MINIREVIEW

Stable Isotopes of Minerals as Metabolic Tracers in Human Nutrition Research

KRISTINE Y. PATTERSON AND CLAUDE VEILLON[1]

USDA, Building 307, BARC-East, Beltsville Human Nutrition Research Center, Beltsville, Maryland 20705

Enriched stable isotopes used as tracers have proven to be valuable in studies of the absorption and metabolism of minerals. Unlike radioisotopes, they can be used in high-risk population groups such as infants, children, and pregnant or lactating women. Estimates of mineral absorption can be made from the oral administration of a single tracer or from two tracers, one given orally and the other intravenously (IV). It is possible to determine the metabolism of the mineral with modeling based on the amount of the tracer or tracers in different biological samples. One of the key decisions in studies of this type is determining which enriched isotope and what amount to use. An example is given of calculations to estimate and compare the amounts of tracers needed for an absorption study. Methods for calculating the amounts of tracer in oral and IV doses are presented, and limits of detection and quantitation are discussed in terms of percent of enrichment and related to isotope ratio measurement precision. A general review of the use of mass spectrometric instruments for quantifying various stable isotopes is given. [Exp Biol Med Vol. 226(4):271–282, 2001]

Key words: stable isotope; mass spectrometry; fractional absorption; apparent absorption; tracer; mineral; metabolism; trace-element nutrition

The use of stable isotopes as tracers in metabolic studies began with their incorporation into organic molecules. In the earliest studies, one or more atoms of hydrogen in either organic molecules or water were replaced with deuterium, a heavier isotope of hydrogen with low natural abundance. Later, heavier isotopes of nitrogen and carbon with low natural abundance were also available for substitution (1). Molecules labeled in this fashion are used primarily to study the metabolism of proteins, lipids, or carbohydrates and could be referred to as "isotopomers" (2). In contrast, enriched stable isotopes of minerals are primarily used to study the metabolism of that particular element.

Initially, enriched stable isotopes of minerals were not available, so the early work with mineral tracers was done using radioisotopes, first in animals and then in humans. From a research perspective radioisotopes have many advantages for determining the metabolic fate of an administered mineral tracer. Two of the most important advantages are that by using whole body counting, the amount of tracer absorbed and remaining in the body after a tracer dose can be unambiguously determined, and second, the amount of dose necessary in order to follow the tracer can be very small, making it less likely that the tracer will perturb the system being investigated. Much of the human nutrition research has shifted away from radioisotopes to enriched stable isotopes as the risks from radiation have been recognized. There have been recent radiotracer studies, but these involved only adult subjects (3–7). The advantages and disadvantages of the use of the two types of isotopes are summarized in Table I.

The Manhattan Project during World War II was vital for making the production of enriched stable isotopes possible. Some of the Calutrons at Oak Ridge, Tennessee, initially built to separate the isotopes of uranium, were converted to isotope separation of other elements in 1945 (8). In 1960 the program was expanded specifically for the enrichment of stable isotopes for research, medicine, and industry. Although the separations do not produce material that is entirely one isotope, the enrichment (atom percentage of the isotope in the material) may be as much as or more than 99%. The current inventory at Oak Ridge National Laboratory (ORNL) includes many isotopes that could be used for nutrition studies. A list of available isotopes, including the

[1] To whom requests for reprints should be addressed at USDA, Building 307 Room 223, BARC-East, Beltsville Human Nutrition Research Center, Beltsville, MD 20705. E-mail: veillon@307.bhnrc.usda.gov

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Table I. Advantages and Disadvantages to the Use of Stable Isotopes or Radioisotopes as Metabolic Tracers

	Radioisotopes	Enriched stable isotopes
Safety	Some risk, especially for pregnant or lactating women, and children	No significant risk
As tracers	True tracers since they are not naturally present	Naturally present, sufficient amounts must be given to be detectable
Study time	Half-life of the radioisotope can affect length of study	Tracers may be followed for extended periods of time
Useful isotopes	Radioisotopes of some minerals have short half-lives (^{64}Cu and ^{28}Mg), making human studies difficult	Some minerals are mononuclidic (Co, As, Mn, and I) while others have several isotopes (Zn-5, Se-6, and Ca-6)
Study design	Generally only one radioisotope is given	Multiple isotopes of an element and/or isotopes of different elements can be given simultaneously
Study approvals	Several levels of institutional review	Generally one level of review
Cost	Generally inexpensive	May be expensive, depending on natural abundance
Analysis	Sample analysis must be timely based on half-life of the isotope, sample preparation minimal	Samples can be stored without loss of the tracer; samples may require extensive sample preparation
Detection	Whole-body measurement possible to determine retention; good measurement sensitivity	Whole-body measurement not possible; some measurement techniques may have poor precision

chemical form, percentage of enrichment, and price can be found on the web site (currently www.ornl.gov/isotopes/p_s_list.html). Other suppliers of enriched stable isotopes have been listed elsewhere (9).

Uses of Stable Isotopes

Stable isotopes (enriched stable isotopes) can be used in many situations where radioisotopes have been used. One example of this is in determining blood volume. A unique characteristic of chromium in the +6 oxidation state is that it will bind irreversibly to red blood cells. This has been the basis of a widely used method for determining blood volume using radioactive ^{51}Cr. The method has been modified so that the same measurement can be made in pregnant women with ^{53}Cr that gives equivalent results and with no risk from radiation, but requires a much more difficult analysis (10). Likewise, stable isotope tracers can be used in nutrition studies in place of radioisotopes, but with more complex analytical requirements.

The questions to be answered in nutrition research often relate to the absorption, utilization, and retention of nutrients. Absorption of an element can be estimated by determining what fraction of a stable isotope or isotopes given as a tracer is absorbed. Depending on the method used to determine the value for absorption, it may be referred to as either apparent absorption or fractional absorption (FA). In this report apparent absorption will be defined as absorption determined from a single tracer of an element, and FA is calculated from the ratio of two different isotopes of an element, one given orally and the other intravenously (IV). Utilization and retention may be investigated using one or more stable isotopes to follow metabolism and to determine pool sizes and turnover with kinetic studies and nutrient modeling.

Apparent Absorption

The traditional way to investigate absorption is the balance study. In this type of study a known amount of an element is given orally, and fecal and urinary output of the element are measured. The fundamental drawback to the method is that there is no way to differentiate between the elemental material given as the oral dose and the element already present in the body. With stable isotopes, the tracer dose can be differentiated. Fecal monitoring as described by Jangherboni and Young (11) and King et al. (12) calculates mineral absorbance based on the difference between the amount of isotope given and the amount recovered in the feces. Knudsen et al. (13) compared this fecal monitoring method for zinc with whole-body counting of a radioactive ^{65}Zn dose and concluded that there was no significant difference in results between the two methods, although the variability of the fecal monitoring was greater. Apparent absorption is determined as follows:

$$\text{Apparent absorption (\%)} = \left(\frac{\text{dose} - M^s}{\text{dose}}\right) \times 100 \quad (1)$$

where dose is the amount of tracer given and M^s is the amount of tracer recovered in the feces.

A source of error in fecal monitoring for some elements is that a portion of the absorbed tracer isotope is excreted into the gastrointestinal tract and is subsequently included in the fecal measurement along with the unabsorbed isotope. When a significant amount of the isotope is re-excreted the result is an underestimation of absorption. Davidsson et al. (14) looked at re-excretion of both zinc and calcium tracer in infants, and concluded that correction for this type of loss was not necessary. A study by Lowe et al. (15) also reported

that fecal monitoring, when not corrected for estimated endogenous losses, gave results closer to their more definitive calculation of absorption based on compartmental modeling, although other techniques, which will be discussed later, gave more accurate results. It is clear that correcting apparent absorption calculations for endogenous losses is not simple.

Turnlund (16), in a series of studies, has investigated the apparent absorption of copper in pregnant women and in young men with various dietary treatments (17, 18). In a recent study (19) with two groups of young adult men, one group was given oral doses of stable isotope copper tracer to measure absorption and the other group was given the tracer IV to determine whether there was an effect by the dietary treatment on endogenous fecal losses of copper. The general conclusions (20) regarding copper absorption were that there is variation in absorption efficiency with dietary intake that helps in regulating copper retention by the body. In addition, the amount of copper excreted into the intestinal tract depends on amount of copper absorbed. Copper has only two stable isotopes, with the lower abundance isotope making up about one-third of the natural element. This creates challenges in these types of studies with copper.

Studies using a zinc stable isotope to determine the apparent absorption of zinc have been designed to look at differences resulting from dietary components or age (21). Significant differences in absorption were found when the tracer was given either with a meal or in a fasting state (22). The effect of adding oat bran to a meat based diet (23) and the absorption of zinc from a wheat-milk-based cereal diet in infants (14) have also been studied. In addition to zinc, the infant study also investigated the absorption of Ca by giving ^{42}Ca as well as the ^{70}Zn in the diet. Tracers are given in such small amounts that there should be little if any effect from giving two or even more tracers simultaneously when it is desirable to investigate the absorption of more than one element. This can result in significant savings from reduced subject costs and the time required to conduct a study.

Fecal monitoring is most reliable when the absorbance of an element is relatively large. With low absorbance the relative error from the analytical measurement and from difficulty in obtaining complete fecal samples from subjects is proportionally greater. Chromium, where the reported absorption of inorganic $CrCl_3$ is on the order of 1% or less, is not a good candidate for fecal monitoring (24). An alternate method of estimating chromium absorption is to measure the isotope tracer from an oral dose that has been excreted in the urine. By definition, if the tracer is excreted in the urine, it was absorbed, giving a measure of minimum absorption.

The absorption of nonheme iron is also low, but still high enough that some studies of this type have been done (25–28). An alternate method for determining iron absorption is to measure the amount of an oral dose of iron stable isotope tracer that is incorporated into the circulating erythrocytes (29–33).

Some elements have more than one isotope that can be enriched and used for tracer studies. Minihane and Fairweather-Tait (27) gave different enriched stable isotopes of iron on each of three successive days to investigate the short-term effect of Ca supplementation on iron absorption in adults. One tracer, ^{57}Fe, was given with a low calcium diet, followed the next day with a diet including supplemental calcium carbonate and a second iron tracer, ^{58}Fe, and concluding with a third tracer, ^{54}Fe, on the third day given as a reference dose. Apparent absorption for each of the tracers and, therefore, for each of the dietary situations, was determined by fecal monitoring. While in the short term calcium supplementation reduced iron absorption, they did not see long term effects on hematologic indexes in iron-replete adults consuming moderate to high amounts of calcium. Sequential dosing of iron-stable isotopes has also been used to measure the availability of iron to infants from weaning food given with and without ascorbic acid (29). In this study the bioavailability of the iron increased 2-fold with the ascorbic acid. These types of studies where the treatment and control are measured in the same subject eliminate some of the variability that results from using separate sets of subjects.

In addition to investigating the absorbance of tracers in an inorganic form, it is possible to incorporate stable isotopes into organic molecules or into natural foods. Mangles et al. (34) were able to compare the apparent absorption of ^{76}Se-selenite, an inorganic form of selenium, and ^{74}Se-selenomethione, an organic form, by giving both tracers to subjects simultaneously. Comparison of the absorption of the different chemical forms of selenium can be made in the same subject simultaneously. Stable isotopes that have been taken up and incorporated by natural processes into plant or animal tissue and then given as oral doses are endogenous tracers. Exogenous tracers are stable isotopes, in either an organic or inorganic form, that are given directly to the subject. Unless the goal of the study is to determine the absorption of the exogenous form of the element, it is important that either the added tracer equilibrates with the element present in the dietary treatment to be tested or that an endogenous tracer be formulated. The most difficult challenge in incorporating a tracer into plant or animal tissue is producing material sufficiently enriched in a stable isotope to be of practical use. If the enrichment is too low, it may be difficult or impossible to follow the metabolism of the tracer analytically.

Some foods have been successfully labeled with stable isotopes. Zinc stable isotopes have been incorporated into milk (35, 36), chicken, eggs, and peas (35), calcium into milk (37), selenium into eggs and chicken (38), and molybdenum into soy and kale (39). It appears to be more difficult to label wheat (35, 39). Weaver (40) has reviewed the techniques involved in producing intrinsically labeled material. Studies using extrinsic and intrinsic tracers in milk for both zinc (36) and calcium (37) found no significant differences in apparent absorption. In both studies the extrinsic tracer

was added to the milk and allowed to equilibrate before dosing. Turnlund et al. (39) found equivalent absorption between the extrinsic molybdenum stable isotope and the intrinsic molybdenum label in kale, but decreased absorption from intrinsically labeled soy. By labeling the plant materials with different Mo-stable isotopes and using a third isotope as the extrinsic label, the study could be done with a single dosing and collection period.

FA

An alternate method has been developed for determining absorption using two stable isotope tracers of calcium (41). One isotope is given IV and another isotope is given orally. FA is determined from the relative amounts of the two tracers in a biological sample such as urine. In simple terms, the equation is as follows:

$$\text{FA}\,(\%) = \frac{\text{Tracer } 1_{\text{IV}}}{\text{Tracer } 2_{\text{Oral}}} \times \frac{\text{Tracer } 2_{\text{s}}}{\text{Tracer } 1_{\text{s}}} \times 100 \qquad (2)$$

where Tracer 1_{IV} and Tracer 2_{Oral} are the amounts of the IV tracer and the oral tracer, respectively, given in dosing and Tracer 1_s and Tracer 2_s are the amounts of the tracers in the sample. The sample may be urine, plasma, saliva, or breast milk, depending on the study.

A critical assumption when using this technique is that the injected isotope quickly equilibrates with the natural form of the element in the circulating plasma and with the extraplasma pools. The IV tracer is presumed to be totally absorbed so that by comparing the relative amounts of the two tracers, one oral and the other IV, an estimate can be made of the absorption of the oral tracer. One of the major advantages of this method over fecal monitoring is that it is not dependent on subject compliance and skill in the challenging task of making complete fecal collections.

Abrams et al. (42) compared the FA technique for calcium to the balance method for measuring absorption and found no differences at absorptions less than 25%. A study by Lowe et al. (15), in addition to looking at fecal monitoring, compared the FA technique for measuring zinc absorption from plasma, 24-hour urine collections, and spot urine collections to their definitive absorption calculation based on a compartmental model. FA results from all three samples agreed well with the expected absorbance value. King et al. (43) concluded that ". . . the double isotope tracer method is a more accurate measure of zinc FA than is the fecal monitoring method . . ."

The double isotope technique has been used for studies of calcium absorption in girls (44, 45), as well as girls and women from families with histories of osteoporosis (46), postpartum women during and after lactation (47), and infants (48). The infant study, in addition to IV and oral doses of calcium isotopes, included IV and oral doses of zinc, making is possible to determine the FA of both calcium and zinc. In the same study a stable isotope of iron was also given with the test meal along with the calcium and zinc, followed the next day with a second isotope of iron given as a reference dose. Iron absorption was determined from incorporation of the tracer into erythrocytes. This type of study is an example of the efficient use of multiple stable isotopes.

Most calcium FA studies have used urine or plasma as the biological sample to determine the relative amounts of the tracers. Smith et al. (49), giving small amounts of calcium tracers, found that blood, urine, and saliva gave similar results for FA. In a study with lactating women, good agreement was found in determining FA of calcium from urine, serum, and breast milk (50). In this study calcium FA could be determined from a 50-μL breast milk sample. Saliva and breast milk for lactation studies may offer subject-friendly sampling for calcium studies.

As with calcium, zinc FA has been determined from the appearance of the IV and oral tracers in urine. The timing of sampling in zinc FA studies appears to be important. Friel et al. (51), in a study with four adults, found that it was necessary to sample 40 hours or more post-dose to determine reliable zinc FA values. This technique has been used for absorption studies in infants (48), pregnant and lactating women (52), and premature infants (53).

This dual isotope FA technique has also been used with iron, magnesium, and nickel. Iron stable isotopes were given both IV and orally, and the FA was calculated from the enrichment of the tracers in erythrocytes (54). Recently, the FA technique has also been used to assess magnesium absorption in children (55). Improvements in analytical methodology have made it feasible to use the two minor magnesium-stable isotopes, even though they have natural abundances of about 10%, as tracers.

Excretion

The body has two ways to regulate body stores of minerals, by absorption and excretion. Minerals can be lost in the urine and can be excreted in the gastrointestinal tract, in breast milk for lactating women, and in minor amounts in sweat, sloughing of tissue, and semen. During periods of inadequate calcium intake in girls, O'Brien et al. (44), in a study with stable isotopes, found that absorption increased and that there was also decreased urinary excretion. Decreased urinary excretion of calcium has also been found in postpartum women in comparing Ca loses prior to and during pregnancy (56). It appears that the body regulates calcium, in part, by decreasing losses in urine. The body may also be able to conserve zinc by reducing intestinal losses. In a study of women with either marginal or adequate zinc intakes, FA of zinc as determined with stable isotopes did not differ, but intestinal loss of zinc was lower for the group with marginal intake (57).

Kinetics and Modeling

Absorption and excretion are only part of the information that can be learned using stable isotopes. By adding a stable isotope to a system and following this tracer over

time, mathematical modeling of the data makes it possible to determine pool sizes and the kinetics of metabolic processes (58). These parameters can be investigated under different conditions of nutriture, and in population groups such as infants, children, and pregnant or lactating women, and may lead to more accurate estimates of dietary requirements, as well as a better understanding of mineral deficiency and, possibly, toxicity. By utilizing stable isotope tracer enrichment information over time from accessible samples such as plasma, urine, erythrocytes, feces, breast milk, and saliva, modeling can be accomplished using computer programs. Available programs such as WINSAAM/CONSAM (59, 60) make it possible to develop a compartmental model that fits the data and describes the metabolism of an element using physiologically valid processes.

A number of kinetic studies have investigated the metabolism of zinc. In addition to estimating the size of zinc pools that exchange rapidly with plasma (61), zinc metabolism has been compared in women in a fasting state or post-prandially (62), in control subjects and alcoholic liver disease patients (63), and in adult men fed three different levels of copper in their diets (64). Compartmental models for zinc metabolism have been developed for adults that vary in complexity from two to 14 compartments (62, 63, 65, 66). The models with the fewest compartments are based on plasma tracer data after infusion of an IV stable isotope (63, 65). More complex models can be developed when both an IV and oral tracer are given and measured in plasma, urine, and feces (62) or plasma, urine, feces, and erythrocytes (66). Wastney et al. (67) modified an adult model of zinc kinetics for pre-term infants based on tracer and natural zinc data from plasma, red blood cells, urine, and feces after administration of either an oral or IV stable isotope tracer.

Bone mineralization and turnover in girls and women has been the focus of several studies modeling calcium kinetics using stable isotopes (45, 46, 68). As with zinc, compartmental models were developed. Other minerals for which models have been described are copper (19), magnesium (69), and two chemical forms of selenium (70, 71).

Table II. Stable Isotopes of Several Elements, Natural Abundances, and Enrichment of Stable Isotopes Available from ORNL

Element	Isotope	Natural abundance[a]	Enrichment[b]
Calcium	40	96.941	99.99
	42	0.647	94.49
	43	0.135	83.93
	44	2.086	98.89
	46	0.004	30.89
	48	0.187	97.69
Chromium	50	4.345	96.05
	52	83.789	99.9
	53	9.501	95.74
	54	2.365	94.35
Copper	63	69.17	99.89
	65	30.83	99.70
Iron	54	5.845	97.29
	56	91.754	99.93
	57	2.119	92.44
	58	0.282	82.12
Magnesium	24	78.99	99.92
	25	10.00	97.87
	26	11.01	99.59
Molybdenum	92	14.84	97.37
	94	9.25	91.59
	95	15.92	96.80
	96	16.68	96.76
	97	9.55	94.19
	98	24.13	98.78
	100	9.63	Not available
Selenium	74	0.89	77.71
	76	9.37	97.05
	77	7.63	94.38
	78	23.77	98.8
	80	49.61	99.45
	82	8.73	97.19
Zinc	64	48.63	99.68
	66	27.9	99.29
	67	4.10	93.11
	68	18.75	99.71
	70	0.62	85.03

[a] Rosman KJR, Taylor PDP. Isotopic compositions of the elements, 1997. Pure Appl Chem **70**:217–235, 1998.
[b] Representative enrichments available from ORNL.

Stable Isotope Tracers: Isotope and Amount

Enriched stable isotopes can provide a wealth of information on absorption and metabolism of nutritionally important elements as described above. When planning these types of studies, the questions are: first, which isotope or isotopes of an element should be used, and second, how much should be given. Table II lists the stable isotopes for some of the elements, the natural abundances, and information on enriched stable isotope material available from ORNL. Unfortunately, aluminum, cobalt, manganese, iodine, and arsenic have only one stable isotope (mononuclidic) and cannot be investigated in these types of studies.

Zinc, with five stable isotopes, can be used as an example that shows the planning that can be done prior to initiating a tracer study. Theoretically, any of the enriched stable isotopes could be used as tracers; however, the essential requirement when using a tracer is that it must be detectable in the samples. One thing that must be considered is what increase in the amount of an isotope due to the presence of the tracer is necessary for accurate measurement by the analytical equipment to be used. The enrichment of the tracer may be as much as 99%, but as it mixes with natural zinc in the subject, the enrichment is diluted. The amount of natural zinc with which the tracer equilibrates determines the dilution (and allows calculation of pool sizes), but it also is a factor in determining the amount of tracer to use. An additional important factor to be considered is what affect the amount of tracer given will have on the system. If too much is given, the tracer may have an unplanned effect on the results.

For purposes of illustration, it will be assumed that a 5% enrichment in the samples will be sufficient to make an accurate measurement of the amount of tracer. How to determine the needed enrichment on an analytical basis will be discussed with detection limits later in this report. Estimating the amount of natural zinc in the subject that will equilibrate with the tracer may depend on the design of the study. There are zinc plasma and extraplasma pools that undergo rapid exchange along with urinary and fecal losses that are of the most importance in short term studies, while other pools such as muscle and bone are much larger and turn over slowly. If the experiment is designed to determine FA, requiring sampling of urine or plasma at only 40 hours after dosing, the amount of tracer needed would be less than for a study looking at more complex kinetics over a longer period. If the tracer is given orally, the amount likely to be absorbed is also a factor.

Table III illustrates how amounts of stable isotope tracer needed for a study can be estimated. The amount of zinc, 2.5 mmol, with which the tracer(s) exchange was estimated for this calculation from the work of Miller et al. (61), who estimated that this amount of zinc exchanges in the plasma over a period of a couple of days. Column A gives the millimoles of each of the stable zinc isotopes in 2.5 mmol of natural (unenriched) zinc. Assuming that an increase of 5% in the amount of the isotope can be detected analytically, column B gives the amount of that isotope, in millimoles, that would need to be supplied by the enriched stable isotope tracer. All of the zinc stable isotopes are available from ORNL as enriched material. Column C gives the enrichment of these materials obtainable as ZnO. Based on the abundance of each isotope in the enriched material, the millimoles of the isotope in 1 mg of enriched material is shown in column D. Dividing column B by column D gives the amount in milligrams of enriched stable isotope material needed for an IV tracer in column E. Column F gives the amount of tracer in milligrams needed for an oral tracer at 25% absorption. The current cost from ORNL for the calculated amount of IV and oral tracer is given in columns G and H, respectively. These costs do not include handling charges for the isotopes from ORNL or preparation and testing costs for IV solution preparation, which can be substantial. The term "tracer" as used here refers to enriched stable isotope material including all the stable isotopes, both the highly abundant isotope that may make up as much as or more than 99% of the material and the rest of the stable isotopes usually in very low abundance.

These calculations are given only to illustrate the type of planning that can be done. Depending on the subject population and the type of study, the amounts of tracer needed may be different. Even though the higher abundance isotopes could theoretically be used as tracers, the amounts needed are too large. Sian et al. (22) found that as little as 3 mg of zinc given with a meal and greater than 5 mg given in a post-absorptive state affects FA. In addition, it is likely that large IV doses could have an affect on zinc metabolism and homeostasis. The best isotopes for use in a FA type of zinc study are ^{70}Zn and ^{67}Zn since these would require the least amount of material to achieve enough enrichment of the samples to allow for measurement of the tracers.

Abrams and Wen(55) describe similar calculations for estimating the amount of stable isotopes that would be necessary for determining FA of magnesium. They concluded that an IV dose of 0.29 mg/kg body weight would be sufficient to achieve a 2% enrichment in urine at 71 to 79 hours after dosing, and that an oral dose would need to be approximately 2.5 times as great to compensate for an expected 40% absorption. A 2% enrichment was chosen since this would be more than 10 times the precision of the ratio measurement used to determine the amounts of the isotopes in the urine. This reasoning is based on limits of detection and quantitation for the analyses.

Calculations: Amount of Tracer in a Dose

Before a stable isotope tracer dose can be given, the amount of tracer in it must be quantified. There are two ways that this can be done: by the use of atomic absorption spectrometry (AAS) or by reverse isotope dilution analysis. With AAS the concentration of the tracer in the dose solution is determined by comparison with elemental standards made from material with the stable isotopes in their natural abundances. Even though the standards are made on a

Table III. Example to Illustrate the Calculation of Amounts of Tracer Dose Needed Depending on the Choice of Stable Isotope and Relative Costs

Isotope	A; amount of isotope (mmol)	B; 5% of A (mmol)	C; % Isotope enrichment available	D; Isotope in 1 mg-enriched (mmol)	E; Enr. isotope needed for IV (mg)	F; Enr. isotope needed for oral (mg)	G; Current cost for IV tracer	H; Current cost for oral tracer
64	1.216	0.061	99.86	0.0156	3.90	15.59	$16.33	$65.31
66	0.698	0.035	99.29	0.0151	2.31	9.24	$12.08	$48.32
67	0.103	0.005	94.60	0.0150	0.34	1.37	$16.17	$64.67
68	0.469	0.023	99.71	0.0147	1.59	6.38	$4.51	$18.05
70	0.016	0.001	88.61	0.0144	0.05	0.22	$22.96	$91.85

Note. A is the amount of each of the zinc stable isotopes in 2.5 mmol of natural zinc; B is 5% of A; C is enrichment of the isotope in the stable isotope material available from ORNL; D is the amount of the isotope in 1 mg of the enriched material; E is the estimated amount of enriched stable isotope material needed for an IV tracer; F is the estimated amount of enriched stable isotope material needed for an oral tracer assuming 25% absorption; G and H are the current costs for calculated amount of IV and oral stable isotope tracer needed, respectively.

weight-per-unit-volume basis, the absorbance reading from the instrument is proportional to the concentration of ground-state atoms in the flame. The reading from the AAS must be adjusted using the atomic weights of the unenriched element in the standards and the isotopically enriched element in the dose solution (72) so that the calculation is made on a molar rather than mass basis and converted back to mass, if desired. To illustrate the potential error if the correction is not made, consider solutions of ^{42}Ca- and ^{44}Ca-enriched stable isotopes that are nominally 10 mg mL^{-1}, as determined in comparison with unenriched Ca standards. Ca standard at 10 mg mL^{-1} has a molar concentration of 0.2495 mmol mL^{-1} (atomic weight of natural calcium, 40.08 mg $mmol^{-1}$) A 0.2495 mmol mL^{-1} solution of ^{42}Ca (atomic weight 42.09) would have 10.5 mg ^{42}Ca mL^{-1} and a 0.2495 mmol mL^{-1} solution of ^{44}Ca (atomic weight 43.92) would have 11.0 mg ^{44}Ca mL^{-1}, a 5% and 10% error, respectively.

The second method for quantifying the amount of enriched stable isotope in a solution is by reverse isotope dilution. Isotope dilution is a powerful method for determining the amount of an element present in a sample using a known amount of an enriched stable isotope as an internal standard. The principles of isotope dilution are well described (73–75). In reverse isotope dilution, an unknown concentration of an enriched stable isotope is determined by adding a known amount of the unenriched element. The same equation as that used for isotope dilution is solved for the amount of stable isotope since the amount of the natural element is known. The analysis requires that the ratio between the enriched isotope and an unenriched or reference isotope of the element be measured by mass spectrometry. That information is combined with the amount of the element added and the atomic abundances of the two isotopes in the enriched and unenriched element giving the amount of enriched stable isotope material in the solution. The calculation is made as follows:

$$R_{i/j} = \frac{M_n \times A_i^n + M_s \times A_i^s}{M_n \times A_j^n + M_s \times A_j^s} \quad (3)$$

where:

$R_{i/j}$ = the ratio measured for the reference isotope to the tracer isotope;
M_n = the amount of the natural element added to the sample (in moles: mass × atomic weight);
M_s = the amount of the enriched stable isotope material (in moles: mass × atomic weight);
A is used to designate atomic abundance with the subscripts, indicating the isotope and superscripts the source of the isotope;
i = reference isotope;
j = tracer isotope;
n = natural element; and
s = enriched stable isotope material;

The equation can be solved for M_s, the amount of the enriched stable isotope in the sample, since all other terms are known. Rearranging:

$$M_s = M_n \left[\frac{(R_{i/j} \times A_j^n) - A_i^n}{A_i^s - (R_{i/j} \times A_j^s)} \right] \quad (4)$$

The amount of stable isotope tracer in a dose can be accurately determined by either AAS or by isotope dilution analysis.

Calculation: Amount of Tracer in a Sample

The amount of the enriched stable isotope in a sample can be determined from the ratio of the tracer isotope to a reference isotope measured by mass spectrometry, together with the total amount of the element present in the sample. The total amount of the element is usually determined by atomic absorption spectrometry. The atomic weight of the total amount of the element that includes the tracer or tracers, as well as the unenriched or natural element, is not significantly different from the atomic weight of the natural element since the actual amount of the tracer is usually very small. Unlike measurement of the dose concentrations, adjustment of the analytical results for atomic weight are not usually necessary. The following calculations based on work of Turnlund et al. (25) can be used to calculate the amount of stable isotope tracer:

$$T = m_n + m_t \quad (5)$$

where T is the total amount of the element determined by AAS, m_n is the amount of the natural element, and m_t is the amount of the stable isotope tracer in mass units. The measured ratio, $R_{x/i}$, is equal to the moles of the tracer isotope in the sample divided by the moles of the reference isotope:

$$R_{x/i} = \frac{\left(\frac{m_n}{W_n}\right) \times A_{xn} + \left(\frac{m_t}{W_t}\right) \times A_{xt}}{\left(\frac{m_n}{W_n}\right) \times A_{in} + \left(\frac{m_t}{W_t}\right) \times A_{it}} \quad (6)$$

where:

W_n = the atomic weight of the natural element;
W_t = is the atomic weight of the enriched stable isotope material;
A is used to designate atomic abundance with the subscripts indicating the isotope and the source of the isotope;
x = tracer isotope;
i = reference isotope;
n = natural element; and
t = enriched stable isotope.

Rearranging Equation 4:

$$m_n = T - m \quad (7)$$

This result can be substituted into Equation 6 and then solved for the amount of tracer based on the total amount of the element, the abundances of the isotopes, the atomic weights, and the measured ratio.

$$m_t = \frac{T \times W_t \times [A_{xn} - (R_{x/i} \times A_{in})]}{W_n \times [(R_{i/x} \times A_{it}) - A_{xt}] + W_t \times [A_{xn} - (R_{x/i} \times A_{in})]} \quad (8)$$

The tracer value or values determined can then be used in Equations 1 or 2 to calculate apparent absorption or FA, or can be used for kinetic and modeling investigations. It is important that the calculation include both the abundances of the tracer and reference isotopes, and the atomic weights of the natural element and the enriched material in order to determine an accurate amount. Before any method for calculating the amount of tracer is used, it should be validated by taking a theoretical sample concentration of tracer and unenriched element, calculating a theoretical experimental ratio from the moles of the tracer and reference isotope, and then putting the ratio and other necessary information into the equation to be tested. A valid equation will result in a back calculation of the precise amount of tracer from the initial theoretical situation. In addition, if the calculation is set up correctly in a computer spreadsheet, it is simple to vary the amount of tracer to show that the equation is valid over a range of enrichments. There are equations for calculating tracer amounts in the literature that are not capable of meeting this test.

A second method for determining the amount of tracer is by isotope dilution analysis. This requires that a known amount of an enriched stable isotope, not used as a tracer, be added to the sample as an internal standard. This is referred to as double isotope dilution if the amounts of the natural element and one tracer are to be determined, or triple isotope dilution for natural and two enriched stable isotope tracers. This has been described in detail elsewhere (76). Patriarca et al. (77), in measuring the amount of a nickel tracer by isotope dilution analysis, have taken a somewhat different approach. Rather than measuring both the tracer and natural amount of the element at the same time with double isotope dilution, they first spiked an aliquot of the sample with an amount of internal standard that made it possible to accurately determine the total amount of the natural element present. In a separate analysis they then added an amount of internal standard that allowed for accurate measurement of the tracer using the known amounts of the internal standard and the previously determined amount of the natural element.

Isotope dilution analysis has the potential for making accurate and precise measurements of tracer content of samples. One of the drawbacks is that an additional enriched stable isotope with low abundance must be available for use as the internal standard. For some elements such as copper, where there is only one low-abundance isotope, this is not possible, or if, in the case of magnesium FA, both low-abundance isotopes are used as tracers, there are no more enriched isotopes to use as the internal standard.

Calculations: Limit of Detection and Limit of Quantitation

In using the information on the tracer content of the samples, it is important to know whether or not the data is accurate and precise. As the analytical reading approaches the limit of detection, the uncertainty in the measured quantity increases and, therefore, the uncertainty in the estimation of absorption, kinetics, and pool size also increases. It is important in modeling to consider the uncertainty in the data from which the model is developed. At the most basic level, whether or not the tracer is actually detected in the sample needs to be determined. This is the limit of detection and it can best be expressed for stable isotopes in terms of percentage of enrichment. This is a comparison between the ratio of the enriched isotope to a reference or unenriched isotope of the element in the sample and in unenriched elemental material. It is defined as:

$$\% \text{ enrichment} = \frac{R^* - R}{R} \times 100 \quad (9)$$

where R is the tracer to reference isotope ratio in the unenriched element and R* is the tracer to reference isotope ratio measured in the sample. The limit of detection (LOD) for an analytically determined ratio is when the percentage of enrichment of the ratio is equal to three times the relative standard deviation (%RSD) of the mean ratio measurement and the limit of quantitation (LOQ) would be 10 times (50). Abrams et al. (55) have also stated that the lower limit of optimal enrichment is 10 times the precision of the measurement. For some instruments an isotope ratio can be determined with an uncertainty, %RSD, of as little as 0.02%. In this case, the LOD would be 0.06% and LOQ would be 0.2%. More typical uncertainties may be 0.2% to 1%, giving a LOQ of 2% to 10% enrichment. If the instrumentation used for making the isotope ratio measurements is precise, less enrichment is needed to make an accurate measurement.

Instrumentation for Isotope Ratio Measurements

There are primarily four types of mass spectrometers that have been used to measure stable isotopes for nutrition studies: thermal ionization mass spectrometers (TIMS), inductively coupled plasma mass spectrometers (ICP-MS), fast atom bombardment mass spectrometers (FAB-MS), and gas chromatograph mass spectrometers (GC-MS). There are excellent reviews of the operation and use of these mass spectrometers for nutrition research (78, 79) and this detailed information will not be repeated. In general, TIMS has been used primarily for calcium (41, 44, 45, 80), mag-

nesium (55), zinc (14, 25, 65, 81), iron (31, 54), copper (16–18), and molybdenum (39, 82, 83).

The most recently developed and now widely available of the four types of mass spectrometers is the ICP-MS. Although the most common type of ICP-MS (quadrupole mass filter) is usually less precise than magnetic sector instruments, there is higher sample through-put and less rigorous sample preparation is usually required. One of the difficulties with using the instrument is that there are numerous interferences that can hinder the measurement of specific isotopes. Stürup et al. (84) were able to measure ^{42}Ca:^{43}Ca and ^{44}Ca:^{43}Ca ratios using high resolution magnetic sector ICP-MS to avoid interferences. Patterson et al. (50) were able to measure ^{42}Ca:^{43}Ca and ^{44}Ca:^{43}Ca ratios by operating a quadrupole ICP-MS in a cool plasma mode, but noted that there were still interferences at ^{46}Ca and ^{48}Ca from titanium. As this illustrates, it is important to understand the limitations of the instrumentation to be used when choosing the enriched stable isotopes for tracers. If cool plasma ICP-MS were to be used, ^{46}Ca and ^{48}Ca would not be good choices for tracer isotopes.

There are also interferences for the elements selenium, copper, and iron. Due to the argon plasma of the ICP-MS, the most abundant isotope of selenium, ^{80}Se, is unusable due to the $^{40}Ar_2$ dimer that occurs at the same nominal mass. Buckley et al. (85) reported that enriched ^{76}Se and ^{82}Se could be used as tracers and ^{78}Se as a reference isotope with hydride generation sample introduction for selenium studies.

The two isotopes of copper can be measured provided that sodium is removed from the samples since $^{23}Na^{40}Ar$ interferes with ^{63}Cu. There have been some studies of copper absorption (86, 87) and stable isotope tracers have been used to study the metabolism of copper in subjects with Wilson's disease (88). Like selenium, the most abundant isotope of iron, ^{56}Fe, has a significant interference from a polyatomic argon ion, $^{40}Ar^{16}O$, which occurs at the same nominal mass. Zlotkin et al. (89) were able to determine iron absorption in premature infants, using two of the three minor isotopes as tracers and a third isotope as the reference, measuring the ^{57}Fe:^{54}Fe and ^{58}Fe:^{54}Fe isotope ratios in erythrocytes, while others measured one tracer, ^{58}Fe, and used ^{57}Fe as the reference isotope (32, 33).

The elements magnesium, molybdenum, nickel, and zinc have less serious interferences in ICP-MS analysis. Although ICP-MS is a promising method for analyzing magnesium stable isotope tracers (90), it has not yet been utilized. Molybdenum has seven stable isotopes that can be used for metabolic studies and only one isotope has any serious interference when using ICP-MS for analysis. This appears to be a potentially useful and underutilized analytical method for investigating molybdenum metabolism. The apparent absorption of nickel, with five stable isotopes, has been studied using ICP-MS (77). Zinc-stable isotopes can be analyzed easily by ICP-MS, and the analytical method has been used extensively after separation of zinc from the sample matrix to reduce interferences (13, 23, 36, 52, 53, 62, 91).

The third mass spectrometer used for mineral analysis is a FAB-MS. The most recent reports using this instrument have been for studies of calcium (37, 92) and zinc (22, 51, 57, 61) absorption and metabolism. Like TIMS, sample preparation needs are extensive for this instrument, and the analyses require more time than ICP-MS.

The fourth mass spectrometer, GC-MS, has been used for nutrition studies of two elements, chromium (24) and selenium (34, 70, 93). Analysis requires chelation of the element to a volatile organic compound to create a complex that can be separated by gas chromatography before electron impact ionization and quadrupole mass separation. Chromium and selenium each form kinetically stable chelates that can be chromatographed without breaking down or undergoing exchange.

Conclusion

Enriched stable isotopes used as tracers in nutrition studies offer a risk-free alternative to radioisotopes that are suitable for use in population groups such as infants, children, and pregnant or lactating women. The value of stable isotopes for absorption and metabolic studies is evident from the large and growing number of reports, especially for the elements zinc, calcium, and iron, and to a lesser degree, copper, magnesium, molybdenum, and selenium. One important key to the growth of stable isotope use has been, and will be in the future, the availability of ICP-MS instruments. Although TI-MS is generally capable of better precision in isotope ratio measurements, the precision of the ICP-MS ratio determinations is sufficient for many tracer studies and has the advantage of faster sample through-put and less complicated operation than TI-MS. It is likely that the use of stable isotope tracers for nutrition studies will increase both for the more traditional study designs and, possibly, for studies designed to investigate the chemical forms of the tracers in different metabolic pools.

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