

Disposition of ^{14}C - β -carotene following delivery with autologous triacylglyceride-rich lipoproteins

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Abstract

Following ingestion, a fraction of β -carotene is cleaved into vitamin A in the intestine, while another is absorbed intact and distributed among tissues and organs. The extent to which this absorbed β -carotene serves as a source of vitamin A is unknown *in vivo*. In the present study we use the attomole sensitivity of accelerator mass spectrometry (AMS) for ^{14}C to quantify the disposition of ^{14}C - β -carotene (930 ng; 60.4 nCi of activity) after intravenous injection with an autologous triacylglyceride-rich lipoprotein fraction in a single volunteer. Total ^{14}C was quantified in serial plasma samples and also in triglyceride-rich, and low density lipoprotein, subfractions. The appearance of ^{14}C -retinol, the circulating form of vitamin A in plasma, was determined by chromatographic separation of plasma retinol extracts prior to AMS analysis. The data showed that ^{14}C concentrations rapidly decayed within the triglyceride-rich lipoprotein fractions after injection, whereas low density lipoprotein ^{14}C began a significant rise in ^{14}C 5 h post dose. Plasma ^{14}C -retinol also appeared at 5 h post dose and its concentrations were maintained above baseline for >88 days. Based upon comparisons of ^{14}C -retinol concentrations following an earlier study with orally dosed ^{14}C - β -carotene, a molar vitamin A value of the absorbed β -carotene of 0.19 was derived, meaning that 1 mole of absorbed β -carotene provides 0.19 moles of vitamin A. This is the first study to show that infused β -carotene contributes to the vitamin A economy in humans *in vivo*.

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1. Introduction

β -Carotene, a fat-soluble plant pigment, serves manifold roles in human health, but is best-known as a precursor of vitamin A [1]. In the intestinal mucosa, a fraction of the dietary β -carotene undergoes specific enzymatic cleavage, providing up to 2 moles of retinal (the aldehyde form of vitamin A) per mole of β -carotene. Retinal is metabolized to retinyl esters prior to transport into blood circulation

via the lymphatics. *In vitro* evidence suggests that extra-intestinal tissues, notably the liver, possess greater cleavage capacities than the intestinal mucosa [2]; however, to date the contribution of extra-intestinal metabolism of β -carotene to vitamin A balance has not been measured using a protocol that bypasses intestinal metabolism and therefore isolates post-intestinal metabolic pathways.

Intravenous infusion techniques are often applied to pharmaceuticals with poor oral absorption characteristics in order to bypass intestinal transformations that can alter the pharmacodynamics of the drug [3–5]. This technique is also applicable to investigating the extra-intestinal metabolism of β -carotene, as it allows the study to be conducted

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under conditions that are uncomplicated by intestinal metabolism. Accordingly, vitamin A derived from the infused β -carotene dose would provide strong evidence of extra-intestinal cleavage. One obstacle to performing such an infusion protocol is the selection of the proper dissolution matrix. It is important that the matrix deliver the highly lipophilic β -carotene in a state that would most closely mimic natural *in vivo* metabolism.

The prevailing views for β -carotene transport closely ties its fate to its lipoprotein carriers. β -carotene and any retinyl esters derived from consumption are transported from the gut into circulation via the lymphatic system aboard intestinal chylomicron particles. These particles are removed from circulation by apolipoprotein E (Apo E) and low density lipoprotein (LDL) receptors at the liver and other tissues [3–7]. Some of the hepatic β -carotene is then secreted into circulation with very low density lipoproteins (hepatogenous VLDL), a fraction of which settles into low density lipoproteins (LDL) upon remodeling in the plasma. As a result, most circulating β -carotene is associated with LDL [8].

An optimal physiological infusion protocol must present β -carotene associated with chylomicrons at physiologically relevant concentrations (nmolar) [8]. However, highly specific molecular labels and sensitive detection instruments are necessary to follow such a small exogenous input within the context of preexisting concentrations. ^{14}C tracing by accelerator mass spectrometry (AMS) is the first technology that provides the requisite specificity and sensitivity to perform this procedure [9–12].

We demonstrate the application of AMS for assessing the *in vivo* disposition of a small ^{14}C -labeled dose of β -carotene associated with autologous triglyceride rich lipoproteins (TGRL) which are principally composed of chylomicron remnants [13] and VLDL. The technique as presented involved the isolation of TGRL enriched plasma a day prior to dose administration. This plasma TGRL fraction was then *ex vivo* incubated with ~ 100 nCi (< 1 μg) of ^{14}C - β -carotene, and a portion of this preparation (60.4 nCi; 930 ng; 1.74 μmol) was pulse injected into the donor volunteer. The concentration of the label in the whole plasma, TGRL, LDL, and the bioconversion to plasma retinol were all quantified by AMS. Cumulative urine and stool were also collected to assess excretion. The retinol data is compared to previous data obtained from oral ^{14}C - β -carotene feeding experiments (p.o. administration) to calculate the provitamin A value of the infused β -carotene.

2. Material and methods

2.1. Subject

The clinical portion of the study was approved by institutional review boards of both the University of California, Davis and Lawrence Livermore National Laboratory (LLNL). The participant (male, 37 y; BMI 24.8 kg/m²)

gave written, informed consent for participation in the study. He was normolipidaemic and normoglycaemic, had no history of medical diseases, metabolic disorders, and did not use any medications with known metabolic effects. He was instructed to maintain his usual dietary habits over the course of the study.

2.2. Dose preparation

The labeled β -carotene was synthesized by growing *Dunaliella Salina*, a halotolerant photosynthetic algae, in the presence of ^{14}C -bicarbonate. This photosynthetic organism is known to accumulate large amounts of β -carotene [14]. An innoculum of *Dunaliella Salina* (University of Texas at Austin, LB 1644) was added to 50 mL of seawater medium (50 mL) containing 3 mCi of ^{14}C -sodium bicarbonate (Moravek). The jar was capped with a 0.45 micron filter and grown without shaking for 10 days under fluorescent lighting. At the end of the growth period, the algae were pelleted by centrifugation, the β -carotene was partitioned into hexanes, and then it was purified by multiple reversed phased-HPLC steps according to prior techniques [12]. The final specific activity of the ^{14}C - β -carotene was 34.8 Ci/mol as determined by radiometric counting and spectroscopy. The material was stored in pyrogen-free hexanes (Fisher Scientific).

To prepare the autologous TGRL, the participant consumed a blended meal of whipping cream (1 g/kg body weight), olive oil (0.5 g/kg body weight) and a 100 g banana the day prior to dose administration. Two hours later, 35 mL of venous blood (into 4 \times 10 ml EDTA vacutainers) was drawn by venipuncture from the antecubital vein, and the plasma obtained by centrifugation (500g for 10 min). All further manipulations were performed using aseptic technique and sterile materials. The plasma was transferred to a pyrogen-free screw-cap Teflon tube (Oakridge, Fisher) and centrifuged at 22,000g for 20 min at 10 °C. The cloudy, TGRL layer at the top of the tube was aspirated off (8 mL) by visual inspection and transferred to an EDTA-containing vacutainer tube. A small volume (~ 200 μl) of ^{14}C - β -carotene in hexanes was overlaid, and the hexanes removed under streaming argon (with in-line HEPA filter). The sample was capped and gently rocked for 30 min in the dark at 12 °C. This dose was stored at 12 °C until use the following morning. The total lipid content of the TGRL was determined to be 472 mg/dL using a true triglyceride kit (Sigma). The distribution of the label within the dose was assessed by ultracentrifugation as described below and was found to be: 78% TGRL, 16% LDL, and 4% other (HDL, serum albumin).

2.3. Clinical

The participant reported to the UCDMC Cancer Center at 08:00 am the following morning and was fed the same meal given the preceding day, except the whipping cream component was reduced to 0.5 g/kg. At 09:30 an indwelling

catheter was inserted in a forearm vein and a baseline blood sample was taken 30 min later. Immediately thereafter, a ~5 mL aliquot of the ^{14}C - β -carotene labeled donor TGRL (~4 mL, 60.4 nCi; 930 ng; 1.74 nmol) was pulse-injected (over 10 s) by syringe into the antecubital vein of the contralateral arm. This point marked the beginning of the kinetic study (time = 0 min). Serial blood draws were taken at 10, 20, 30, 40, 50, 60 and 90 min and then 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 10 and 12 and 22 h post infusion. The patency of the catheter was maintained by 2 mL saline flushes. The line was filled with heparin after the 12 h collection. Prior to each collection the line was primed by removing 2 mL of blood, which was discarded. Additional samples were collected in the morning (fasted state) out to 88 days post dose. No adverse reactions to any of the procedures were observed. Complete fecal and urine collections were started 24 h in advance of dosing and daily collections continued through 10 days post dose. Stool samples were collected in sterile bags (Stomacher collection bags, 4 mil, Fisher Scientific).

Plasma was separated by centrifugation and immediately stored at $-70\text{ }^{\circ}\text{C}$. The separation of the TGRL and LDL was performed on a microultracentrifuge (Sorvall S120-AT2 rotor on a Sorvall Discovery M120; Kendro Laboratory Products, Newton, CT) according to standard methods described by the manufacturer. For the procedure, 550 μL of 0.195 M NaCl was underlaid in 1.2 ml of fresh plasma in a 2.0 ml polyallomer centrifugation tube (Sorvall). The tubes were sealed and centrifuged at 120,000 rpm (average 530,611g) for 23 min at 4 s to float the TGRL. The TGRL was aspirated off (0.6–0.8 ml) from the top of the NaCl layer, placed into a tared vial, and stored at $-70\text{ }^{\circ}\text{C}$. The centrifuge tube was then refilled with 550 μL of 0.195 M NaCl/2.44 M NaBr and spun at 120,000 rpm (529,000g) for 125 min to float the LDL fraction, which was collected and stored as the TGRL. The urine and stool were prepared according to previously described methods [15], and the carbon content of urine, stool, plasma, and lipoprotein fractions were determined using the DUMAS method at the UC Davis core analytical laboratory (DANR).

3. Plasma retinol isolation

The incorporation of ^{14}C into the retinol pools required thorough cleanup methods to prevent contamination by much larger concentrations of labeled β -carotene. The extraction and AMS analysis procedure was performed in duplicate. The procedure was described by us previously with some modification [15]. A 200 μL aliquot of plasma was precipitated with 200 μL of 0.025 N KOH in ethanol and retinol was partitioned into mixed hexanes ($2 \times 1\text{ mL}$). The extract was transferred to a test tube, supplemented with 5 μg of butylated hydroxytoluene, and concentrated under streaming argon to ~200 μL . This volume was applied to an aminopropyl bonded silica extraction cartridge (500 mg, 2.8 mL, Alltech Associates) that had

been preequilibrated with methanol followed by hexanes. β -Carotene was eluted from the cartridge with a 2 mL wash of 90/10 hexanes/ethyl acetate. Retinol was then eluted with 700 μL of methanol and collected in an amber vial containing 5 μg of butylated hydroxytoluene. The methanol fraction was then removed under streaming argon, reconstituted in 50 μL of hexanes, and injected onto Normal-Phase HPLC system (SB-CN, Zorbax Rapid Resolution, $4.6 \times 75\text{ mm}$, 3.5 μm particle size with guard cartridge; Agilent Technologies, Palo Alto, CA, USA). The separation was developed using a binary pumping system (Agilent 1100) under the following gradient conditions: solvent A: hexane; solvent B: 80/19/1 hexane/ethyl acetate/MeOH; 0–5 min: 90/10 A/B; 6 min 70/30 A/B; 9 min 70/30 A/B; 10 min: 90/10 A/B; flow rate 1.2 ml/min. The eluent was monitored for retinol at 325 nm. Retinols (^{14}C -retinol and endogenous) eluted from 3.8 to 4.3 min; this entire eluent fraction was collected in an amber vial loaded with 5 μg butylated hydroxytoluene, concentrated to dryness under streaming argon, and reconstituted in 50 μL of CH_3CN . This resuspension was then injected (50 μL injection) onto a gradient reversed phase-HPLC system (Zorbax Eclipse XDB- C18; $3.0 \times 150\text{ mm}$; 3.5 μm particles size; Agilent) using the following conditions: Solvent A: 70/25/5 $\text{CH}_3\text{CN}:\text{H}_2\text{O}:\text{MeOH} + 0.01\%$ ammonium acetate; Solvent B 50/50 $\text{CH}_3\text{CN}:\text{2-propanol} + 0.01\%$ ammonium acetate; 0–2.5 min 100% A; 7 min 100% B; 11 min 100% B; 13 min 100% A; flow rate 0.5 ml/min.

4. AMS analysis

All samples were dried in quartz inserts under reduced pressure, combusted to CO_2 , and then reduced to graphite using methods developed at Lawrence Livermore National Lab [16,17]. Carbon-14 measurements were made at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory [18]. Data are expressed as fmol ^{14}C - β -carotene/mL for plasma, and as attomoles of ^{14}C per mL or percentage of administered dose for stool and urine, respectively.

5. Calculations

Elimination in urine and stool were determined by analysis of total ^{14}C above background in each sample collection. Half-lives were estimated by linear regression analysis of the log-transformed data. The radioactivity remaining in the plasma compartments after dosing was expressed as a fraction of the activity administered where the plasma volume was estimated using the equation: $\text{vol} = \text{mass} \times 31.47 + 627$. In the retinol plots (infusion and oral), area-under the curve concentration time plots (AUC) were determined by linear interpolation of the data points using Origin (Microcal, Northampton, MA). Oral ^{14}C -retinol data was obtained from a prior study [12] in which the vitamin A value of orally administered β -carotene was determined in the same volunteer. The ^{14}C -retinol

data were normalized to the dose (200 nCi and 60.4 nCi for the oral and infusion experiments, respectively).

6. Results

The ^{14}C plasma data for 88 days post dosing is presented in Fig. 1 in log–log form. The Y axes on the left presents the data as equivalents of ^{14}C - β -carotene, whereas the second Y axis (right) presents the concentration data as dose fraction, where a value of 1 indicates that entire dose is accounted for in the plasma compartment. The data were fitted with a two term exponential equation of the form $Y = 1.2218 + 33.9558 e^{(-X^{1*0.00596})} + 29.3292 e^{(-X^{2*0.00175})}$; $X^2 = 2.2031$, $R^2 = 0.98934$ with derived plasma decay half lives for the ^{14}C of 4.8 days and 16.5 days.

Fig. 2 shows the time course of ^{14}C in the TGRL and LDL fractions. Concentrations of ^{14}C in the TGRL decayed to baseline levels within 5 h (300 min) with a $t_{1/2}$ 122 min; conversely, the levels of ^{14}C in the LDL levels were relatively static over this same period. Early concentrations of ^{14}C in the LDL are attributed to the non-TGRL β -carotene in the starting dose. After >5 h post dose, however, label concentration rose, to reach a higher concentration plateau starting at roughly 10 h post dose. This plateau was maintained for approximately 36 h, with some variation, after which, the ^{14}C concentration consistently decayed.

A visual representation of fecal and urinary excretion data is shown in Fig. 3 for the 240 h (10 days) post dose. The points represented 5 h-interpolations of the actual data to improve visualization. After 10 days, 16% of the administered dose was recovered in the urine and stool combined. Output via the urinary route exceeded that of the fecal route.

Fig. 4 shows the concentration of ^{14}C in total plasma retinol after HPLC purification. The data from the present infusion experiment is shown alongside retinol results of a

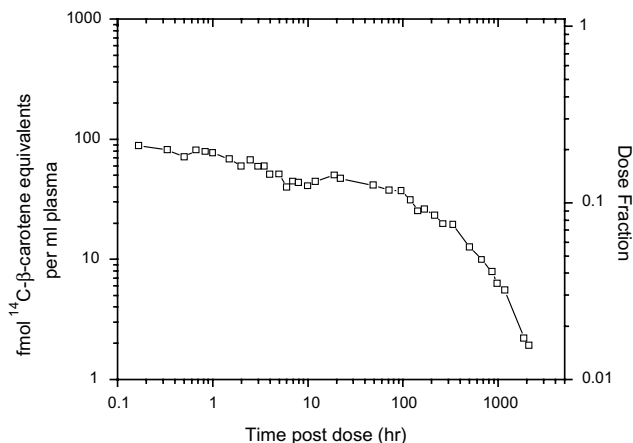


Fig. 1. The plasma concentration of ^{14}C - β -carotene is shown as both ng-equivalents of carotene (left axis) and as fraction of total administered dose (right axis). The time scale is given in log form to show both early and late detail.

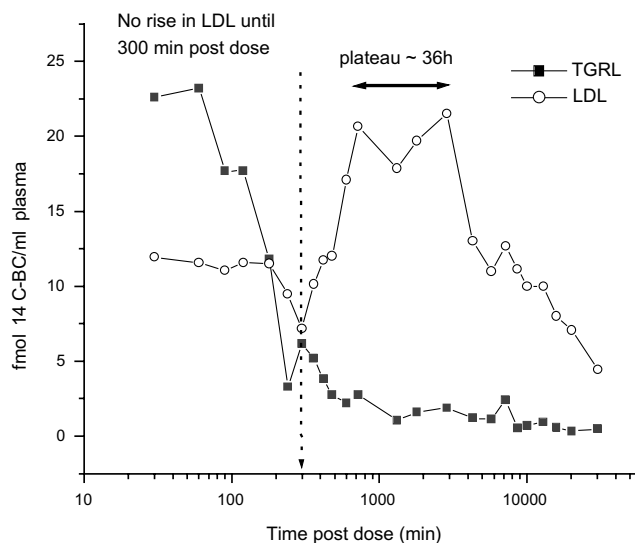


Fig. 2. The time course of ^{14}C in the TGRL and LDL fractions.

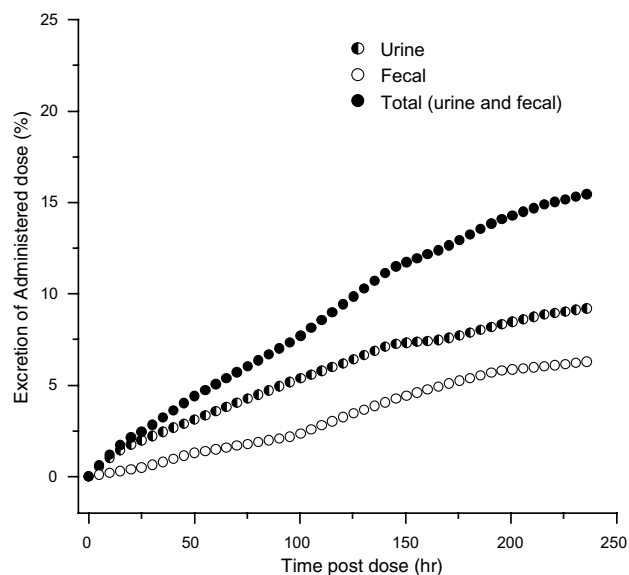


Fig. 3. Fecal and urinary excretion of ^{14}C - β -carotene is shown for 10 day post dose.

previously conducted ^{14}C - β -carotene oral feeding experiment in the same subject [12]. A break in the Y -axes from linear to log scale was performed to show the full range of the data from the oral feeding experiment. ^{14}C -retinol concentrations peaked between 120–170 h post infusion whereas retinol derived from orally administered β -carotene peaked much earlier. The AUC_{0-2112} (attomole $^{14}\text{C} \cdot \text{h/mL}$) for both the oral and infusion experiment were calculated to be 1.81×10^6 and 5.14×10^6 for the infusion and oral experiments, respectively. Using the reported molar vitamin A value in the oral experiment of 0.53 moles [12] the ratio of the retinol derived from infusion and oral exposures ($\text{AUC}_{\text{infusion}}/\text{AUC}_{\text{oral}}$), the molar vitamin A value of the infused β -carotene was predicted to be 0.19, meaning the 1 mole of β -carotene provides 0.19 moles of vitamin A as retinol.

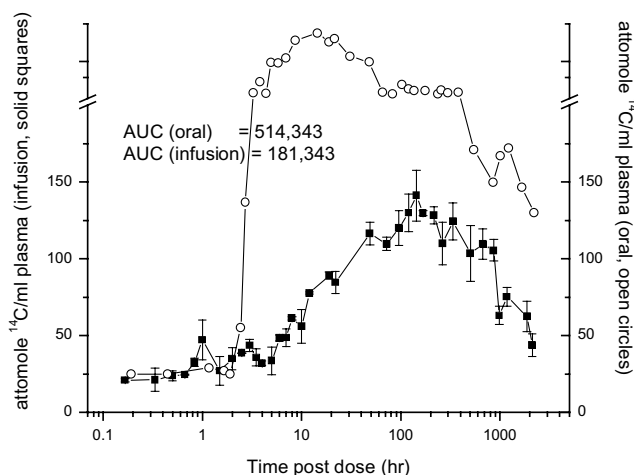


Fig. 4. The concentration of ^{14}C in total plasma retinol obtained by HPLC purification is shown as a function of time post dose.

7. Discussion

The concept of extra-intestinal cleavage of β -carotene to retinol is not entirely new, but to date the importance of such a mechanism is largely inferential. The human liver contains five-fold greater activity towards the directed central cleavage of β -carotene to vitamin A than intestine, and the limited available evidence also suggests that β -carotene is a precursor of retinol in the human prostate [2,19,20]. Moreover, the fact that primates absorb significant quantities of intact β -carotene while many other species (i.e. rats, pigs) do not, might suggest a post intestinal role for β -carotene in humans. Nonetheless, no previous human studies have directly tested this capability, due to a lack of sensitive infusion and tracing techniques. AMS sensitivity towards ^{14}C labeled biochemicals affords the possibility to trace endogenous molecules and their minor metabolites following dilution in a large endogenous pool. Our data in the present study provides *in vivo* evidence that ^{14}C -retinol is a product of absorbed ^{14}C - β -carotene metabolism.

In addition to quantifying retinol production from β -carotene, we were also able to propose a new means for quantifying TGRL clearance rates using the ^{14}C - β -carotene as a surrogate marker of TGRL metabolism. This information is of clinical importance due a purported association with cardiovascular disease [21,22]. This concept of a surrogate marker has its origins in the similar application of non-labeled retinyl esters in oral fat loading tests, in which retinyl esters associated with chylomicrons and their remnants are used to quantify their rate of clearance fate [23–28]. Interpretation of the resulting plasma concentration plots from these studies is complex: the intestinal input is drawn out over several hours and the kinetic behavior is a convolution of both input and removal kinetics. As a result, complex compartment models are required to interpret the data. Our method offers a simpler means of assessing clearance rates without these complications.

The observed TGRL clearance rate ($t_{1/2}$ 122 min) in our study was considerably longer than the expected based

upon prior literature (~ 5 min) [13]. A deficiency of our study was the lack of multiple time points in the first 30 min post dose; however, the fact that $<10\%$ of the administered dose was recovered in the TGRL at the 30 min time point indicates that at least 3 half lives of clearance had occurred, suggesting that there is a rapidly removed component with a half-life of less than 10 min. More frequent sampling in the first 30 min region would have more clearly resolved this rapid clearance phase. Additional experiments must be performed before the utility of this method for quantifying fat clearance can be fully evaluated. The apparent clearance rate of 122 min is nonetheless of potential significance, since the slow clearance of lingering particles represents the underlying etiological mechanism that may explain atherogenicity [29,30].

The concentration of ^{14}C in LDL above baseline did not substantially rise until ~ 300 min post dose, consistent with the time lag between chylomicron/VLDL uptake and resecretion of β -carotene with hepatic VLDL that rapidly remodels to longer-lived LDL. This protocol is well suited for lipoprotein kinetic studies that enlist physiological kinetic modeling to explain observed behavior [31,32].

Classical balance methods, which compare compound intake with total recovery in urine and feces, are not useful in this circumstance, unless potential losses in the gut and bile can also be ascertained. Evaluating fecal excretion kinetics after intravenous administration enables the biliary loss to be assessed. In the present study, the ^{14}C label recovered in the stool is attributed to loss via the biliary route either as β -carotene, its catabolites, or conjugates of derived vitamin A. Surprisingly, the urine was the dominant route of label loss. The urinary route is not accessible to intact β -carotene due to aqueous insolubility in normal urine, suggesting that substantial catabolism of β -carotene to water-soluble products occurred.

In this report, we used AMS to quantify the natural kinetics of a non-perturbing dose ^{14}C - β -carotene associated with autologous TGRL, and also followed its subsequent bioconversion to retinol. The techniques described here open up new avenues of study for examining the fate of small doses of drugs, nutrients, toxins, or *ex vivo* labeled endogenous molecules at levels that greatly reduce both chemical and radiation exposure. AMS is expected to play an increasingly important role in drug selection as well as nutrient and phytochemical research as the merits of this technology become more widely known.

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