Quantification of the bioavailability of riboflavin from foods by use of stable-isotope labels and kinetic modeling¹⁻³

Jack R Dainty, Natalie R Bullock, Dave J Hart, Alan T Hewson, Rufus Turner, Paul M Finglas, and Hilary J Powers

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. Am J Clin Nutr 2007;85:1557–64.

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

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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 included for 7 of the remaining 17 volunteers for whom sample analysis problems were encountered. The number of data sets that were included in any statistical or mathematical analysis is 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 g 13C-labeled riboflavin in 200 mL semi-skimmed milk (fat content 1.7 g/100 mL) with a simultaneous intravenous injection of 200 g 13C-labeled riboflavin or, 308 g labeled total flavins as 15N-labeled spinach soup with a simultaneous intravenous injection of 200 g 13C-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 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, 50, 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 x 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 x g at 4 °C. The supernatant fluid and theuffy coat and cell debris were discarded. A 100-µL volume of cells was added to 100 µL 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 13C-labeled riboflavin

[2,4,6-13C3]Barbituric acid. This was prepared from [13C]urea and [1,3-13C2]diethyl malonate according to the literature procedure (13).

[2,4,10a-13C6]Riboﬂavin and [1,2,2',3',4,4',5,10a-13C7]riboflavin. These were prepared from the labeled barbituric acid and either unlabeled ribose or 13C5-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) chloroform:acetone:concentrated ammonia, 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 [13C3]riboflavin), and 407 (Na adduct of [13C4]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 ~98% of the desired isotopomer.

Preparing the intravenous 13C-labeled dose
13C-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 dark 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 13C milk dose
A total of 12 mg 13C-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 µg 13C-labeled riboflavin). Samples were defrosted overnight at 4 °C before use. The unlabeled riboflavin content of the milk was measured as 1.67 µg/mL. FAD and FMN constituted 0.40 µg/mL (see “Blood sample flavin analysis” below), and each test meal consisted of 200 mL milk. Each volunteer therefore consumed 334 µg unlabeled and 400 µg labeled riboflavin from the milk dose.

Preparing the intrinsically labeled 15N 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 hydroponicum, 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 (15N) 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 d (more detail of the procedure can be found in a previous publication; 16), yielding 4.55 kg of leaf material. The 15N-labeled spinach was washed, dried, mixed, and frozen at –20 °C for shipment to Sheffield University for processing into soup within 7 d. After being defrosted, the spinach leaves were finely chopped with a food processor. To make the soup, 42.5 g fresh whole 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) and 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 15N-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 were prepared for flavin isotope ratio determination by HPLC/elecrospray 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 Quatro 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 isotopeomers at m/z 399 (M), 400 (M + 1), 401 (M + 2 – spin had 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].

Urine sample analysis

Unlabeled

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.
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 be taken 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 \( ^{13} \text{C}-\text{milk or } ^{15} \text{N}-\text{spinach} \) to that from the intravenous (IV) label that appears in the urine in the 48 h after dosing.

\[
\text{True absorption (abs}_{\text{true}} = \left( \frac{\sum_{0}^{t=48h} \text{oral label in urine}}{\sum_{0}^{t=48h} \text{oral label}} \right) \times \left( \frac{\text{dose of IV label}}{\sum_{0}^{t=48h} \text{IV label in urine}} \right) \]

Compartmental model

Isotopic data from plasma analysis were analyzed by using the SAAM II 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 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 \( k(i,j) \), 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 \( k(0,1), k(1,2), \) 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 \( M/T \), 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 \( \text{abs}_{\text{apparent}} = \frac{M}{\text{dose of oral label}} \) (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

\[
\text{First-pass effect} = 1 - \left( \frac{\text{abs}_{\text{apparent}}}{\text{abs}_{\text{true}}} \right) \]

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

| TABLE 1 | Subject characteristics \( ^{1} \) |
| --- | --- | --- | --- | --- |
| Age | Height | Weight | BMI |
| y | cm | kg | kg/m² |
| Mean | 36.5 | 164.2 | 59.6 | 22.1 |
| SEM | 3.38 | 1.91 | 2.00 | 0.65 |
| Minimum | 21 | 149 | 47 | 19.07 |
| Maximum | 60 | 178 | 74 | 25.39 |

\( ^{1} n = 17 \) subjects for whom data were available for the kinetic analysis.
TABLE 2
Biomarkers of riboflavin status (plasma flavin concentrations) at baseline

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

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

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 µg (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-µg 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 µg (10.4%; n = 14) and that from milk was 38 ± 4.90 µg (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 ≈42 min after the spinach dose and ≈30 min after the milk 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 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 d$^{-1}$; $k(1,2)$: 54.5 ± 8.6 d$^{-1}$; and $k(2,1)$: 102.4 ± 17.3 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 d$^{-1}$ ($P = 0.076$); $k(1,2)$: 40.7 ± 22.9 compared with 25.3 ± 8.4 d$^{-1}$ ($P = 0.959$); and $k(2,1)$: 20.0 ± 5.4 compared with 55.3 ± 14.3 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 (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 I). 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 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 the 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 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: 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.