

Traditional and accelerator mass spectrometry for quantitation of human folate pools

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Folate is a structurally diverse family of molecules that occur in low concentrations in biological tissues and fluids and therefore present complex challenges to the analytical chemist. As a result, techniques that quantify folates in biological specimens have emerged as a high scientific priority. In this manuscript, isotopic methods using traditional mass spectrometry for the measurement of whole blood total folate and ¹⁴C-accelerator mass spectrometry (AMS) for human kinetic studies are presented. Additionally, recent developments with the intrinsic labeling of spinach folate from a [¹⁴C]-*para*-aminobenzoic is discussed.

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Discussion

The measurement of folate concentrations in serum or whole blood (WBF) which, using either hematocrit or hemoglobin (Hb) concentration, can be used to express a result either per ml of packed red cells (RCF) or per g Hb, are commonly used indices of folate status. While a higher predictive value is assigned to RCF (or whole blood/g Hb) determinations, the measurement is fraught with difficulties. As a result, the measurement is often passed over in favor of the more analytically reliable serum measurement. The UC Davis group has developed an internally standardized GC/MS method for total whole blood folate that is relatively free of matrix effects and recovery inefficiencies (Dueker *et al.*, 2000; Lin, Dueker, & Clifford, 2003; Lin, Dueker, Jones, & Clifford, 2002). Folates are released from the matrix of whole blood (100 µL) and cleaved to *p*-aminobenzoic acid (*p*ABA) by acid hydrolysis in the presence of [¹³C₆]*p*ABA as an internal standard. After some clean-up, volatile derivatives are quantified by GC/MS operated in the selected ion mode. This method is well suited for field studies of blood folate concentrations as it eliminates several conventional requirements including: specific affinity resins, fastidious sample handling procedures, as well as careful storage conditions. The results of a method validation against three competing methods is presented in Table 1 (taken from Dueker *et al.*, 2000). These results (and on-going work from our laboratory) find strong correlation between GC/MS method and the microbiological assay based upon *L. caseii* growth curves performed in the micro-titer plate format (in the hands of an experienced technician). Mean values, however, were significantly lower for other methods we evaluated (Quantaphase II and chemiluminescence ACS 180). While less convenient than automated or kit assays, our method has value where a high degree of confidence is required in the results. It is also not disturbed by anti-folates such as methotrexate, making it the only option for certain chemotherapy and immunotherapy patients.

While status information is obtained from static measurements, absorption and disposition information is obtained from kinetic tracer studies. Stable isotopes have been applied to this topic since the mid-1980s and recently reviewed (Gregory & Quinlivan, 2002); in the late 1990s ¹⁴C-Accelerator Mass Spectrometry (AMS) was described as

Table 1. Whole blood folate of 31 healthy volunteers using four analytic methods

Assay method	Folate concentrations (nmol/g hemoglobin)		
	Mean \pm SD	Minimum	Maximum
<i>L. casei</i>	4.16 \pm 1.49 ^a	2.19	7.86
Chemiluminescence	3.25 \pm 0.79 ^b	1.49	4.80
Quantaphase II	2.61 \pm 0.71 ^c	1.33	4.70
GCMS	4.26 \pm 1.05 ^a	2.34	7.17

Values with different superscript letters are different at $p < 0.001$ using a paired *t*-test.

a sensitive and versatile new tool for quantifying folate metabolism *in vivo*. AMS is a type of tandem isotope ratio mass spectrometer that measures the ratios of $^{14}\text{C}/\text{C}$ to parts per quadrillion or down to as few as 10^5 ^{14}C atoms. Accordingly, attomole levels (10^{-18}) of ^{14}C or labeled chemicals are quantified in milligram-sized biological specimens (Vogel, Turteltaub, Finkel, & Nelson, 1995; Vuong, Buchholz, Lamé, & Dueker, 2004). Details of the operation of a new model AMS instrument housed at Lawrence Livermore National Lab is provided in Fig. 1. While there is a real concern with access and the cost associated with these instruments, barriers to entry are rapidly disappearing. There are seven facilities in the United States, four of which engage in biomedical tracing to some degree, and numerous laboratories also exist in Europe, Asia, and Australia/New Zealand (Vogel, Ognibene, & Buchholz, 2003). The instruments are expensive but have dropped below \$1 million for the lower voltage

compact spectrometers. This cost is less than high-field research grade NMR systems, which are relatively abundant at institutions and large research organizations. The measurement costs vary with facility access and required sample preparation, but, in general, measurement costs are comparable to other high-resolution mass spectrometry techniques. Numerous private AMS labs are expected to emerge over the next several years; a forerunner of this movement is evident in the establishment of Vitalea Science in 2003 (www.vitaleascience.com), an AMS analytical enterprise that serves both for academic and pharmaceutical markets (also a coauthor of this manuscript).

The UC Davis group has been investigating the human metabolism of folate using the AMS platform. In these studies, a small oral dose of [^{14}C]folic acid (35 μg or $< 1/10$ the RDA) is administered with water, and the appearance and disappearance of the ^{14}C label in the plasma, urine, stool, and red blood cell was determined for > 200 days post-dosing (Clifford *et al.*, 1998; Lin *et al.*, 2004). The subsequent results demonstrated folic acid followed similar absorption patterns in plasma of all volunteers (Fig. 2), despite a range of bioavailability from 65 to 97% estimated from fecal balance determinations. The small sample requirements (25 μL plasma) enabled the collection of a high density of samples during the absorptive period (20 sample in the first 16 h), facilitating development of compartmental models. Application of compartmental modeling to the data indicated substantial biliary recycling of the absorbed folate and supported the non-exchangeable

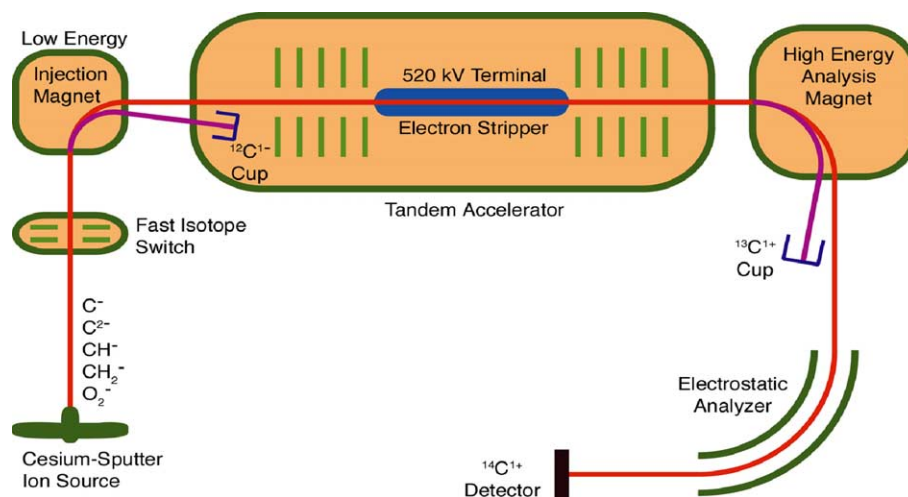


Fig. 1. Schematic of compact AMS instrument: biological samples for ^{14}C -AMS are typically combusted to CO_2 and then reduced to graphite. These samples are then bombarded by 3–10 keV Cs ions in a cesium-sputter ion source. This process physically knocks atoms and molecules out of the sample and contributes an electron to a fraction of the ejected particles, forming negative elemental or molecular ions. Singly ionized negative ions are accelerated to 40 keV and then filtered by a low energy mass spectrometer that alternately switches between ions of mass about ^{14}C , i.e. ^{14}C , ^{13}CH , and $^{12}\text{CH}_2$ and mass 13, for the stable isotope measurements, into the accelerator. Negative ions are accelerated through 520 kV in the first stage of a tandem accelerator. At the end of this first acceleration stage these ions pass through an electron stripper, a small volume of gas constrained by differential pumping. Here, particles undergo collisions, losing valence electrons. Depending on the accelerator potential, charge states of +1 to +4 are created. These positive ions then accelerate away from the positive potential to ground potential in the second half of the accelerator. The loss of electrons in the collision cells destroys all molecules, leaving only nuclear ions at relatively high energies (1.04 MeV for C). Positive ions exiting the accelerator are analyzed with a magnet and an electrostatic analyzer. An off-axis Faraday cup measures the $^{13}\text{C}^{1+}$ current after the analyzing magnet. A solid state particle detector counts individual $^{14}\text{C}^{1+}$ ions. The footprint of the compact Bio-AMS system is approximately 50 m^2 .

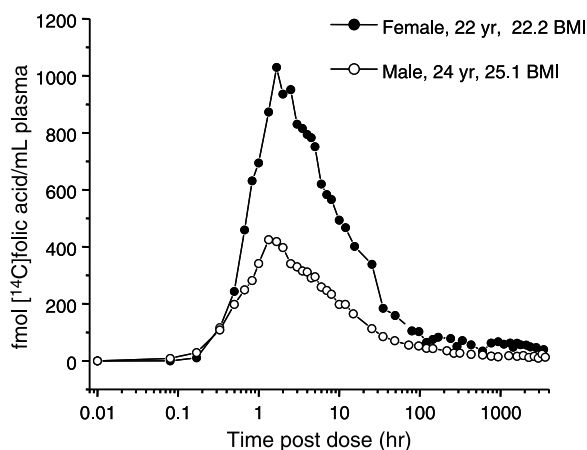


Fig. 2. Biokinetics of a $[^{14}\text{C}]$ folic acid tracer dose in female and male subject using accelerator mass spectrometry detection.

residence of folate, after incorporation during erythropoiesis, into the circulating red blood cells (Buchholz *et al.*, 1999). Occasional plasma specimens were extracted by acetonitrile precipitation of the plasma proteins and the analyte concentrations determined by HPLC with AMS analysis of the eluent fraction (Fig. 3). From this analysis, 5-methyl- H_4THF was confirmed to be the major metabolite; a small amount of parent folic acid (PteGlu) was also observed. The limit of detection was 2 attomoles of ^{14}C analyte in microliter-sized sample. These studies were conducted using 13.3 MBq of radioactivity and therefore posed no quantifiable radiation hazard to the subjects.

For most of the population, the intake of folate from food alone is seen as inadequate to promote optimal health. One strategy to combat this perceived deficiency is to raise the folate content of foodstuffs. There are growing efforts directed towards manipulation of plant biochemistry to create products of higher folate concentrations than normally present (Basset *et al.*, 2004; Quinlivan *et al.*, 2003). To support this endeavor, it is important to understand the

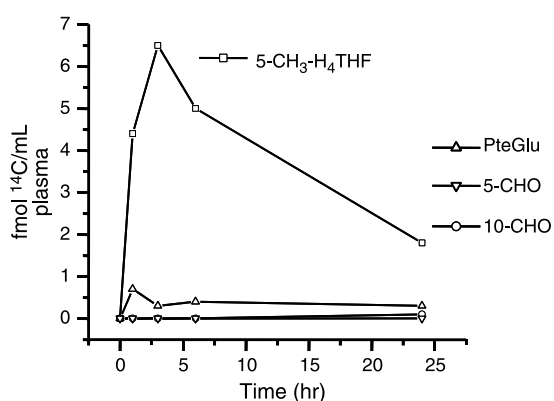


Fig. 3. Concentration time-course of plasma folates. Absorbed $[^{14}\text{C}]$ PteGlu was quickly metabolized, and 5- CH_3 -THF was the major metabolite in plasma. The C_{max} for 5- CH_3 -THF was at 3 h post-dose. Trace amounts of $[^{14}\text{C}]$ folic acid (PteGlu) but not 5-CHO-THF or 10-CHO-THF were detected.

availability of folate when contained within the intact plant matrix (as opposed to an isolate or supplement). This problem is best addressed through the use of isotopes that permit selective detection of the ingested folate from endogenous pools. While stable isotopes have been applied to this task (Finglas *et al.*, 2002), our interest in AMS prompted us to attempt to intrinsically label folate with a ^{14}C tag in a common food plant. Folate is particularly well-suited target since ^{14}C -*p*-aminobenzoic acid (*p*ABA) could be specifically incorporated into newly synthesized folate. Building upon the work of Cossins (Imeson & Cossins, 1997) we fed a water solution of $[^{14}\text{C}]$ *p*ABA (200 μCi) to two young spinach plants via the roots. In one plant, a cold *p*ABA 'chaser' followed the labeled uptake. The plants were then allowed to grow for 2 weeks in a light box with temperature control. Labeled incorporation into the folates *versus* non-folate pools was determined by assessment of the radioactivity content of tissue extracts separated on a well-characterized HPLC system. Up to 80% of the labeled was present as folate (mainly mono-, di-, and penta-glutamyl forms) when a cold 'chaser' was given in one sample (5 mg leaf sub-sample; Fig. 4). Other samples showed that the results will vary (based upon the experiment and sampling site); regardless of the variance, however, these data show a product suitable for human studies.

Isotopes are valuable tools for quantifying folate concentrations. While stable isotope methods continue to advance, understanding folate metabolism *in vivo* will be advanced by the use of appropriately ^{14}C -tagged folates and Accelerator Mass Spectrometry detection. The field is still in its infancy but rapidly growing. Over the next several years, AMS will emerge as an indispensable research tool

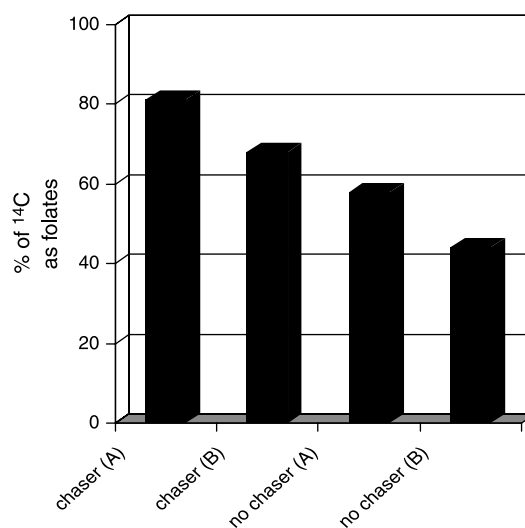


Fig. 4. The figure displays the percent of ^{14}C recovered in 5 mg leaf tissue extracts analyzed by reversed phase HPLC. Two experiments were performed in parallel: one plant was fed ^{14}C -*p*ABA via its roots followed by a cold *p*ABA chaser. The other plant received no chaser. Although not statistically determined, the results show greater incorporation of the *p*ABA labeled into folate when a cold chaser followed the labeled dose.

not only for folate but also for the spectrum of phytochemicals, vitamins and other food components.

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