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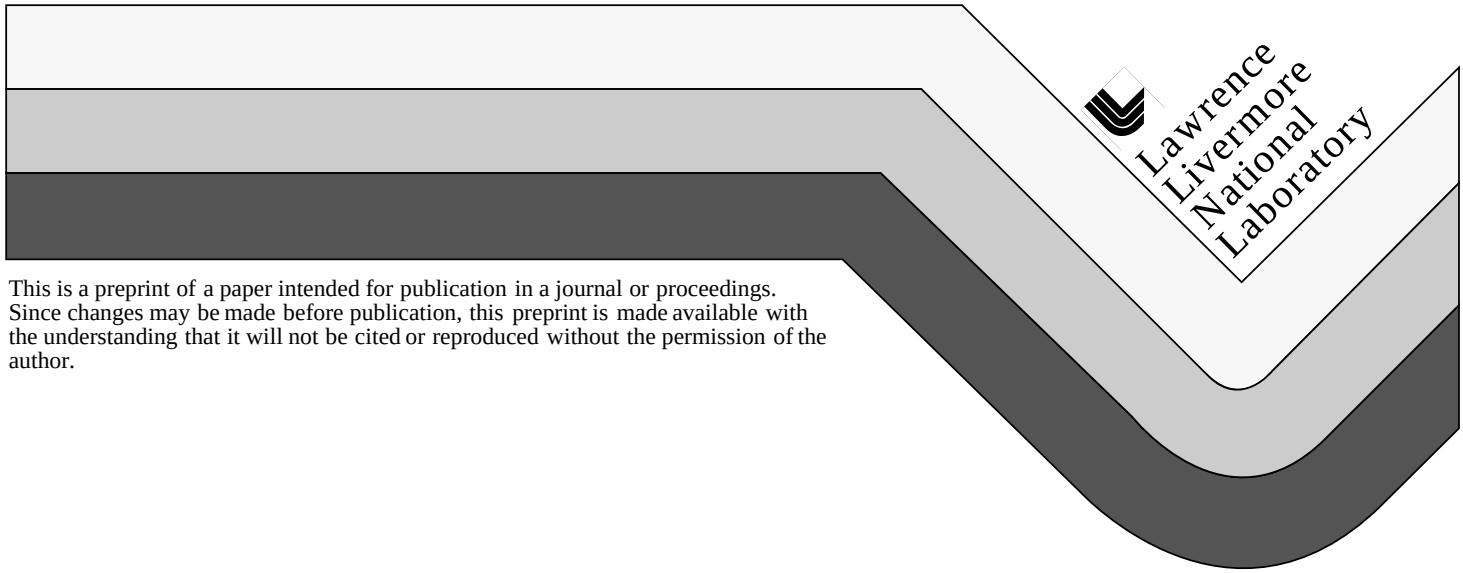

HUMAN FOLATE METABOLISM USING ^{14}C-ACCELERATOR MASS SPECTROMETRY

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Folate is a water soluble vitamin required for optimal health, growth and development. It occurs naturally in various states of oxidation of the pteridine ring and with varying lengths to its glutamate chain. Folates function as one-carbon donors through methyl transferase catalyzed reactions. Low-folate diets, especially by those with suboptimal methyltransferase activity, are associated with increased risk of neural tube birth defects in children, hyperhomocysteinemic heart disease, and cancer in adults. Rapidly dividing (neoplastic) cells have a high folate need for DNA synthesis. Chemical analogs of folate (antifolates) that interfere with folate metabolism are used as therapeutic agents in cancer treatment.

Although much is known about folate chemistry, metabolism of this vitamin *in vivo* in humans is not well understood. Since folate levels in blood and tissues are very low and methods to measure them are inadequate, the few previous studies that have examined folate metabolism used large doses of radiolabeled folic acid in patients with Hodgkin's disease and cancer (Butterworth *et al.* 1969, Krumdieck *et al.* 1978). A subsequent protocol using deuterated folic acid was also insufficiently sensitive to trace a physiologic folate dose (Stites *et al.* 1997). Accelerator mass spectrometry (AMS) is an emerging bioanalytical tool that overcomes the limitations of traditional mass spectrometry and of decay counting of long lived radioisotopes (Vogel *et al.* 1995). AMS can detect attomolar concentrations of ^{14}C in milligram-sized samples enabling *in vivo* radiotracer studies in healthy humans. We used AMS to study the metabolism of a physiologic 80 nmol oral dose of ^{14}C-folic acid (1/6 US RDA) by measuring the ^{14}C-folate levels in serial plasma, urine and feces samples taken over a 150-day period after dosing a healthy adult volunteer.

MATERIALS AND METHODS

L-[^{14}C(U)]-Glutamic acid was purchased from Moravek Biochemicals. Other chemicals were obtained from Sigma Chemical Co. and were reagent grade or better.

Pteroyl-[^{14}C(U)]-glutamic acid (^{14}C-folic acid) was synthesized according to the method of Plante *et al.* (1980) with some modifications. To 0.789 mmol pteroic acid was added 10 mL trifluoroacetic acid (TFA). Upon dissolution, 10 mL trifluoroacetic anhydride was added and the solution refluxed for 1.5 h. The solvent was evaporated and the oil suspended in 10 mL water by vigorous shaking. The white precipitate was collected on filter paper and dried to yield 0.75 mmol of N^{10}-

trifluoroacetylpteroic acid. To make the ethyl ester of the ^{14}C-glutamic acid, 250 μCi [^{14}C(U)]-glutamic acid (S.A. 200 mCi/mmol) was diluted with 0.130 mmol glutamic acid and dried. The solid [^{14}C(U)]-glutamic acid was suspended in 3 mL 1% acetyl chloride in ethanol and incubated for 2 h at 60˚C. The solvent was removed to yield [^{14}C(U)]-glutamate diethyl ester-HCl. The diethyl-glutamate was coupled to the N^{10}-trifluoroacetylpteroic acid by a mixed anhydride synthesis using isobutylchloroformate in dimethylformamide. The free acid form was restored by hydrolysis in 0.2N NaOH and the pH adjusted to 3.5 with acetic acid. The solution crystallized overnight at 4˚C. The yellow solid was collected by filtration and dried.

The yellow solid was dissolved in TFA and pteroyl-[^{14}C(U)]-glutamic acid (Fig. 1) was isolated by reverse-phase HPLC using a semi-prep column (Ultacarb 5, ODS 30, 250 x 10 mm, Phenomenex, Torrence, CA) and isocratic mobile phase of 850 mL 0.1% TFA in water with 150 mL CH_3CN; pumped at 1.0 mL/min. Eluant was removed under vacuum and pteroyl-[^{14}C(U)]-glutamic acid stored at 4˚C. Pteroyl-[^{14}C(U)]-L-glutamic co-eluted with standards in reverse-phase and normal phase HPLC systems. The specific activity of the pteroyl-[^{14}C(U)]-L-glutamic acid was 1.25 mCi/mmol .

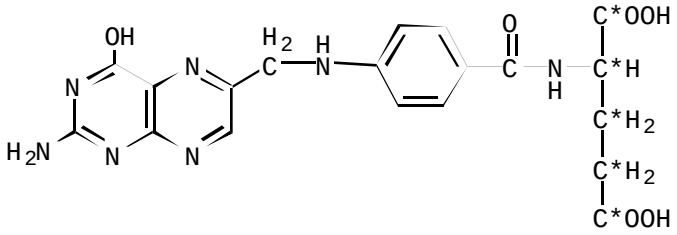

Fig. 1. Pteroyl-[^{14}C(U)]-L-glutamic acid

Pre-dose urine and feces were collected a day before dosing. Pre-dose blood was drawn just before an 80 nmol dose of the ^{14}C-folic acid (100 nCi) in ~ 25 mL water was drunk by the fasting volunteer. After dosing, 24 blood samples were drawn day 1 and 18 more over the next 42 d. Urine and feces were collected for 42 days.

Plasma and erythrocytes were separated by centrifugation. Feces were homogenized in 1L 0.5M KOH. Sample aliquots were packaged in a clean room at UC Davis and transported to Lawrence Livermore National Laboratory (LLNL) for AMS. Total carbon was measured with Carlo Erba 1500 NCS analyzer.

Samples were combusted and reduced to graphite (Vogel 1992) for AMS measurement (Vogel and Turtletaub, 1992, Creek *et al.* 1994). Natural ^{14}C was subtracted from measured isotope ratios. Excess isotope concentration was converted to folate equivalents (parent compound and all metabolites) using the specific activity of the folic acid, its molecular weight and tissue carbon content.

RESULTS AND DISCUSSION

AMS measured isotope ratios to ±3% for all samples. Concentration detection limits for plasma, urine and feces were 0.4, 0.04 and 0.12 fmol ^{14}C folate/mL plasma, based on double the uncertainty in the background. These respective concentrations correspond to absolute detection limits of 0.008, 0.004 and 0.006 fmol ^{14}C folate in neat plasma, urine and feces.

The ^{14}C first appeared in plasma 10 min after dosing, peaked at 1-2 h, and was seen in plasma 42 days after dosing (Fig. 2). The asymmetric shape of the plasma ^{14}C-folate peak suggests a more slowly responding metabolite (^{14}C-5-methyltetrahydrofolic acid) may also be present. This compound is being isolated by HPLC and its ^{14}C will be assayed by AMS.

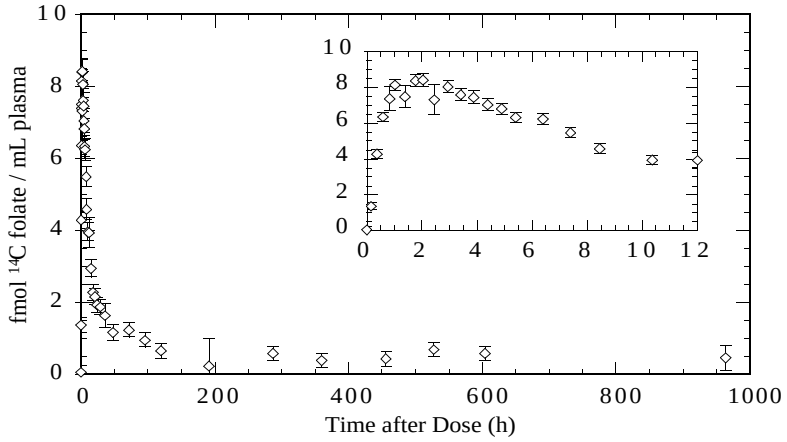

Fig. 2. Changes in plasma ^{14}C-folate concentration by time since dosing.

Based on the recovery of 9 nmol ^{14}C-folate in the first two stools, dose bioavailability was ~90%. Cumulative urine and feces recoveries were 9 and 11 nmol ^{14}C-folate, accounting for a quarter of the dose by day 42. At that time urine and feces losses were 0.14 and 0.03 nmol ^{14}C-folate/d.

CONCLUSION

High sensitivity of AMS allowed determination of minute quantities of folate not previously measureable in humans. Synthesized pteroyl-[^{14}C(U)]-L-glutamic acid had a modest specific activity (1.25 mCi/mmol) that could be followed in blood for six months after a physiologic dose. AMS avoids need for high specific activities and pharmacological doses as needed with scintillation counting. The volunteer received an effective radiation dose of only 1.1 mrem. The low levels of ^{14}C require

fastidious lab practices to avoid contamination. Generated lab wastes may legally be non-radioactive in many cases. Results will better define folate needs for optimum health.

ACKNOWLEDGMENT

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