

Human vitamin B₁₂ absorption measurement by accelerator mass spectrometry using specifically labeled ¹⁴C-cobalamin

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There is a need for an improved test of human ability to assimilate dietary vitamin B₁₂. Assaying and understanding absorption and uptake of B₁₂ is important because defects can lead to hematological and neurological complications. Accelerator mass spectrometry is uniquely suited for assessing absorption and kinetics of carbon-14 (¹⁴C)-labeled substances after oral ingestion because it is more sensitive than decay counting and can measure levels of ¹⁴C in microliter volumes of biological samples with negligible exposure of subjects to radioactivity. The test we describe employs amounts of B₁₂ in the range of normal dietary intake. The B₁₂ used was quantitatively labeled with ¹⁴C at one particular atom of the dimethylbenzimidazole (DMB) moiety by exploiting idiosyncrasies of *Salmonella* metabolism. To grow aerobically on ethanolamine, *Salmonella enterica* must be provided with either preformed B₁₂ or two of its precursors, cobinamide and DMB. When provided with ¹⁴C-DMB specifically labeled in the C2 position, cells produced ¹⁴C-B₁₂ of high specific activity (2.1 GBq/mmol, 58 mCi/mmol) (1 Ci = 37 GBq) and no detectable dilution of label from endogenous DMB synthesis. In a human kinetic study, a physiological dose (1.5 μg, 2.2 kBq/59 nCi) of purified ¹⁴C-B₁₂ was administered and showed plasma appearance and clearance curves consistent with the predicted behavior of the pure vitamin. This method opens new avenues for study of B₁₂ assimilation.

dimethylbenzimidazole | ethanolamine | metabolic engineering | *Salmonella* | Schilling test

Vitamin B₁₂ (B₁₂) is a compound of significant nutritional and clinical importance (1). The classical manifestations of B₁₂ deficiency include pernicious anemia, a type of megaloblastic anemia, and neurological dysfunction (2). The Schilling urinary excretion test (3, 4) indirectly measures B₁₂ absorption and has been applied when B₁₂ insufficiency is identified and malabsorption is the suspected cause. The test involves ingestion of a physiological quantity of B₁₂ labeled with gamma-emitting cobalt, followed by administration of a pharmacological parenteral flushing dose of unlabeled B₁₂ to force urinary excretion of radioactivity, which is measured during a 24-h period. The Schilling test is currently the only accepted method for assessing B₁₂ absorption. Despite its utility, the method is semiquantitative and has methodological and practical problems; it is now rarely prescribed, despite the prevalence of B₁₂ malabsorption in older adults (2, 5). We describe a method that has the potential to reinvigorate interest in diagnosis of the underlying causes of vitamin B₁₂ deficiency. The test has several advantages over the Schilling test: It poses a negligible radiation exposure to the subjects and medical workers and can be performed from a capillary-sized blood sample, without the requirement for a flushing dose of B₁₂ or for collection of radioactive urine for an extended period.

The absorption test described uses carbon-14-labeled vitamin B₁₂ (¹⁴C-B₁₂) coupled to sensitive detection of the ¹⁴C-B₁₂ by accelerator mass spectrometry (AMS). AMS was originally developed for carbon dating in archaeological or earth science samples; however, in the past decade or so, its sensitivity has been exploited for tracing of biological systems (6). In contrast to liquid scintillation counting, which records decay events of a radioisotope, AMS is a direct atom counter that was developed for quantifying long-lived isotopes such as ¹⁴C (half-life of 5,370 yr). It is a tandem isotope ratio mass spectrometer that provides the relative abundance of the ¹⁴C atom with respect to total carbon (¹⁴C/C) down to parts per quadrillion (1 in 10¹⁵) (6–8). Thus, it is possible to quantify attomole (10⁻¹⁸ mol) amounts of ¹⁴C in a milligram-sized biological sample at high precision (typically <2% imprecision). The remarkable combination of sensitivity and precision of AMS allows quantitation of ¹⁴C-B₁₂ from small biological samples and reduces the exposure of subjects to a negligible radiation risk.

The key to the success of the B₁₂-absorption test we describe is the synthesis of ¹⁴C-B₁₂ by an efficient, microscale method that produces ¹⁴C-B₁₂ specifically labeled at the carbon 2 position of the dimethylbenzimidazole (DMB) moiety of B₁₂. This B₁₂ is produced by *Salmonella enterica*, a bacterium that normally produces B₁₂ *de novo* only under anaerobic conditions and uses it to support growth on ethanolamine. Cells cannot grow aerobically on ethanolamine because they fail to synthesize two B₁₂ precursors, cobinamide and DMB, but they retain the ability to assemble B₁₂ from these precursors supplied exogenously. When isotopically labeled DMB is supplied, B₁₂ is produced with no detectable isotope dilution. The processes leading to the biosynthesis of ¹⁴C-B₁₂ by *S. enterica* are shown in Fig. 1. The ¹⁴C-B₁₂ synthesized by this method was used for a quantitative AMS-based assay of human B₁₂ absorption.

Results

Metabolic Engineering of *S. enterica* for Biosynthesis of ¹⁴C-Labeled B₁₂. The method used to produce labeled B₁₂ relies on two idiosyncrasies of B₁₂ metabolism in *S. enterica*. Under aerobic conditions on minimal medium with ethanolamine as the sole carbon source, *S. enterica* produces neither cobinamide (the corrinoid precursor) nor DMB (the lower ligand of B₁₂) but retains the ability to assemble B₁₂ when these precursors are provided exogenously.

Conflict of interest statement: A U.S. patent entitled "Assay for Vitamin B₁₂ Absorption and Method of Making Labeled Vitamin B₁₂" has been filed by P.J.A., S.R.D., J.W.M., R.G., J.R.R., C.C., and B.A.B.

Abbreviations: AMS, accelerator MS; DMB, dimethylbenzimidazole; Th, Thompson.

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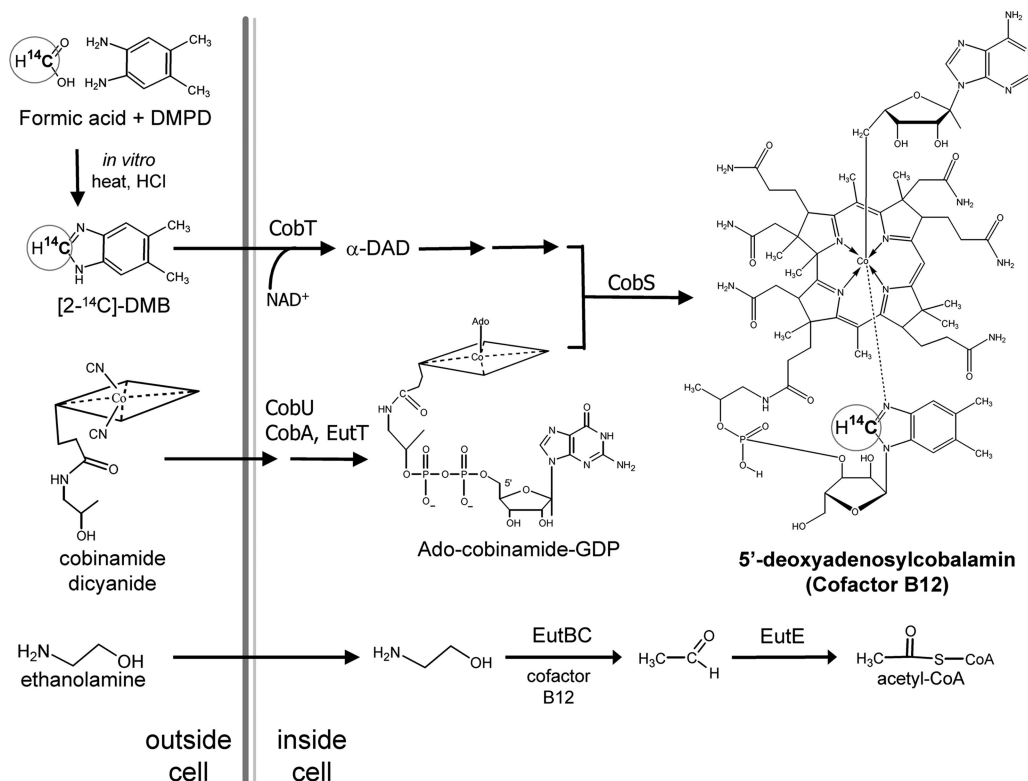


Fig. 1. Synthesis of cofactor B₁₂ from cobinamide and ¹⁴C-DMB during growth on ethanolamine. *S. enterica* enzymes CobTUSC and EutT catalyze the synthesis of adenosylcobalamin (25, 26). Growth on ethanolamine proceeds by means of ethanolamine ammonia lyase EutBC, which requires adenosylcobalamin to catalyze the formation of acetaldehyde from ethanolamine.

The 17-gene ethanolamine (*eut*) operon (9) of *S. enterica* is shown in Fig. 2. Induction of *eut* and the growth conditions pertinent to this work are shown in Table 1. Induction of the *eut* operon was monitored by assay of β-galactosidase produced by a *lacZ* gene inserted within a transcribed region of the *eut* operon that is distal to all genes of the operon. The basal level of *eut* transcription produces 10 Miller units of β-galactosidase activity from an operon fusion and increases ≈50-fold when the operon is induced by the combination of B₁₂ plus ethanolamine. Surprisingly, the B₁₂ precursor cobinamide can replace B₁₂ as an inducer but does not permit growth on ethanolamine (Table 1). This failure to produce B₁₂ is due to a lack of DMB because B₁₂ production and growth on ethanolamine is restored if DMB is provided in addition to cobinamide. Thus, during aerobic growth on ethanolamine, *S. enterica* fails to make both cobinamide and DMB but can synthesize B₁₂ if these precursors are provided. The CobA, U, S, T, C, and EutT enzymes, which catalyze conversion of cobinamide plus DMB to B₁₂, are produced at levels sufficient to assemble B₁₂ and permit aerobic cell growth on ethanolamine. These conditions allow efficient conversion of labeled precursors into B₁₂ without label dilution. The growth conditions used appear to avoid feedback repression of B₁₂

synthesis, presumably because B₁₂ is sequestered and bound by the EutBC enzyme.

Synthesis and Purification of ¹⁴C-Labeled DMB and Incorporation into B₁₂. High specific activity ¹⁴C-labeled DMB was synthesized by condensation of ¹⁴C-labeled formic acid with dimethylphenylenediamine (10) (Fig. 1). The reaction product, [2-¹⁴C]-DMB, was purified by HPLC to eliminate unwanted reaction products. When this labeled DMB and unlabeled cobinamide were provided to *S. enterica*, the cells produced B₁₂ and grew aerobically on ethanolamine.

Purification and Mass Spectral Analysis of ¹⁴C-B₁₂. Adenosylcobalamin was extracted from bacteria in the presence of cyanide so that highly stable cyanocobalamin (vitamin B₁₂) would be formed. These extracts were purified by HPLC and analyzed by two methods of mass spectrometry (MS) to establish chemical identity. The ¹⁴C radiolabel coeluted precisely with the single chromatographic peak of the vitamin B₁₂ standard (Fig. 3a). The

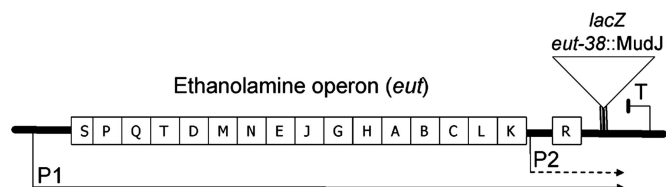


Fig. 2. Organization of the 17-gene *eut* operon. Genes are transcribed from left to right from the primary promoter P1. P2 is a weak constitutive promoter.

Table 1. Induction of *eut* and growth on ethanolamine

Inducer	Mean ± SD	Growth
1. None	10 ± 1	–
2. EA	9 ± 3	–
3. DMB	14 ± 6	–
4. CBI	7 ± 1	–
5. EA + DMB	12 ± 3	–
6. EA + CBI	447 ± 30	–
7. EA + CBI + DMB	313 ± 24	+
8. EA + B ₁₂	486 ± 16	+

Induction of the *eut* operon in the presence of ethanolamine and glycerol is compared with growth on ethanolamine as sole carbon source.

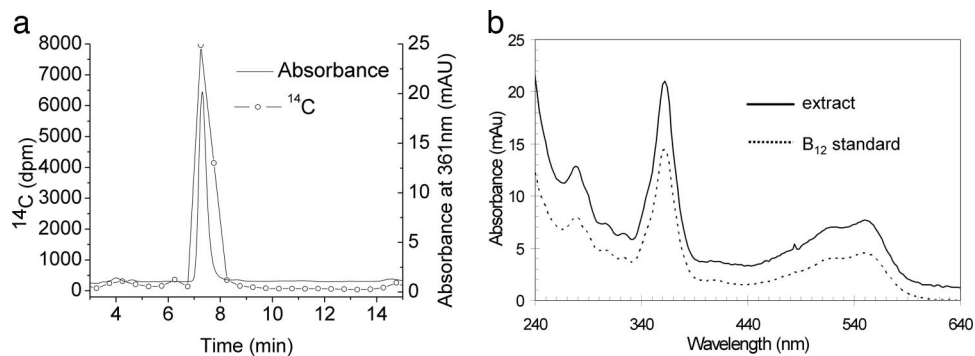


Fig. 3. Comparison of synthesized ^{14}C -B $_{12}$ with authentic B $_{12}$. (a) Coelution of the ^{14}C radiolabel with B $_{12}$. (b) Spectrum of standard vitamin B $_{12}$ and that of the putative ^{14}C -B $_{12}$.

UV/visible absorption spectrum of the purified ^{14}C compound was consistent with that of the B $_{12}$ standard spectrum (Fig. 3b). The putative ^{14}C -labeled cyanocobalamin (B $_{12}$) was also analyzed by two forms of MS; the MS/MS product spectra of the vitamin B $_{12}$ standard, (M+H) $^{+}$ at $m/z = 1,355.8$ Thomson (Th), and the ^{14}C -B $_{12}$, (M+H) $^{+}$ at $m/z = 1,357.8$ Th, are shown in Fig. 4. There is a 2 Th mass difference between the molecular ions of the standard and ^{14}C -labeled cobalamins; the fragmentation pattern confirms that this difference is due to a single ^{14}C -label on the 5,6-DMB moiety. The molecular ion (M+H) $^{+}$ of the unlabeled B $_{12}$ was compared with the ^{14}C -labeled com-

pound also by high resolution MS. The measured values for both the unlabeled ($m/z = 1,355.5741$ Th) and ^{14}C -labeled cobalamin ($m/z = 1,357.5787$ Th) agreed in molecular mass with the predicted values ($m/z = 1,355.5752$ Th and $m/z = 1,357.5782$ Th, respectively). Based on the identified decomposition products of the MS/MS experiments, the accurate mass measurement data, and the HPLC data, we conclude that the product of the directed biosynthesis is ([^{14}C]-5,6-DMB)cyanocobalamin.

Specific Activity of ^{14}C -B $_{12}$. The theoretical specific activity for a compound with one atom of ^{14}C at 100% incorporation is 2.308

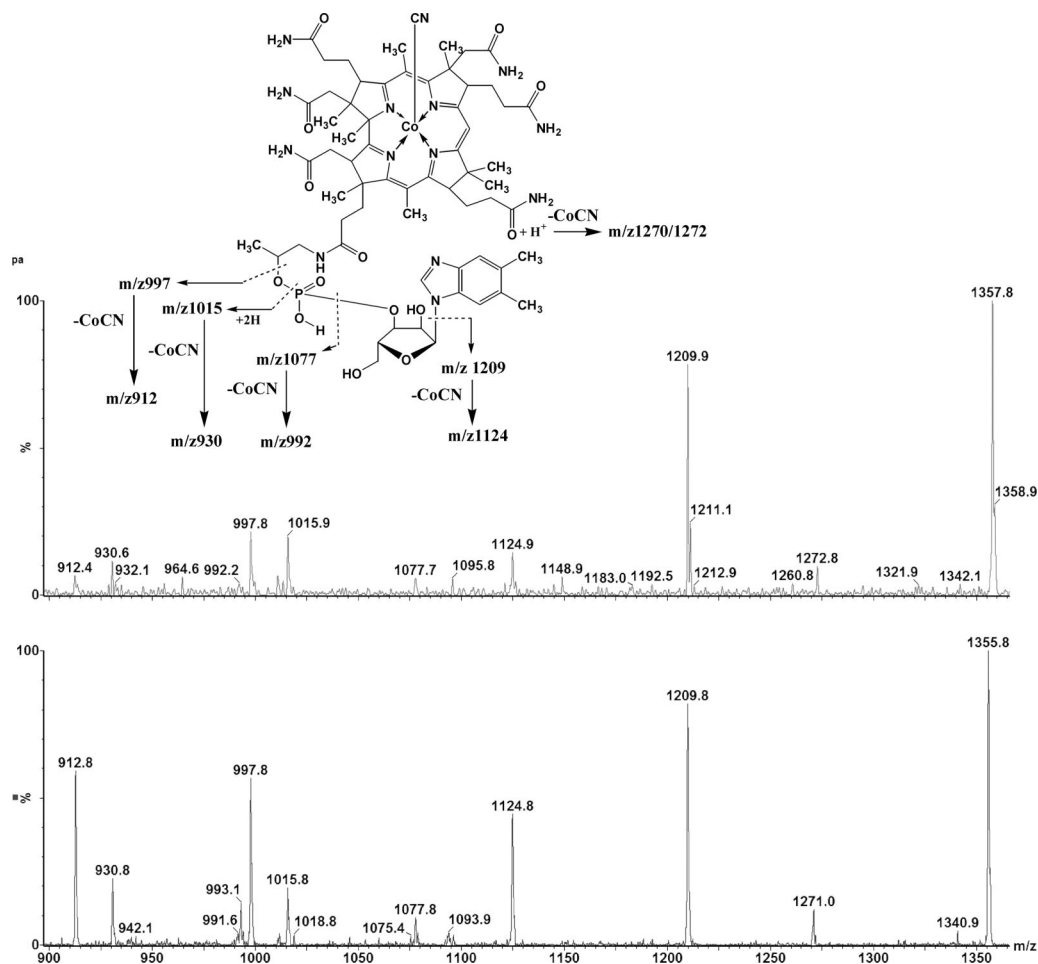


Fig. 4. MS/MS spectra in product mode showing the decomposition products of the molecular ions (M+H) $^{+}$ of B $_{12}$ and ^{14}C -B $_{12}$.

1.5- μg dose of the ^{14}C - B_{12} was administered orally and detected by AMS as it appeared in the bloodstream. Normally, the release of B_{12} from intestinal mucosa cells into the portal vein occurs ≈ 2 h after the oral consumption of the vitamin, and release into systemic circulation takes an additional 1 h (18, 19). Consistent with this finding, the ^{14}C from the labeled B_{12} appeared in the plasma of the human subject 3 h after the dose. A concentration of 5–6 fmol of ^{14}C - B_{12} per ml was observed in peak samples (5–12 h) (Fig. 5). This time window would make it possible to assess B_{12} absorption from a single capillary blood sample. The current Schilling urinary excretion test, which has been the standard method of assessing B_{12} absorption since its introduction in 1953, is now rarely prescribed because it requires the administration of radiocobalt B_{12} followed by an intramuscular flushing dose and 24-h total urine collection. By this method, patients with normal B_{12} absorption excrete 8–40% of the labeled B_{12} in the urine, compared with patients who have malabsorption, from whom there is little or no recovery of labeled B_{12} from urine (4). By contrast, the method we describe makes it possible to follow the fate of the vitamin at near ambient levels of radiation exposure by using microliter-sized blood specimens and without a flushing dose. Relatively little of the dose was recovered in either urine or stool; 84% of the administered dose was retained in body tissues after 7 days. This observation is consistent with the very slow body elimination of B_{12} (0.1% loss per day after initial excretion of the unabsorbed dose) due to efficient enterohepatic recycling. The 6.8% loss in the 24-h urine was unexpected in the absence of a parenteral flushing dose, based on previous experiments carried out using radiocobalt B_{12} . Examination of the chromatographic behavior of ^{14}C -labeled compounds in the urine revealed that the predominant urinary products were not vitamin B_{12} . The accumulation of ^{14}C - B_{12} degradation products in the urine may be due to acid hydrolysis of some of the ^{14}C - B_{12} dose in the stomach or bacterial degradation in the gut with consequent release of DMB. Absorption of free DMB and its metabolism, such as through hepatic conjugation to glucuronide, may result in urinary excretion of the product. However, further analysis is necessary to determine the exact nature of the products detected in the urine.

In summary, we describe the biosynthesis of ^{14}C -labeled B_{12} and human absorption kinetics by using near-ambient levels of radiation that pose little or no risk from exposure. The sensitivity of AMS reduces the needed sample size to only tens of microliters of blood and minimizes exposure to radiation. We believe that combined use of ^{14}C -labeled B_{12} and AMS detection has the potential to be a powerful clinical diagnostic tool and an improved method for studying the underlying causes of B_{12} -uptake disorders, including the development of a sensitive and quantitative test for B_{12} absorption in humans.

Methods

Synthesis and Purification of ^{14}C -DMB. Radiolabeled DMB was synthesized by using a procedure modified from that of Phillips (10). Into a 10-ml boiling flask containing 500 μl of sodium phosphate buffer (pH 7.4, 100 mM) was added ^{14}C -formic acid (1 mCi; 0.0182 mmol, 1.85–2.22 GBq/mmol) (Moravek Biochemicals, Brea, CA). The material was dried under reduced pressure, and the solids were dissolved in 1 ml of 4 M HCl. To initiate the reaction, 15.15 mg (111 μmol) of *o*-dimethylphenylenediamine was added and the contents taken to a vigorous boil by using a reflux system with the condenser maintained at -10°C by using a recirculating chiller. After 2 h of heating, the reaction solution was neutralized by dropwise addition of concentrated ammonium hydroxide to a pH of ≈ 7 . The reaction product was then loaded onto a solid-phase extraction cartridge (1 g of Bond Elut C18 from Varian), which had been primed with 3 ml of methanol and 3 ml of deionized water. The column was

washed with 2 column volumes of deionized water, and the bound ^{14}C -DMB was eluted with 3 ml of methanol.

The solvent was removed under streaming nitrogen, and the product was dissolved in 0.5 ml of absolute ethanol. The product was then purified by multiple injections onto an isocratic reverse-phase HPLC by using an Agilent 1100 chromatograph (Agilent Technologies, Palo Alto, CA) fitted with an Adsorbosphere HS C18 column (150 mm \times 4.6 mm; Alltech Associates). The isocratic mobile phase, 34:33:33 water:methanol:acetonitrile, was pumped at a flow rate of 0.80 ml/min; the absorbance of the outflow was monitored at 284 nm. The peaks, which had similar retention and spectral characteristics to a purchased DMB standard (Sigma), were pooled and evaporated to dryness under reduced pressure, dissolved in absolute ethanol, and stored at -70°C . Radioactivity was determined by liquid scintillation counting.

Microorganism Cultivation. The strain used for reporting β -galactosidase activity was TT10674, genotype *eut58::mudA*. The strain used for ^{14}C - B_{12} biosynthesis was *S. enterica* (serovar Typhimurium) strain TT24733, genotype *cbiD24::MudJ*. The labeling medium consisted of no carbon E medium (20) supplemented with 40 mM ethanolamine, 250 nM dicyanocobinamide, and 500 nM ^{14}C -DMB synthesized as described above. Approximately 130 ml of labeling medium was added to a sterile, 500-ml conical flask. The medium (130 ml in a 500-ml flask) was inoculated by a 100-fold dilution of a *S. enterica* culture grown in no carbon E medium supplemented with 20 mM glycerol. Cultures were incubated in the dark for 48 h at 30°C with shaking at 250 rpm.

Extraction and Purification of ^{14}C -Cyanocobalamin from Cells. Bacterial cells were pelleted by centrifugation for 20 min at $6,000 \times g$. Supernatants were removed, and cell pellets were washed three times with no carbon E medium (20). The pellets were resuspended in 5 ml of methanol and 500 μl of 50 mg/ml sodium cyanide, vortex mixed, and placed in a 60°C water bath for 12 h with intermittent vortex mixing. The samples were then centrifuged at $20,000 \times g$ for 1 h. The supernatants were removed from the pellet and evaporated to dryness. Dried samples were resuspended in water and filtered (0.22 μm) to remove any insoluble material. A first step in purification of the ^{14}C - B_{12} was performed by extraction on C18:0 solid-phase extraction cartridges (5-g bond Elut C18 from Varian). The corrinoids were eluted with 50:50 water:methanol, and the solvent was evaporated to dryness. Purification to homogeneity was carried out by HPLC on an Agilent series 1100 HPLC equipped with a diode array detector and fitted with an Agilent Zorbax Eclipse XDB C18 (3.5 μm) column (150 mm \times 3.0 mm). Solvent A was 90/10 water/methanol, and solvent B was methanol with initial conditions of 82/18 A/B. At 12 min, a linear gradient was started that reached 25/75 A/B after 16 min. The flow rate was held constant at 0.360 ml/min. Extracts were run in multiple injections, and the peak corresponding to ^{14}C -cyanocobalamin was collected and pooled for each run. The solvent was evaporated to dryness, and samples were resuspended in water for storage at -70°C .

MS. High-resolution MS: accurate mass measurement. Exact mass measurement experiments were performed, in positive mode, on a Micromass liquid chromatograph orthogonal acceleration time-of-flight mass spectrometer (Waters-Micromass, Manchester, U.K.). The cone and desolvation gas were set to 50 and 650 liters/h, respectively. Resolution was 8,000, measured at 803-Th mass, based on the definition of full width at half maximum (FWHM). Sample source conditions were as follows: capillary voltage, 3,250 V; sample cone voltage, 30 V; extraction cone voltage, 6 V; source temperature, 100°C ; and desolvation temperature, 250°C . Transfer optics settings were as follows: radio frequency (rf) lens, 250 V; rf dc

offset-1, 4.0 V; rf dc offset-2, 6.0 V; aperture, 2.0 V; acceleration, 200.0 V; focus, 1.0 V; and steering, -0.3 V. Analyzer settings were as follows: multichannel plate (MCP) detector, 2,430 V; ion energy, 32.0 V; tube lens, 4.0 V; grid-2, 20.0 V; time-of-flight flight tube, 4,599 V; and reflectron, 1,713 V. The pusher cycle time was 50 μ s. Data files were acquired in continuum mode, and spectra were stored from $m/z = 100$ to 1,600 with a 1.1-sec scanning cycle consisting of a 1.0-sec scan and a 0.1-sec interscan time. Typically, 20–30 individual spectra were summarized. TOF calibration: effective length of the flight tube (L_{teff}) value was set to 1,122.7250 in positive mode by using molecular ions of leucine-enkephalin (L9133 from Sigma) at 556.2771 Th. System calibration was performed by using poly-DL-alanine (P9003 from Sigma), which was also used as an internal standard for accurate mass measurement. To obtain accurate masses, the following procedure was performed: Savitsky–Golay smoothing, by using a ± 4 -channel window repeated twice, and centering, by using the center value at the 50% height of the peak. Samples were introduced into the mass spectrometer through direct-flow injection by using the Waters Alliance 2795 HPLC system for solvent delivery at the flow rate of 250 μ l/min; mobile phase CH₃CN/H₂O (1/1) was used. MASSLYNX 4.0 SP3 software (Waters-Micromass) was used for instrument control, data acquisition, and data evaluation.

MS/MS experiments. Positive ion MS/MS experiments were performed in product mode on a Quattro Premier (Waters-Micromass) triple quadrupole mass spectrometer with a configuration of quadrupole-traveling wave collision-cell quadrupole (QtQ) equipped with an atmospheric pressure ionization (API) interface sprayer. The instrument was operated with the following instrumental conditions: source temperature, 120°C; desolvation temperature, 200°C; capillary voltage, 3.1 kV; cone voltage, 50 V; extraction cone, 5 V; and radio frequency lens, 0.5 V. The drying and cone gas was nitrogen. The cone gas flow was set to 50 liters/h, and the desolvation gas was set to flow to 700 liters/h. Quadrupole-1 parameters were as follows: low mass (LM) resolution, 14.0; high mass (HM) resolution, 14.0; ion energy, 0.4–0.6 V; entrance, 7 V; and exit, 16 V. Quadrupole-2 parameters were as follows: LM resolution, 15.5; HM resolution, 15.5; and ion energy, 2.0–2.5 V. Multipliers were set at 550 V. The collision gas was argon (99.9999%) (Airgas, Radnor, PA) with a pressure of $3.6\text{--}4.6 \times 10^{-3}$ mbar (1 bar = 100 kPa) in the collision cell. MS/MS experiments were performed at a collision energy of 50–70 eV (in the case of single charged ions) and 30–40 eV (in the case of double charged ions). MS/MS data were acquired in continuous mode. The scanning speed was in all cases 0.025 sec/decade with a 0.1-sec interscan time. The sampling density was set at 16/Da. Infusion experiments were

performed on an integral syringe pump controlled from MASSLYNX, with a flow rate of 20 μ l/min, directly connected to the interface. Data acquisition and instrument control was performed by using MASSLYNX 4.0 SP 3.

Subjects and Human Experimental Design. The subject was a healthy male aged 40 years with a body mass index (BMI) of 27.5. The subject began complete fecal and urine collection 24 h in advance of the ¹⁴C-B₁₂ dose and continued complete 24-h collections until day 7. On the day of dose administration, the subject was fitted with an i.v. catheter in a forearm vein. Blood was drawn into 7-ml tubes containing EDTA. A baseline blood sample was drawn (at 7 a.m.), and the ¹⁴C-B₁₂ dose consumed that corresponded to 2.2 kBq of radioactivity (1.5 μ g) was administered in 50 ml of drinking water in a paper cup. The volunteer was allowed to have water ad libitum thereafter, with a light meal taken 2 h after dosing. Blood samples (5 ml) were drawn at frequent intervals for the first 15 h after dosing and daily thereafter. Other meals were controlled for time and content on the dose-administration day. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Review Boards at the University of California Davis and Lawrence Livermore National Laboratory. Informed consent was obtained from the subject.

AMS Analysis. Aliquots of plasma (30 μ l), urine (80 μ l), and a stool slurry (80 μ l) were dried, combusted to CO₂, and reduced to filamentous carbon by using procedures described in refs. 21 and 22. No other processing preceded the graphitization step; thus, quantitative recovery was ensured. The ¹⁴C measurements were performed at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory (23). Measurements were conducted to <3% instrument imprecision, and, in general, signal acquisition was complete to the desired statistical precision in 3–5 min per sample. The radiation exposure of the subject, due to the ¹⁴C-B₁₂ dose, was equivalent to or less than exposure due to 16 h of intercontinental plane flight (7, 24).

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