

Intrinsic Erythrocyte Labeling and Attomole Pharmacokinetic Tracing of ^{14}C -Labeled Folic Acid with Accelerator Mass Spectrometry

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Long-term physiologic tracing of nutrients, toxins, and drugs in healthy subjects is not possible using traditional decay counting of radioisotopes or stable isotope mass spectrometry due to radiation exposure and limited sensitivity, respectively. A physiologic dose of ^{14}C -labeled folic acid (35 μg , 100 nCi) was ingested by a healthy adult male and followed for 202 days in plasma, erythrocytes, urine, and feces using accelerator mass spectrometry. All samples and generated wastes were classified nonradioactive and the subject received a lifetime-integrated radiological effective dose of only 11 μSv . Radiolabeled folate appeared in plasma 10 min after ingestion but did not appear in erythrocytes until 5 days later. Approximately 0.4% of the erythrocytes were intrinsically labeled with an average of 130 ^{14}C atoms during erythropoiesis from the pulse of plasma [^{14}C]folate. An appropriate radiocarbon-labeled precursor can intrinsically label DNA or a specific protein during synthesis and obtain limits of quantitation several orders of magnitude below that of stable isotope methods. © 1999

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Key Words: intrinsic labeling; accelerator mass spectrometry; AMS; pharmacokinetics; folate; physiologic dose.

Metabolism studies of pharmaceuticals, toxins, and nutrients traditionally use radioisotope labels and liquid

scintillation counting (LSC)² to trace parent compound and major metabolites. Recent improvements in mass spectrometry have increased interest in stable isotope labeling as an alternative that avoids radiation exposure, regulatory overhead, and waste disposal complications, but sensitivity limitations often necessitate pharmacological doses. A physiologic dose is often difficult to trace with stable or radioactive isotopes in a healthy human due to low concentrations in the easily sampled biological pools. Accelerator mass spectrometry (AMS) (1) has the sensitivity to overcome the limitations of traditional mass spectrometry for stable isotope detection and of decay counting for radioisotope detection in measuring the fate of a physiologic dose of an isotope-labeled compound.

AMS counts electrostatically accelerated nuclei in a simple particle detector. Molecular isobars are completely disassociated in the charge changing process and any atomic isobars are discriminated in the detector. AMS is particularly efficient in detecting long-lived isotopes ($10\text{ y} < t_{1/2} < 100\text{ My}$). The rarity of long-lived radioisotopes yields a much lower background than that achievable with stable isotopes. For example, the naturally occurring carbon isotopic abundances are ^{12}C (98.9%), ^{13}C (1.1%), and ^{14}C ($1.2 \times 10^{-10}\%$). The natural background of ^{14}C is 10 orders of magnitude lower than the rare stable isotope ^{13}C . The decrease in background yields much greater sensitivity for ^{14}C AMS over ^{13}C MS. Decay-counting long-lived isotopes is not efficient, counting 0.1% of the decays of a ^{14}C sample takes 8.3 years.

² Abbreviations used: LSC, liquid scintillation counting; AMS, accelerator mass spectrometry; DRI, dietary reference intake; LLNL, Lawrence Livermore National Laboratory; LOQ, limits of quantitation; RBC, red blood cells; IRMS, combustion isotope ratio mass spectrometry.

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High sensitivity opens other avenues of research. Selection of an appropriately radiolabeled precursor can be used to label DNA or specific proteins during synthesis. Cell proliferation and life cycles can be monitored through the turnover of the radiocarbon signal. Although nutrients are more difficult to detect than toxins and drugs due to the endogenous pool, we selected B-vitamin folic acid for a demonstration in human nutrient kinetics because its detection is confounded by low concentrations in tissues (~ 20 nM in plasma) and its role in disease prevention is topical. The role of folate in erythropoiesis provided a simple example of intrinsic labeling of a specific cell type.

Folate is a generic term for compounds that have nutritional properties and chemical structures similar to those of folic acid (pteroylglutamic acid), the synthetic pharmaceutical form of the vitamin. It is crucial for nucleotide synthesis, cell division, and gene expression (2). Recent evidence linking low plasma folate concentrations with increased risk of neural tube birth defects (3–6), coronary heart disease (7–9), and cancer (10, 11) has led to an increase in the consumption of this vitamin, frequently as a dietary supplement. Despite its physiologic role and widespread use, the dynamics of folate metabolism in healthy humans, the factors that affect it, and the consequences of altering these dynamics are poorly understood.

The current understanding of folate metabolism is based on a physiologic dose consumed by a woman in remission of Hodgkin's disease (12) and an early depletion/repletion study in a healthy man (13). Other folate tracer studies utilized nonphysiologic doses with radiocarbon (14) or tritium (15) labels. Pharmacological doses saturated normal folate metabolic pathways, causing much of the material to be excreted without providing natural kinetic data (14–16). Physiologic doses of labeled folate have been traced using large amounts of tritium in patients in poor health (17) and using deuterium (18), which suffered from problems of poor sensitivity, isotope exchange, and isotope effects (19). All these analytical methods failed to measure the minute concentrations of labeled folates and folate catabolites in biologic fluids and tissues (12, 14–18). We used AMS to study the metabolism of a physiologic oral dose of [^{14}C]folic acid by measuring the [^{14}C]folate levels in serial plasma, erythrocyte (RBC), urine, and feces samples taken over a 202-day period from an informed healthy 57-year-old male volunteer. The erythrocyte life cycle was also clearly monitored by intrinsically labeling the RBCs with the pulse of [^{14}C]folate.

MATERIALS AND METHODS

Materials

Pteroyl-[^{14}C (U)]glutamic acid ([^{14}C]folic acid) was synthesized from L-[^{14}C (U)]glutamic acid (20) by modifying the method of L. T. Plante, K. L. Williamson, and E. J. Pastore (1980) (21). The synthesized [^{14}C]folic acid was isolated and checked for purity by coelution with standards using normal and reversed-phase HPLC. Its specific activity was 1.25 mCi mmol^{-1} . A physiologic (80 nmol, 35 μg) oral dose of [^{14}C]folic acid ($\sim 1/12$ current U.S. dietary reference intake (DRI)) was given in water and rinsed down with ~ 200 mL water.

Sample Collection

Predose blood, urine, and feces were collected to ensure the subject did not possess ^{14}C concentrations above natural levels, 109 fmol ^{14}C g^{-1} carbon. Additional predose blood was drawn just before a healthy adult male consumed a 3.7 kBq (100 nCi) dose of pteroyl-[^{14}C (U)]glutamic acid ([^{14}C]folic acid) in ~ 125 mL water after fasting overnight. After dosing, 24 blood samples (8 mL each) were drawn on day 1 and 18 more over the next 42 days. All urine and feces were collected for 42 days. Blood, urine, and feces were collected periodically over the next 160 days.

AMS Sample Preparation

All AMS sample preparation is carried out in disposable plastic or glassware to avoid contamination. Plasma and erythrocytes were separated by centrifugation. Platelets and leukocytes were removed but not measured. Erythrocytes were resuspended to the original blood concentration and lysed in ^{14}C -free 18 M Ω water. Feces were homogenized in 1-L 0.5 M KOH. Sample aliquots were packaged in a clean room at UC Davis and transported to Lawrence Livermore National Laboratory (LLNL) for AMS. Total carbon content of each sample was measured in triplicate with a Carlo Erba (now ThermoQuest) 1500 NCS analyzer. Samples were combusted and reduced to graphite for measurement using Vogel's method (22). Sample volumes were selected to achieve 1–2 mg total carbon: plasma, RBC suspension, fecal extract, and urine volumes were 20, 40, 50, and 100 μL , respectively.

AMS Measurement and Analysis

All predose samples contained natural levels of ^{14}C in all tissues. Carbon isotope ratios were measured to $\pm 3\%$ for all samples. Excess ^{14}C concentration over natural abundance was converted to folate equivalents (parent compound and all metabolites) using the spe-

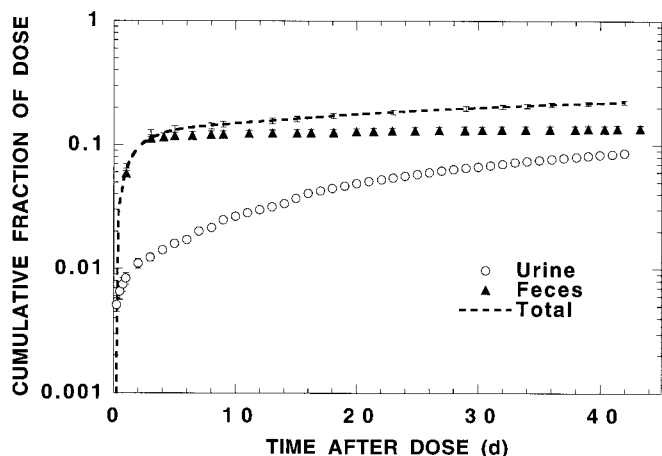


FIG. 1. Cumulative clearance of [^{14}C]folate through urine and feces. Error bars (SD, $n \geq 3$) for data fall within the symbols in most cases. Ninety percent of the dose was absorbed and $>75\%$ was retained after 42 days. Urine was the major route of excretion after absorption.

cific activity of the labeled folic acid, its molecular weight, and tissue carbon content.

RESULTS

Absorption and Elimination

Once folate is absorbed, the body exhibits a strong preference to retain it through internal recycling. Daily turnover is small ($\sim 200 \mu\text{g}$) compared to the total body pool ($\sim 60 \text{ mg}$). The isotope label was located in the tracer molecule to prevent catabolism and exhalation as $^{14}\text{CO}_2$ (14), allowing complete excretion of labeled folate to be monitored in urine and feces. Cumulative urine and fecal ^{14}C losses (Fig. 1) indicate that 90% of the dose was absorbed and $>75\%$ was retained in the body after 42 days. This contrasts with pharmacological doses that were only 20–65% retained after 6–24 h (14, 15). Gastrointestinal tract epithelial cell loss and folate turnover in the enterohepatic pool due to bile loss were measured in the feces without differentiation of the two contributions. Since folate is utilized in nucleotide synthesis, high turnover epithelial tissue is a major route for folate loss. The terminal phase of the sum of cumulative urine and fecal losses gives a whole body clearance half-life of 300 days, based on an exponential extrapolation of the data in Fig. 1. A linear extrapolation of these data predicts total body clearance in 360 days. These extrapolations do not account for clearance from deep pools with slow turnover (e.g., long-lived cells) and indicate that absorbed folate is retained substantially longer than the 100-day half-life reported previously (12). Limits of quantitation (LOQ) for urine and feces were 0.004 and 0.005 fmol [^{14}C]folate, based on predose background plus double the uncertainty for each tissue. The urine retained a signal above the LOQ

throughout the 202-day sampling period but the feces extract fell below the LOQ between days 42 and 175.

Plasma

The ^{14}C rapidly appeared in plasma 10 min after dosing, peaked at 1–2 h, and was seen in plasma for 61 days. The asymmetric peak shape in labeled folate concentration (Fig. 2) indicated at least two compartments or metabolic pools, one quickly clearing with an $\sim 5\text{-h}$ half-life, and a second in equilibrium with the endogenous pool having an $\sim 60\text{-d}$ half-life. The diseases associated with low folate concentrations are affected by imbalances in folate metabolism. While these data provide metabolic kinetics, metabolite profiles will be required to determine the distinct differences between health and disease states. The plasma LOQ was 0.004 fmol [^{14}C]folate, based on predose background plus double its uncertainty, and this sensitivity is adequate for further fractionation of the plasma by HPLC to identify specific metabolites. Limits of quantitation of HPLC fractions should be lower than neat plasma because the contemporary ^{14}C background from proteins can be replaced with a low level ^{14}C petroleum-derived carbon carrier, probably extending the range of sampling beyond 100 days.

Intrinsic Erythrocyte Labeling

The erythrocytes did not contain labeled folate until 120 h after dosing (Fig. 3), clearly showing that mature RBCs do not absorb folate. Folic acid is incorporated during a single stage of erythropoiesis (23), and the 8.8-h FWHM plasma pulse of [^{14}C]folate labeled only those

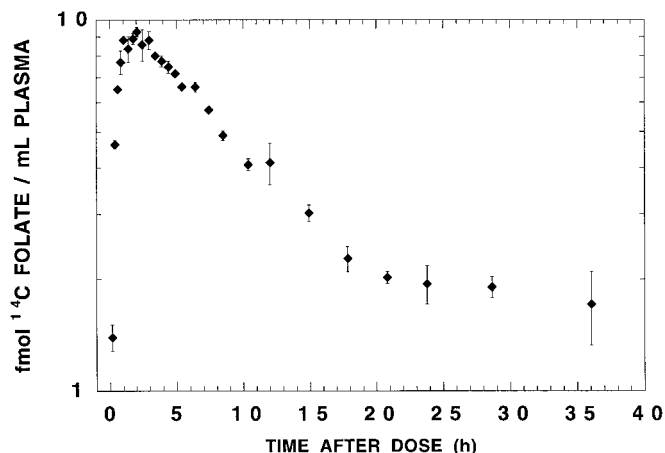


FIG. 2. Absorption and early clearance of [^{14}C]folate in plasma. Asymmetric peak suggests metabolite(s) grow in after delay. The signal declined slowly and remained above the LOQ (0.19 fmol [^{14}C]folate mL^{-1} plasma) through day 61. Error bars represent the standard deviation between measurements of at least 3 separate aliquots ($20 \mu\text{L}$) of plasma from the same blood draw.

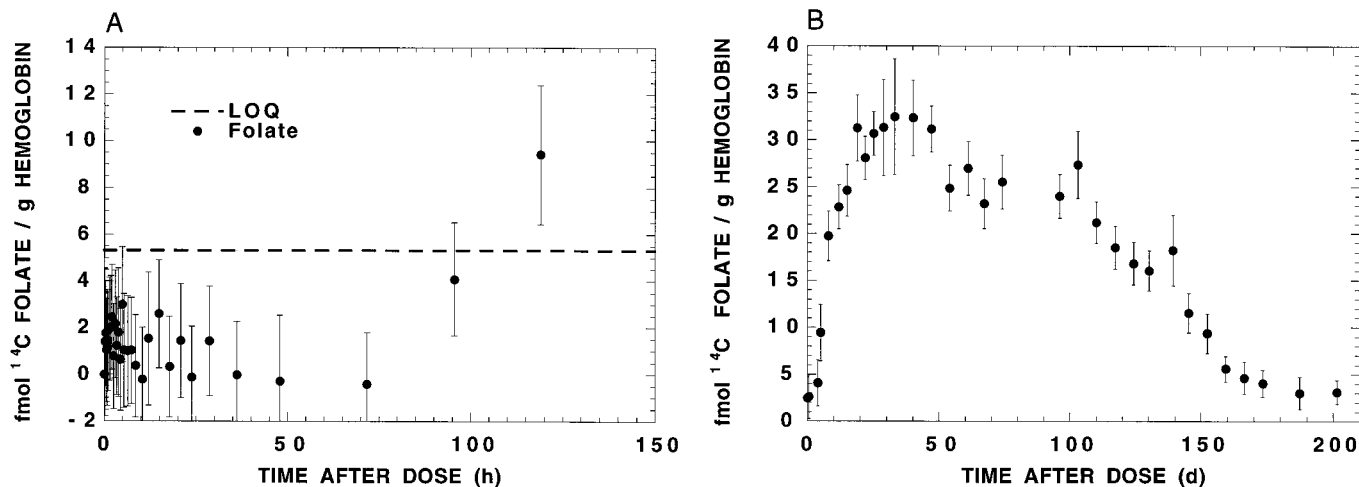


FIG. 3. Uptake of folic acid in erythrocytes and elimination of labeled red blood cells. Concentrations of [^{14}C]folate were normalized to hemoglobin to eliminate any variability in the RBC suspensions graphitized for AMS. Error bars represent the standard deviation between measurements of 3 or more separate aliquots of RBC suspension from the same blood draw. The 40- μL aliquots contained 4–8 μg hemoglobin. (A) Intensive early sampling defined the plasma profile (Fig. 2) but no RBC sample contained a ^{14}C signal above the LOQ ($5.3 \text{ fmol } [^{14}\text{C}]\text{folate} (\text{g hemoglobin})^{-1}$) until 120 h after dosing. (B) The erythrocyte life cycle was clearly traced with the ^{14}C signal which remained above the LOQ for 5–160 days after dosing.

erythrocytes completing that stage during the pulse. Erythrocyte ^{14}C levels displayed a broad maximum from 8 to 103 days after the dose and dropped to half the peak level at 130 days after dose, as the ^{14}C -labeled erythrocytes aged and were eliminated. The RBC lifetime was approximately 125 days for the study subject using the cohort method, similar to the accepted lifetime of 120 days derived previously (24, 25). The pulse of labeled folic acid intrinsically labeled $\sim 0.4\%$ of the erythrocytes *in vivo* and revealed their cellular kinetics directly. This estimate of labeling is based on the peak width of the [^{14}C]folate plasma pulse, the measured lifetime of the erythrocytes, and the assumption that erythrocyte production is constant when averaged over 6-h increments. Each labeled RBC contains ~ 130 ^{14}C atoms above natural background (40 labeled folate molecules) on average, with a maximum value probably close to 500 ^{14}C atoms RBC^{-1} due to the plasma pulse. These values for labeling are based on the specific activity of the glutamic acid used in the folic acid synthesis (20), the measured isotope ratios and carbon concentrations of the RBC suspensions, and an average 5×10^6 $\text{RBC } \mu\text{L}^{-1}$ of whole blood. AMS can exploit an appropriately labeled precursor (e.g., vitamin, amino acid, sugar) at physiological dose to intrinsically label DNA or a specific cell type and follow detailed kinetics *in vivo* with negligible radiological hazard and very high sensitivity.

DISCUSSION

Stable isotope precursors have been used to label DNA and fatty acids, but natural backgrounds of these isotopes limit sensitivity. Deuterium was recently used

to study T lymphocyte kinetics in HIV-seropositive and -seronegative volunteers using a 60-g intravenous infusion of [$6,6\text{-}^2\text{H}_2$]glucose over 48 h (26). Our data show that AMS is capable of obtaining similar information with 10^{-6} the dose in a single bolus. Goodman and Brenna described a gas chromatography–combustion isotope ratio mass spectrometry (GC–IRMS) method using ^{13}C that might achieve a LOQ 10 times that of ^{14}C AMS for molecules possessing 99% ^{13}C (27). Only 0.7% of the dose folic acid molecules contained ^{14}C atoms in our study, a level of labeling much easier to achieve through chemical synthesis or natural production in an isotope enriched atmosphere. An *in vitro* study using [$1\text{-}^{13}\text{C}$]glycine precursor to measure DNA synthesis rates utilizing a chemical reaction interface IRMS called for a better mass spectrometer capable of detecting orders of magnitude lower enrichment (28). The limiting factors in these techniques—high natural abundances of the stable isotopes; instrument noise, retention of molecular isobars, and poor detection efficiency for molecular MS; and baseline instability for IRMS—are avoided by using a low abundance isotope with AMS detection.

The rapid initial clearance of the [^{14}C]folate from the plasma spike and the 5-day delay in appearance of labeled erythrocytes provides a simple clinical method for monitoring defective erythropoiesis. A single drop of blood is sufficient material for analysis by AMS, and two such drops a few hours (for plasma concentration) and a few days (for RBC concentration) after ingestion of labeled folic acid measures the incorporation of folate into RBCs during production in the marrow.

Although this study included only one adult male, the good health of the subject and high sampling density permitted the most detailed analysis of the metabolism of a physiologic folate bolus, revealed sampling strategies for differentiating between healthy and diseased state metabolism of folate, and laid the foundation for detailed metabolism studies of other nutrients. Previous studies involved subjects in ill health and did not have sufficient sampling densities for detailed kinetics evaluations.

The attomole LOQ of [^{14}C]folate achieved in neat tissue with AMS were 10^{-5} that of the standard method, the microbiological folate assay (29). The validity of red cell folate assays has also been questioned due to variable results depending the oxygen content of the hemoglobin (30). The high sensitivity of AMS allows the use of small amounts of labeled compound with modest specific activity. All samples and generated wastes contained less than $1.85 \text{ kBq } ^{14}\text{C g}^{-1}$ (50 nCi g^{-1}) and were classified nonradioactive (31), greatly simplifying handling and disposal. This folate study gave the subject a lifetime-integrated radiological effective dose of only $11 \text{ } \mu\text{Sv}$ (1.1 mrem), the same dose received during 2 h of transcontinental plane flight. The negligible radiological risk to subjects, the elimination of radioactive wastes, the low LOQs, and the high precision make AMS an ideal analysis method for nutrient and drug metabolism studies.

Decisions about RDAs, the new DRIs, nutrient supplementation during food production, and special needs for specific subpopulations are based on epidemiological studies that infer significant relations between nutrition and health from statistical analyses (4–9, 32, 33). Fates and effects of micronutrients in healthy or diseased humans, as outlined here, offer a mechanism to understand the biochemical bases of epidemiological trends.

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