehensive model of human copper metabolism.

1 From the Institute of Food Research, Norwich Research Park, Norwich, United Kingdom (LJH, JRD, WJH, VJB, JAH, and SJF-T); Rowett Research Institute, Aberdeen, United Kingdom (JHB); Sofia University, Faculty of Chemistry, Sofia, Bulgaria (TIV); and the Fisheries Research Services Marine Laboratory, Aberdeen, United Kingdom (IMD)

2 Supported by the Biotechnological and Biological Sciences Research Council and the European Union (Marie Curie Fellowship, Mass School Programme, contract number HPMT-CT-2000-00140).

3 Address reprint requests to JR Dainty, Institute of Food Research, Colney Lane, Norwich, NR4 7UA, United Kingdom. E-mail: jack.dainty@bbsrc.ac.uk. Received August 19, 2004. Accepted for publication November 19, 2004.
SUBJECTS AND METHODS

Subjects

Six healthy men aged 34–57 y (i = 39 y) were recruited to the study. A 10 mL screening blood sample was taken to exclude volunteers whose biochemical and hematologic indexes fell outside the normal range. Other exclusion criteria included use of medications or nutritional supplements and smoking. The aims and procedures of the study were explained to the subjects during a visit to the Human Nutrition Unit at the Institute of Food Research, and written informed consent was obtained. The Norwich District Ethics Committee approved the protocol, and the study was conducted in accordance with the Helsinki Declaration of 1975 as revised in 1983.

Study design

Subjects took part in 2 d of experiments at the Human Nutrition Unit but were otherwise free-living during the course of the study. On the first experimental day, which followed an overnight fast (10 h), the subjects received a 0.5-mg intravenous infusion of a highly enriched copper-65 stable isotope ($^{65}$Cu = 99.4%). The dose was administered over 60 min via a cannula inserted into a vein in the forearm. The subjects were given a light breakfast 1 h after completion of the infusion. Fecal and urine samples were subsequently collected over the next 14 and 7 d, respectively. No blood samples were taken. A minimum of 4 wk later, the subjects attended the Human Nutrition Unit for a second time. After an overnight fast, the subjects were given a 3-mg oral dose of highly enriched copper-65 stable isotope ($^{65}$Cu = 99.7%). Blood samples were collected over 5 h via a cannula inserted into a vein in the forearm. After removal of the cannula, the subjects were given lunch before they returned home. Single blood samples were collected each morning from fasted subjects for the following 4 consecutive days and 7 d postdosing. Each subject made complete fecal and 24-h urine collections for 14 and 7 d, respectively, after dosing. No fecal markers were given but the subjects were made aware of the importance of compliance and the necessity to report any missed samples. The subjects kept a record of all food and beverages consumed for 3 d before and 3 d after the second experimental day. Diary entries were recorded by using household measures and coded with the most appropriate food code selected from UK food-composition tables by using DIET CRUNCHER (Way Down South Software, Dunedin, New Zealand; Internet: www.waydownsouthsoftware.com) nutritional analysis software. Estimated amounts were calculated from values derived from average food portion sizes (8).

Dose preparation and administration

The intravenous doses were prepared by Ipswich Hospital Pharmacy Manufacturing Unit by dissolving 67 mg [$^{65}$Cu]copper (II) chloride (Trace Sciences International, Richmond Hill, Canada) in 100-mL sterile saline. The doses were portioned into 2-mL sterile glass ampules and stored at 4 °C until used. The doses were tested for sterility by Ipswich Pharmacy Quality Control Department, and copper concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS). The intravenous doses were administered by a qualified nurse issued with the appropriate approvals by a medical doctor. Before infusion, the 2-mL copper dose (0.25 mg/mL) was mixed with 50 mL sterile normal saline and infused with an Omnifuse pump (Graseby Medical Ltd, Watford, United Kingdom).

Isotopically enriched oral doses of copper chloride were prepared from [$^{65}$Cu]elemental copper (Chemgas, Boulogne, France) as previously described (6), and the concentration was accurately determined by ICP-MS. The solution was divided into individual doses, which were stored in plastic vials at −20 °C until required. The oral dose was administered in 50-mL water, and the subjects were asked to consume the dose as quickly as possible.

Blood sample collection and analysis

A baseline blood sample (10 mL) was collected on the second experimental day, 15 min before the oral dose was administered; subsequent blood samples (10 mL) were collected at 0, 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, and 300 min. Five additional blood samples were collected 24, 48, 72, 96, and 168 h after dosing. Blood was collected into trace element–free lithium heparin–containing tubes (Sarstedt, Leicester, United Kingdom) mixed gently by inversion, and centrifuged at 15 000 × g for 10 min at room temperature. The supernatant fraction of the plasma was transferred to acid-washed Nalgene cryogenic vials (Nalge Company, Rochester, NY), frozen on dry ice, and stored at −80 °C.

The copper that was not bound to ceruloplasmin, known as directly reacting copper, was extracted from the samples by using a dialysis-chelax method (9) with the following modifications. Human plasma (5 mL) was dialyzed against 100 mL of 175 mmol ammonium phosphate buffer/L (pH 7.0) for 4 h and then 2 × 100 mL of 175 mmol ammonium phosphate buffer/L (pH 7.0) containing 50 mmol histidine/L for 16 h with the use of Spectrapore dialysis tubing (10 mm flat-width cellulose ester membrane, MWCO 5000; Spectrum Europe BV, DG Breda, Netherlands). Copper in the histidine-containing dialysates was extracted by using 2 mL Chelex-100 minicolumns, which were prepared according to the manufacturer’s instructions (Bio-Rad, Hemel Hempstead, United Kingdom). The pH of the dialysates was adjusted to 8.0 with ammonium hydroxide before being applied to the columns. After washing with 1 mol ammonium acetate/L and 18.2 MΩ water, the divalent metals (including copper) were eluted with the use of 10 mL of 2.5 mol nitric acid/L. Total plasma copper and retentate copper were extracted by adding 0.8 mL water and 0.1 mL concentrated ultrapure nitric acid to a 0.1-mL sample. Precipitates were removed by centrifugation at 10 000 × g for 15 min, and the supernatant fractions were retained. All solutions were then analyzed for copper by atomic absorption spectrometry and for copper isotope ratios by ICP-MS. The methods used to remove the contaminant copper from the reagents used throughout the procedure and to quantify the remaining contaminant copper were described previously (9, 10).

Copper isotope ratios were measured with a Perkin-Elmer (Norwalk, CT) Elan 6100 DRC ICP-MS instrument (10). Instrumental performance was checked daily with the use of multielement standard solutions. The ratio of cerium oxide to cerium signals was maintained between 2.2% and 3.5% to control the formation of oxides, and the formation of double-charged ions was controlled by keeping the signal for Ba$^{2+}$/Ba below 3%. Isotope ratios were first corrected for fractionation by reference to the published isotope ratio for $^{63}$Cu/$^{65}$Cu (11). The isotope ratio for a prepared standard was obtained before each measurement and used to derive an isotope ratio correction factor.
Fecal sample preparation and analysis

All equipment used during sample processing was acid-washed before use. Fecal samples were autoclaved, freeze-dried, ground to a fine powder with the use of a mortar and pestle, and subsampled. Samples were prepared for ICP-MS analysis by using a combination of 2 methods (2, 12). Briefly, portions of fecal samples were ashed at 450 °C for 48 h in a muffle furnace (Vulcan 3-550; Jencons Scientific Ltd, United Kingdom). Samples were then prepared for the ion-exchange extraction of copper by taking up 0.2 g fecal ash in a 1:1 mixture of water (Milli-Q; Millipore, Billerica, MA) and concentrated ultrapure nitric acid (Merck Ltd, Lutterworth, United Kingdom), drying on a hot-plate, and reashing overnight at 500 °C. The resultant ash was taken up in 2 mL of 6 mol hydrochloric acid/L (Aristar grade; Merck Ltd) and left overnight before being centrifuged at 3000 rpm for 10 min. The supernatant fraction was removed and dried down under hot lamps until only 1 mL solution remained.

Copper was subsequently extracted from the supernatant fraction by ion-exchange chromatography using analytic-grade anion-exchange resin (AG1 × 8, 200-mesh chloride; Biorad Ltd, Hemel Hempstead, United Kingdom). The resin was soaked in deionized water for ≥24 h before use, and 2 mL presoaked resin was packed into acid-washed glass columns (1-mL pipette tips; Sarstedt, Nümbrecht, Germany). The columns were connected to a peristaltic pump (Watson Marlow, Falmouth, United Kingdom) via polyethylene tubing (1 mm internal diameter, 2 mm outside diameter) with a flow rate of 1 mL/min. The ion-exchange system was purged of any contaminant copper by washing with 2.5 mol HCl/L (Aristar grade; Merck Ltd) for 10 min. Minerals were eluted from the columns for 1 h with a solution of 2 mol HNO₃/L. The resin was regenerated into the chloride form by pumping a 6 mol HCl/L solution through the column for 1 h. Samples were subsequently loaded directly onto the resin, and the columns were flushed with a 6 mol HCl/L solution for 15 min. Copper was eluted with a 2.5 mol HCl/L solution into polytetrafluoroethylene vials in 15-mL fractions. Fractions were dried down under a hot lamp and reconstituted for MC-ICP-MS analysis in 2% (by vol) ultrapure nitric acid. Total copper concentrations were measured by atomic absorption spectrometry (model 3300; Perkin-Elmer).

Kinetic data analysis

Estimation of endogenous losses

In a previous publication we described a method to estimate endogenous losses of copper from a labeled oral dose, which involved the simultaneous administration of the rare earth marker holmium (3). That article showed that copper and holmium share identical excretory patterns; therefore, it was deemed unnecessary in the present study to give holmium as a marker to indicate when all the unab sorbed copper had been excreted. This point comes when the mole fraction of labeled copper in the feces falls below 0.02 (2% of the total copper in the feces). After this time, all of the subsequent stool samples contain only labeled copper that has been absorbed and then excreted. By plotting a straight line through these subsequent points in a graph of mole fraction of labeled copper in feces versus time and extrapolating back to the time of label administration (t = 0), the mole fraction of all labeled copper absorbed and then excreted can be estimated. The process for converting this into mass of copper is detailed in the article (3). The procedure for estimating the loss of the intravenous dose into the feces is simple; it is the cumulative appearance of the intravenous label over the period of fecal collection.

Simple model for predicting copper absorption

A previous publication (5) details the mathematical approach taken for this simple model. In summary, it is assumed that a certain fraction of the copper from the oral dose is absorbed at a constant rate over a clearly defined absorptive time and appears in the plasma, where it is eliminated according to a rate constant.

Compartmental model for investigating copper metabolism

Data from plasma and feces were analyzed by using the SAAMII (SAAM Institute Inc, Seattle, WA) program (14) and the compartmental model (Figure 1).

Definitions. The compartments represent discrete amounts of copper that behave identically. A compartment is a theoretical space in a system. A model can be viewed as a hypothesis to be tested against experimental data, and the structure of the model is then altered until a satisfactory fit to the data occurs. The accessible compartments in our system were 2 and 5, which represent the plasma. Transfer of copper between compartments \( k_{ij} \) (fraction/time) is defined as the fraction of compartment \( j \) moving into compartment \( i \) per unit time.

Data fitting. Two parameters, the apparent volume of distribution of the accessible compartments (\( V \)) and the fractional transfer rate from compartments \( 1 \) to \( 7 \) (\( k_{1j} \)), were held constant for all volunteers. Both of these parameters were difficult to estimate from our data; therefore, \( V \) was fixed at 5 L according to information received from a personal communication (N Lowe, 2003) and \( k_{1j} \) set to 10.0 d⁻¹. The other parameters were given initial estimates consistent with published data on human copper.
Oral and intravenous (IV) isotope excretion in feces and estimated absorption of the oral dose in feces and plasma

**TABLE 1**

<table>
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<tr>
<th>Subject no.</th>
<th>Amount of IV dose excreted</th>
<th>Amount of oral dose excreted</th>
<th>Apparent absorption</th>
<th>True absorption based on excretion of IV dose</th>
<th>True absorption based on excretion of oral dose</th>
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<td><strong>32</strong></td>
<td><strong>35</strong></td>
<td><strong>33</strong></td>
<td><strong>48</strong></td>
<td><strong>49</strong></td>
<td><strong>8</strong></td>
</tr>
<tr>
<td>SD</td>
<td><strong>5</strong></td>
<td><strong>2</strong></td>
<td><strong>3</strong></td>
<td><strong>5</strong></td>
<td><strong>4</strong></td>
<td><strong>2</strong></td>
</tr>
</tbody>
</table>

\(^1\) Refers to the percentage of the oral dose that was absorbed and then excreted into the feces as estimated with the method of Harvey et al (3).

\(^2\) Refers to the simple model developed by Dainty et al (5).

During the fitting process, the parameters are allowed to vary until a minimum of the objective function is reached. The software then returns the mean and the SD of the parameters. The 3 data sets (plasma copper concentrations from the oral dose, copper from the oral dose that appeared in the feces, and copper from the intravenous dose that appeared in the feces) were combined within SAAMII. The fecal data were entered as a cumulative total. It is assumed that within the time frame of this experiment, none of the oral or intravenous isotope would have been incorporated into bone and subsequently released and returned to the accessible compartment. The final model structure was arrived at by a process of trial and error but with the guiding principle that it must contain the fewest compartments to adequately describe the data (Principle of Parsimony). The final model parameters are nonuniquely identifiable, which means that they have more than one but a finite number of solutions. Several compartmental structures were attempted based on known physiology and metabolism, but the final structure (Figure 1) was chosen because it seemed to capture most of the known features of copper metabolism with the minimum SD on the parameters. The mass of copper in each compartment was estimated by using SAAMII in “system” mode with an exogenous input into the gut compartment equivalent to the calculated daily intake from the diet.

**RESULTS**

From the dietary assessment, the volunteers habitually consumed 1.4 ± 0.4 mg Cu/d. This value is in good agreement with the mean unlabeled copper excreted in feces over the course of the study (1.6 ± 0.2 mg Cu/d; \(P = 0.25\)) and indicates that the subjects were in steady state when they consumed their habitual diets. The analysis of total copper in urine resulted in trace amounts being detected, and these data are not reported or used in any of the kinetic modeling.

The oral and intravenous isotope excretion and oral absorption estimates are summarized in **Table 1**. The quantity of intravenous copper isotope excreted (32 ± 5%) was not significantly different (\(P = 0.27\)) from the estimate of excretion of the absorbed oral copper label (35 ± 2%). The mean apparent absorption from the oral dose was 33 ± 3%. When a correction was made to the apparent absorption for the quantity of oral dose absorbed and then excreted, the mean true absorption was estimated to be 49 ± 4%. This was not significantly different (\(P = 0.48\)) from the true absorption (48 ± 5%) when the correction was made by using the estimation of the quantity of the intravenous dose excreted. True absorption as calculated by the simple model was 8 ± 2%, which was significantly different (\(P < 0.01\)) from that estimated by fecal monitoring.

The results of the compartmental modeling are shown in **Table 2**. The exchangeable pool size was estimated to be 43 ± 30 mg, with most of the copper being located in compartment 6 (84%). True absorption was estimated to be 49 ± 4% [true absorption = \((k_{2,1} + k_{3,1})/(k_{2,1} + k_{3,1} + k_{1,1})\)]. Plasma copper was present in 2 compartments, the vast majority (99%) being in compartment 5; the remaining 1% was found in compartment 2. Most of the rate constants (\(k_{3,j}\)) that were fitted by the model had a CV (CV = 100 × SD/\(k_{3,j}\)) <25%, although \(k_{1,3}\), \(k_{1,6}\), and \(k_{3,8}\) had greater uncertainty (CV: ≈50% on average). Rate constant \(k_{6,5}\) for subjects 4...
and 6 was fixed because of fitting problems and has no uncertainty associated with it. The same applies to \( k_{s,1} \) for subject 5. The mean model fit to the average of the 6 subjects’ experimental data is shown in Figure 2. The first peak concentration \( C_{\text{max}} = 0.028 \pm 0.008 \mu g/mL \) in the labeled plasma occurred \( \approx 90 \) min after the dose before steadily decaying and then peaking again \( C_{\text{max}} = 0.021 \pm 0.005 \mu g/mL \) 2–3 d later. An example of the distribution of labeled copper between components of human plasma in subject 4 is shown in Figure 3. The directly reacting copper was the first to peak, at \( \approx 80 \) min, followed by the ceruloplasmin-bound copper \( \approx 3 \) d later.

**DISCUSSION**

The recovery of the intravenous labeled copper in the feces appears to validate our previously published method (3) in which the appearance of orally labeled copper in feces is used in conjunction with a linear model as a predictor of endogenous losses. In a subsequent article (4) we speculated that the linear model may not be adequate and that a mono- or bi-exponential fit may be necessary. However, the data presented in this article indicate that a linear fit is valid under our conditions and can be easily applied because of its simplicity.

The attempt to quantify copper absorption with the use of a simple mathematical model (single compartment) was unsuccessful. True absorption estimated from the simple model was only 8%, whereas it was 48–49% when measured by fecal monitoring. This difference can probably be explained by the large first-pass effect in the liver that newly absorbed copper undergoes on passing through the portal vein. This effect is not incorporated into our simple model.

The more complicated compartmental model (Figure 1) was developed by using the well known Principle of Parsimony, which states that one should not increase, beyond what is necessary, the number of entities required to explain anything. Therefore, this model had the minimum number of parameters to produce the observed behavior of the experimental data. The parameters are nonuniquly identifiable, which means that different starting estimates for the parameters will result in the program optimizer converging to different estimates for the parameters. This requires the starting estimates to be based on sound physiologic principles, otherwise the final estimates will be meaningless even though the optimizer may converge to what appears to be a good fit. Convergence also depends on how much noise or uncertainty is in the data, and, unfortunately, it was necessary to fix rate constant \( k_{(6, 5)} \) for subjects 4 and 6 and \( k_{(4, 3)} \) for subject 5 to fit the remaining parameters. This is an indication that the model suffers from some a posteriori identifiability issues when the data are noisy. The uncertainty on most of the parameters was acceptable for modeling of this type (15). The experimental data that was simultaneously fitted to the model was the fecal appearance of the oral and intravenous isotopes and the plasma appearance of the oral isotope in the form of total labeled copper concentration. Originally, we planned to use the ceruloplasmin-bound labeled copper plasma concentration and the directly reacting labeled copper plasma concentration as model inputs rather than the total labeled copper plasma concentration. Unfortunately, the labeled ceruloplasmin-bound and directly reacting copper plasma concentrations proved to be very noisy data sets (see Figure 3 for an example), so only the total labeled copper concentration was used.

One of the main findings from our compartmental model (Figure 1) was the necessity of including a pathway \( (k_{s,1}) \) from the gut to the liver. This so-called first-pass effect accounts for the surprisingly small quantity of newly absorbed copper that appears in the plasma and explains why the simple model of predicting

### Table 2

Model parameters for each subject

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Subject 1</th>
<th>Subject 2</th>
<th>Subject 3</th>
<th>Subject 4</th>
<th>Subject 5</th>
<th>Subject 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>delay 7 (d)</td>
<td>1.377 ± 0.053</td>
<td>1.687 ± 0.135</td>
<td>0.985 ± 0.018</td>
<td>1.399 ± 0.043</td>
<td>1.445 ± 0.034</td>
<td>1.409 ± 0.034</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>0.175 ± 0.017</td>
<td>0.365 ± 0.055</td>
<td>0.438 ± 0.249</td>
<td>3.659 ± 0.079</td>
<td>0.449 ± 0.055</td>
<td>0.284 ± 0.039</td>
</tr>
<tr>
<td>( k_{s,6} ) (d⁻¹)</td>
<td>0.028 ± 0.006</td>
<td>0.006 ± 0.017</td>
<td>0.056 ± 0.005</td>
<td>0.068 ± 0.005</td>
<td>0.047 ± 0.005</td>
<td>0.039 ± 0.004</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>3.749 ± 0.895</td>
<td>1.480 ± 0.153</td>
<td>1.942 ± 0.369</td>
<td>3.624 ± 0.605</td>
<td>2.522 ± 0.099</td>
<td>2.266 ± 0.237</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>6.570 ± 1.010</td>
<td>11.232 ± 0.824</td>
<td>5.499 ± 0.389</td>
<td>6.839 ± 0.762</td>
<td>7.113 ± 0.381</td>
<td>6.640 ± 0.335</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>39.410 ± 10.639</td>
<td>9.012 ± 1.544</td>
<td>18.970 ± 3.860</td>
<td>26.862 ± 6.171</td>
<td>13.447 ± 0.664</td>
<td>12.143 ± 1.409</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>0.267 ± 0.137</td>
<td>0.171 ± 0.173</td>
<td>0.188 ± 0.126</td>
<td>2.552 ± 0.179</td>
<td>0.514 ± 0.026</td>
<td>0.207 ± 0.046</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>2.491 ± 0.844</td>
<td>4.012 ± 1.623</td>
<td>4.002 ± 1.471</td>
<td>25 (fixed)</td>
<td>4.000 ± 1.528</td>
<td>5 (fixed)</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>0.387 ± 0.087</td>
<td>0.337 ± 0.104</td>
<td>3.029 ± 1.893</td>
<td>14.975 ± 3.373</td>
<td>1 (fixed)</td>
<td>1.174 ± 0.238</td>
</tr>
<tr>
<td>delay 4 (d)</td>
<td>0.680 ± 0.168</td>
<td>0.889 ± 0.270</td>
<td>2.514 ± 0.437</td>
<td>0.685 ± 0.322</td>
<td>0.985 ± 0.189</td>
<td>1.451 ± 0.197</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>1.452 ± 0.056</td>
<td>1.186 ± 0.095</td>
<td>2.031 ± 0.037</td>
<td>1.430 ± 0.044</td>
<td>1.385 ± 0.033</td>
<td>1.419 ± 0.035</td>
</tr>
<tr>
<td>( k_{s,1} ) (d⁻¹)</td>
<td>2.943 ± 0.729</td>
<td>2.251 ± 0.685</td>
<td>0.796 ± 0.138</td>
<td>2.920 ± 1.371</td>
<td>2.031 ± 0.390</td>
<td>1.379 ± 0.187</td>
</tr>
<tr>
<td>( M_1 ) (μg)</td>
<td>136</td>
<td>97</td>
<td>187</td>
<td>99</td>
<td>171</td>
<td>148</td>
</tr>
<tr>
<td>( M_2 ) (μg)</td>
<td>13</td>
<td>16</td>
<td>19</td>
<td>13</td>
<td>32</td>
<td>28</td>
</tr>
<tr>
<td>( M_3 ) (μg)</td>
<td>2495</td>
<td>1758</td>
<td>401</td>
<td>56</td>
<td>1137</td>
<td>904</td>
</tr>
<tr>
<td>( M_4 ) (μg)</td>
<td>656</td>
<td>524</td>
<td>3056</td>
<td>570</td>
<td>1120</td>
<td>1540</td>
</tr>
<tr>
<td>( M_5 ) (μg)</td>
<td>4130</td>
<td>4140</td>
<td>1319</td>
<td>1292</td>
<td>3416</td>
<td>1330</td>
</tr>
<tr>
<td>( M_6 ) (μg)</td>
<td>34892</td>
<td>93553</td>
<td>21621</td>
<td>12330</td>
<td>24365</td>
<td>26957</td>
</tr>
<tr>
<td>( M_7 ) (μg)</td>
<td>1873</td>
<td>1638</td>
<td>1842</td>
<td>1384</td>
<td>2470</td>
<td>2086</td>
</tr>
<tr>
<td>Total mass (μg)</td>
<td>44197</td>
<td>101727</td>
<td>28446</td>
<td>15745</td>
<td>32711</td>
<td>32993</td>
</tr>
</tbody>
</table>

1 Rate constants \( (k_i) \) refer to the fractional transfer of copper from compartment \( j \) to compartment \( i \) in unit time. Masses \( (M_i) \) refer to the quantity of copper located in compartment \( i \). Delay elements \( (\text{delay}) \) refer to the average time that copper spends inside compartment \( i \).

2 \( \bar{x} \pm \text{SD (all such values)}. \)
copper absorption on the basis of plasma appearance data does not work. The ratio of $k_{3,1}$ to $k_{3,1}/L_{1151}$ would indicate that $74\%$ of absorbed copper is removed by the liver on the first pass before undergoing a delayed entry into the plasma bound to ceruloplasmin ($80\%$) or excreted back into the gastrointestinal tract ($20\%$). The other copper excretory path back into the gut in our model is via compartment 6, which we speculate as containing muscle and other soft tissue. This excretory pathway is consistent with known copper physiology, which suggests that a large flux of copper ($2.0–2.8 \text{ mg/d}$) is excreted via salivary, gastric, duodenal, and pancreatic routes (16). The estimated copper flux from our model is $1.4 \text{ mg/d}$ via these routes and from biliary excretion ($k_{1,3}/L_{1154}$ mass of compartment 3) is $1.0 \text{ mg/d}$, which, again, is lower than the value (ie, $2.5 \text{ mg/d}$) reported by Linder et al (16).}

Our estimates of copper pool sizes are also lower than those previously reported. It is thought that a $70$-kg man contains $\approx 110 \text{ mg Cu}$ (17), but our model has estimated this to be $\approx 43 \text{ mg}$. One explanation for this low value may be that the time frame of our blood sampling was a relatively short (7 d) period; therefore, we would not have seen any exchange of the labeled copper with skeletal copper. This seems likely because it is known from calcium kinetic studies that even 14 d of plasma sampling does not allow detection of exchange or equilibrium of labeled calcium with the native element in bone matrix (18). It is thought that almost one-half of the copper stored in the body is contained in the skeleton, so our estimate of total body copper (43 mg) almost certainly excludes any copper in this region.

The sampled compartments (2 and 5) represent the plasma. It was decided to fix the volume of distribution of this space to 5000 mL based on unpublished data (N Lowe, 2003). This value was the mean of 8 subjects who each received an intravenous dose of labeled copper and then had 12 blood samples taken over 90 min, which allowed an estimate to be made of the apparent volume of distribution by a standard pharmacokinetic method (19). The ratio of the volume of distribution to the total copper contained in compartments 2 and 5 represents the predicted plasma concentration of copper. The model predicted that the distribution of copper in the plasma was $99\%$ in compartment 5 (identified as ceruloplasmin-bound copper) and $1\%$ in compartment 2 (identified as directly reacting copper). This compares with $94\%$ ceruloplasmin-bound copper as measured by Cartwright and Wintrobe (20), $97\%$ measured by Buckley et al (21), and $96\%$ measured by Inagaki et al (22).

The half-life ($t_{1/2}$) of ceruloplasmin copper in this study was estimated to be $\approx 27 \text{ d}$, which compares well with the value of $20 \text{ d}$ that was found in 10 "normal" control subjects in the study.
by Lyon et al (23). The $t_{1/2}$ of the directly reacting copper was estimated to be 60 min, which is higher than the values reported by Buckley et al (21) in 2 subjects after an intravenous infusion ($t_{1/2} = 11$ min) and estimated indirectly by Scott and Turnlund (7) in 5 subjects after an oral dose ($t_{1/2} = 26$ min) during a metabolic period when the subjects were consuming 1.68 mg Cu/d. Given the small sample size of these studies, there is still some uncertainty as to the true population mean of $t_{1/2}$ for directly reacting copper. Interestingly, the indirect estimate from the Scott and Turnlund model after the intravenous dose of labeled copper ($t_{1/2} = 41$ min for the directly reacting copper) suggests that it depends on the route of administration. We cannot comment on this on the basis of our study because no blood samples were taken after the administration of the intravenous dose.

In conclusion, the previously published method (3) for estimating endogenous losses of copper from an oral dose has been shown to be consistent with that from an intravenous dose. The use of a simple model for estimating the absorption of an oral dose of copper from its appearance in plasma does not warrant further investigation. Fecal monitoring remains the only feasible method for estimating copper absorption. A more complicated compartmental model showed that newly absorbed copper undergoes a substantial first-pass effect in the liver, where $\approx 74\%$ of the copper is initially removed from the circulation. The exchangeable pool of copper in the body was estimated to be 43 mg; this value is well below the total body copper concentration of 110 mg reported from autopsy data but consistent with the fact that most copper in the body cannot be quantified by using stable isotopes and compartmental modeling because it is contained in very slowly exchanging bone.

LJH was responsible for the study design and overall supervision of the sample analysis. JRD was involved in the study design and data interpretation and was responsible for all mathematical modeling and statistics. WHJ was responsible for volunteer recruitment, collection of samples, and some sample analyses. VJB was responsible for most of the sample analyses. JHB was involved in the study design and the supervision of some of the sample analyses. TIV was involved in the plasma sample analysis at the Rowett Research Institute. JAH analyzed all of the fecal samples on the ICP-MS at the Marine Lab. SJF-T was involved in the study design. All authors contributed to writing the final manuscript. None of the authors had any financial or personal interest in any company or organization sponsoring the research.

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REFERENCES

ORIGINAL COMMUNICATION

[6S]5-methyltetrahydrofolate or folic acid supplementation and absorption and initial elimination of folate in young and middle-aged adults

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1Department of Clinical Chemistry, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands; 2Department of Internal Medicine, Institute for Cardiovascular Research, VU University Medical Center, Amsterdam, The Netherlands; and 3Institute of Food Research, Norwich, UK

Objectives: To assess the effects of supplementation with the diastereoisomer of 5-methyltetrahydrofolate ([6S]5-methylTHF), as an alternative supplement for folic acid, on folate absorption and elimination, in two age groups.

Design: A randomized, double-blind intervention study.

Subjects: A total of 12 young (<30 y) and 12 middle-aged (≥50 y) healthy volunteers were recruited.

Methods: Volunteers were randomized to receive daily supplementation with 400 μg folic acid or equimolar amounts of [6S]5-methylTHF during 5 weeks. Before and after supplementation, absorption and initial elimination were calculated following oral [2H2]folic acid test doses using isotope kinetics in plasma.

Results: Folic acid absorption was lower in the middle-aged as compared to the young adults, both before (P = 0.03) and after (P = 0.05) supplementation. In the young adults, absorption decreased by 22% after [6S]5-methylTHF and increased by 21% after folic acid (P = 0.02). In the other age group, no such changes were found. The folate rate constant of elimination increased after folic acid supplementation in the young (+50%; P = 0.05) but not in the middle-aged (+18%; P = 0.5) adults.

Conclusions: Young adults show increased folate turnover after folic acid supplementation relative to the effect of [6S]5-methylTHF supplementation. Similar differences are not observed in middle-aged adults, in whom folic acid absorption was found to be lower as compared to the young adults.

Sponsorship: Financial support was received from the European Union 5th Framework Programme (Grant QLRT-1999-00576).

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Keywords: [6S]5-methyltetrahydrofolate; folic acid kinetics; homocysteine; elderly

Introduction

Supplementation with folic acid, the synthetic form of the B vitamin, has been shown to reduce the incidence of neural tube defects during pregnancy and to reduce elevated plasma total homocysteine (tHcy) levels, a risk factor for cardiovascular disease and other illnesses (Homocysteine Trialists’ Collaboration, 1998, 2002; Oakley, 1999). Mandatory fortification of grain products is in effect in the United States and elsewhere, and is known to reduce the incidence of neural tube defects and tHcy levels in the population (Savage & Lindenbaum, 1995; Jacques et al, 1999; Flynn & Enright, 2000; Halliday & Riley, 2000; Honein et al, 2001). In many other countries, folic acid fortification has not been implemented, in part because of the concern that high levels of folic acid intake prevent the timely diagnosis of vitamin B12 deficiency (Dickenson, 1995; Savage & Lindenbaum, 1995; Flynn & Enright, 2000; Dhar et al, 2003). This is of particular concern in people over the age of 60 y, in whom...
decreased cobalamin status and associated elevated tHcy have been documented (Savage & Lindenbaum, 1995; Stabler et al., 1997; Carmel et al., 1999). Whether gut absorption and initial metabolism of folic acid changes over time upon supplementation, and whether this is different among natural folate monoglutamyl forms are not known.

The most abundant natural folate in human plasma is the diastereomer ([6S]) of 5-methyltetrahydrofolate (5-methylTHF). On theoretical grounds, the use of [6S]5-methylTHF in supplements or as a fortificant is less likely to mask the anemia of vitamin B_{12} deficiency, as 5-methylTHF (and not folic acid) requires the cobalamin-dependent methionine synthetase reaction for conversion into tetrahydrofolate (Shane, 1997). Tetrahydrofolate is the precursor of folate forms that support hematopoiesis (ie methylenetetrahydrofolate and formyltetrahydrofolate). In the presence of cobalamin deficiency, supplemented [6S]5-methylTHF is unable to stimulate erythropoiesis because vitamin B_{12} is necessary for conversion of 5-methylTHF to tetrahydrofolate. However, folic acid is converted to dihydrofolate and tetrahydrofolate (Scott & Weir, 1994). Since [6S]5-methylTHF has become commercially available in recent years, its effect on folate status in the blood and reduction of plasma Hcy levels have been studied (Venn et al., 2002, 2003; Lamers et al., 2004). However, data on its bioavailability and elimination as compared to folic acid are limited (Prinz-Langenohl et al., 2003; Pentieva et al., 2004).

Bioavailability, the appearance of 5-methylTHF in plasma after oral folic acid ingestion, may be different between these compounds.

[6S]5-methylTHF could possibly be used for food fortification or in supplements as an alternative for folic acid, provided [6S]5-methylTHF has beneficial effects equal to those of folic acid with respect to prevention of neural tube defects (in offspring of women at the age of reproduction) and reducing cardiovascular disease risk (in the middle-aged and elderly). Ageing is associated with changes in gut function. Age-associated changes (eg in intestinal luminal pH and microbial flora) could possibly affect absorption of different folate forms. Whether [6S]5-methylTHF and folic acid supplementation have different effects on folate absorption at different ages is at present unknown. The aim of the study was to compare the effects of supplementation with [6S]5-methylTHF or folic acid on folic acid bioavailability in healthy young and middle-aged adults.

Subjects and methods

Subjects

Volunteers were recruited from Amsterdam and suburbs, through advertisements in public places and local written media. The respondents were invited to participate in the study if they were either aged ≥ 18 and < 30 y (young adults) or ≥ 50 y (middle-age adults) and were neither taking medication nor vitamin supplements. Respondents were not eligible if they had any type of chronic or acute disease, consumed ≥ 2 U alcohol/day, had lost body weight (> 5 kg in the previous 2 months), were either pregnant or planning a pregnancy, or were found to have a deficiency of folate (total serum folate level measured with immunologic assay < 5.9 nmol/l) or vitamin B_{12} (serum level < 150 pmol/l) on screening. A total of 12 young and 12 middle-aged volunteers were recruited. The size of these groups was calculated based on data on folate absorption in women of childbearing age (Finglas et al., 2002b), assuming that a clinically relevant difference of 20% in bioavailability would be detected with power 90% (and type I error 5%). The ethics committee of the VU University Medical Center approved the study, and written informed consent was obtained from all participants.

Study protocol

A 5 weeks, double-blind, randomized trial was conducted comparing oral supplementation with either folic acid (400 µg once daily) or [6S]5-methylTHF (454 µg once daily). Experiments with an oral test dose of [2H]{\textsubscript{2}}folic acid were performed before and at the end of the intervention period. During preintervention testing, volunteers visited the VU University Medical Center outpatient clinic. Height, body weight and blood pressure were measured, and a blood sample was taken after an overnight fast to determine serum folate and vitamin B_{12}. For the first experiment (d 1), the participants reported to the hospital in the morning (at ~ 0800), after an overnight fast of 11–14 h. An intravenous catheter was placed in a dorsal hand vein for blood sampling. Baseline blood samples were drawn for tHcy, vitamins, methymalonic acid and 677C → T polymorphism. At 0830 (time 0), an oral test dose of 202 µg (= 454 nmol) [2H]{\textsubscript{2}}folic acid ([3\textsuperscript{'}, 5\textsuperscript{-}H]{\textsubscript{2}}folic (monoglutamic) acid, 98% isotopic purity; Professor JF Gregory III, University of Florida, Gainesville, FL, USA) was dissolved in water and administered in a drink (Kok et al., 2004). Venous blood was drawn at time 0, 20, 40, 60, 90, 120, 150, 180, 240, 300, 360 and 480 min for total folate and for 5-methylTHF concentrations and enrichments in plasma. Volunteers had access to water, and used a light folate-free lunch (after time 240 min). Participants were assigned to one of two interventions ([6S]5-methylTHF or folic acid) according to a computer-generated randomization list, and received a batch of supplements (see below). They were asked to take, from d 1 to d 34, two capsules in the morning of each day and to record this on a diary form. The volunteers were encouraged to continue their customary diet. The second experiment was performed on d 35, using the same [2H]{\textsubscript{2}}folic acid test dose protocol as described above. Compliance was checked by inspection of the diary forms and counting of the number of capsules at the end of the intervention period.

The supplements were manufactured by Eprova (Schaffhausen, Switzerland) as hard gelatin capsules containing a blend of magnesium stearate and microcrystalline cellulose.
and either 227 μg [65]S-methylTHF as calcium salt (208 μg (454 nmol) for the [65]S-methylTHF moiety) or 200 μg (454 nmol) folic (monoglutamic) acid. The supplements were identically packed in coded batches of 70 capsules, and stored in bags covered with aluminum foil. The randomization code was kept at the Institute of Food Research, Norwich, UK (PF). Stability tests of the supplements (microbiological assay; Institute of Food Research) performed at the beginning, middle and end of the intervention studies showed a recovery of 98% of both folate forms.

Analytical methods
Blood samples were placed on ice, and were transferred into EDTA-containing tubes for determination of the concentrations of total folate, tHcy and methylmalonic acid and concentration and enrichment of 5-methylTHF, or transferred into glass tubes for determination of serum vitamin B₁₂ and pyridoxine. The samples were centrifuged at 4 °C within 15 min (1000 × g, 10 min) and stored at −80 °C. A blood sample was also taken for determination of the 677C→T polymorphism of MTHFR, transferred into EDTA-containing tubes and stored at −80 °C.

Plasma total (ie free and protein bound) Hcy was measured with a microparticle enzyme immunoassay (IMX analyser; Abbott, Chicago, IL, USA) (interassay CV 4%). Methylmalonic acid was measured as its dicyclopenteryl ester by stable isotope dilution capillary gas chromatography–positive chemical ionization mass spectrometry (interassay CV 5–8%) (Rasmussen, 1989). Methylmalonic acid and tHcy samples were tested in a single run to eliminate day-to-day assay variation. Serum vitamin B₁₂ concentrations were measured with radioassay (ICN, Costa Mesa, CA, USA) (interassay CV 4%) and serum vitamin B₉ (pyridoxal-5-phosphate) with the use of fluorescence high-performance liquid chromatography (interassay CV 7%) (Ubbink et al., 1985).

Total folate was determined by means of automated chemiluminescence (ASC:180 Automated Chemiluminescence Systems, Chiron Diagnostics, Halstead, UK) (interassay CV 5%). The concentration and isotopic enrichment of 5-methylTHF were measured as previously described (Kok et al., 2004). Briefly, [1³C₃]S-methylTHF (Institute of Food Research, Norwich, UK) was added to the plasma sample as internal standard. The samples were deproteinated and protected against oxidation, and folates were extracted with folate binding protein affinity columns and a concentration step. Liquid chromatography tandem mass spectrometry was performed on a triple quadrupole tandem mass spectrometer (API 3000, Applied Biosystems, Foster City, CA, USA) in the positive ion mode following multiple reaction monitoring of precursor fragment transitions (interassay CVs: for unlabeled 5-methylTHF concentration 3–8% and for [²H₂]5-methylTHF enrichment 1–2%). Enrichment was expressed as tracer-to-tracer ratio (TTR).

The 677C→T polymorphism of MTHFR was assessed in DNA obtained from the buffy coat of EDTA blood (Tsai et al., 2000).

Calculation of folate kinetics
Changes in labeled 5-methylTHF plasma concentrations are approximated in a single compartment model of the fast folate pool (Finglas et al., 2002a; Kok et al., 2004). Following an oral dose of [²H₂]folic acid, a measurement protocol of > 6 h allows for the completion of the absorptive process and characterization of the decay of the plasma concentration. In the model, apparent absorption and true absorption can be estimated (in the latter, we assume that when folate is absorbed, a first pass effect in the liver is present at a fraction of 0.65 of the dose; Finglas et al., 2002a) as well as the rate constant of elimination (k). The model, assumptions and calculations are described in Appendix A1.

Statistical analyses
Compartmental modeling was performed with SaamII (version 1.1.1), obtained from the Saam Institute (Seattle, USA), and true absorption, apparent absorption (assuming no first pass effect) and the rate constant of elimination from the pool were retrieved under goodness of fit conditions. Differences between the [65]S-methylTHF and folic acid supplementation and between age groups were analyzed with unpaired Student’s t-tests or, when data were skewed, Mann–Whitney U-tests. For paired observations, paired Student’s t-tests or Wilcoxon matched pairs rank sum tests were performed. Correlation analysis was performed with the Spearman’s rank test. ANOVA was performed to test the effects of interactions between age groups and folate intervention. To account for potential confounders, in particular baseline differences in folate status and absorption and elimination kinetics, multiple regression analyses were performed. Because MTHFR genotype and folate status interact with plasma tHcy, these analyses were performed with and without inclusion of MTHFR genotype. Normality was assessed by visual inspection of distributions and Kolmogorov–Smirnov tests. Values are presented as means ± s.d. or median (range). Data were analyzed with SPSS (version 9.0, SPSS, Chicago, IL, USA). Differences were considered significant if P < 0.05 (two-tailed).

Results
All volunteers completed the study according to the protocol’s criteria for compliance. There were no significant differences in baseline plasma tHcy, 5-methylTHF, total folate and other characteristics between the volunteers receiving [65]S-methylTHF or folic acid (Tables 1 and 3). Baseline vitamin B₉ was slightly lower in the middle-aged volunteers in the folic acid subgroup, but there was no significant difference between groups (overall
Table 1  Characteristics of human volunteers in both supplementation groups

<table>
<thead>
<tr>
<th>Age &lt; 30 y</th>
<th>Age &gt; 50 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>[6S]-methylTHF (n = 6)</td>
<td>Folic acid (n = 6)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>3/3</td>
</tr>
<tr>
<td>Age (y)</td>
<td>26 ± 4</td>
</tr>
<tr>
<td>Body weight (kg)</td>
<td>66 ± 10</td>
</tr>
<tr>
<td>Vitamin B₆ (nmol/l)</td>
<td>50 ± 19</td>
</tr>
<tr>
<td>Vitamin B₁₂ (pmol/l)</td>
<td>202 ± 46</td>
</tr>
<tr>
<td>Methylenalonic acid (µmol/l)</td>
<td>0.19 (0.14–0.22)</td>
</tr>
</tbody>
</table>

Values are means ± s.d. or median (range).
No significant differences were present between age and intervention groups.

Table 2  Folic acid absorption and initial metabolism before and after 5 weeks supplementation with [6S]-methylTHF or folic acid in young and middle-aged adults

<table>
<thead>
<tr>
<th>Age &lt; 30 y</th>
<th>Age &gt; 50 y</th>
</tr>
</thead>
<tbody>
<tr>
<td>[6S]-methylTHF (n = 6)</td>
<td>Folic acid (n = 6)</td>
</tr>
<tr>
<td>Apparent absorptionb (% dose)</td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>42 ± 10</td>
</tr>
<tr>
<td>After</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>Mean % change</td>
<td>−21*</td>
</tr>
<tr>
<td>True absorption% (% dose)</td>
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</tr>
<tr>
<td>Before</td>
<td>119 ± 29</td>
</tr>
<tr>
<td>After</td>
<td>93 ± 28</td>
</tr>
<tr>
<td>Mean % change</td>
<td>−22*</td>
</tr>
<tr>
<td>Rate constant of elimination (h⁻¹)</td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.104 ± 0.021</td>
</tr>
<tr>
<td>After</td>
<td>0.090 ± 0.032</td>
</tr>
<tr>
<td>Mean % change</td>
<td>−9</td>
</tr>
</tbody>
</table>

*Values are means ± s.d. Results were obtained with kinetic modeling of [²H₂]5-methylTHF concentrations.
*Assuming no first pass effect.
*Assuming a first pass effect of 65% of tracer dose (Finglas et al, 2002b). Significant difference between folate interventions (for change from baseline): *P < 0.05 (Mann-Whitney U-test).

ANOVA, P = 0.18, independent t-tests for separate groups: 0.12 ≤ P ≤ 0.92. Body weight was slightly higher in the middle-aged as compared to young adults (independent t-test, P = 0.06).

At baseline, true absorption of folic acid was significantly lower in the middle-aged as compared to the young adults (110 ± 34 vs 84 ± 20%, respectively; independent t-test, P = 0.03), and tended to be different after supplementation (107 ± 56 vs 80 ± 35%, P = 0.05). The rate constant of elimination was not different between age groups (data not shown). Results for the intervention studies are presented in Table 2. In the young adults, folic acid true absorption increased significantly after folic acid supplementation, as opposed to a decrease in absorption after [6S]-methylTHF supplementation (paired t-test, difference between interventions: 43%, P = 0.02). Absorption in the middle-aged adults did not change significantly after either supplement. For both age groups combined, the means of percentage change in absorption were significantly different between [6S]-methylTHF and folic acid supplementation (−16 vs +13%, respectively; P < 0.05). The rate constant of elimination showed a trend to increase after folic acid supplementation in the young adults (+50%; paired t-test, P = 0.052), and was unchanged after [6S]-methylTHF supplementation. In the middle-aged adults, changes after either supplement were small. For all volunteers combined, changes in folic acid absorption were positively associated with changes in the rate constant of elimination (Spearman’s r = 0.38, P = 0.06).

Multiple regression analyses were performed with ‘absorption after supplementation’ as the dependent variable and ‘baseline absorption, baseline 5-methylTHF status, age category and intervention type’ as independent variables. For both age groups combined, the only positive predictor of ‘absorption after supplementation’ was intervention with folic acid (beta-coefficient = +8.9% compared to [6S]-methylTHF; P = 0.04). Separate multivariate analyses of the two predefined age categories confirmed that this positive effect of folic acid supplementation on true absorption was entirely attributable to the young age group (beta-coefficient +13.2%; P = 0.02) and was not present in the elderly (beta-coefficient 5.0; P = 0.50). Results were identical for apparent absorption. Additional adjustment for baseline
vitamin B6 levels and MTHFR genotype did not change these results. In addition, similar multiple regression analyses for ‘rate of elimination’ were also in agreement with the results of the bivariate analyses presented in Table 2.

Effects of supplementation on blood concentrations of 5-methylTHF and tHcy are shown in Table 3. After supplementation, plasma 5-methylTHF concentration was higher in all subgroups (Mann–Whitney U-tests, 0.03 ≤ P ≤ 0.08). Plasma total folate was also higher after supplementation in all subgroups (Mann–Whitney U-tests, 0.03 ≤ P ≤ 0.05), with a higher increase in the young adults as compared to the middle-aged adults (Mann–Whitney U-test, P = 0.015). Both folate interventions resulted in lower tHcy levels, with no apparent overall differences between the two age groups. Fasting tHcy showed a more pronounced decrease in [6S]-methylTHF-supplemented volunteers (Mann–Whitney U-test, P = 0.07), which was entirely attributable to the younger age group (Mann–Whitney U-test, P = 0.03). The change in plasma 5-methylTHF concentrations did not correlate with the change in fasting tHcy. There was no statistical significance present for the interaction between age and folate intervention with regard to changes on tHcy, 5-methylTHF and total folate levels.

**Discussion**

In young adults, but not in the middle-aged, we found a significant difference in absorption and initial elimination of a folic acid test dose between supplementation with the diastereoisomer of 5-methylTHF as compared to folic acid. Absorption was lower in the middle-aged as compared to the young adults. These findings suggest different age-dependent mechanisms for these folate forms.

With respect to methodology, we studied initial metabolism of 5-methylTHF following labeled folic acid absorption after oral loading. As shown by others (Krumdieck et al, 1978; Clifford et al, 1998), the results indicate that folic acid bioavailability in the young adults is nearly complete, but variation of the estimate existed due to small sample size in each subgroup. The elimination constant in our study is higher than reported in radioactive folic acid studies conducted in two healthy adults (Krumdieck et al, 1978; Clifford et al, 1998). Exchange with hydrogen of the deuterium in the folic acid tracer may result in underestimation of the 5-methylTHF enrichment (Gregory & Quinlivan, 2002). The extent of this isotope effect under the study conditions is very limited. With our study model, similar values for k are obtained for [3'S-3H2]folic acid and [15C6]folic acid ingested as test doses (Kok et al, 2004). The rate constant of elimination used in our study represents elimination from the plasma, not excretion from the body.

In the young adults in the present study, 400 μg folic acid supplementation elicited an increased initial turnover of folic acid relative to supplementation with [6S]-methylTHF. Previous studies on increasing folate intake and folate absorption and elimination have not been conclusive. In a study in two groups of young women at total folate intake levels of 450 or 850 μg/day (part of it as folic acid supplements), a trend to a lower residence time was reported in women with the higher folate intake (Gregory et al, 2001). In another study performed in 32 elderly women (60–85 y) randomized into two groups after experimental depletion at a folate intake of 118 μg/day, total urinary folate catabolite excretion was significantly increased after 7 weeks of repletion in women receiving a folate intake of 400 μg/day but not in those receiving 200 μg/day (Wolfe et al, 2003). It should be noted that these studies were conducted in women of different age groups and used different experimental protocols, making comparison of the results hazardous. In the present study, folic acid absorption was 20% lower in the middle-aged as compared to the young volunteers. Under these dissimilar baseline conditions, the middle-aged adults in the present study exhibited no
changes in absorption and initial folic acid elimination upon supplementation with either supplement.

What could be the physiological explanation for these findings? The intestinal folate transport system has similar avidity for the monoglutamyl forms of folic acid and 5-methylTHF (Selhub et al., 1984; Mason & Rosenberg, 1994). However, in terms of 5-methylTHF appearance in plasma, the metabolic routing of orally administered folic acid is different from that of oral 5-methylTHF. For a [6S]-methylTHF supplement, the 5-methylTHF transported over the brush border of the enterocyte only needs to be transported to the portal circulation and pass through the liver to appear in the systemic circulation. In contrast, for the folic acid supplement, the folic acid appearing in the enterocyte must undergo reduction and one-carbon substitution. At low doses, folic acid is excreted into the systemic circulation mainly as 5-methylTHF. However, even at an oral dose of 200 μg, part of the folic acid reaches the liver and systemic circulation in its unmethylated form (Sweeney et al., 2002; Kok et al., 2004). Folic acid that reaches the portal circulation requires reduction and one-carbon substitution in the liver (or in other tissues). In the liver, folic acid undergoes these transformations only if unbound to folate binding protein (Henderson, 1990). From the vantage point of appearance in the plasma 5-methylTHF pool, there are now several theoretical options to explain the increased folate appearance rate after folic acid supplementation relative to [6S]-methylTHF supplementation.

The first option is that in the folic acid supplementation group, exposure of enterocytes and hepatocytes to unmethylated folic acid during the 5 weeks supplementation resulted in upregulation of enzyme activity for dihydrofolate reductase, the rate-limiting enzyme in the conversion of folic acid into 5-methylTHF. Enzyme induction by folic acid exposure (Kamen et al., 1985) is a possible mechanism because dihydrofolate is the product of the first step in folic acid metabolism and also the endogenous substrate for dihydrofolate reductase in its conversion to tetrahydrofolate. In the folic acid supplementation group, this may have resulted in faster conversion of the labeled folic acid from the test dose into 5-methylTHF on d 35 as compared to d 0. Since enzyme induction would not occur in the participants supplemented with [6S]-methylTHF, a difference in post-supplementation absorption characteristics between both supplementation groups may be explained. However, to explain the differences between both age groups with respect to absorption and initial elimination after supplementation, this option would require the assumption that the dihydrofolate reductase upregulation by folic acid supplementation is age dependent.

The second option follows the observation that folate binding protein has a higher affinity for folic acid than for reduced folates (Henderson, 1990). The exposure to folic acid supplementation may saturate the hepatic folate binding protein pool with folic acid, so that on the second test day less unmethylated labeled folic acid arriving in the hepatocytes is able to bind to this protein. Conversely, exposure to [6S]-methylTHF supplementation saturates the hepatic folate binding protein with reduced folates but not with folic acid. Thus, bioavailability for the second folic acid test dose could be lower in the volunteers who received [6S]-methylTHF supplementation as compared to those with folic acid supplementation. In order to explain the difference in absorption between young and middle-aged volunteers, this explanation would require an age-dependent difference in the characteristics of the folate binding protein pool or its interaction or saturation with folates.

The third option follows the reasoning that the explanation for age-related differences in folic acid absorption derives from changes in the gut that are associated with age. Ageing in humans is associated with a decrease in gastric acid production and a rise in pH of the intestinal lumen content. A mildly acidic luminal environment greatly enhances folate transport (Smith et al., 1970). Atrophic gastritis is common in the elderly and results in folic acid malabsorption, which can be corrected by oral administration of hydrochloric acid (Russell et al., 1984, 1986; Krasinski et al., 1986). In a dynamic in vitro model simulating human gastric passage, folate binding proteins have been shown to maintain binding of folic acid in the gut lumen whereas binding of 5-methylTHF diminished, resulting in a high fraction of unbound 5-methylTHF (Verwei et al., 2004). Interaction between age, small bowel luminal pH and folate binding protein affinity to folate forms may thus explain both the difference in folic acid absorption between young and middle-aged adults in our study and the differential effect of supplementation with different folate forms on folate absorption in these age groups. It should be noted that in the present study, no further data are available to test these optional hypotheses, and other explanations remain possible.

The limitations of the study merit discussion. Firstly, our measurement protocol has the limitation that, without sampling of the portal vein or liver, the two components of folate bioavailability (absorption and first pass effect) cannot be quantified. No estimations of intracellular or long-term folate metabolism or excretion in the urine were made (Sites et al., 1997). The hepatic first pass effect was assumed to be constant (65% of the dose) in our study, but it should be noted that it may vary between individuals (between-subject CV approximately 10%; Kok et al., 2004). At baseline, means for true absorption ranged between 78 and 101% in three subgroups but was 119% in the young volunteers in the [6S]-methylTHF supplementation group. Since absorption cannot exceed 100%, the latter finding indicates either measurement or modeling error. Background plasma folate, enrichment curves and modeling goodness of fit were therefore different in the young volunteers in this subgroup; simulations showed that true absorption of 100% was obtained if first pass effect in the subgroup was assumed to be 54% of the dose. Although we have no reason to suspect background
hepatic folate stores or first pass effect in the young volunteers receiving \([6\text{S}]-\text{methylTHF}\) supplementation to be different from the other volunteers, we cannot exclude this possibility. Whether the first pass effect changes with supplementation and is different between two orally administered folate forms is unknown, but if present this would affect the calculation of true absorption. With the present knowledge, we cannot further explore these validity issues of the model. Secondly, as a complication of the study design (which included two age groups and two interventions, with six volunteers per subgroup), the possibility to detect differences within- and between-study groups and to formally test for interaction effects (which requires many more subjects than testing for group differences) was reduced. Changes were observed in folic acid absorption and rate constant of elimination after supplementation (range between −20 and +50% within subgroups; see Table 2). Wolfe et al (2003) found a significant increase in urinary folate excretion after supplementation with 400 μg/day folic acid in folate-depleted elderly women, and this is in agreement with the trend (±18%) for higher rate constant of elimination from plasma in our middle-aged volunteers supplemented with 400 μg/day folic acid. Nonetheless, statistical significance was present for lower absorption in the middle-aged adults and intervention-related differences in the younger adults.

Thirdly, the measurement of folate forms in plasma was incomplete. Folic acid ingestion increases not only 5-methylTHF but also other reduced folate concentrations. We have not measured dihydrofolate and tetrahydrofolate levels and of 5-methylTHF (measured by chemiluminescence) but also other reduced folate concentrations. The dynamics of folic acid metabolism in an adult given a small tracer dose of C-14-folic acid. (1999): Serum cobalamin, homocysteine, and methylmalonic acid concentrations in a multiethnic elderly population: ethnic and sex differences in cobalamin and metabolite abnormalities. J. Clin. Nutr. 70, 904–910.


European Journal of Clinical Nutrition

References


**Appendix A1**

**Estimation of in vivo folate absorption and elimination**

Changes in $S$-methylTHF isotopomer plasma concentrations are approximated in a single-compartment model (Kok et al, 2004). The appearance of labeled folic acid approximates an infusion rate ($R$) over the absorption period $T$. The definition of $R$ is

$$R = \frac{\text{mass of dose that is absorbed}}{\text{time period for absorption}} = \frac{M}{T} \quad (A.1)$$

With $V$ the apparent volume of distribution for folate ($\sim 389 \text{ ml/kg body weight}$; Loew et al, 1987), $k$ the rate constant of elimination from the compartment and $t$ the time after ingestion of the test dose (dose$_{oral}$), the labeled $S$-methylTHF concentration ($C$) can be approximated as

$$C = \frac{M}{VTk}(1 - e^{-k(t - t_{lag})}) \quad (0 < t < t_{max}) \quad (A.2)$$

and

$$C = \frac{M}{VTk}(1 - e^{-kT}) e^{-k(t - t_{lag})} \quad (t > t_{max}) \quad (A.3)$$

Here, $t_{lag}$ is the time during which the plasma enrichment remains at baseline. The value of $T$ (ie $t_{max} - t_{lag}$) is established from the plasma $S$-methylTHF enrichment curve. $M$ can be calculated by fitting equations (A.2) and (A.3) to the time series of labeled $S$-methylTHF concentrations. The apparent absorption can then be calculated as

$$\text{apparent absorption} = \frac{M}{\text{dose}_{oral}} \quad (A.4)$$

A first pass effect is present when folate is absorbed. Assuming that in humans it is a fraction of 0.65 of dose$_{oral}$ (Finglas et al, 2002b), true absorption can be estimated as

$$\text{true absorption} = \frac{M}{1 - \text{first pass effect}} \quad (A.5)$$
SHORT COMMUNICATION

Estimation of iron absorption in humans using compartmental modelling

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Objective: To evaluate whether a compartmental model could estimate iron absorption as accurately as the well-validated technique of plasma area under the curve using labelled test meals.

Design: The study is a randomised cross-sectional intervention.

Setting: The study was carried out at the Human Nutrition Unit at the Institute of Food Research, Norwich, UK.

Subjects: A total of nine female volunteers, aged 33 ± 8 y.

Interventions: Volunteers were given an oral dose (approximately 5 mg) of Fe-57 as iron sulphate in an orange juice test drink and simultaneously infused Fe-58 (approximately 200 μg) as iron citrate over 90 min. Multiple blood samples were taken for the following 6 h. The samples were analysed by mass spectrometry and iron absorption was estimated using a mathematical model based on the appearance of Fe isotopes in plasma and the area under the curve technique.

Results: The geometric mean (±1 s.d., 1 s.d.) absorption of the model estimate is 16% (9, 31) and the area under curve estimate is 18% (8, 29).

Conclusions: Results indicate that a compartmental model can be used to estimate labelled iron absorption although it is unlikely that this new method will be used in favour of an existing one. Further studies are now needed with unlabelled iron to assess whether the technique could have application in the assessment of total (haem + nonhaem) iron absorption from food.

Sponsorship: European Union Marie Curie Fellowship and Biotechnology and Biological Sciences Research Council (BBSRC), UK.

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Keywords: compartmental modelling; iron absorption; stable isotopes; plasma iron concentration; area under the curve

Introduction

The aim of several previous studies investigating iron absorption methodologies in human volunteers has been to correlate the peak increase in serum iron concentration with the absorption of iron from a test meal (Hallberg & Solvell, 1960; Ekenved et al, 1976). This has failed because the underlying assumption behind it that the rate of elimination of iron from serum does not vary markedly between individuals is false. Where this has been recognised, an intravenous (i.v.) labelled iron has been given in conjunction with an oral test dose to calculate iron absorption (Gonzalez et al, 2001). The drawback to the latter method is that the oral and i.v. doses must behave in the same (kinetic) way once in the systemic circulation, but there is some evidence that this does not always happen (Winchell, 1968).

Compartmental modelling can overcome these problems because it can be used to estimate the quantity of iron absorbed from an oral dose without the need to give a simultaneous i.v. dose of iron. It is a well-established technique for estimating fluxes and pool sizes, and does not require invasive sampling of body tissues (Carson et al, 1983; Green & Green, 1990; Jacquez, 1996). Its use in iron metabolism is based on radioisotope work, published mainly in the 1960s, which resulted in the development of complex multicompartmental models (Pollycove & Mortimer, 1961; Sharney et al, 1965; Hosain et al, 1967; Najean et al, 1967). A pilot study (Dainty et al, 2003) has indicated that simple,
single compartmental modelling shows promise in estimating unlabelled iron absorption; however, bias due to modelling assumptions needs further investigation. The current paper attempts to address this issue by comparing the estimate of iron absorption made from the plasma area under the curve (AUC) method (Barrett et al, 1994) with that from the compartmental model using labelled test doses.

**Subjects and methods**

Volunteers were recruited through advertisements placed around the Norwich Research Park. Nine healthy women between the ages of 20 and 45 y volunteered for the study. Their health was assessed through prestudy screening, which included a blood test and a medical questionnaire. None of the subjects were taking dietary supplements, was pregnant or a smoker. The study was approved by the Norwich and Norfolk District Ethics Committee and all recruits signed informed consent forms before entering the study. After an overnight fast, the subjects had an i.v. cannula inserted into each arm. The experimental protocol and dose preparation are similar to that described by Dainty et al (2003). In all, 12 serial blood samples were taken at \( t = 0, 20, 40, 60, 80, 120, 145, 175, 200, 260, 320 \) and 380 min after the simultaneous oral and intravenous dose. A single focussing multicolonlector inductively coupled plasma mass spectrometer (Isotope, Micromass, UK) with a desolvating sample introduction system and microconcentric nebulizer (Aridus and TI H, both from Cetac) was used for isotope analysis. All samples were run in duplicate and calibrated against IRMM014 (Rosman & Taylor, 1998).

**Model analysis**

The majority of the analysis is contained in a recently published paper (Dainty et al, 2003) and is not reproduced here. Briefly, the model estimates the quantity of iron that has been absorbed from a test meal by assuming that it crosses the gut wall as a constant infusion in the plasma and is cleared. By fitting equations to the labelled plasma iron concentration data, it is possible to estimate the quantity of iron absorbed from the test meal (\( M_{\text{oral}} \)). Using the same model for the i.v. infusion, it is possible to validate some of its assumptions by estimating the ‘i.v. recovery’, which is defined as the calculated mass coming from the i.v. dose, \( M_{\text{i.v.}} \), divided by the actual mass of labelled iron in the i.v. dose (\( \text{dose}_{\text{i.v.}} \)). The ratio of these two masses should be an independent validation of the model’s suitability for predicting absorption from the oral dose.

\[
i.v. \text{ recovery} = \frac{M_{\text{i.v.}}}{\text{dose}_{\text{i.v.}}} \tag{1}
\]

**Statistical analysis**

All data are presented as arithmetic mean ± s.d. and are based on nine volunteers \( (n = 9) \) unless stated otherwise. A paired, two-tail, Student’s \( t \)-test was carried out to assess the significance of numerical differences arising from different routes of isotope administration. Differences were considered significant if \( P < 0.05 \). A graphical technique was used to assess the agreement between estimation of iron absorption from the AUC method and the model method (Bland & Altman, 1986). The difference in iron absorption between the two methods is plotted against the average value of iron absorption found using both techniques. Two methods for measuring the same quantity are said to have good agreement provided (1) variations within the mean of the difference in iron absorption ±2 s.d. are not significant and (2) the average bias of one method relative to the other is small.

**Results**

Absorption data, plasma half-lives and i.v. recovery data are shown in Table 1. The geometric mean (−1 s.d., +1 s.d.) absorption for the AUC method is 18% (10, 32) and that for the model is 16% (8, 29). When the model is applied to the labelled i.v. plasma concentration data, it can be seen that there is a small underestimation of the true i.v. dose that was infused into the subjects (i.v. recovery = 95 ± 11%). The half-life \( (t_{1/2}) \) of the labelled iron in the plasma from the oral dose is \( 2.67 ± 0.36 \text{ h} (k = 0.26 ± 0.04 \text{ h}^{-1}) \) and is not significantly different \( (P = 0.43) \) from that for the i.v. dose \( (t_{1/2} = 2.60 ± 0.36 \text{ h}, k = 0.27 ± 0.04 \text{ h}^{-1}) \). Figure 1 is a graphical depiction of the agreement between the two methods. The mean difference between the estimation of %Fe absorption is calculated at −1.8% (ie the model method gives a lower estimate) and the 95% confidence interval is estimated as being between −7.5 and 3.9 units of difference (% iron absorption).

**Discussion**

In the present paper, we have attempted to show that a single compartment model can be used to estimate the

<table>
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<th>Subject no.</th>
<th>AUC (absorption %)</th>
<th>Model (absorption %)</th>
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<th>i.v. recovery (%)</th>
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</tr>
<tr>
<td>7</td>
<td>30</td>
<td>24</td>
<td>2.3</td>
<td>2.3</td>
<td>82</td>
</tr>
<tr>
<td>8</td>
<td>12</td>
<td>12</td>
<td>2.7</td>
<td>2.8</td>
<td>104</td>
</tr>
<tr>
<td>9</td>
<td>9</td>
<td>7</td>
<td>2.2</td>
<td>2.6</td>
<td>77</td>
</tr>
<tr>
<td>Mean</td>
<td>18\textsuperscript{c}</td>
<td>16\textsuperscript{c}</td>
<td>2.7</td>
<td>2.6</td>
<td>95</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Half-life of labelled iron in plasma \( (t_{1/2}) = \text{log}_2/\text{elimination rate constant, } k.\)

\textsuperscript{b}I.v. recovery = \( m_{\text{i.v.}}/\text{dose}_{\text{i.v.}} \) (Eq. (1) in Subjects and methods section).

\textsuperscript{c}Geometric mean.
Estimation of iron absorption
B Sarria et al

Figure 1 Bland and Altman plot showing mean difference between methods (—) and 95% confidence interval (•••).

absorption of extrinsically labelled nonhaem iron from a test drink. For a new measurement technique to be accepted, it must be shown to perform as well as existing methods and to offer some advantages. Inspection of the Bland and Altman analysis (Figure 1) suggests that there is a small bias (mean difference = -1.8%) between the two methods for calculating iron absorption. This finding is reinforced by the geometric mean iron absorption values, 18% for the AUC method and 16% for the model. The underestimation in i.v. recovery (Table 1) also supports these observations and the reasons for this have already been discussed in an earlier publication (Dainty et al, 2003) and can be summarised as an oversimplified model structure that does not take account of labelled iron that disappears and then refuxes back into the plasma compartment during the experimental period. Despite these concerns, the model method appears to compare well with the AUC method although the width of the 95% confidence interval needs further discussion.

In their original paper (Bland & Altman, 1986), a key question was posed as to how much disagreement can be tolerated between ‘old’ and ‘new’ measurement techniques before a new method is considered to be acceptable. Using the model method in the present study, we may expect an estimate of iron absorption to be somewhere between 22 and 10% instead of the ‘true’ value of 18% as estimated by the AUC technique. In most nutritional studies, this would clearly be unacceptable and the model method would not be used. However, agreement between the two techniques would almost certainly have been improved with a larger sample size. For this kind of comparative study it is recommended that 50 data points be collected (Altman, 1991) and this would lead to a much more definitive statement regarding the agreement of the two methods. The promising nature of the results from this pilot study suggests that such an exercise should be undertaken because, while it is clear that the model method for predicting labelled iron absorption is not perfect, the method could be useful for unlabelled iron studies, where there is presently no method for quantifying the absorption of total iron from single test meals. Further work is needed to assess the quantity of unlabelled iron that would be needed in a test meal to ensure adequate characterisation of the plasma response curve, and to establish whether the method is applicable to a range of test meals with differing iron release properties.

Acknowledgements
We wish to thank John Eagles and Jurian Hoogewerff for processing the samples on the mass spectrometer and Treasa Nic Suibhne, Fiona O’Neill and Gosia Newman for help in the laboratory. Sources of support: Beatriz Sarria was funded by a European Union Marie Curie Fellowship. The other authors are funded by the Biotechnology and Biological Sciences Research Council (BBSRC), UK.

References
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 in vivo gastrointestinal passage of dairy products containing FBP, up to 43 % of the ingested FBP was found in ileostomal effluent. Folate absorption was similar for (6S)-5-CH₃-H₄folate fortificant from fermented milk and for folic acid from fortified bread. Folic acid, ingested as food fortificant in bread, was significantly less absorbed compared with an isolated supplement. We conclude that all tested foods were suitable matrices for folate fortification. However, dairy products, fortified with the new candidate fortificant (6S)-5-CH₃-H₄folate, are suitable if no active FBP is present.

(6S)-5-Methyltetrahydrofolate/folic acid: Folate-binding proteins: Fortified foods: Human folate absorption

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, AU(C)ₜₒ₋ₜ₂₀, area under the (plasma concentration) curve, superscript time range (in min); Cᵢₒ, plasma folate concentration, subscript defines time (in min); FBP, folate-binding proteins; (6S)-5-CH₃-H₄folate; (6S)-5-methyltetrahydrofolate; tᵢ, t₂₀, 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 proctocolectomy 12–37 years earlier as a result of ulcerative colitis with a maximal resection of 5–10 cm (except one volunteer: 25 cm) of the 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>Intramuscular injection</td>
<td>Injection solution (1 ml) prepared from (6S)-Ca-5-CH₃-H₄ folate (Merck Eprova AG, Schaffhausen, Switzerland) according to Witthöft et al. (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>Commercial fermented milk product (Filmjök®; Arla Foods, Stockholm, Sweden; 0.5% fat), addition of (6S)-Ca-5-CH₂-H₄ folate injection solution 30 min prior to consumption; 400 g portion.</td>
</tr>
<tr>
<td>U</td>
<td>Fermented milk</td>
<td>Commercial fermented milk product (Filmjök®; Arla Foods; 0.5% fat), addition of (6S)-Ca-5-CH₂-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>F</td>
<td>Fermented milk with FBP</td>
<td>Pasteurized skinned 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>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 (Jäma, 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-CH₂-H₄ folate acid/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-CH₂-H₄ folate, (6S)-5-methyltetrahydrofolate; FBP, folate-binding proteins.
* Concentrations in µg as free 5-CH₃-H₄folic acid.
† Range for n = 8, another = 433 µg/321 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 Jastrebova et al. (2003) using a HP 1100 series system equipped with a multi-wavelength detector and a fluorescence detector (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 (multi-wavelength 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 obtained. 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

FBP concentrations in dairy products and stomal effluent samples were determined by a two-site ELISA developed for milk according to Højjer-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 (C₅₆₀₀) were still above baseline concentrations (C₀), the AUC beyond t₅₆₀₀ to infinity (AUC₅₆₀₀−∞) 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:

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

\[
C = \frac{M}{VTk} \left( 1 - e^{-kT} \right) e^{-k(t - t_{lag})} \quad (t > t_{max}) \quad (2)
\]

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-CH$_3$-H$_4$folate curve (above C$_0$) over time, M can be calculated. For bread, folate absorption was estimated using the first-order absorption process using the Bateman function:

$$C = \frac{MK_a}{V(K_a - K_e)}(e^{-K_ate} - e^{-K_et})$$  \hspace{1cm} (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 $K_a$ and $K_e$ are rate constants of absorption and elimination, respectively. The apparent folate absorption was calculated according to: apparent absorption (%) = $100 \times M/Dose_{total}$.

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 error. Wilcoxon signed rank test was used to compare effects of treatments on log-transformed AUC–dose-corrected ratios. For details of treatments and procedures, see Table 1 and p. 182.

**Results**

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

After ingestion of test foods and pharmaceutical preparations containing 5-CH$_3$-H$_4$folate and folic acid, post-dose plasma 5-CH$_3$-H$_4$folate concentrations increased above fasted baseline levels, but no folic acid was detected. AUC–dose-corrected ratios after intramuscular injection of pharmaceutical (6S)-5-CH$_3$-H$_4$folate (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-CH$_3$-H$_4$folate concentrations over time was observed. Resulting AUC from $t_0$ to $t_{\text{end}}$ 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) ($P=0.0137$ and $P=0.0056$, respectively). Apparent folate absorption from bread (B) tends to be larger than from pasteurized milk (P) ($P=0.067$).

Only small quantities of intact 5-CH$_3$-H$_4$folate from below 1 to 20 µg were excreted into urine during 10 h post-dose (data not shown). Highest amounts of intact 5-CH$_3$-H$_4$folate 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-CH$_3$-H$_4$folate, 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-CH$_3$-H$_4$folate (1.6–6.0 µg/10 h) were excreted, being in the same magnitude as absolute 5-CH$_3$-H$_4$folate excretion after intramuscular injection (I) (0.7–11.2 µg/10 h) and after ingestion of folic acid-fortified bread (B) (1.7–15.4 µg/10 h, n 8, for one volunteer peak masked). Relative 5-CH$_3$-H$_4$folate excretion increased significantly after ingestion of all test foods containing 5-CH$_3$-H$_4$folate (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-CH$_3$-H$_4$folate) after absorption of 5-CH$_3$-H$_4$folate and folic acid from test foods**

(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>0.099$^a$</td>
<td>0.034–0.154</td>
</tr>
<tr>
<td>O</td>
<td>0.065$^d$</td>
<td>0.033–0.121</td>
</tr>
<tr>
<td>C</td>
<td>0.075$^d$</td>
<td>0.023–0.094</td>
</tr>
<tr>
<td>U</td>
<td>0.055$^a$</td>
<td>0.027–0.087</td>
</tr>
<tr>
<td>F</td>
<td>0.030$^e$</td>
<td>0.015–0.078</td>
</tr>
<tr>
<td>P</td>
<td>0.020$^a$</td>
<td>0.014–0.035</td>
</tr>
<tr>
<td>B</td>
<td>0.039$^e$</td>
<td>0.016–0.052</td>
</tr>
<tr>
<td>Y</td>
<td>0.143$^a$</td>
<td>0.056–0.177</td>
</tr>
</tbody>
</table>

$^a$ For details of treatments and procedures, see Table 1 and p. 182.

**Table 3. Apparent 5-methyltetrahydrofolate (5-CH$_3$-H$_4$folate) 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>86$^a$</td>
</tr>
<tr>
<td>F</td>
<td>62$^{ab}$</td>
</tr>
<tr>
<td>P</td>
<td>55$^a$</td>
</tr>
<tr>
<td>B</td>
<td>74$^{ab}$</td>
</tr>
<tr>
<td>Y</td>
<td>80$^a$</td>
</tr>
</tbody>
</table>

$^a$ For details of treatments and procedures, see Table 1 and p. 182.

$^a$ Median values with unlike supersetcript letters were significantly different ($P<0.05$) (Tukey pair-wise comparison among treatments on log-transformed apparent folate absorption).
being significantly higher on days P and Y compared with day U (P=0.0214 and P=0.0371, 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 2).

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 than 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 folic acid 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 matrices. Median AUC–dose-corrected ratios are significantly reduced in the presence of FBP (Table 2). Also apparent 5-CH3-H4folate absorption from pasteurized milk with FBP (P) is smaller than from fermented milk without FBP (U) (Table 3) and conversely relative folate excretion with ileostomal effluent is higher for P than U (Table 4). The present findings suggest that FBP is reducing folate absorption. Folate is better absorbed from fermented milk than from pasteurized milk due to the absence of native FBP.

The present study is the first to prove intestinal ‘survival’ of FBP in man. Using in vitro methods simulating the upper human intestinal tract, Verwei et al. (2003) and Arkbäge et al. (2003) reported that between 0 and 34 % of FBP from the dose was recovered after ‘digestion’ of milk and yoghurt fortified with (6S)-5-CH3-H4folate or folic acid. Interestingly, this reflects our findings in vivo with FBP survival (Table 5).
Arkåge 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. Of interest is 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 ‘biofortification’. 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 (Finglas 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.

**Acknowledgements**

We are most grateful to the volunteers for their enthusiastic participation in the study. The project was funded by the European Union under Key Action 1: Food Nutrition and Health (QLK1-1999-00 576). The gift of (6S)-Ca-5-methyltetrahydrofolate and folic acid pharmaceutical preparations from Merck Eprova AG, Schaffhausen, Switzerland, the preparation of some dairy products by Arla Foods, Stockholm, Sweden and
References


Quantification of the bioavailability of riboflavin from foods by use of stable-isotope labels and kinetic modeling

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ABSTRACT

Background: Discrepancies have been reported between estimates of the prevalence of riboflavin deficiency based on intakes of riboflavin and estimates based on measures of riboflavin status. One reason for this may be an overestimate of the bioavailability of riboflavin from foods, about which relatively little is known.

Objective: We aimed to quantify the bioavailability of riboflavin from milk and spinach by using stable-isotope labels and a urinary monitoring technique and by a plasma appearance method based on kinetic modeling.

Design: Twenty healthy women aged 18–65 y were recruited for a randomized crossover study performed with extrinsically labeled (13C) milk and intrinsically labeled (15N) spinach as sources of riboflavin. An intravenous bolus of labeled riboflavin was administered with each test meal to assess the apparent volume of distribution of riboflavin in plasma.

Results: No significant differences were noted in riboflavin absorption from the spinach meal and from the milk meal according to either the urinary monitoring technique (60 ± 8.0% and 67 ± 5.4%, respectively; P = 0.549) or the plasma appearance method (20 ± 2.8% and 23 ± 5.3%, respectively; P = 0.670).

Conclusions: A large fraction of newly absorbed riboflavin is removed by the liver on “first pass.” The plasma appearance method therefore underestimates riboflavin bioavailability and should not be used to estimate riboflavin bioavailability from foodstuffs. Urinary monitoring suggests that riboflavin from spinach is as bioavailable as is riboflavin from milk.

KEY WORDS Intrinsic label, stable isotopes, compartmental models, absorption, riboflavin, riboflavin bioavailability

INTRODUCTION

Recent national surveys of the dietary intake and nutritional status of various groups in the United Kingdom have reported some discrepancy between the estimated intake of riboflavin in the diet and measures of riboflavin status. The National Diet and Nutrition Survey of British Adults (1) reported biochemical riboflavin deficiency in between 54% and 80% of the population, depending on age, whereas the National Diet and Nutrition Survey of Young People aged 4–18 y (2) reported biochemical riboflavin deficiency in 95% of adolescent girls. In view of the evident interaction between riboflavin status and iron handling (3), this may be significant for the 50% of 15–18-y-old girls with iron intakes less than the lower reference nutrient intake (mean − 2 SDs). It has been suggested that a high prevalence of biochemical riboflavin deficiency in the elderly may reflect a less efficient absorption of riboflavin with increasing age, but this has not been substantiated (4, 5). In all cases, calculated dietary intakes relative to dietary reference values gave lower estimates of riboflavin deficiency than did biochemical data. These discrepancies suggest either an overestimate of bioavailability or an inappropriate biochemical threshold for deficiency.

Published mechanistic studies of riboflavin absorption have used the pure free compound in experimental systems in vitro (6, 7). Although these studies provide mechanistic insight into the absorption of riboflavin, they do not address questions relating to availability from foods, in which this vitamin occurs predominantly as the phosphorylated derivatives FMN and FAD. No data suggest that the nature of the food matrix limits riboflavin absorption, although milk and eggs contain free riboflavin bound to specific binding proteins, which is in contrast with most foodstuffs, which contain predominantly FAD and riboflavin phosphate bound tightly to enzymes.

A previous study of riboflavin bioavailability used doses of riboflavin many times greater than usual dietary intakes (8). In the present study, we determined the bioavailability of riboflavin from foods in human subjects by using stable isotopes. Riboflavin was synthesized with different labels (milk; 13C; spinach; 15N), which allowed us to administer physiologic doses (<0.5 mg) and trace the absorbed riboflavin in plasma and urine. It is well known in trace element work that the dual-stable-isotope approach can estimate the absorption of a labeled dose from plasma or urine samples (9). This approach can be complemented by mathematical modeling, as recently applied to folate metabolism (10–12), which yields several important kinetic parameters, including the riboflavin pool size, volume of distribution, rate of elimination, and mean residence time in the plasma.

SUBJECTS AND METHODS

Subjects

Twenty female volunteers (aged 18–65 y) were recruited from the Sheffield, United Kingdom, area. Exclusion criteria were

1 From the Institute of Food Research, Norwich, United Kingdom (JRD, DJH, and PMF); the Human Nutrition Unit, University of Sheffield, Sheffield, United Kingdom (NRB, RT, and HJP); and the Biomedical Research Centre, Sheffield Hallam University, Sheffield, United Kingdom (ATH). 2 Supported by the Biotechnology and Biological Sciences Research Council (BBSRC). 3 Reprints not available. Address correspondence to H Powers, Human Nutrition Unit, University of Sheffield, Sheffield, United Kingdom. E-mail: h.powers@sheffield.ac.uk. Received October 30, 2006. Accepted for publication January 19, 2007.
smoking, history of cardiovascular disease, use of vitamin supplements, use of drugs known to interfere with folate or riboflavin metabolism, and pregnancy. Although all subjects completed the study, data sets from 3 volunteers were excluded from the article because of a failure of the mass spectrometer and the loss of the samples. The subjects did not differ significantly from those whose data were analyzed, in terms of either demographic characteristics or indexes of riboflavin status. Partial data sets are those whose data were analyzed, in terms of either demographic characteristics or indexes of riboflavin status. Partial data sets are indicated in the tables, figures, and results.

Ethical considerations

The study protocol was explained in group interviews, and detailed written information was provided. Each participant was asked to give written informed consent to both screening procedures as well as study participation. Ethics approval for this study was obtained from the Sheffield University Ethics Committee.

Study design

Each subject was admitted to the study unit on 2 occasions, 4 wk apart, in a randomized crossover design. Twenty-four–hour urine samples were collected for 3 d over the study, beginning 24 h before the test dose. Participants received the following test doses: 400 \( \mu\)g \(^{13}\text{C}\)-labeled riboflavin in 200 mL semi-skimmed milk (fat content 1.7 g/100 mL) with a simultaneous intravenous injection of 200 \( \mu\)g \(^{13}\text{C}\)-labeled riboflavin or, 308 \( \mu\)g labeled total flavins as \(^{15}\text{N}\)-labeled spinach soup with a simultaneous intravenous injection of 200 \( \mu\)g \(^{13}\text{C}\)-labeled riboflavin.

Test meal and blood sampling protocol

On the morning of the study, a 10-mL baseline blood sample was taken (0 h) after the subjects had fasted overnight fast and immediately before riboflavin administration. The intravenous vitamin was given as a bolus into a forearm vein within 2 min. This was followed immediately by oral administration of either the spinach soup or milk. Next, 10-mL blood samples were withdrawn from a permanent cannula in the opposite arm at 10, 15, 20, 25, 30, 40, 60, and 80 min and thereafter at 2.0, 2.5, 3.0, 3.5, 4.0, 5.0, 6.0, and 7.0 h.

Blood samples were centrifuged immediately at 2000 \( \times \) \( g \) for 5 min at 4 °C. Plasma was removed by aspiration and divided into aliquots for storage at –80 °C. The remaining cells were washed with an equal volume of ice-cold 1 mol phosphate-buffered saline/L (pH 7.0) and centrifuged for 5 min at 2000 \( \times \) \( g \) at 4 °C. The supernatant fluid and theuffy coat and cell debris were discarded. A 100-\( \mu\)L volume of cells was added to 100 \( \mu\)L sterile, distilled water, and the tubes were mixed briefly to hemolyse the cells before storage at –80 °C for the later measurement of erythrocyte glutathione reductase activation coefficient (EGRAC). Between the blood samples at 3.0 and 4.0 h, all subjects were given a low-riboflavin lunch consisting of canned vegetable soup and 2 slices of white, unfortified bread, with a total riboflavin content of 0.06 mg.

Dose preparation and administration

Synthesizing the \(^{13}\text{C}\)-labeled riboflavin

\([2,4,6-{^{13}\text{C}} \text{C}]\text{Barbituric acid. This was prepared from } [^{13}\text{C}]\text{urea and } [1,3-{^{13}\text{C}} \text{C}_2]\text{diethyl malonate according to the literature procedure (13).}\]

\([2,4,10\alpha-{^{13}\text{C}} \text{C}]\text{Riboflavin and } [1',2',3',4',4',5',10\alpha-{^{13}\text{C}} \text{C}]\text{riboflavin. These were prepared from the labeled barbituric acid and either unlabeled ribose or } {^{13}\text{C}}\text{C}_6\text{ribose according to the following procedure, which is essentially that previously described in the literature (14). The identity and purity of the products was initially determined by absorbance measurements at 267 nm, 373 nm, and 444 nm and the ratios 373/267 and 444/267. Further analysis of the products by HPLC with fluorescence detection (15) showed no measurable contaminants.}\]

The purity of the samples was confirmed by thin-layer chromatography on silica gel in 2 solvent systems: 1) chloriform: acetone:concentrated ammonium, 2:2:1, RF 0.35, and 2) butanol: ethanol:water, 7:2:1, RF 0.30. The purity of the synthetic material was confirmed by the appearance of a single spot with the same RF as authentic riboflavin. Electrospray mass spectrometry showed a major peak for [M\(^+\)Na\(^+\)] in each case at mass-to-charge ratios (m/z) of 399 (Na adduct of natural abundance riboflavin), 402 (Na adduct of \([^{13}\text{C}]\text{riboflavin}), and 407 (Na adduct of \([^{13}\text{C}_6]\text{riboflavin) respectively. Channels (m/z) at 400, 401, 403, 404, 405, 406, and 408 were also monitored, and total peaks were \(<2\% of the major peak. It was concluded that the doses were \approx98\% of the desired isotope.}\]

Preparing the intravenous \(^{14}\text{C}\)-labeled dose

\(^{14}\text{C}\)-Labeled riboflavin prepared in phosphate-buffered saline, pH 7.4, underwent sterility testing with a British Pharmacopoeia membrane filtration method and was dispensed in 4.0-mL aliquots into brown glass vials for intravenous injection. All procedures were carried out by the Pharmacy Department of the Royal Hallamshire Hospital, Sheffield, United Kingdom.

Preparing the extrinsically labeled \(^{13}\text{C}\) milk dose

A total of 12 mg \(^{13}\text{C}\)-labeled riboflavin was added to 6 L semi-skimmed milk. The mixture was stirred magnetically for 1 h at room temperature to ensure thorough homogenization. The milk was then frozen at –20 °C in 200-mL aliquots (equivalent to 400 \( \mu\)g \(^{13}\text{C}\)-labeled riboflavin). Samples were defrosted overnight at 4 °C before use. The unlabeled riboflavin content of the milk was measured as 1.67 \( \mu\)g/mL. FAD and FMN constituted 0.40 \( \mu\)g/mL (see “Blood sample flavin analysis” below), and each test meal consisted of 200 mL milk. Each volunteer therefore consumed 334 \( \mu\)g unlabeled and 400 \( \mu\)g labeled riboflavin from the milk dose.

Preparing the intrinsically labeled \(^{15}\text{N}\) spinach dose

Spinach plants were grown hydroponically. Primed seeds (var. Ballet) were sown in 9-cm pots, 2 to a pot, in a support of vermiculite and pearlite (50:50) in a greenhouse. Temperature was partially controlled by automatic ventilation (opening at 18 °C) and by applying a shade coating to the glass (Coolglass; PBI Agrochemicals, Waltham Abbey, United Kingdom). Initially, the pots were placed in the hydroponic unit, watered with unlabeled nutrient solution, and covered with a polythene sheet.
until germination. When the plants had obtained 2 leaves, the weaker plant in each pot was removed. Labeled \(^{15}N\) nutrient was then provided by using a nutrient flow technique. Nutrient solution flowed through the trays from a header tank to a sump tank and was returned by a pump. Air was bubbled through the solution in the header tank by an air pump (Fisher Scientific, Loughborough, United Kingdom).

The crop was harvested after 56 days (more detail of the procedure can be found in a previous publication; 16), yielding 4.55 kg of leaf material. The \(^{15}N\)-labeled spinach was washed, dried, mixed, and frozen at –20 °C for shipment to Sheffield University for processing into soup within 7 days. After being defrosted, the spinach leaves were finely chopped with a food processor. To make the soup, 42.5 g fresh clove garlic (crushed) and 850 g finely chopped white onions were heated in 175 mL olive oil. All of the chopped spinach was added to this, along with 850 mL reconstituted vegetable stock (from powder). The mixture was heated briefly for 2 min until warmed (approx 60 °C) throughout and was then divided into 283-g portions and stored at –20 °C in sealed containers. Each portion contained 202 g wet weight spinach. Before the day of the study, the spinach soup was defrosted overnight. A total of 200 mL semi-skimmed milk was added, to be comparable with that used in the milk dose. This mixture was heated briefly to serving temperature (≈85 °C) immediately before consumption. A portion of soup thus consisted of 200 g raw spinach cooked and blended with 200 mL milk.

**Spinach flavin analysis**

Extraction and enzymic deconjugation of riboflavin in spinach was carried out, followed by determination of flavin content by HPLC. The detailed procedure can be found in an earlier publication (16). The riboflavin content was 0.86 μg/g raw spinach, and the FMN content was 0.68 μg/g raw spinach. The riboflavin content of the milk was measured as 1.67 μg/mL. Therefore, for every portion of soup, each volunteer consumed 334 μg unlabeled riboflavin (200 mL of semi-skimmed milk) and 308 μg \(^{15}N\)-labeled total flavins (free riboflavin + FMN) from the spinach.

**Blood sample flavin analysis**

Unlabeled

**Plasma flavin concentrations.** Plasma samples were analyzed for flavin content (FAD, FMN, and riboflavin) by HPLC by using a method modified from a previous publication (15). Briefly, 150 μL chilled 10% trichloroacetic acid was added to 300 μL plasma or standards. Precipitated protein was removed by centrifugation at 20,780 × g for 5 min. A total of 100 μL of the supernatant fluid was injected onto an HPLC column [PLRP-S, 100A, 5 μmol/L, 250 × 4.6 mm (internal diameter); Polymer Laboratories, United Kingdom] in accordance with the method described previously (17).

Red blood cell erythrocyte glutathione reductase activation coefficient. The EGRAC assay was performed on lysed red blood cells (18) as a measure of functional riboflavin status. The assay is a measure of the degree of unsaturation of glutathione reductase with FAD, such that the greater the value for the activation coefficient, the less the enzyme is saturated with its cofactor FAD and the poorer the riboflavin status.

Labeled

**Samples.** Samples were prepared for flavin isotope ratio determination by HPLC/electrospray ionization + mass spectrometry in batches of one volunteer day. Frozen plasma samples were thawed and mixed. An amount of 2 mL (or as much as was available, if less than this) was placed in a centrifuge tube and was mixed by vortexing while 1 mL (or one-half the plasma volume) of water was added, followed by 1 mL (or one-half the plasma volume) of 10% trichloroacetic acid solution, and the tube was mixed thoroughly to precipitate proteins. Samples were then centrifuged and the supernatant fluid was placed into 30 mg/1 mL Strata-X solid-phase extraction cartridges (Strata-X is a surface-modified styrene divinylbenzene polymer from Phenomenex UK, Macclesfield, United Kingdom). Columns were first conditioned with 2 mL methanol and 2 mL water. The supernatant fluid was then added, and the columns were washed with 2 × 2-mL water washes and sucked dry. Flavins were eluted with 2 × 500-μL portions of 1:1 methanol:water and were collected directly into an autosampling vial. The flow rate was regulated at 500 μL/min by drawing the sample through the columns with a peristaltic pump (Watson Marlow Bredel, Falmouth, United Kingdom).

**HPLC–mass spectrometry**

HPLC separation was carried out by using a Jasco 1500 system (Jasco UK Ltd, Great Dunmow, United Kingdom) according to a previously published method (19). Briefly, the mobile phase consisted of 65:35 water:methanol, and an isotropic flow rate of 0.80 mL/min into a 4 × 250 mm Chromosorb RP18 column (VWR International Ltd, Lutterworth, United Kingdom) was maintained at 30 °C. The flow was split post-column in a 5:1 ratio by using an ASI 600 fixed ratio splitter (Presearch, Hitchin, United Kingdom), with the higher flow being monitored by a fluorescence detector (excitation wavelength = 450 nm, emission wavelength = 510 nm), and the lower flow going to the mass spectrometer.

Mass spectra were obtained by using a Micromass Quattro II (Waters/Micromass, Manchester, United Kingdom) in positive ion electrospray mode with a Z-spray ion source. Selected ion monitoring liquid chromatograph–mass spectrometry chromatograms were recorded by monitoring the sodium adducts of the riboflavin isotopomers at m/z 399 (M), 400 (M + 1), 401 (M + 2 – spinach oral dose), 402 (M + 3 – milk oral dose), 403 (M + 4), and 407 (M + 8 – intravenous dose) with a dwell time of 0.1 s per mass and a cycle time of 0.03 s. Spectra and chromatograms were processed with MassLynx software [version 3.4; Micromass Ltd (Waters Group), Manchester, United Kingdom].

**Urinary flavins were measured by use of the HPLC method described for the plasma samples.**

**Labeled**

Frozen urine samples were thawed and shaken. A 1-mL aliquot of urine was applied directly to a preconditioned Strata-X column, and the same procedure as for plasma was followed throughout.
“True” mass absorbed from gut
Liver
“Apparent” mass M appears in plasma at rate R in time T
Liver “first-pass” effect, where a large fraction of riboflavin is removed

Riboflavin concentration, C
Volume of distribution, V

k_{1,2}
k_{2,1}

k_{0,1}
rate constant of elimination to other tissues or excretion

FIGURE 1. Circles represent compartments, and arrows represent transfer of riboflavin from one compartment to another or out of the system. Compartment 1 was the accessible (sampled) compartment and is associated with the volume of distribution (V).

Kinetic data analysis

Urinary monitoring

The definition of the true absorption of a nutrient is that fraction of the ingested nutrient that is absorbed across the gastrointestinal tract and not eliminated (eg, via the biliary tract) back into the gastrointestinal tract for removal as part of fecal waste. For most minerals and vitamins, there is some elimination via the bile, and therefore a measure of true absorption needs to take account of this. By giving a simultaneous intravenous label with an oral label and measuring the dose-corrected ratio of the appearance of the 2 labels in urine, true absorption can be quantified. The main assumption behind this is that the 2 labels are excreted in urine (and via the bile) at the same rate. Therefore, riboflavin “true” absorption is estimated from the dose-corrected ratio of labeled riboflavin from the oral label (15C-milk or 15N-spinach) to that from the intravenous (IV) label that appears in the urine in the 48 h after dosing.

True absorption (abstrue) = \( \sum_{0}^{t=48h} \) oral label in urine/dose of oral label \( \times \) (dose of IV label/ \( \sum_{0}^{t=48h} \) IV label in urine) \( (I) \)

Compartmental model

Isotopic data from plasma analysis were analyzed by using the SAAMII program (SAAM Institute Inc, Seattle, WA; 20) and the compartmental model (Figure 1).

Definitions. The compartments represent discrete amounts of riboflavin that behave identically. A compartment is a theoretical construct that may in fact combine material from several different physical spaces in a system. A model can be viewed as a construct that may in fact combine material from several different physical spaces in a system. A model can be viewed as a hypothesis to be tested against experimental data, and the structure of the model is then altered until a satisfactory fit to the data occurs. The accessible compartment in our system was compartment 1, which represents the plasma. Transfer of riboflavin between compartments \([k(i, j), \text{fraction/time}]\) is defined as the fraction of compartment \(j\) moving into compartment \(i\) per unit time.

Data fitting. One parameter, the apparent volume of distribution of the accessible compartment \((V)\) was proportional to the volunteers’ body weight and held constant during the fitting process (see below). The other parameters \([k(0,1), k(1,2), \text{and } k(2,1)]\) were given initial estimates consistent with published data on human riboflavin metabolism. During the fitting process, the parameters are allowed to vary until a minimum of the objective function is reached. The software then returns the mean and SD of the parameters. Only the labeled riboflavin concentration in the plasma was used in the fitting process. The final model structure was arrived at by a process of trial and error but with the guiding principle that it must contain the fewest compartments to adequately describe the data (Principle of Parsimony). The final model parameters are uniquely identifiable, which means that they have one solution only.

Modeling the absorptive process. The appearance of riboflavin from the gut into the plasma is assumed to be zero order and can be modeled as a constant infusion of rate \(R\) over time \(T\). The rate \((R)\) is defined as \(\text{MIT, where } M\) is the apparent mass of riboflavin seen in the plasma from the oral dose. The time \((T)\) is defined as the time to peak riboflavin concentration from the time that labeled riboflavin first appears in the plasma. The apparent mass of riboflavin seen in the plasma from the oral dose \((M)\) is one of the parameters that is estimated in the fitting process and therefore allows the apparent absorption of the oral dose to be estimated from the plasma data.

Apparent absorption \((\text{absapparent}) = \frac{M}{\text{dose of oral label}} \) \( (2) \)

First-pass effect. This is defined as the fraction of newly absorbed riboflavin that is removed by the liver on the “first pass” before it enters the systemic blood system. From Equations 1 and 2, the first-pass effect can be estimated as

First-pass effect = \(1 - \left(\frac{\text{absapparent}}{\text{abstrue}}\right)\) \( (3) \)

Volume of distribution in the compartment \((V)\). The change in plasma concentration of the riboflavin from the intravenous dose was fitted to the compartmental model, and all parameters were

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<th>Age</th>
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</table>

\( n = 17\) subjects for whom data were available for the kinetic analysis.
Biomarkers of riboflavin status (plasma flavin concentrations) at baseline

<table>
<thead>
<tr>
<th></th>
<th>Visit 1</th>
<th></th>
<th>Visit 2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 17)</td>
<td></td>
<td>(n = 17)</td>
</tr>
<tr>
<td></td>
<td>EGRAC</td>
<td>Riboflavin</td>
<td>FAD</td>
</tr>
<tr>
<td>Mean</td>
<td>1.36</td>
<td>10.51</td>
<td>45.59</td>
</tr>
<tr>
<td>SEM</td>
<td>0.04</td>
<td>1.42</td>
<td>1.78</td>
</tr>
<tr>
<td>Minimum</td>
<td>1.17</td>
<td>3.73</td>
<td>35.65</td>
</tr>
<tr>
<td>Maximum</td>
<td>1.59</td>
<td>22.92</td>
<td>60.71</td>
</tr>
</tbody>
</table>

\(^1\) EGRAC, erythrocyte glutathione reductase activation coefficient.
\(^2\) Significantly different from visit 1, \(P < 0.05\) (ANOVA).

allowed to vary freely. By pooling the estimates from all the volunteers, the volume of distribution in the accessible compartment (\(V\)) was calculated to be 549 ± 284 mL/kg body wt.

Statistics

Statistical analysis was performed by using the statistical package R (21). Analysis of variance (ANOVA) models in conjunction with Tukey’s highly significant difference post hoc test were used to analyze the data. Initial models were set up to include the potential explanatory variables and all possible two-way interactions; backwards elimination was then used to reduce the model to one consisting only of terms with a significant effect on the response. Model diagnostics were checked and, if necessary, remedial action was taken (outlier exclusion, data transformation, or nonparametric modeling). Repeated-measures models were also tried. These were primarily so that the interaction between food and visit could be examined. When the interaction was not present, the repeated-measures models are similar to the ANOVA model and so usually only the ANOVA model is presented.

All data in the text are presented as means ± SDs. Data points in figures are means ± SEMs unless stated otherwise in the figure caption. Differences were considered significant at \(P < 0.05\).

RESULTS

The 20 subjects recruited were between 21 and 60 y of age. Descriptive data for the 17 subjects whose data were included in the analyses are shown in Table 1. Riboflavin status measurements at baseline for volunteers at each of the 2 occasions are shown in Table 2. Mean EGRAC values were significantly higher (\(P < 0.05\)) at visit 2, and plasma FAD values were significantly lower, which suggests a moderate decline in riboflavin status over the 4-wk study even though the subjects received the test meals in random order and the samples were analyzed randomly. Nevertheless, median EGRAC values at both visits were close to those reported in the recent National Diet and Nutrition Survey of UK adults, which suggests that this cohort was representative of the population with respect to riboflavin status.

The variation in urinary excretion of total (labeled + unlabeled) flavins is shown in Table 3. There was no significant difference for any flavin within or between test meals across the time periods. In our analyses, ≈70% of total excretion was made up of free riboflavin, with the remainder being mostly FAD.

The volunteers excreted 30 ± 1.59 \(\mu g\) (\(n = 32\), data not tabulated) of the labeled riboflavin from the intravenous dose in the urine in the first 24 h, which constituted 15% of the original 200-\(\mu g\) dose. No trace of labeled riboflavin from the intravenous dose was found in urine after 24 h. The excretion of labeled riboflavin from the spinach was 3214 \(\mu g\) (10.4%; \(n = 14\)) and that from milk was 38 ± 4.90 \(\mu g\) (9.5%; \(n = 15\)) over the 48 h after dosing. Most (>75%) was excreted in the first 24-h period.

The total (labeled + unlabeled) plasma concentrations of FMN, FAD, and riboflavin are shown in Figure 2. The riboflavin concentration increased markedly after both milk and spinach test meals, and the peak plasma concentration occurred at 8–12 min after the milk dose and 42 min after the spinach dose. Neither the milk nor the spinach test meal elicited a clear response in plasma concentration of FAD or FMN. The small fluctuations in concentrations of these metabolites over the 420 min after the test meals are also shown in Figure 2.

Absorption and kinetic parameters are shown in Table 4. There was no significant difference in riboflavin true absorption (\(P = 0.549\)) between the spinach meal (60 ± 8.0%) and the milk meal (67 ± 5.4%) according to the urinary monitoring technique. The modeled plasma data also indicated that there was no significant difference in riboflavin apparent absorption (\(P = 0.670\))

Table 3

Total (labeled + unlabeled) flavins in urine

<table>
<thead>
<tr>
<th>Test meal</th>
<th>FAD (n = 17)</th>
<th>FMN (n = 17)</th>
<th>Riboflavin (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−24 h</td>
<td>0–24 h</td>
<td>24–48 h</td>
</tr>
<tr>
<td>Milk</td>
<td>334 ± 29.37</td>
<td>381 ± 40.78</td>
<td>366 ± 37.38</td>
</tr>
<tr>
<td>Spinach</td>
<td>373 ± 35.7</td>
<td>371 ± 31.6</td>
<td>365 ± 26.9</td>
</tr>
</tbody>
</table>

\(^1\) All values are \(\bar{x} ±SEM\). No significant differences in flavins were found at any time point for any test meal (ANOVA).
between the spinach meal (20 ± 2.8%) and the milk meal (23 ± 5.3%). The peak concentration of labeled riboflavin in the plasma ($C_{\text{max}}$) was not significantly different ($P = 0.536$) after the milk (1.5 ± 0.2 ng/mL) and spinach (1.3 ± 0.2 ng/mL) meals. Furthermore, there was no significant difference ($P = 0.497$; data not tabulated) in the fraction (first-pass effect) of newly absorbed riboflavin from the milk meal (0.66 ± 0.06) or the spinach meal (0.57 ± 0.08) that was removed by the liver on the first pass before entering the systemic blood system. The time to maximum plasma concentration ($T_{\text{max}}$) was significantly longer ($P = 0.026$) after the spinach meal (34 ± 2.3 min) than after the milk (24 ± 2.5 min), but there was no significant difference ($P = 0.435$) in the time taken (delay) for labeled riboflavin to first appear in the plasma (spinach: 11 ± 1.3 min; milk: 10 ± 1.3 min).

The predicted plasma riboflavin concentrations with use of the 2-compartment model are shown in Figure 3 for the intravenous data. There was a rapid decline in riboflavin concentration from the intravenous dose over the first 20 min, and after 3 h the label was barely measurable. Rate constants from the 2-compartment model (data not tabulated) were $k(0,1): 234.7 ± 71.4 \text{ d}^{-1}$; $k(1,2): 54.5 ± 8.6 \text{ d}^{-1}$; and $k(2,1): 102.4 ± 17.3 \text{ d}^{-1}$.

The predicted plasma riboflavin concentrations from the 2-compartment model are shown in Figure 4 for the test meal data. Rate constants from the 2-compartment model differed between test meals (spinach versus milk) as $k(0,1): 18.5 ± 3.6$ compared with $36.7 ± 8.4 \text{ d}^{-1}$ ($P = 0.076$); $k(1,2): 40.7 ± 22.9$ compared with $25.3 ± 8.4 \text{ d}^{-1}$ ($P = 0.959$); and $k(2,1): 20.0 ± 5.4$ compared with $55.3 ± 14.3 \text{ d}^{-1}$ ($P = 0.041$). The volume of distribution ($V$) of riboflavin in the sampled (plasma) compartment was estimated to be $549 ± 71$ mL/kg body wt, which is equivalent to ≈35 L for a subject with a body mass 64 kg.

**DISCUSSION**

The kinetics of riboflavin have been investigated before, but the present study is unique for several reasons. This was the first study to use stable-isotope labels, which allows small, physiologic doses to be administered. A recently published study used very high, nonphysiologic doses of riboflavin, up to 60 times what is usually found in meals (8). The use of unlabeled riboflavin necessitated correction for baseline plasma and urinary riboflavin, which introduces uncertainty, especially because riboflavin is prone to changes in plasma concentration as the result of circadian rhythms. The use of foods is also unique, and the labeling of the spinach and milk meant that the labeled riboflavin from these meals would be handled identically to the unlabeled vitamin. This is important when it comes to interpreting the results and extrapolating them to other foodstuffs.

Milk represents an important source of riboflavin in Western diets. In the United Kingdom, for example, milk and milk products are estimated to provide about one-third of the dietary riboflavin in the adult population (1, 2). Because the riboflavin in milk is present predominantly in the free form, it could be assumed that riboflavin in milk would be highly bioavailable. The results from this study suggest that riboflavin from spinach is absorbed to the same extent, and this could have important implications for public health messages for good sources of riboflavin in the diet. The urinary monitoring technique is a gold standard method in mineral research for measuring bioavailability (22) and gives an estimate of so-called true absorption (see Eq 1). The method works from the premise that the route of administration of riboflavin (oral or intravenous) is immaterial because the body does not discriminate between riboflavin administered by either route, in terms of excretion via the urine, as long as there are no differences in the chemical form in which the vitamin is transported in the plasma. This is generally true for most
minerals, but some evidence suggests that more care is needed for certain vitamins. For example, the form of folate that appears in the plasma after an oral dose is 5-methyltetrahydrofolate and not folic acid, which is the form that was used as the intravenous dose in some previous studies (23, 24). We were careful to ensure that our intravenous dose contained only free riboflavin, because this is the predominant form that appears in the plasma from an oral dose immediately after absorption (8). This was also confirmed in our study from inspection of the change in concentration of total riboflavin, FMN, and FAD in Figure 2 and the lack of any labeled FMN and FAD in the plasma after the milk and spinach test meals (data not shown). In this study, most of the flavins excreted in the urine occurred as riboflavin, the remainder being mainly FAD. Other flavin metabolites have been characterized in urine, and various estimates have been made of the contribution they make to urinary flavins (25, 26), but uncertainty still exists as to the relative concentrations of such metabolites that are not of microbial origin, especially under conditions of physiologic intakes of riboflavin.

The apparent riboflavin absorption was estimated from the plasma appearance of the oral dose by using the model shown in Figure 1 and Eq 2. The principles behind this approach are similar to previous work in folate (10, 11, 27) and iron (28, 29) modeling. The apparent absorption of the riboflavin test doses is lower than the true absorption estimated by the urinary monitoring method and the reasons for this will be discussed later. The apparent absorption of riboflavin from spinach and milk is not statistically different, which supports the conclusions drawn from the urinary monitoring method.

Only some of the absorbed riboflavin appears in the plasma, and it is likely that the remainder is sequestered by the liver on the first pass through the portal vein from the gut (see Eq 3). This first-pass effect has also been seen in human studies of folate (11), vitamin C (30), and vitamin B-6 (31) and in an animal study of biotin (32). This suggests that it is a common feature of water-soluble vitamins and plays some important role in homeostasis and metabolism. Interestingly, the folate and vitamin C articles (11, 30) also reported a large release of unlabeled folate and vitamin C (respectively) into the plasma after a labeled test dose. This has implications for those studies that try to interpret the plasma response from large unlabeled doses of water-soluble vitamins to make predictions about absorption. Our study design does not allow us to test whether riboflavin follows the same pattern, but because we are quantifying absorption by using labeled test doses, it does not detract from our conclusions.

The delay in first appearance in plasma was not significantly different between the milk (10 ± 1.3 min) and the spinach (11 ± 1.3 min), but the time to peak plasma concentration was different, with the riboflavin from the milk peaking earlier (24 ± 12.5 min) than that from the spinach (35 ± 12.3 min). This was despite the fact that more riboflavin from the milk (93 ± 21 mg) than from the spinach (63 ± 8.5 mg) entered the plasma pool. The implication from this is that the riboflavin from the milk was absorbed faster across the gut wall than that from the spinach, but this was
unlikely to have been due to the more complicated food matrix present in the soup because it was thoroughly homogenized and of the same consistency as the milk. Instead, the greater delay in peak time was likely due to the need for the gut wall to convert the large fraction of FMN in the spinach into free riboflavin for transport in the plasma.

Although it is unlikely that the conversion of spinach FMN to riboflavin happened in any place other than the gut wall, it is possible that some conversion occurs in the liver. Differences in rate constants between the milk and spinach data indicate that riboflavin from milk and spinach are handled differently after being absorbed. This could just be due to the time delay in conversion at the gut wall, but it is impossible to be certain without taking blood samples from the hepatic portal vein shortly after consumption of the test doses. However, the first-pass effects were no different after the spinach and milk meals, which indicates that the liver receives the same form of riboflavin after each meal, thereby providing further support for the gut wall being the major site of FMN conversion to riboflavin.

Thus, the results of the present study suggest that an average of 60–65% of flavins are absorbed from milk or spinach, that no significant differences exists in the handling of flavins from either of these food sources, and that because of an evident first-pass effect in the liver, the plasma appearance method is an inappropriate tool for estimating the bioavailability of riboflavin. Estimates of true absorption suggested that some individuals may be poor absorbers, but generally absorption was good and it is unlikely that an overestimation of the bioavailability of riboflavin explains the apparently lower prevalence of riboflavin deficiency when estimated from dietary intakes than by biochemical status indexes. It is more likely that the biochemical threshold for deficiency has been set too low and needs to be reevaluated.

The authors’ responsibilities were as follows—JRD, PMF, and HJP: concept and design of the study; NRB, DJH, and RT: the experimental and analytic aspects of the study; ATH: synthesizing the labeled riboflavin; DJH: growing and labeling the spinach; NRB, RT, and HJP: volunteer recruitment, preparation of labeled test meals, and sample collection; JRD: all mathematical analysis and writing of the first draft of the manuscript. All authors contributed to the writing of the final manuscript. The authors had no conflicts of interest to report.

REFERENCES


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Review Article

Folic acid metabolism in human subjects revisited: potential implications for proposed mandatory folic acid fortification in the UK

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Following an introduction of the importance of folates and the rationale for seeking to estimate fractional folate absorption from foods (especially for countries not having a mandatory folic acid fortification policy), scientific papers covering the mechanisms of folate absorption and initial biotransformation are discussed. There appears (post-1983) to be a consensus that physiological doses of folic acid undergo biotransformation in the absorptive cells of the upper small intestine to 5-methyltetrahydrofolic acid (as happens for all naturally-occurring reduced 1-carbon-substituted folates). This ‘validates’ short-term experimental protocols assessing ‘relative’ folate absorption in human subjects that use folic acid as the ‘reference’ dose. The underlying scientific premise on which this consensus is based is challenged on three grounds: (i) the apparent absence of a 5-methyltetrahydrofolinic acid response in the human hepatic portal vein following absorption of folic acid, (ii) the low dihydrofolate reductase activity peculiar to man and (iii) the implications derived from recent stable-isotope studies of folate absorption. It is concluded that the historically accepted case for folic acid being a suitable ‘reference folate’ for studies of the ‘relative absorption’ of reduced folates in human subjects is invalid. It is hypothesised that the liver, and not the absorptive cells of the upper small intestine, is the initial site of folic acid metabolism in man and that this may have important implications for its use as a supplement or fortificant since human liver’s low capacity for reduction may eventually give rise to saturation, resulting in significant (and potentially deleterious) unmetabolised folic acid entering the systemic circulation.


Introduction

Folic acid (pteroylmonoglutamic acid), though occurring rarely in nature, is the most oxidised and stable form of a vitamin used extensively for supplements and food fortification purposes. Folate is a generic term for the related family of water-soluble B-group vitamins found widely in foodstuffs, mainly reduced methyl and formyl folylpolyglutamates1, that have similar nutritional properties and chemical structures to those of folic acid2,3. Reduced tetrahydrofolates, carrying 1-carbon substitutions at positions 5 and/or 10, are crucial for methionine and nucleotide biosynthesis4,5. A significant reduction in the incidence and recurrence of neural tube defects, such as spina bifida, has been shown when women undertake periconceptual supplementation with folic acid6,7. Low folate status is associated with elevated plasma homocysteine, a risk factor for CVD and stroke8,9, and has been linked to dementia and Alzheimer’s disease10. Low folate status is additionally associated with altered methylation of DNA that may affect gene expression and uracil induced genomic instability, both of which may increase cancer risk11,12.

Fortification of food with physiological levels of folic acid may be expected to reduce the prevalence of folate-related diseases. Some countries have mandatory folic acid fortification of flour programmes (USA 1.4 mg/kg from 1998; Canada 1.5 mg/kg from 1998; Chile 2.2 mg/kg from 2000), but many European countries do not permit the fortification of foodstuffs with folic acid at all. Only a thorough knowledge of the fractional absorption of folate from a variety of folate supplements, fortified foods and natural food folates would allow us to answer the question of whether optimal folate status is easily achievable in countries that do not have a mandatory folic acid fortification policy. An international workshop concluded that the absorption of different folate vitamers from foods and isolates is not well understood13.

Mechanism of folate absorption

In human subjects, dietary folate polyglutamates are deconjugated at the mucosal epithelial cell brush border by folylpolyglutamate carboxypeptidase (EC 3.4.17.21) to the corresponding monoglutamate forms14. Folic acid and reduced monoglutamate folates are absorbed mainly in the proximal

Abbreviations: AUC, area under the curve; DHF, dihydrofolic acid; DHFR, dihydrofolate reductase.
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small intestine (jejunum) by a saturable, carrier-mediated, pH and energy-dependent transport mechanism which, unlike other epithelial tissues, appears to be unique in its lack of hierarchy of transport, having a similar affinity for both oxidised (e.g. folic acid) and reduced folate forms. Oral doses of folic acid in excess of about 260–280 μg (589–634 nmol) have been reported to lead to the direct appearance of untransformed folic acid in the systemic circulation of man. This indicates a saturation point, is evidence that intestinal conversion is not a prerequisite for transport, and is arguably indicative of the dividing line between physiological and non-physiological oral doses of folate. Absorbed folate, which may undergo biotransformation in the absorptive mucosa (see next section), is subsequently transferred via the mesenteric veins to the hepatic portal vein and carried to the liver where an extensive amount (liver ‘first-pass’) is removed, but a lower one for the removal of 5-methyltetrahydrofolic acid which allows a reasonable fraction of this folate form to proceed uninterrupted into the systemic circulation where it usually appears, under fasting conditions, as the predominant plasma folate form for passage to tissues. Fulates removed by the liver, some of which may have undergone further biotransformation, are partially released into the bile allowing significant re-absorption of folate from the small intestine and subsequent delivery of 5-methyltetrahydrofolic acid, via systemic circulating plasma, to tissues. This process of ‘enterohepatic recirculation’ is responsible for maintaining baseline plasma folate levels. Physiological doses of pure folate compounds seem to be well absorbed (≥90%) in both man and rats.

Site of biotransformation of newly absorbed folates, and folate transport into the hepatic portal vein

A high-quality study, with catheterisation of the hepatic portal vein of three human adult volunteer patients, indicated that the absorptive mucosa simply rearranges the 1-carbon substitution of physiological doses of 5-formyltetrahydrofolic acid to 5-methyltetrahydrofolic acid before transport to the serosal side, and transports 5-methyltetrahydrofolic acid unchanged. Using everted sacs of rat small intestine, one group reported no reduction (potentially via dihydrofolate reductase to H₂-folic acid and then to H₄-folic acid) and subsequent methylation of physiological doses of folic acid to 5-methyltetrahydrofolic acid. In contrast, others not only reported significant degrees of mucosal biocconversion but also reported concurrently that folic acid above the physiological range was passively transported, appearing in circulating plasma in an untransformed state (i.e. that the mechanism of absorption was ‘saturable’). An explanation for the inability of some investigators to demonstrate the intestinal conversion of folic acid to 5-methyltetrahydrofolic acid in an alkaline medium was given by Strum. He suggested that this may reflect that the enzyme dihydrofolate reductase (DHFR) has an acidic pH optimum for reducing folic acid to H₂-folic acid (in contrast to the much wider acid-to-slightly-alkaline pH range for the reduction of H₂-folic acid to H₄-folic acid) and that whilst the intestinal conversion of folic acid to 5-methyltetrahydrofolic acid is extensive at pH 6.0 it is negligible at pH 7.5. Any previous argument and doubts over the degree to which physiological doses of folic acid could be reduced and methylated in mucosal epithelial cells appeared to be mitigated following publication of the high quality study of Tani & Iwai. These authors reported on their in situ investigation of absorption and conversion of folic acid in the small intestine of the rat in vivo, with mesenteric vein sampling after jejunal loops were injected directly with a solution of [³H]folic acid, and HPLC investigation of [³H]compounds being transferred into the portal blood. They argued compellingly that their in vivo technique was much more useful for studying intestinal functions because more physiological conditions were maintained compared to other techniques in vitro. They also commented that the correct ratio of conversion of folic acid was not reported in previous papers since, for low concentrations of [³H]folic acid injected into jejunal loops, as high as about 90% of [³H]compounds that they identified unequivocally in portal blood was [³H]5-methyltetrahydrofolic acid.

The paper of Tani & Iwai appeared to be a watershed. Whilst pre-1983 there was already a reasonable degree of agreement that a significant portion of physiological doses of folic acid would undergo conversion to reduced forms in the intestine, it was being accepted post-1983 as the norm, with any lingering doubts muted. In particular, specifically those doubts generated some years earlier by the reported appearance of high concentrations of untransformed folic acid in the hepatic portal vein of human subjects following oral administration of doses of folic acid that were, arguably, non-physiological. This watershed was reflected in the consensus of subsequent scientific ‘Review Articles’ which invariably either overtly stated or implied that when the mucosal extracellular folic acid concentration is low (physiological) the small intestine efficiently reduces and methylates folic acid and, as with absorbed naturally-occurring reduced and 1-carbon-substituted folates, subsequently transfers essentially only 5-methyltetrahydrofolic acid to the hepatic portal vein, thus, from 1983, short-term experimental protocols assessing ‘relative’ folate absorption in human subjects, which centred on methods comparing the serum/plasma response to a single oral test-dose relative to that of an equal ‘reference’ dose of folic acid, appeared to have been ‘validated’ since all consumed folates would broadly enter the hepatic portal vein as 5-methyltetrahydrofolic acid.

Short-term protocols for the estimation of ‘relative’ folate absorption

Short-term experimental protocols, which do not allow assessment of slow-turnover folate pools in the body, are specifically designed to elicit an understanding of the kinetics of initial absorption, metabolism and transport of absorbed folates. They cannot predict whole-body folate turnover. A short-term protocol involves monitoring the appearance and subsequent clearance of the 5-methyltetrahydrofolic acid response in the fast-turnover plasma pool arising from absorbed and biotransformed oral test folates. However, since there is extensive but unquantified removal of newly absorbed folate from the hepatic portal vein (liver ‘first-pass’), ‘absolute absorption’ cannot be estimated from plasma response. Instead, in an attempt to circumvent this problem, these protocols estimate
Folic acid metabolism in human subjects

669

‘relative absorption’ comparative to a similar ‘reference’ dose of folic acid.

Work with radiolabelled folates has been confined mainly to animal models (usually the rat) that may not be comparable to human subjects. Application of pioneering work with stable-isotope folates in human subjects has generally lacked analytical sensitivity when used in single-dose protocols, especially when administered in the physiological range53. To date, the vast majority of short-term protocols attempting to assess ‘relative’ folate absorption in human subjects have centred on methods comparing the serum/plasma response to a single unlabelled oral test-dose relative to that of an equal unlabelled ‘reference’ dose of folic acid10,35. This may entail either measurement of the rate of increase, or the maximum increase, in plasma folate concentration over 2–3 h17,36–40, or measurement of the dose-normalised rise in plasma folate concentration AUC (the area under the curve of the increase in plasma 5-methyltetrahydrofolate acid concentration above fasting baseline level) over 6 h or more41–47. Short-term protocols using unlabelled test folates have not just been confined to the field of nutrition and have also been used in pharmacokinetic studies48.

Comparison of the dose-normalised AUC between test (food folate or isolate) and ‘reference’ folic acid has been accepted as a valuable indicator of absorption, provided the post-dosing plasma measurement test period is long enough to capture ≥80 % of the whole AUC49. However, it is dependent on the premise that the initial absorption and metabolism of folic acid satisfies the following four conditions: Condition-1, that physiological doses of folic acid are absorbed by the same mechanism as reduced folates, and with a similar affinity;

Condition-2, that physiological doses of folic acid are initially reduced and then methylated in the epithelial cells of the small intestine and that essentially only 5-methyltetrahydrofolic acid is exported from the mucosa to the hepatic portal vein, as is the case for absorbed physiological doses of all naturally-occurring reduced folates; Condition-3, that the kinetics of plasma 5-methyltetrahydrofolic acid response to folic acid is similar, if not equal, to that elicited for reduced folic acid ‘reference’ dose unusable50. Concurrent examination of the plasma 13C-labelled 5-methyltetrahydrofolic acid response to 13C-labelled (6S)-5-formyltetrahydrofolic acid and 13C-labelled folic acid also unmasked an underlying serious discrepancy in plasma responses to these two folates50. This was possibly due to a limitation in the rate of initial mucosal reduction of folic acid to H2-folic acid, prior to further reduction to H4-folic acid and subsequent methylation, which may result in a slower transport of absorbed folate to the serosal side53. This is in complete contrast to Condition-3 above and thus renders direct estimates of ‘relative absorption’ using even labelled-AUC, which are derived definitively from the oral test-doses, invalid.

To overcome this, the application of suitable mathematical modelling54,55, which makes complete allowance for any differences in the kinetics of plasma labelled 5-methyltetrahydrofolic acid response, can be used to estimate ‘apparent absorption’. Apart from unlabelled 5-methyltetrahydrofolic acid (the lone folate form we detected in the baseline fasted plasma samples of our unfortified and unsupplemented volunteers), it is important to note that the only enriched folates appearing in plasma after ingestion of physiological doses of labelled 13C6 or 15N(1–7)folates were 13C6 or 15N(1–7)5-methyltetrahydrofolic acid. This was confirmed by liquid chromatography–MS selective ion monitoring for folate monoglutamate forms on the negative [M-H]- ion folate masses: from m/z 440 (folic acid, M + 0; having the smallest folate monoglutamate mass) to m/z 479 (5-formyltetrahydrofolic acid, M + 7; having the largest folate monoglutamate mass)50,55.

Absorption and site of initial metabolism of folic acid in human subjects

The underlying scientific basis on which the post-1983 consensus is based (that physiological doses of folic acid undergo biotransformation to 5-methyltetrahydrofolic acid in the absorptive mucosal cells of the small intestine) is now challenged on three grounds: the apparent absence of a plasma 5-methyltetrahydrofolic acid response in blood sampled directly from the human hepatic portal vein following the mucosal absorption of folic acid; the evidence of an extremely low dihydrofolate reductase activity that seems peculiar to man; the implications derived from mathematical modelling of plasma labelled 5-methyltetrahydrofolic acid responses in human subjects to oral physiological doses of isotopically-labelled folates.

Absence of a 5-methyltetrahydrofolic acid response in the hepatic portal veins of human subjects following an oral dose of folic acid

Whitehead & Cooper1, who gave an oral dose of 1000 µg (2266 nmol) folic acid, and Meilikian et al.32, who gave a lower oral dose of 500 µg (1132 nmol) folic acid, both reported that human subjects transport folic acid unaltered into the hepatic portal vein. Both groups took blood samples from the hepatic portal vein, the hepatic vein and the systemic blood system for at least 2 h post-dosing. They concluded that folic acid was absorbed unaltered and removed by the liver which they proposed as the initial site of subsequent reduction and methylation. Critics would point to a number of perceived problems with this work: (i) the dose...
of folic acid used was non-physiological; (ii) the studies were carried out in patients with either liver disease (cirrhosis) or cancer; (iii) analysis of folate concentrations were undertaken by differential microbiological assay (L. casei and S. faecalis) in order to distinguish non-methyl folate from total folate. Nevertheless, it cannot be overemphasised that it is not the presence of copious amounts of untransformed folic acid in the hepatic portal vein that is noteworthy, since that would be an inevitable consequence of using a non-physiological dose. On the contrary, it is the almost complete absence of an assayable 5-methyltetrahydrofoleric acid response in the hepatic portal vein that is remarkable, particularly when a dose of 500 mg (1132 nmol) was used. This is only twice the dose of 260–280 μg (589–634 nmol) reported much later to be the threshold at which folate acid may even start to appear in the plasma. Hence, at the very least, one may have expected at least half of the dose used by Melkian et al. to have appeared as 5-methyltetrahydrofoleric acid.

Heterogeneity in the dihydrofolate reductase activity between animals and human subjects

Though initial work with crude enzyme preparations of DHFR had taken place earlier, it was perhaps the work of Zakrzewski & Nichol with highly purified chicken liver DHFR that first provided evidence for a single enzyme reducing folic acid and dihydrofolic acid (DHF; H2-folic acid)56. These authors reported that this enzyme reduced folic acid significantly slower than DHF with a more acidic optimum pH (4.4–4.8 v. 5.2–5.6, respectively), and that there was an measurable activity above pH 7.0 for folic acid but still reasonable activity for DHF. These conclusions, which indicate the initial reduction of folic acid to DHF to be the rate limiting step in the biotranformation of folic acid, were confirmed by Mathews & Huennekens53. Human placental DHFR also showed two pH optima (with unmeasurable activity for folic acid above pH 7.0) with the activity for reducing folic acid seventy-five times lower than DHF at pH 6.257. Nylen et al.58 reported a distinct lack of dihydrofolate reductase activity in human biopsy and autopsy samples compared to cultured cells. Kamen et al.59 noted dihydrofolate reductase activity in normal human tissue and human tumour and leukaemia cells in vivo that was several-hundred times lower than present in human cell lines grown in vitro, or normal animal liver tissue. One hypothesis put forward for the discrepancy was that the higher level of DHFR activity with in vitro cell lines could be due to the high levels of folic acid used historically in culture medium. The apparent heterogeneity in the dihydrofolate reductase activity between animals and human subjects was confirmed in a later paper which concluded that low levels of DHFR activity are a feature peculiar to man, shared in part only by closely related primates (the great apes), and that 78% of 105 American postmenopausal women have been observed to have measurable concentrations of unmetabolised folic acid in samples of their fasting plasma, strongly suggests that DHFR activity in the human mucosa, as well as the liver, must also be quite low. Our reasoning is as follows. A portion of the human systemic blood flow is continuously diverted via the splenic and mesenteric arteries where it eventually arrives at the hepatic portal vein (formed from the superior mesenteric and splenic veins) thus allowing the potential for clearance (and subsequent reduction and then methylation) of any systemically circulating folic acid by the human liver, which we now know to have a limited DHFR capacity. When systemically circulating folic acid is presented to the human liver some of it can reappear in bile as unchanged folic acid55. The process of enterohepatic re-circulation will allow for the significant re-absorption of biliary folic acid from the small intestine20,21; a recent multi-compartment kinetic model indicating a 24-fold higher flux of bile folate than that previously thought66. Though human liver is known to have a limited DHFR capacity in comparison to the rat, it cannot be argued that it is still theoretically possible for human subjects to have a good mucosal DHFR activity because enterohepatic re-circulation works in tandem with continuous partial systemic blood flow via the hepatic portal vein through the liver. If mucosal DHFR activity in human subjects was much better than that seen in human liver, then the clearance of excess folic acid from the systemic system would not be observed to be quite slow, as it could then be processed alternatively by the mucosal cells of the upper small intestine. The overall implication is that, in comparison to the rat, man is not likely to have the capacity to significantly reduce folic acid in the absorptive mucosa. Thus, most of a physiological dose of folic acid may inevitably be transported by the absorptive mucosa into the hepatic portal vein in an untransformed state to be sequentially removed by the liver for processing (with a much higher affinity than the 5-methyltetrahydrofolic acid usually presented20. This would result in a delayed release (via entero-hepatic re-circulation) into the systemic blood system of 5-methyltetrahydrofolic acid, its biotransformed metabolite11. A delayed plasma response to folic acid compared to a reduced folate was confirmed by the use of isotopically labelled folates50, though whether this was due to a delay in mucosal or liver biotransformation could not be concluded.
Mathematical modelling of plasma labelled 5-methyltetrahydrofolic acid responses to oral physiological doses of isotopically-labelled folates

The advent of stable-isotope-labelled folates allows folate absorption to be tracked not only with sensitivity but, more importantly, with specificity. This way, folate can be followed and differentiated from the natural store of folate already present in the body. The recent use of mathematical modelling,55 which bypasses the need for adherence to Condition-3 because it completely takes into account differences in the kinetics of plasma response, infers that sequestration of physiological doses of folic acid to the liver is the main cause of a delayed plasma response. This conclusion is incompatible with current theory that only 5-methyltetrahydrofolic acid enters the hepatic portal vein after oral ingestion of physiological doses of any folate form. Mathematical modelling of stable-isotope-labelled plasma 5-methyltetrahydrofolic acid response was used to estimate the ‘apparent absorption’ of single oral physiological doses of [13C6]folic acid, (6S)-[13C6]5-formyltetrahydrofolic acid and [13N6]3-intrinsically-labelled spinach folates in fasting human adults.55 The ‘apparent absorption’ of reduced folates was significantly higher than for the ‘reference’ dose of folic acid; generating ‘relative absorptions’ significantly in excess of 100% for both 5-formyltetrahydrofolic acid (158%) and spinach-folate (183%). This was unexpectedly in excess of 100% for both 5-formyltetrahydrofolic acid (158%) and spinach-folate (183%). This was unexpected, and biologically impossible, since the ‘true absorption’ of physiological doses of [14C]folic acid in human subjects has been reported to approximate 90% or more. It was concluded, contrary to current theory, that a significant fraction of absorbed [13C6]folic acid may be entering the hepatic portal vein unchanged in contrast to Condition-2, to be more effectively removed by the liver than reduced 5-methyltetrahydrofolic acid, prior to subsequent biotransformation and (limited) enterohepatic recirculation.

Implications for the use of folic acid as a ‘reference folate’

The essential absence of a plasma 5-methyltetrahydrofolic acid response in human blood sampled directly from the hepatic portal vein following a dose of folic acid,41,32, the extremely low dihydrofolate reductase activity that now seems peculiar to man,60,62,63, the recent implications derived from mathematically-modelled ‘apparent absorption’ of isotopically-labelled folates55, and the observation of unmetabolised folic acid in plasma of fasted American females64 are key observations. Collectively, they arguably justify the proposition that, unlike the rat (the chief historical experimental animal model), absorbed physiological doses of folic acid are essentially transferred to the hepatic portal of man in an unmetabolised state to be subsequently removed and metabolised by the liver. The main implication of this is that the currently accepted case for folic acid being a suitable ‘reference folate’ for studies of the ‘relative absorption’ of reduced folates in man, particularly in short-term experimental protocols, is invalid.

If subsequent experimentation, using sensitive liquid chromatography–tandem MS techniques67,68, confirms that physiological doses of stable-isotope-labelled folic acid mainly enter the hepatic portal vein of man in an unmetabolised state, then folic acid should be avoided as the ‘reference’ folate in ‘short-term’ studies of the absorption of reduced folates. We suggest that it could be replaced by (6S)-5-methyltetrahydrofolic acid (the natural folate form found in circulating blood plasma). Furthermore, it is questionable whether folic acid should be used as the ‘reference’ folate in longer-term dietary intervention studies where surrogate biological markers of nutritional status or metabolic wellbeing are used as arguments to the relative absorption of folate from basal diets supplemented with natural ‘high-folate’ foods versus those supplemented with an equal amount of folic acid supplied in either supplement form or as folic acid-fortified foods. This is because changes in surrogate markers may be influenced by differential tissue distribution of supplemented naturally-occurring reduced folates and folic acid between the liver and other body tissues. This may explain the recent observation that erythrocyte folate concentrations increase more after supplementation with (6S)-5-methyltetrahydrofolic acid than with folic acid.69

Implications for the use of folic acid as a fortificant or supplement

If the initial primary site of folic acid metabolism in human subjects is actually the liver then, because of liver’s apparent poor dihydrofolate reductase activity, it would seem entirely logical to hypothesise that regular daily intake of physiological doses of folic acid may eventually result in its chronic appearance in plasma of the systemic circulatory blood system. This may even happen at quite modest physiological doses since poor liver dihydrofolate reductase activity could give rise to eventual saturation of the liver folate-mono glutamate pool with regular intake of doses well below that of the acute threshold dose (260–280 μg; 589–634 nmol) that has been noted to result in its subsequent appearance in plasma.17 Such an hypothesis could go a long way to explaining observations of the systemic appearance of unmetabolised folic acid observed in both fasting and non-fasting American subjects exposed to what is debatably quite a modest policy of mandatory folic acid fortification.64,70

Though there have been a variety of concerns expressed regarding the potential negatives of mandatory fortification policies, some of these may arguably result from the generality of an inappropriate exposure to high concentrations of folate per se, rather than specifically to folic acid (pteroylmonglutamic acid); the current exclusive fortificant folate form. The following discussions are solely restricted to potential concerns in human subjects for which there is either metabolic or direct observational arguments that derive uniquely from a systemic exposure to unmetabolised folic acid.

Potential masking of the anaemia of B12 deficiency

Effects on cognitive function. Concern that the US policy of mandatory fortification may to a significant degree mask the anaemia of vitamin B12 deficiency, primarily in the elderly population, appears to have been unwarranted as there is no evidence of an increase in the proportion of subjects with low vitamin B12 concentration but without anaemia.71 Whether fortification at 280 μg/100 g flour (double that of the US), as proposed for the UK, would have an impact is unknown. A review on folic acid and cognition in...
older persons, which recognised that there may be positive benefits derived from folic acid fortification, concluded that the potential harm is the greater concern 22.

A high intake of folate (mainly as a result of the intake of folic acid) has been associated with accelerated cognitive decline in older persons, with the hypothesis that this may be related to low vitamin B₁₂ status. In contrast, results from the Folic Acid and Carotid Intima-media Thickness (FACIT) Trial on B₁₂ replete adults aged 50–70 years suggested that folic acid supplementation may improve domains of cognitive function that tend to decline with age 72. A contemporary paper 73, examining American seniors exposed to mandatory folic acid fortification, confirmed that when vitamin B₁₂ status is normal, high serum folate is associated with protection against cognitive impairment. However, in seniors with low vitamin B₁₂ status it also confirmed the hypothesis that high serum folate is associated with cognitive impairment; with the suggestion that this effect may be due to unmetabolised folic acid in the circulation. Thus folic acid supplementation/fortification may be a ‘double-edged sword’ capable of exhibiting polar ‘Jekyll and Hyde’ characteristics, depending on vitamin B₁₂ status. An accompanying editorial succinctly listed some of the challenging research questions that may need to be addressed if this dichotomous interaction between folic acid and B₁₂ is confirmed 74.

Effect on cancer. Arguably, it is important here to distinguish between the relationship between (naturally-occurring) folate and cancer and the potential effects of appreciable concentrations of systemically-circulating unmetabolised folic acid and cancer. Low folate status may be a risk factor for cancer, possibly through uracil induced genomic instability 75 and/or altered methylation of DNA 76, and having an adequate folate status (achievable through sufficient intake of folate, including folic acid) may thus be beneficial.

As compared to the activity in fresh human cells ex vivo, similar cells cultured in vitro with folic acid as the source of folate can have their DHFR activity increased 100-fold or more 59. If an up-regulation of DHFR activity can be also induced in vivo this may be accompanied by increased thymidylate synthase activity since the transcription of both these genes is co-regulated by the same E2F-1 transcription factor 77,78. Mathematical modelling indicates that this would increase pyrimidine production (the rate-limiting step for DNA synthesis) without significantly affecting the rest of folate metabolism 79. It could thus be hypothesised that, in contrast to an increased exposure to the naturally circulating folate (65)-5-methyltetrahydrofolic acid, exposure to unmetabolised folic acid may increase cells’ capacity for division, thus predisposing to an ‘accelerating’ effect which may be detrimental in the context of cancer. Of course if, with prolonged exposure to folic acid, DHFR is inducible in small intestine mucosa and liver then it could potentially somewhat mitigate systemic exposure to unmetabolised folic acid. However, direct evidence of the appearance of folic acid in serum of both fasting and non-fasting American adults many years after the 1998 introduction of mandatory folic acid fortification indicates that any such alleviation must be of limited consequence.

Recent research findings, in human subjects, suggest that folic acid supplements may increase the risk of multiple colorectal adenomas (Aspin–Folate Polypl Prevention Trial)80 and that plasma folate concentrations are associated with colorectal cancer in a U-shaped manner such that high, as well as low, folate concentrations may increase colorectal cancer risk 81. Additionally, a recent paper, reporting on a prospective study of US subjects, suggests that a high intake of folate ‘generally attributable to supplemental folic acid’ may increase the incidence of breast cancer in postmenopausal women 82.

In respect of hyperplasia, supplementation with folic acid at only 1 mg/d adversely increases the risk and rate of in-stent restenosis in men, and the need for target-vessel revascularisation 83. For the vast majority of patients undergoing coronary intervention, stenting (as opposed to balloon angioplasty) was reported as the current method of choice; for which proliferation of smooth-muscle cells is one of the most important mechanisms leading to restenosis. It was concluded that previously reported positive effects of folic acid on coronary restenosis appeared to be predominantly in patients who were treated with balloon angioplasty alone, where thrombus formation within the intimal cracks and vascular remodelling are of predominant importance to the process of restenosis; these changes being potentially more susceptible to the folate-induced effects of homocysteine lowering.

Effect on anti-folate chemotherapy. A further area of concern is the potential for negative effects on chemotherapy using the anti-folate drug, methotrexate. Its mode of action is to limit folate availability to cells by reducing (by substrate competition) the activity of dihydrofolate reductase, an enzyme whose gene could also conceivably be up-regulated through exposure to high concentrations of folic acid. Post-hoc analysis from two randomised, controlled studies has indicated that folic acid reduces the degree of improvement of methotrexate-treated rheumatoid arthritis patients 84. Additionally, a recent study in a US population has indicated that high serum folate levels above about 50 nmol/litre (approximately 22 ng/ml), a similar concentration above which unmetabolised folic acid makes up about 15 % of total folate 70, significantly increases the failure of ectopic pregnancy treatment with single-dose methotrexate 85.

Effect on multiple births. We accept the conclusions of Li et al. that previous claims, from European studies, that folic acid supplementation may increase the risk of multiple births may have been affected by the use of ovarian stimulation 86. However, more and more individuals (currently about 14 % in Europe) seek medical advice for infertility, of which about half undergo in vitro fertilisation. It has recently been reported that high folate status (attributable to folic acid supplementation) increases the likelihood of multiple births after in vitro fertilisation, with its associated increased risks of maternal and infant mortality and morbidity, at a rate similar to that seen in the USA after mandatory folic acid fortification 87.

Effect on immune function. New research has indicated that the concentration of unmetabolised folic acid (but not the concentration of natural circulating folate, (65)-5-methyltetrahydrofolic acid) correlates to a reduction in the cytotoxicity of natural killer cells 84. Since experimental and clinical evidence supports a role of natural killer cells in tumour cell destruction 88, and that they may be considered a first line of host defence against carcinogenesis, it was hypothesised that this would suggest another way in which excess folic acid (but not circulating high concentrations of ‘natural’ 5-methyltetrahydrofolic acid) might promote existing premalignant and malignant lesions.
Conclusions

Our recent results from stable-isotope-folate absorption studies and a re-appraisal of historical literature, strongly suggests that the initial site of folic acid biotransformation in human subjects is the liver, and not the mucosal absorptive cells of the upper small intestine. Such a conclusion invalidates the historical use of folic acid as a ‘reference dose’ in studies of the ‘relative absorption’ of reduced folates in man. It can be concluded that previous attempts to gauge the absorption of synthetically-produced ‘nature-identical’ (6S)-5-methyltetrahydrofolic acid (the natural circulating folate form, which arguably should be the form generally used as the ‘reference folate’ in most future acute and long-term folate-absorption studies), by expressing the plasma response ‘relative’ to that from an equal dose of folic acid, are untenable. This is not only because unlabelled folate test doses have been used, but because the use of labelled folates and mathematical modelling cannot overcome the inherent flaw of using folic acid as the ‘reference’ folate, as we currently argue in this paper. It is suggested that the absolute absorption of oral physiological doses of (6S)-5-methyltetrahydrofolic acid in human subjects can only be estimated using 14C-label protocols and accelerator MS, as it has for folic acid.

Additionally, since human subjects uniquely amongst mammals and birds have a reduced dihydrofolate reductase activity (and a poor ability to reduce folic acid), it is hypothesised that even a modest regular daily intake of physiological doses of folic acid could eventually saturate the preliminary liver folate-monoglutamate pool. This would result in the subsequent chronic appearance of unmetabolised folic acid in the systemic circulatory blood system which arguably, according to circumstance, increasingly looks as though it can induce polar ‘Jekyll and Hyde’ health effects. Before mandatory folic acid fortification is introduced to the UK, it is suggested that a thorough appraisal of all potential concerns that derive uniquely from the systemic circulation of unmetabolised folic acid should be addressed methodically to ascertain a true picture of risk/benefit of fortification.

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Sodium and Bone Health: Impact of Moderately High and Low Salt Intakes on Calcium Metabolism in Postmenopausal Women

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ABSTRACT: High salt intake is a well-recognized risk factor for osteoporosis because it induces calciuria, but the effects of salt on calcium metabolism and the potential impact on bone health in postmenopausal women have not been fully characterized. This study investigated adaptive mechanisms in response to changes in salt and calcium intake in postmenopausal women. Eleven women completed a randomized cross-over trial consisting of four successive 5-wk periods of controlled dietary intervention, each separated by a minimum 4-wk washout. Moderately low and high calcium (518 versus 1284 mg) and salt (3.9 versus 11.2 g) diets, reflecting lower and upper intakes in postmenopausal women consuming a Western-style diet, were provided. Stable isotope labeling techniques were used to measure calcium absorption and excretion, compartmental modeling was undertaken to estimate bone calcium balance, and biomarkers of bone formation and resorption were measured in blood and urine. Moderately high salt intake (11.2 g/d) elicited a significant increase in urinary calcium excretion (p = 0.0008) and significantly affected bone calcium balance with the high calcium diet (p = 0.024). Efficiency of calcium absorption was higher after a period of moderately low calcium intake (p < 0.05) but was unaffected by salt intake. Salt was responsible for a significant change in bone calcium balance, from positive to negative, when consumed as part of a high calcium diet, but with a low calcium intake, the bone calcium balance was negative on both high and low salt diets.

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Key words: salt, calcium absorption, calcium metabolism, bone biomarkers, postmenopausal women

INTRODUCTION

Diets high in sodium alter calcium metabolism by increasing urinary calcium excretion (calciuria), and a high salt intake is therefore assumed to be a risk factor for osteoporosis. However, the evidence is mainly based on acute salt-loading studies, which have little relevance to long-term diets, and the results of bone turnover studies are inconsistent.1–3 There is therefore a need to clarify the effects of salt and calcium on calcium metabolism at dietary levels that are commonly consumed. The published literature on sodium and calcium metabolism indicates that the average loss of calcium is 1 mmol Ca (40 mg) per 100 mmol (2290 mg) of sodium, and without any adaptive compensatory mechanisms, a daily loss of 40 mg calcium would depleted 10% of the skeleton within a decade.2,3 Interindividual variation in sodium-induced calciuria in cross-sectional and some salt-loading studies are explained by differences in intake of other dietary factors known to affect urinary calcium excretion, such as protein3 and potassium.4,5 Differences in sodium-induced calciuria in short-term controlled dietary interventions (7–14 days) suggest that genotype-related salt-sensitivity may also play a role.6,7 To maintain homeostasis, urinary calcium losses may be compensated for by an increase in the efficiency of calcium absorption and/or an increase in the rate of bone resorption. The conclusion from a 2-yr longitudinal study in postmenopausal women was that high sodium intakes were associated with increased bone loss at hip but not spine7 and from the regression analysis, it was estimated that reducing the daily urinary sodium excretion from 3450 (8.8 g salt) to 1725 mg (4.4 g salt) would have an effect on BMD equivalent to an increase in daily calcium intake of 891 mg, indicating a potentially powerful effect of sodium on bone loss at the hip. However, the overall evidence from cross-sectional and prospective studies on salt reduction and BMD is inconsistent, and there seems to be no published data on the relationship between sodium intake and fracture risk.8 With regard to calcium balance, it has not yet been conclusively showed that upregulation of calcium absorption compensates (partially or fully) for sodium-induced calciuria. The null hypothesis for our study was that the level of dietary intake (moderately high or low) of sodium or calcium would not affect the efficiency of calcium absorption or bone calcium metabolism. State-of-the-art stable isotope labeling techniques were used to determine calcium absorption and excretion in a controlled cross-over metabolic study in postmenopausal women.

The authors state that they have no conflicts of interest.

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MATERIALS AND METHODS

Subjects

Sixteen female subjects, at least 5 yr postmenopausal and <75 yr of age, were recruited. Study exclusion criteria included current use of hormone replacement therapy (HRT) or <2 yr since the use of HRT, osteoporosis or other diagnosed bone disease, rheumatoid arthritis, osteoarthritis, chronic illness, renal, gastrointestinal or hormonal disorders, hypertension, regular use of diuretics or antacids, smoking, and unwillingness to stop taking vitamin/mineral supplements for the study duration. Blood pressure was measured at the prestudy stage to ensure subjects were not hypertensive. Following British Heart Foundation recommendations, systolic blood pressure of 140 mm Hg and diastolic blood pressure of 90 mm Hg were the thresholds above which subjects were excluded. Eleven of the volunteers completed all four dietary interventions.

Ethical considerations

Ethical approval for the study was obtained from the Norwich Research Ethics Committee. Each participant gave written informed consent.

Study design

The study was a randomized, repeat cross-over trial of the effect of moderately high or low salt intake (11.2 g salt [4.4 g/d sodium] and 3.9 g salt [1.6 g/d sodium], respectively) on calcium and bone metabolism in postmenopausal women, consuming either a moderately high or low or high calcium diet (1284 and 518 mg/d, respectively); there were four successive 40-d dietary intervention periods, each separated by a minimum 4-wk washout period when subjects consumed their habitual diet. An identical basal diet intake was restricted to 90 g/d. To make the diets palatable without the UK legislative addition of calcium, and the milk used for the high salt diet. A total of 13 batches of the menu selections on offer were prepared. Of these, four were analyzed by AAS to confirm the sodium and calcium content of the menu. Salt was also added to some of the cooked meals during batch cooking for the high salt diet. A kettle. During the high sodium intervention, additional sodium (250 mg/d) was provided from high salt bread. Salt was added to the low calcium, high sodium), −Ca−Na (low calcium, low sodium), +Ca+Na (high calcium, high sodium), and +Ca−Na (high calcium, low sodium).

A dual-tracer stable isotope technique (oral 42Ca and IV 43Ca) was used to measure calcium absorption after 30 days on each of the four dietary interventions. Kinetic measurements were made to undertake compartmental modeling to estimate bone resorption and balance. On the morning of day 30, after an overnight fast of at least 10 h, subjects attended the Human Nutrition Unit (HNU) and were given a standard breakfast (containing 183 mg calcium) of toast, butter, jam, and 100 ml of semiskimmed milk, extrinsically labeled with 20 mg 45Ca (as CaCl2). The glass was rinsed several times with deionized water, and the washings were consumed to make sure the full dose of oral isotope was ingested. Approximately 30 min later a solution of 44Ca (4.5 mg 45Ca as CaCl2) was infused over 20 min through a cannula inserted into the antecubital vein of the other arm. During this period, volunteers were only allowed to consume deionized water (MilliQ; Millipore, Watford, Herts, UK). After removal of the cannula, volunteers were given a standard light meal, and further 10-ml fasting blood samples were collected on days 31, 34, and 38, and a final blood sample (40 ml) was collected on day 40. Calcium absorption was measured from isotope ratios in fasting serum samples obtained on days 31, 34, 38, and 40. Subjects collected 24-h urine samples on 2 consecutive days before each intervention, on day 16 (midintervention) and days 30–40 after the isotope administration. Urine samples before each intervention were analyzed for calcium, sodium, phosphorus, and potassium. Urines collected on days 16 and 30–40 were analyzed for calcium and sodium and those collected on days 39 and 40 were analyzed for potassium and phosphorus. Individual fecal samples were collected into autoclavable bags from day 30 through to day 40 and analyzed for total calcium and isotopic enrichment.

Venous blood on days 1 and 40 was analyzed for markers of bone formation (bone alkaline phosphatase [B-ALP] and osteocalcin [OC]) and calcitropic hormones [PTH, 1,25-dihydroxyvitamin D [1,25(OH)2D], and 25-hydroxyvitamin D [25(OH)D]]. Volunteers collected 24-h urine samples in preweighed acid-washed 2.5-liter bottles 2 days before each intervention and during the 10 days after isotope administration. Aliquots of the urine collected before each intervention and during the last 2 days of intervention were pooled and analyzed for markers of bone resorption (N-terminal telopeptide [NTX], pyridinoline [Pyr], deoxypyridinoline [Dpyr]) and sodium, calcium, potassium, and phosphorus.

Blood pressure was determined on three different occasions before acceptance onto the study and monitored on a weekly basis during each of the four dietary interventions. All measurements were carried out by trained investigators using a fully automated blood pressure monitor (Omron 711; Omron Matsusaka).

Experimental diets

The diet was comprised of a 7-day rotating menu, calculated to be low in sodium (1.5 g/d) and calcium (500 mg/d). Volunteers selected 7 menus from a total of 12 available menus. All meals (breakfast, lunch, dinner, and snacks) during each intervention period were prepared in the Human Nutrition Unit (HNU) and delivered to subjects’ homes three times a week. A low calcium intake was achieved by preparing all bakery products with white flour without the UK legislative addition of calcium, and the milk intake was restricted to 90 g/d. To make the diets palatable and hence ensure compliance up to eight portions of fruits and vegetables were provided each day. A low calcium water supply was obtained by providing volunteers with a water filter, a supply of replacement filter cartridges and a kettle. During the high sodium intervention, additional sodium (0.7 g/d) was provided from high salt bread. Salt was also added to some of the cooked meals during batch cooking for the high salt diet. A total of 13 batches of the menu selections on offer were prepared. Of these, four were analyzed by AAS to confirm the sodium and calcium content of the low and high salt diets. The intake of other nutrients was calculated with “Diet Cruncher” (Way Down South, Watford, Herts, UK).
software, Dunedin, New Zealand) using the McCance and Widdowson’s food composition tables. No additional food was allowed and subjects completed a food intake diary each day to monitor dietary and fluid intake.

Supplements

Calcium (125 mg calcium as CaCO₃) and sodium supplements (333 mg sodium as NaCl) were provided by Penn Pharmaceutical Services (Tredegar, Gwent, UK). Vitamin D supplements (10 μg cholecalciferol) were provided by Solgar Vitamins UK (Newcastle, Staffordshire, UK). For the high calcium and/or sodium diets, subjects were requested to take two calcium and/or sodium capsules with each main meal (i.e., three times a day, providing a total of 750 mg calcium/d or 2.0 g sodium/d from supplements) for the full 40-day intervention period. Vitamin D supplements were taken once every day, at breakfast, starting 4 wk before each intervention period and supplementation was continued until the end of each 40-day intervention period. A daily guide for supplement intake was included in the food intake diary and compliance was monitored by written self-reported intake.

Preparation of isotope labels

The ⁴³Ca tracer for intravenous infusion (52.1% enrichment) was prepared by the Ipswich Hospital Pharmacy (Ipswich, UK) as follows: an accurately weighed amount of ⁴³Ca-enriched CaCO₃ was dissolved in concentrated HCl (molar ratio of 3:1), the solution adjusted to pH 6, and made up to volume with sterile water to give a final concentration of 0.75 mg/ml. Individual subsamples of the solution were transferred to glass ampoules, sealed, and heat sterilized, and the sterility of the solution was verified. For oral administration, ⁴³Ca-enriched CaCO₃ (90.8% enrichment) was converted to CaCl₂ by adding 6 M HCl in a 2:1 molar ratio. The pH was similar to that of milk (pH 6.7). For each subject, an aliquot of the extrinsic label containing 20 mg CaCO₃ was added to 200 g semiskimmed milk and allowed to equilibrate overnight at 4 °C.

Dietary analysis

All meals and snacks that formed part of the 7-day rotating menu cycle were homogenized (Ultra-Turrax T-50 homogenizer; IKA) and freeze-dried. Aliquots were ashed in a muffle furnace for 48 h at 450 °C (Vulcan, 3–550; NEY Dental International, Bloomfield, CT, USA), dissolved in 5 ml of a 5% HCl solution, and analyzed for calcium by atomic absorption spectroscopy (AAS; model 3300; Perkin-Elmer, Norwalk, CT, USA) after further dilution with 0.1% lanthanum chloride. For the determination of sodium by atomic emission spectroscopy (AES; model 3300; Perkin-Elmer) further dilutions were carried out using 18-μl water. All samples were analyzed in duplicate, and a certified typical diet reference material (Typical Diet 1548a; NIST, Gaithersburg, MD, USA) was analyzed with each batch of samples.

Urine analysis

On receipt, all urines were weighed, mixed, and aliquots stored. Sodium and potassium were measured by AES and AAS, after appropriate dilution, respectively (model 3300; Perkin-Elmer). All samples were analyzed in duplicate together with certified urine controls with each batch (Lyphochek, Quantitative Urine Control; Bio-Rad). Calcium isotopes and total calcium were measured by ICP-MS (IsoProbe; GV Instruments, Manchester UK) using the isotope dilution technique (⁴⁰Ca spike). UV digestion (Model 707; Metrohm), and oxalate precipitation. Phosphorus was measured using the ABX Diagnostics Phosphorous Kit (Shefford, Bedfordshire, UK) on a COBAS MIRA automated analyzer. All samples were analyzed in triplicate, and ABX Diagnostics Human Control N was run for quality control purposes. The intra-assay variation was 3.7%. Interassay variation was avoided by analyzing all samples from each individual in the same run.

Fecal analysis

Individual fecal samples collected for 10 days after dosing were weighed, autoclaved, freeze-dried, thoroughly homogenized, and ashed in a muffle furnace. When more than one fecal sample was collected on the same day, the individual’s collection was pooled for that day and processed as described above. Again the samples were spiked with Ca-48 isotope solution, but this time were digested with 0.1 M HNO₃ at 80 °C. After centrifugation of the digest, the supernatant was subjected to the oxalate precipitation method as used for urine and serum samples.

Biochemical markers of bone metabolism

Urinary Pyr and Dpyr were measured in triplicate using a three-step procedure. Urine was first hydrolyzed with an equal volume of 12 M HCl at 110 °C for 18 h, and the cross-links were extracted by CF1 cellulose chromatography with the use of an internal standard (acyetylated pyridinoline; MetraBiosystems, Wheatley, Oxon, UK) and measured using a reverse-phase HPLC method with fluorescent detection. The acetylated pyridinolone was used in accordance with the method described by Calabrese et al. and Robins et al. The cross-links content of urine was quantified by external standardization using a commercially available pyridinoline/deoxypyridinoline HPLC calibrator (MetraBiosystems). The intra-assay CVs for Pyr and Dpyr, measured as the variation between 10 chromatograms obtained between column regenerations, were 6% and 7%, respectively. Interassay variation was avoided by analyzing all samples from each individual in the same run.

Urinary NTX was measured in urine samples by an ELISA (Osteomark; Ostex International). The intra-assay CV was 1.6%. Interassay variation was avoided by analyzing all samples from each individual in the same run.

Blood analysis

Total calcium and calcium stable isotopes were quantified in serum. After an overnight fast, blood was collected into sterile trace element free tubes (Vacutainer; Becton Dickinson, Rutherford, NJ, USA) and allowed to clot for a minimum of 30 min. The serum was removed after centrifugation at 1500g for 10 min and stored at −18 °C before analysis. After UV digestion (Model 707; Metrohm) and
oxalate precipitation, calcium isotopes were determined by inductively coupled plasma mass spectrometry (ICP-MS; IsoProbe, GV Instruments, Manchester, UK). Total calcium was determined by isotope dilution.

PTH was measured in plasma from blood collected in EDTA tubes, the plasma having been removed after centrifugation and stored at °C. Intact PTH was measured using a chemiluminescent immunometric assay (IMMULITE; Diagnostic Products, Los Angeles, CA, USA). Quality control was monitored through participation in an External Quality Assessment Scheme (DEQAS).

1,25(OH)2D and 25(OH)D was measured in serum. 25(OH)D was identified and quantified using HPLC equipped with a PDA-detector, and a radioimmunoassay (65100E; DiaSorin, Stillwater, MN, USA) was used for the quantification of 1,25(OH)2D as described elsewhere. The accuracy of the analysis was monitored by participation in the Vitamin D External Quality Assessment Scheme (DEQAS, Charing Cross Hospital, London, UK).

Serum OC and B-ALP were measured by an ELISA (BRI-Diagnostics, Dublin, Ireland and MetraBiosystems, respectively). The intra-assay CVs were 11% and 4.5%, respectively. All samples from each individual were analyzed in the same run.

**Kinetic modeling**

A compartmental model, developed in SAAMII (SAAM Institute, Seattle, WA, USA), was used to fit the experimental data and estimate parameters of interest (Fig. 1), similar to that published by Neer et al. and Wastney et al. but containing just a single soft tissue/bone compartment. The main calcium fluxes were from bone (V 0+), to bone (V b), to urine (V u), to feces (V f), and calcium absorption from the diet (V a). Several of these fluxes were used to estimate bone calcium balance, V bal = V b - V a + V u + V f.

**Statistical analysis**

Statistical analyses were performed using the R data analysis software. Linear models (ANOVA) and paired t-tests were used to determine effects of the different diets on a range of physiological responses. Because the four intervention diets were characterized by combinations of “low” and “high” dietary intake of sodium and calcium, the input variables to the linear models were two-level factors describing sodium and calcium intake and the interaction of these factors. Where appropriate, these factors had a third level, corresponding to the baseline intakes. Volunteer identification was included as a factor variable in all models so that each volunteer could act as her own control. The appropriateness of all final models was checked for outliers (including leverage and influence) and normal errors. Where necessary, outliers were excluded and/or data transformations performed, and the models were refitted. Statistical analysis of the effect of diet on bone biomarkers at the end of each intervention was performed on the calculated percent change from baseline. At no point was it necessary to use nonparametric models. Tukey’s honest significant differences were calculated to determine the differences in the levels of sodium or calcium in the linear models. All results were considered significant if p < 0.05.

The kinetic data from the low and high calcium dietary intervention periods was separated and analyzed independently. Within these two treatments, the effect of low versus high salt intake was assessed by paired t-tests. All results were considered significant if p < 0.05.

For some of the outcome measures, baseline values were obtained before each of the four diets. The assumption that all the baseline values were identically distributed could be tested in these cases. Where this assumption was not met, the data were tested to see whether the sequence of the diets was significant. The possibility that sequence of diet may affect outcome was also investigated for calcitropic hormones and bone biomarkers.

**RESULTS**

**Characteristics of the subjects**

The mean age was 64 yr (range, 59–73 yr), and the mean body mass index (BMI) was 24.7 kg/m2 (range, 20.9–32.1 kg/m2). According to the WHO definition, 4 of the 11 volunteers were overweight (BMI > 25 kg/m2). Menopause was defined as at least 12 mo since the last menstrual cycle. All participating women reported to be at least 5 yr postmenopausal. The average systolic and diastolic blood pressure for all four intervention periods was 115 and 72 mm Hg, respectively (data not shown). There was no significant difference in blood pressure when consuming the high and low salt diets.
**Intervention diet**

The calculated mean energy intake was 1979 kcal, and the percentage of food energy from fat, carbohydrates, and protein was 34%, 54%, and 15%, respectively. The diet met the UK recommended nutrient intake (RNI)(17) for all vitamins except retinol (intake = 384 μg/d; RNI = 600 μg/d) and vitamin D (intake = 2.1 μg/d; RNI for women <60 yr = 0 μg/d and >60 yr = 10 μg/d); the mean intake of these two nutrients was also less than the RNI in women 50–64 yr of age in the latest National Diet and Nutrition Survey in Great Britain (retinol = 449 μg/d and vitamin D = 3.5 μg/d).(18) The mean analyzed calcium content of the 12 menus devised for the low calcium diet was 518 ± 49 (SD) mg and for the moderately high calcium diet the intake was increased to 1284 mg/d by the addition of calcium supplements. The mean sodium content of the menus offered for the low and high salt diets was 1557 ± 237 and 4422 ± 253 mg sodium, respectively. The low salt diets provided a mean of 3.9 g salt (1.5 g sodium) and the high salt diets a mean of 11.2 g salt (4.422 g sodium) per day. The high salt interventions required the ingestion of additional salt in six capsules per day, each capsule containing 342.3 mg sodium, and several subjects complained of nausea and vomiting during the first 2 days of the high salt intervention. In response, advice was given on methods of supplement ingestion that would avoid any adverse reactions (e.g., mixing of the salt supplement into the food and dissolving the capsules in hot drinks before consumption). Once adopted, these guidelines prevented further episodes of nausea, and none of the subjects withdrew from the study because of persisting intolerance to the salt supplements. Following Good Clinical Practice (GCP) guidelines, all adverse events were reported to the local Ethics Committee.

**Calcitropic hormones**

Because this cross-over trial spanned a minimum of 36 wk, vitamin D supplementation of the subjects was essential to avoid any effect of seasonal variation that might have masked changes in intraindividual absorption efficiency. The mean serum 25(OH)D3 concentration was 69.6 ± 11.5 nM (range, 47.8–92.5 nM; data not shown). There were no significant differences in 25(OH)D3 levels at the start and end of each intervention and no differences in 1,25(OH)2D3 at the end of each intervention period. Differences in these parameters were also nonsignificant when analyzed according to sequence of diet consumed. Although subjects received identical vitamin D supplements, the 25(OH)D3 concentration ranged from 48–93 nM, which suggests that interindividual differences in absorption may have been affected by differences in vitamin D status; however, because each subject acted as her own control, this would have had no bearing on the overall findings from the study.

The overall mean serum PTH concentration was 3.5 ± 1.4 pM, which compared well with that reported by others for this age group.(19,20) In response to sodium, average PTH levels (3.9 pM) in subjects consuming the high sodium diets were statistically significantly different (p = 0.007; data not shown).

**Urinary sodium, calcium, potassium, and phosphorus excretion**

Results for all four controlled dietary interventions and for the habitual diet are summarized in Table 1. Urinary sodium excretion was significantly higher on the high sodium diets compared with the low sodium diets (p < 0.0001) and the habitual diet (p < 0.0001). The difference in sodium excretion between the low and high salt diet was key to the success of the study and reflects compliance. There was no significant difference in sodium excretion between the two low sodium diets (p > 0.05) and the two high sodium diets (p > 0.05). The average sodium excretion from the low (–Ca–Na and +Ca–Na combined) and high (–Ca+Na and +Ca+Na combined) sodium diets was 1402 ± 161 (equivalent to 3.6 g salt) and 3778 ± 460 mg/d (equivalent to 9.6 g salt), respectively, which represents a 2.7-fold difference between the low and high salt diets.

The mean calcium urinary excretion in subjects consuming their habitual diet was 164 ± 91 mg/d (Table 1). Two of 11 subjects excreted calcium in excess of 250 mg/d (>4 mg/kg/d; data not shown). The average calcium excretion for diets –Ca–Na, +Ca–Na, –Ca+Na, and +Ca+Na was 123, 159, 141, and 192 mg/d, respectively (Table 1). Both high sodium and high calcium intake independently provoked a significant increase in urinary calcium excretion (p <
The average level of biochemical markers of bone resorption measured in 48-h urine pools at the end of each dietary intervention is given in Table 3. Statistical analysis
of percentage change from baseline of Pyr and Dpyr measured at the end of each dietary intervention showed a significant increase after the low calcium interventions (Fig. 2). There was a (nonsignificant) decrease in NTX with the high calcium diet, whereas Pyr and Dpyr excretion increased with the low calcium diets. The resorption markers differed in their sensitivity to the changes in sodium intake. There was a significant change in levels of NTX in response to the high compared with the low sodium diets (p = 0.031), but this was not observed for Dpy or Pyr.

Although there was no effect of diet on the bone formation marker OC, there was a significant increase in percent change from baseline for B-ALP on the -Ca+Na diet (+14 ± 12%, p = 0.011), suggesting an increase in bone formation activity and overall bone turnover after the increase in salt and decrease in calcium intake.

**DISCUSSION**

A marginally negative bone balance is observed in postmenopausal women because of the dominance of bone resorption over bone formation, but large interindividual differences have been reported in women adapted to low and high calcium intakes. The particular strength of the data presented here is the randomized cross-over study design that used four very-well-controlled diets that provided different intakes of calcium and sodium within the range that is commonly observed in Western-style diets. This study investigated the effect of sodium on calcium excretion at two levels of calcium intake in the same individuals using a cross-over design, thereby eliminating the confounding effect of genotype. The calcium and salt levels reflect those consumed by a significant proportion of the UK postmenopausal population. This is an important feature of the study design because many others have used higher salt loads that were likely to elicit a response that would only be seen at the extremes of intake.

The responses to sodium-induced calciuria are similar to those reported by others and summarized by Cohen and Roe, but we observed a significantly more pronounced calciuria on the moderately high (an increase of 33 mg/d) compared with the moderately low calcium diet (an increase of 18 mg/d, p < 0.001), which shows greater conservation on the lower calcium diet. One previously published study based on a parallel group design also reported a trend toward higher calcium excretion on a high calcium diet, but it was not statistically significant.

A significantly positive association between urinary calcium and sodium at baseline (habitual diet) was observed (p < 0.05), which supports observations from cross-sectional and cohort studies. However, one quarter of the volunteers did not respond to the sodium challenge by increasing their urinary calcium output. Shortt et al. reported that some individuals do not respond to a sodium challenge by increasing urinary calcium excretion and suggested that interindividual differences may be related to salt sensitivity. Furthermore, it is well documented that the magnitude of the estimated increase in calcium excretion per 100-mmol rise in urinary sodium can vary considerably; 2-fold differences in sodium-induced calciuria at low and high calcium intakes are common.

The low calcium diets also induced a significantly higher excretion of phosphorus (mean difference, 92 mg; p < 0.001), which is indicative of increased bone turnover because bone resorption is associated with a loss of both calcium and phosphorus in the urine. Potassium can modify the renal handling of calcium and sodium, and therefore it is an important covariate in metabolic studies on calcium. The average potassium excretion in this study was similar to that obtained by the Dietary Approaches to Stop Hypertension (DASH) trial (i.e., ~3000 mg/d). The DASH trial observed no effect on sodium-induced calciuria (~8 g salt/d) between diets low in potassium (1700 mg/d) and calcium (450 mg/d) compared with diets high in potassium (4700 mg/d) and calcium (1250 mg/d). This contradicts results obtained by Sellmeyer et al. who found that a doubling of potassium excretion from 2720 to 5500 mg/d was effective in reducing the sodium-induced calciuria in postmenopausal women consuming a rather high mean intake of 12 g salt/d. The basal diet in our study contained up to 8 g salt/d. The calcium test loads should have represented the calcium content of the intervention diets.
This would mean that our test dose of 183 mg was appropriate for the low calcium diet but that 450 mg calcium should have been given as the test dose for the high calcium diet. According to work by Heaney et al.,(31) calcium absorption from a diet containing 1250 mg/d of calcium (our high calcium intervention diet) should be ~20%, rather than the 23% that we observed with our test dose of 183 mg. Taking the 20% absorption figure obtained from the work of Heaney et al. and applying it to our model would, theoretically, cause minor reductions in the calcium fluxes shown in Table 2 (high calcium diet). Given this possibly lower absorption on the high calcium diet and the subsequent effect on the calcium fluxes, it would be inappropriate to examine the changes in the kinetic parameters that may be caused by calcium intake between the low and high calcium diets. Therefore, resulting kinetic data were separated into the two dietary calcium groups (Table 2) and analyzed separately to determine the effect of the salt intervention. This is a valid comparison because the difference in salt intake did not significantly alter calcium absorption (low Na: 23 ± 5%, high Na: 24 ± 5%; p = not significant), and therefore the only variable that changes within either the low or high calcium intervention groups is salt intake, which must be responsible for any changes in the kinetic parameters.

Our kinetic data suggest that salt has a significant effect on urinary calcium excretion (p < 0.001) and bone calcium balance (p < 0.05) only on the high calcium diets. More calcium is excreted in the urine as a result of the increased salt intake, and bone calcium balance changes from being positive on the low salt diet to negative on the high salt diet. There was a trend for more endogenous calcium to be excreted into the feces, but this was not significant (p = 0.082). The low calcium diets show similar trends, but they are not significant; more calcium is excreted in the urine as a result of the increased salt intake (p = 0.037), and bone calcium balance becomes more negative when moving from a low to high salt diet (p = 0.196). Extending periods of low calcium intake, as used in this study, have been shown to maximize mechanisms to conserve calcium, which may explain why the salt challenge on the low calcium diet seems to be less pronounced and did not reach significance in terms of calcium kinetics.

An earlier study in postmenopausal women consuming a moderate intake of calcium (816 mg/d) showed no detectable effect of high or low salt diets on the rate of bone resorption, but this is not surprising given the fact that the study was only an 8-day duration.(29) Longer-term studies are needed to evaluate effects on bone turnover in response to diet(30) as well as drug therapy(32) when assessed by bone biomarkers. Notwithstanding this requirement, the bone calcium balance predicted by our kinetic model is supported by the findings of reduced levels of markers of bone resorption in response to the high calcium diets. For each of the three markers of bone resorption, the observed significant changes from baseline in response to calcium were associated with a power of >90%. If we assume a minimum 10% difference from baseline in response to sodium to be significant, the power of identifying such a difference in our sample was 20% for NTX, 80% for Pyr, and 57% for Dpyr.

The observed percent change for NTX in response to sodium was actually 19%, with an associated power of 57%. In a longer-term study (6 mo) of postmenopausal black women, a low sodium diet (2 g/d) reduced bone turnover, as indicated by lower serum concentrations of aminoterminal propeptide of type 1 collagen.(33) This was accompanied by a lower urinary excretion of both sodium and calcium, but there were no changes in calcitropic hormones. Conversely, in younger men and women (21–39 yr of age) given a low salt diet for 7 wk, there were no detectable changes in markers of bone metabolism.(34) However, this was a parallel design study, which is not as robust as the cross-over design used in this study.

In conclusion, it seems that both dietary calcium and sodium play a major role in the maintenance of bone health in postmenopausal women. Low calcium intake (518 mg/d) was associated with negative bone calcium balance with both high and low salt diets, but with a moderately high calcium intake (1284 mg/d), the bone balance was positive when the salt intake was low (3.9 g/d) but not when it was moderately high (11.2 g/d).

ACKNOWLEDGMENTS

The authors thank Angela Twaitie and Veronica Kellas for assistance with food deliveries, sample collections, and processing, and John Eagles for mass spectrometry analysis. We are also grateful for the care and supervision of the volunteers provided by the staff of the IFR Human Nutrition Unit (Aliceon Blair, Lesley Maloney, Linda Oram, Yvonne Clements, and Nicola Hewitt). SFT and BT were responsible for the study design and manuscript preparation (with JRD) and the conception and funding of the study in collaboration with AF and KDC; BT supervised the study; CAAS, GM-N and KDC undertook the human intervention and the majority of sample preparation and biochemical analysis; JAH was responsible for the mass spectrometric analysis; JRD undertook the modelling and stable isotope calculations; JJ carried out the vitamin D and PTH analysis; KDC and AF supervised the bone biomarker analysis; RJJ provided statistical support. This project was funded by the UK Food Standards Agency, the European Commission Quality of Life Fifth Framework Programme QLK1-CT 1999-00752 and the Biotechnology and Biological Sciences Research Council.

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Appendix C: Letters from co-authors
18th February, 2010

Mr. Jack Dainty
Institute of Food Research (IFR),
Colney Lane,
Norwich NR4 7UA,
UK.

To Whom It May Concern

Re: Jack R. Dainty

Prior position in the scientific field of human nutrition: During 4 decades of research into the human metabolism of folate, the in vivo kinetics and quantification of relative gut absorption of oral 5-methyl tetrahydrofolate, the main folate component available from food, remained unclear. Early work by V. Herbert (Herbert & Zalusky, 1962) and S.E. Steinberg (Steinberg et al. 1979) had provided estimates of in vivo plasma disappearance and the role of the enterohepatic system, respectively. In 2003, in vivo stable isotope studies by J.F. Gregory III and co-workers had provided modelling results for folate absorption of 30% of the oral dose, which was much lower than estimations of true absorption obtained in animal studies.

Contribution: Jack Dainty made a major contribution to the field by developing a mathematical model which integrated the different components, including estimated liver metabolism and initial elimination. However, his proposed model required prior estimates of the laboratory error as well as in vivo (plasma) variation coefficients from human volunteer studies. These model parameters were derived from in vivo experiments in 5 human volunteers comparing stable isotope labelled folic acid ingested from a drink versus a capsule (Kok RM et al. 5-Methyltetrahydrofolate acid and folic acid measured in plasma with LC-tandem-MS: application to folate absorption and metabolism. Anal Biochem 2004;326;129-38). The novel and sophisticated mass spectrometry method and the mathematical modelling were described in the same paper, providing for the first time reliable estimations of bioavailability of folic acid and internal validation of the mode of administration. Jack Dainty’s contribution was vital for the human experiment’s interpretation: he made and wrote a major contribution to the methods’ section, and did the modelling and interpreted the results of the human volunteers’ study. The paper is a rare example of a novel mathematical application in this field of human physiology and novel biochemical method development.
Re: Jack R. Dainty

The new development was applied in a study which, for the first time, directly measured the absorption and elimination of the biologically active form of 5-methyltetrahydrofolate, [6S]5-methyltetrahydrofolate, in an intervention study in human volunteers. (de Meer K et al. [6S] 5-Methyltetrahydrofolic acid or folic acid supplementation and absorption and initial elimination in young and middle-aged adults. Eur J Clin Nutr 2005;59: 1409-16. Mr. Dainty’s contribution in this study equals the first author’s contribution. The study showed the robustness of the model as applied to human vitamin supplementation experiments. Jack Dainty’s estimates of ‘true’ absorption of folate was close to 100% of the dose (as indeed would be expected from animal gut balance studies), while the ‘apparent’ absorption was around 32% (a value close to the published data in Gregory III et al. 2003).

Overall Assessment: These findings are important for the fundamental understanding and quantification of metabolism of folates in human health and disease, and opened the field for trials using isotopes of these substrates (as currently underway, see clinicaltrials.gov.org)

Yours faithfully,

Dr K. De Meer
Paediatric Consultant
ROCKHAMPTON HOSPITAL
To whom it may concern

Re: Jack R. Dainty

Kinetics of gastro-intestinal transit and carotenoids absorption and disposal in ileostomy volunteers fed spinach meals.

Background:
Prior to the publication of this paper there was considerable confusion over the interpretation of the finding that volunteers fed carotenoids in the same form showed quite different plasma responses. This lead to the belief that there were 'responders' and 'non responders'. However, it was recognised that 'response' was a result of both absorption and disposal and neither had been properly quantified.

Jack Dainty made a major contribution to the design of the experiment, modelling the data and writing the paper. By reconciling both the mass balance (intake-excretion) and the TRL curves it was possible to establish that all the volunteers responded similarly and that the differences seen in previous studies could be ascribed to plasma kinetics rather that a lack of absorption per se.

This finding is of fundamental importance in that it explains recovery from vitamin A deficiency without the assumed prerequisite of elevated plasma beta-carotene.

Richard Faulks  
5th Jan 2010
Re: joint papers relating to Jack R Dainty’s PhD by publication


To whom it may concern,

I can confirm that I am one of the co-authors on the papers (above) and have worked with the candidate (Jack Dainty) for the past 10 years. I can also confirm that he has provided substantial input into the folate research performed at IFR during the past decade. In particular, he has been solely responsible for all the mathematical modelling work in this area which has given us crucial insight into the bioavailability and metabolism of folic acid. In papers [1] and [3] (above), Jack helped in the design of the studies which enabled us to combine stable isotope labelled folates with mathematical modelling for the first time. He analysed all the data and used compartmental modeling to help us gain new knowledge regarding the way that folic acid (in particular) is metabolised, in humans. He made a substantial contribution to the preparation of the manuscripts that have subsequently become highly cited in the field. In addition, he presented the folate modelling work at a highly regarded conference (Experimental Biology) in New Orleans in 2002. Paper [6] is a summary of the work we have carried out in folate and Jack was responsible for the important re-evaluation of the "old" literature as well as development of new hypotheses, and preparation of the paper for publication. Papers [2] and [4] represent work that Jack was involved in during an EU folate project and were only possible because of his modelling skills. The design of the experiment was carried out mainly by the Dutch group but Jack had an influence on the design (e.g. sampling times and curation of the experiment) to allow him to model the subsequent data. Analysis was carried out by him and he also helped prepare the manuscripts although not to the same extent as papers [1], [3], and [6]. Paper [5] was the output from a BBSRC responsive mode proposal that Jack co-wrote with myself and Hilary Powers at Sheffield University. He is responsible for the majority of the design of this novel study which was the first experiment performed with stable isotope labelled riboflavin. The analysis was performed by him and he wrote the first draft of the manuscript which was published in the highest impact factor journal in the field.

Yours faithfully,

[Signature]

Paul Finglas
Head, Food Databanks & Exploitation Platform Leader
Contribution made by Jack Dainty to 6 co-authored papers


   a) Design of investigation – 0%
   b) Conduct of research – 10%
   c) Analysis of outcome – 25%
   d) Preparation of the work for publication – 20%


   a) Design of investigation – 0%
   b) Conduct of research – 10%
   c) Analysis of outcome – 80%
   d) Preparation of the work for publication – 80%


   a) Design of investigation – 10%
   b) Conduct of research – 10%
   c) Analysis of outcome – 40%
   d) Preparation of the work for publication – 50%


   a) Design of investigation – 30%
   b) Conduct of research – 25%
   c) Analysis of outcome – 80%
   d) Preparation of the work for publication – 60%

   a) Design of investigation – 20%
   b) Conduct of research – 40%
   c) Analysis of outcome – 75%
   d) Preparation of the work for publication – 70%


   a) Design of investigation – 5%
   b) Conduct of research – 10%
   c) Analysis of outcome – 25%
   d) Preparation of the work for publication – 20%

[Signature]

Professor Susan Fairweather-Tait
UEA School of Medicine, Health Policy & Practice

18th February 2010
17 February 2010

To whom it may concern,

I am pleased to acknowledge Jack Dainty's contribution to the following papers on which I am a co-author.


In each case Jack contributed to various aspects of each publication as follows:

**Study design**: Jack was specifically involved in determining the study design outlined in each paper in terms of calculations related to copper stable isotope doses and the required duration of sample collection from study participants. This required an understanding of human copper metabolism *in vivo* and the relationship to the feasibility and reliability of mass spec. analyses of biological samples.

**Data analysis**: Jack was instrumental in developing algorithms and computer programs which facilitated analysis of all the mass spec data related to stable isotopes and rare earth elements discussed in these publications. He had responsibility for the majority of data analyses related to copper stable isotope absorption and excretion reported in these papers. In paper 8, he was additionally responsible for undertaking all kinetic modelling (using SAAMII software) of the copper data generated from the human intervention study.

**Manuscript preparation**: Jack was responsible for writing all aspects of the manuscripts related to his contributions outlined above. He was also involved in proof-reading and commenting on the completed manuscripts prior to submission.

I am happy to provide further information if required.

Yours faithfully,

Linda Harvey PhD

Norwich Research Park, Colney, Norwich NR4 7UA, UK

[www.ifr.ac.uk](http://www.ifr.ac.uk) Tel: +44(0) 1603 256000 GTN 8626 6000 Fax: +44 (0)1603 507723
Dear Sir/Madam

re: Jack Dainty: application for PhD by publication

First authorship of:

Jack Dainty was a named researcher on the grant proposal to BBSRC (D19615) which secured the funding underpinning the research leading to this publication. Mr Dainty’s input to discussions prior to the submission of the grant proposal was absolutely essential to the development of ideas and finalisation of the submitted application, without his input it would not have been possible to include the bioavailability component of the study. Once the funding had been secured Mr Dainty used his expertise in steering the progress of the bioavailability work, and contributed to regular Steering Committee meetings in Sheffield to facilitate this. Finalisation of protocol detail for the use of stable isotope-labelled riboflavin was ultimately Mr Dainty’s responsibility, as was the sample handling, chemical analysis and kinetic modelling of data relevant to riboflavin bioavailability. Mr Dainty was responsible for interpreting the analytical data and developing the mathematical model of riboflavin bioavailability. Mr Dainty prepared the first draft of this manuscript; his expertise in this area was again essential at this point. The manuscript was published in the Journal with the highest impact factor in the nutritional sciences, testimony to the high quality of the study and the data generated.
This work would not have been possible without Mr Dainty's important contribution.

Please do not hesitate to contact me if you require further information.

Yours faithfully

Hilary J Powers
Professor of Nutritional Biochemistry
16 February 2010

Re: Jack Dainty PhD – contribution to publications

I am writing to confirm that I was a co-author of the 2003 publication ‘Quantification of unlabelled non-haem iron absorption in human subjects: a pilot study’ that was published in the British Journal of Nutrition, Volume 90, pages 503-506. The paper described an investigation of a single compartment mathematical model for predicting iron absorption from oral doses of unlabelled ferrous sulphate. Samples were collected as part of a larger study of in vitro solubility and in vivo absorption of different iron compounds that may be used in fortification of breakfast cereals. Jack Dainty developed the mathematical model using data generated by the study and interpreted the value of the new model against absorption calculated using a stable isotope technique. He drafted the majority of the paper and made the major contribution to the Introduction section, the description of calculations used in the Materials and methods section and the Results and discussion section. The paper would not have been possible without his major involvement and that is reflected in his role as first and corresponding author.

Yours Sincerely,

Mark Roe
To whom it may concern

Re: Jack R. Dainty

doi:10.1038/sj.ejcn.1602030
Published online 14 July 2004

Estimation of iron absorption in humans using compartmental modelling

Compartmental modelling can overcome the limitations that other iron absorption techniques present. Nowadays, it is the only method that can be used in unlabelled iron studies. In the past, complex multicompartmental models have been developed, however Jack Dainty designed a single compartmental model which showed good agreement with the well validated area under the curve method. In order to carry out the comparison of the two techniques, he participated in designing the most adequate human study using an oral and intravenous doses of iron, supervised the running the experiments, performed all the required mathematical calculations as well as the analysis of the outcome and consequently wrote the majority of the present publication. Further studies have been carried which assess whether the single compartmental model could have application in the assessment of total (haem+nonhaem) iron absorption from food and corroborate the usefulness of the method.

Beatriz Sarria
Feb. 15th 2010
To whom it may concern:

I confirm by this letter the contribution made by Jack Dainty to


in respect of the (a) design of the investigation, (b) conduct of the research, (c) analysis of the outcome, (d) preparation of the work for publication, which was carried out at the Institute of Food Research during 2000 to 2004 with funding from the UK Food Standards Agency, the European Commission Quality of Life 5th Framework Programme (QLK1-CT 1999-00752) and the Biotechnology and Biological Sciences Research Council (BBSRC).

Jack Dainty was involved in the conception of this project, specifically the isotope dose calculation for study participants based on kinetic models. He contributed to the writing of the original grant proposal, was solely responsible for the compartmental modelling of the data obtained, and for most of the statistical data analyses. Jack made a major contribution to the interpretation of the results, and the preparation and submission of the manuscript to the Journal.

Jack Dainty’s expertise was essential to the successful completion of this research project. It was a pleasure to work with him.

Yours sincerely,

[Signature]

Dr. Birgit Teucher
Research Scientist
Project co-ordinator for EPIC-Heidelberg
Uppsala, 29.01.2010

To whom it may concern

Herewith I confirm that Jack Dainty, IFR, contributed to the joint original publication entitled


by carrying out the kinetic modelling/analysis for determination of apparent folate absorption and by preparation of parts of the manuscript.

Cornelia Witthöft
(Assoc Prof)
RE: Mr Jack R. Dainty's application for PhD by publication


*Journal of Nutrition* 135, 619-623.

*British Journal of Nutrition* 98, 667-675.

To whom it may concern,

As corresponding author for the above mentioned peer-reviewed scientific publications, I am pleased to confirm that Mr Jack Dainty provided substantial contributions to the design, conduct, analysis and interpretation of results, and preparation of papers [1] and [2] for publication. Additionally, I confirm that for the Review Article [3] Mr Jack Dainty provided substantial contributions to the design, critical and insightful (re-)evaluation of the literature, to the development of new hypotheses, and preparation of the paper for publication.

I have worked with Jack for over a decade and have not only welcomed his thoughtful and perceptive mathematical modelling input into vitamin folate research, but also have recognised (as have other long-term researchers in this field) that his input was a vital key that unlocked the historical and crystallized view of (‘pro-vitamin’) folic acid absorption and initial site of biotransformation to ‘vitamin’ in humans.

Jack’s input into the reappraisal of older literature and the development of new hypotheses has helped invigorated research on folic acid absorption/metabolism — which has high political interest in the current era with its heated arguments over the risk/benefit of population-wide policies of mandatory folic acid fortification. Already, two aspects of our views have been corroborated; (i) that humans, as opposed to animals, may have difficulty in the initial but vital biotransformation of folic acid ‘pro-vitamin’ to tetrahydrofolic acid ‘vitamin’ (Bailey SW & Ayles JF. PNAS 2009,8;15424-9); (ii) that the 60-year-old historical use of folic acid as the ‘reference folate’ in longer-term (ca. 12-16 weeks) dietary supplementation studies of the comparative bioavailability of folates from foods or synthetic (but ‘nature-identical’) folates using changes in folate status (plasma and red cell concentrations) is scientifically untenable (Wright AJA et al. Br J Nutr 2010 ‘in press’).

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Furthermore, on the basis of our revised hypotheses, we have secured a Biotechnology and Biological Sciences Research Council; 3-year (2008-2011) BBSRC Research Grant Award in excess of £1 Million for a 3-centre collaboration "Where is the initial site of folic acid biotransformation in humans?" [Ref: BB/F014457/1] on which Jack is a 'named researcher'. I look forward to Jack's input into the project that will provide definitive answers, and will inter alia (a) carry out an in vivo kinetic investigation of absorption and initial biotransformation, using stable-isotope-labelled folic acid and 5-formyltetrahydrofolic acid (a 'natural' reduced folate), in 'stable' Transjugular-Intrahepatic-Portal-Systemic Shunt' (TIPSS) patients (Newcastle University) — which should allow sampling direct from the hepatic portal vein prior to folates transported from the absorptive mucosal cells being removed by the liver on '1st-pass'; and (b) carry out an in vivo kinetic estimation of the sampled plasma 'volume-of-distribution', using i.v administered stable-isotope-labelled 5-methyltetrahydrofolic acid, in healthy volunteers (IFR).

I look forward to Jack's continued input, not only into folate research but also into the absorption/initial metabolism of other water-soluble vitamin and non-vitamin bioactive phytochemical compounds; his lucid thinking helping to transform the phenomenological into the hypothesis-driven.

Anthony J. A. Wright
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Appendix D: Full list of publications


