Lietz G, Furr HC, Gannon BM, Green MH, Haskell M, Lopez-Teros V, Novotny JA, Palmer AC, Russell RM, Tanumihardjo SA, Van Loo-Bouwman CA. Current Capabilities and Limitations of Stable Isotope Techniques and Applied Mathematical Equations in Determining Whole-Body Vitamin A Status. *Food and Nutrition Bulletin* 2016, 37(2), S87-S103.

DOI link to article:

http://dx.doi.org/10.1177/0379572116630642

Date deposited:

25/07/2016

Article

Food and Nutrition Bulletin
2016, Vol. 37(2S) S87-S103

DOI: 10.1177/0379572116630642
fnb.sagepub.com
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Current Capabilities and Limitations of Stable Isotope Techniques and Applied Mathematical Equations in Determining Whole-Body Vitamin A Status

Georg Lietz, PhD[1], Harold C. Furr, MNS, PHD[2], Bryan M. Gannon, PhD[2], Michael H. Green, PhD[3], Marjorie Haskell, PhD[4], Veronica Lopez-Teros, MS, PhD[5], Janet A. Novotny, PhD[6], Amanda C. Palmer, PhD[7], Robert M. Russell, MD[8], Sherry A. Tanumihardjo, MS, PhD[2], and Carolien A. Van Loo-Bouwman, PhD[9]

Abstract

Background: Retinol isotope dilution (RID) methodology provides a quantitative estimate of total body vitamin A (VA) stores and is the best method currently available for assessing VA status in adults and children. The methodology has also been used to test the efficacy of VA interventions in a number of low-income countries. Infections, micronutrient deficiencies (eg, iron and zinc), liver disease, physiological age, pregnancy, and lactation are known or hypothesized to influence the accuracy of estimating total body VA stores using the isotope dilution technique.

[1] Newcastle University, Newcastle, United Kingdom
[2] University of Wisconsin-Madison, Madison, WI, USA
[3] Department of Nutritional Sciences, The Pennsylvania State University, University Park, PA, USA
[4] Program in International and Community Nutrition and Department of Nutrition, University of California, Davis, CA, USA
[5] Universidad de Sonora, Hermosillo, Mexico
[6] Beltsville Human Nutrition Research Center, United States Department of Agriculture, Beltsville, MD, USA
[7] Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA
[8] Tufts University, Boston, MA, USA
[9] Radboud University Medical Center, Nijmegen, the Netherlands

Corresponding Author:
Georg Lietz, Human Nutrition Research Centre, School of Agriculture, Food and Rural Development, Newcastle University, Newcastle NE1 7RU, United Kingdom.
Email: georg.lietz@ncl.ac.uk

Objective: Our objectives were to review the strengths and limitations of RID methods, to discuss what is known about the impact of various factors on results, and to summarize contributions of model-based compartmental analysis to assessing VA status.
Methods: Relevant published literature is reviewed and discussed.
Results: Various equations and compartmental modeling have been used to estimate the total body VA stores using stable isotopes, including a newer 3-day equation that provides an estimate of total body VA stores in healthy adults. At present, there is insufficient information on absorption of the isotope tracer, and there is a need to further investigate how various factors impact the application of RID techniques in field studies.
Conclusions: Isotope dilution methodology can provide useful estimates of total body VA stores in apparently healthy populations under controlled study conditions. However, more research is needed to determine whether the method is suitable for use in settings where there is a high prevalence of infection, iron deficiency, and/or liver disease.

Keywords
model-based compartmental analysis, prediction equations, retinol isotope dilution, vitamin A status

Introduction

Vitamin A (VA) is an essential nutrient that is required for normal vision, reproduction, growth, and immune health. Vitamin A deficiency (VAD, defined as plasma retinol concentrations <0.7 μmol/L) affects an estimated 190 million preschool-aged children and 19.1 million pregnant women globally, which correspond to 33.3% of the preschool-aged population and 15.3% of pregnant women in populations at risk of VAD.[1]

Eradication of VAD remains an important goal for public health professionals worldwide, and providing VA is one of the most cost-effective health interventions known.[2] Evaluation of such interventions is important to ensure that optimal levels of total body VA are achieved but not exceeded,[3] especially in countries where more than one public health intervention to control deficiency is in place.[2,4,5] Concerns have been raised about inadvertent chronic excessive VA intakes due to high-dose VA supplementation combined with concurrent use of VA-fortified foods, micronutrient powders (MNPs), and voluntarily fortified commercial products.[2,4] Elevated hepatic VA concentrations (>1 μmol/g liver) have been reported in a small study of Nicaraguan schoolchildren 1 year after implementation of sugar fortification[3] and in Zambian children exposed to supplementation and fortification.[6] To determine whether public health intervention programs place some individuals at risk of excessive VA intake, sensitive biomarkers are needed to evaluate the effectiveness and safety of VA interventions across the full spectrum of VA status, especially because plasma concentrations of retinol show limited responsiveness to changes in status in populations with adequate or excessive VA intakes.[3,7] The retinol isotope dilution (RID) technique can be used to (a) assess total body stores of VA, (b) detect quantitative changes in response to interventions, (c) assess the efficacy of provitamin A food-based interventions, and (d) estimate VA requirements.[8] The benefits and limitations of the technique and associated mathematical calculations, as well as the independent method of compartmental analysis, are discussed in this review.

Evolution of Stable Isotope Dilution Techniques for Determining VA Status in Population Groups

Over time, many methods have been proposed for estimating VA status, but none of these are fully satisfactory.[9] Most of the body's VA is stored in the liver in well-nourished individuals,[10] and thus, direct measurement of liver VA concentration is a good indicator of VA status in these individuals. In contrast, in rats with low VA

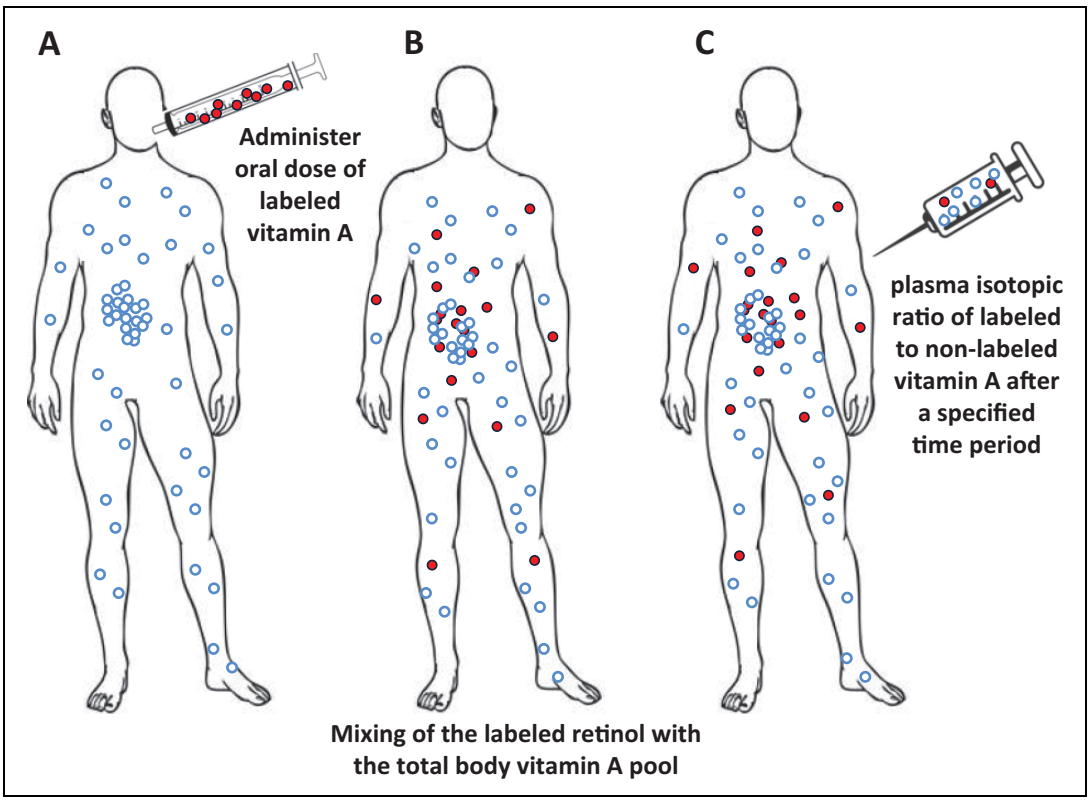

Figure 1. The principle of the retinol isotope dilution (RID) technique. A, A known dose of stable isotope-labeled vitamin A is administered orally. B, This is followed by an adequate mixing period during which the labeled vitamin A mixes with the endogenous nonlabeled vitamin A pool. C, Finally, a blood sample is obtained for measurement of the plasma or serum isotopic ratio of labeled to nonlabeled retinol.

status, about 90% of total body VA is stored in nonhepatic tissues,[11] and therefore, liver VA concentrations would not reflect the total body VA stores. The contribution of nonhepatic tissues to VA status in humans with low VA status is currently not known. Furthermore, direct sampling of liver can only be accomplished in exceptional circumstances.[12,13] Despite these limitations, liver VA concentrations are still being used to define VA status, and the most common cutoff is >0.07 μmol/g (>20 μg/g) as adequate.[9,14]

Beyond liver VA concentrations, the distribution of plasma retinol concentration in a population has traditionally been used to provide useful information about the VA status of that population and their response to VA interventions[15,16]; use of this index is discussed further in paper 2 of this series. However, plasma retinol is under homeostatic control and is maintained over a wide range of liver VA stores, and thus, it is not a sensitive indicator of VA status in individuals. Nevertheless, at the population level, a shift in the distribution of plasma retinol concentrations can be used to assess changes in VA status over time or in response to an intervention,[7] if there is a high prevalence of low serum retinol concentrations initially. In addition to liver and plasma VA measurements, there are functional assessments of VA status, such as impaired dark adaptation and night blindness. Night blindness is assessed by interview, and dark adaptation can be assessed objectively in field settings, but both may be problematic to assess accurately, especially in population groups most at risk, such as very young children. Finally, the relative dose–response and modified relative dose–response tests have proven useful in identifying individuals with inadequate liver VA stores but do not provide

quantitative measures of total body VA stores.[17,18]

Isotope dilution methods have been used to evaluate VA status. The RID technique is responsive to supplementation with VA and has been used successfully in apparently healthy adults and children in low-income countries to assess the efficacy of various VA interventions.[3,19-25] In apparently healthy Bangladeshi men, total body VA stores responded in a dose-dependent manner to 3 different daily doses of VA (0, 1.5, or 3.0 mg/d for 75 days), suggesting that the RID technique could be used to detect quantitative changes in response to interventions.[20] Furthermore, the RID technique has been used to assess the efficacy of provitamin A food-based interventions in both clinical and community settings in low-income countries.[6,21-24] In addition to assessing the efficacy of interventions, the RID technique has been used to estimate VA requirements in Bangladeshi men[26] and, more recently, in women from the United States.[27] Thus, the RID technique is clearly useful for evaluating interventions in controlled studies of apparently healthy adults and children.

The RID method is based on the oral administration of a small dose of tracer-labeled VA followed by the determination of the tracer to tracee (unlabeled VA) ratio in plasma using mass spectrometry. The ratio is measured after a suitable period for mixing of the tracer with the total body VA pool (Figure 1). Currently, a blood sample is collected at 11 to 26 days after dosing with stable isotope-labeled VA.[28,29] However, reevaluation of earlier studies, coupled with recent work, indicates that total body stores of VA can be assessed by measuring the tracer to tracee ratio in serum at 3 days after administration of a physiological dose of stable isotope-labeled VA.[30] The stable isotopes that are used as tracers in the RID technique can be either ^{2}H or ^{13}C. The size of the dose depends on the target group (infants, children, or adults) and the limit of detection of the analytical method being used to measure the ratio of tracer to tracee in plasma. In early studies, pharmacological doses of $^{2}H_4$-retinyl acetate were used in adults (20-45 mg).[12,13,31] However, smaller physiological doses are desirable for evaluating nutritional status by RID techniques because this minimizes perturbations of endogenous retinoid pools and limits departure from steady-state kinetics during metabolism of the tracer.[32] Furthermore, administration of smaller doses reduces costs and thus facilitates use of the technique in studies with larger sample sizes.[8]

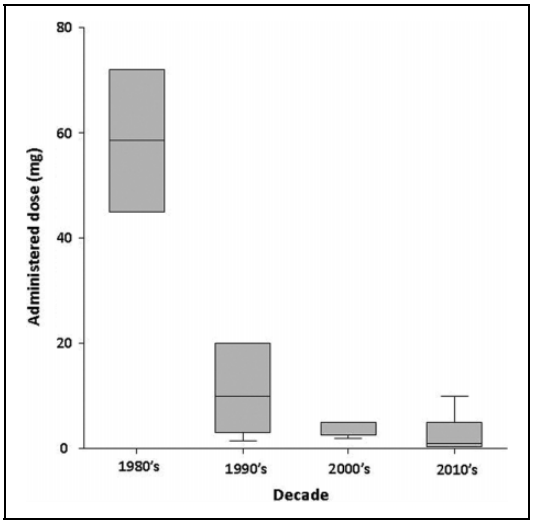

Figure 2. Sizes of vitamin A tracer doses administered for isotope dilution studies (compiled from published reports[3,6,12,13,19-22,25-29,31,38,41-48]).

Various types of mass spectroscopy are used to measure enrichment of stable isotope-labeled VA in plasma,[33] including gas chromatography–mass spectrometry and gas chromatography followed by electron capture negative chemical ionization/mass spectrometry,[32,34] gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS; used for $^{13}C/^{12}C$ determination),[35-37] or more recently, liquid chromatography coupled with tandem triple quad mass spectrometry with atmospheric pressure chemical ionization in positive ion mode.[38-40] The analytical sensitivity of mass spectrometers has increased greatly in the past 3 decades, and it is now possible to measure isotopic enrichment in plasma after administration of a small, physiologic oral dose of stable isotope-labeled VA (Figure 2).

Calculation of Total Body Stores of VA Using Stable Isotope Data

Three methods are used for calculating VA status using stable isotope data. Two isotope dilution

methods are based on analytical equations (''Olson'' and ''mass balance'' equations as described subsequently and in Table 1), and the third uses model-based compartmental analysis. Background information, assumptions, and mathematical adjustments used for both analytical equation methods will first be discussed, followed by a brief review of the modeling approach.

The RID technique was first used to estimate body VA status in rats,[49,51,52] as summarized by Bausch and Rietz.[53] When the prediction equation developed by Rietz et al was applied to human data, the agreement between calculated and measured liver VA stores (determined from liver biopsy samples) was not satisfactory, and an alternate equation, often referred to as the ''Olson equation,'' was proposed (see Equation 1 in Table 1).[12] The application of this equation was corroborated by Haskell et al in a study in Bangladeshi adults.[13] Most subsequent human studies have used variations of this equation for calculating total body or liver stores of VA (reviewed by Furr et al[33]). The mass balance equation (Equations 2-4 in Table 1) is the basis for the $^{13}C_2$-RID method calculations.[36] This technique requires a baseline blood sample in at least a few individuals to determine the natural abundance of ^{13}C and a subsequent blood sample to analyze ^{13}C-enrichment by GC/C/IRMS.[6,27,35,36] It is important to note that work is ongoing to refine constants of the presented equations to verify their accuracy and utility in predicting VA stores under different circumstances.

Both analytical equations are best applied to evaluate VA status of groups rather than individuals due to the fact that large interindividual variations in VA metabolism have been observed.[8,54] Importantly, several mathematical adjustments are needed to convert the tracer data to VA pool size estimates. First, an adjustment is required because some of the tracer dose never enters the endogenous VA pool. Thus, a correction factor is necessary to account for the inefficiency of the absorption and storage of the tracer dose. In rats and sheep, 50% of an oral VA dose was recovered in liver at the time of sampling,[53] which is why this value has been used to correct the equations for use with human data. Incorrect estimation of tracer absorption and storage can lead to overestimation of total body stores if absorption is estimated as higher than actual and underestimation if absorption is estimated as lower than actual. Reported tracer absorption in healthy children ranges from 76.5% to 99.2%, whereas retention of tracers (retention here means absorbed tracer minus loss of tracer through bile and urinary excretion during the first several days after dose administration) has been reported to be between 71.1% and 82.2%.[41,55] Lower retention was attributed to excretion of 5.4% to 17.0% of the administered tracer dose in the urine.[41,55] Furthermore, average absorption and retention were significantly reduced (to 74.3% and 57.6%, respectively) in Indian children with infections (ie, respiratory infection, enteric fever, and gastroenteritis), with 10% to 70% of the administered label appearing in the feces.[55] Thus, it seems prudent to adjust for reduced absorption efficiency under conditions of infection. However, because there are insufficient data to determine appropriate correction factors for different types and severity of infections, individuals with symptomatic illness should be excluded from studies using RID techniques.

A second correction is required to account for the ongoing metabolism of VA because some of the tracer dose will be metabolized during the mixing period. Although adequate time for mixing among metabolic pools of VA must be allowed, sampling should occur as soon as possible after dose administration to minimize these effects. Using a VA fractional catabolic rate (ie, the percentage of the available VA body pool that is lost per day; 0.5%/day for adults[56]), either 98.5% or 89.5% of the absorbed dose is still present at 3 or 21 days after administration of the tracer, respectively. However, a higher fractional catabolic rate of 2.2%/day was found in Peruvian children,[42] leaving 93.4% or 53.8% of the absorbed dose still present at 3 or 21 days, respectively. Thus, earlier time points are beneficial in determining total body stores of VA because more of the tracer will still be present and smaller sample volumes can be used for laboratory analysis. Moreover, shorter study periods will likely reduce the burden on study participants.

Table 1. The Olson and Mass Balance Equations for Estimating Total Body Stores of VA Using Stable Isotope Methodology.

Olson equation[a]

1. Total liver reserves $= F \times$ dose $(S \times a \times [(H{:}D) - 1])$

Equation 1 is the form of the isotope dilution equation published by Furr and colleagues in 1989.[12] F is a factor to express efficiency of storage (estimated to be 0.5[49]); S is the ratio of specific activity of retinol in plasma to specific activity of VA in liver (taken as 0.65 from a rat study[50]); a is the fraction of absorbed tracer dose remaining in body at time t after dosing ($a = e^{-kt}$, using an estimated rate of catabolism of VA for adults; $k =$ [ln 2]/140 days $= 0.00495$ per day); $H{:}D$ is the isotope ratio of nondeuterated (tracee) to deuterated (tracer) retinol in plasma; and the term "-1" is used to correct for the contribution of the mass of the tracer dose to the total VA stores (not needed when the tracer dose is small)

Mass balance equation[a]

2. $(F_a \times a) + (F_b \times b) = (F_c \times c)$

3. $c = a + b$

4. $b = a\frac{F_a - F_c}{F_c - F_b}$

5. Total body reserves $= b \times e^{-kt}$

Equation 4 is the form of the mass balance equation used for calculating VA stores by Tanumihardjo in 2000.[36] F_a is the isotope abundance of ^{13}C in the isotopic dose; a is the amount of the absorbed isotopic dose (dose $\times$ absorption efficiency); F_b is the isotope abundance of ^{13}C in the endogenous VA pool; b is the amount of the endogenous VA pool at baseline; F_c is the isotope abundance of ^{13}C in the total VA pool ($F = {}^{13}C/[{}^{12}C + {}^{13}C]$) after dosing; c is the amount of the total VA pool after dosing; $k = \ln(2)$/metabolic half-life of retinol in days; $t =$ time after dosing in days. The values of F_c and F_b are determined by GC/C/IRMS before (F_b) and after the dose (F_c). The terms b and c are unknowns. Equations 2 and 3 can be rearranged to solve for b in terms of known and measured factors, yielding Equation 4

Abbreviations: GC/C/IRMS, gas chromatography/combustion/isotope ratio mass spectrometry; VA, vitamin A.

[a] The Olson and mass balance equations are presented as published previously, but their accuracy and utility for different circumstances are still being evaluated.

A third correction factor is required to account for the differential distribution of tracer and tracee in body tissues and plasma. In other words, the ratios of tracer molecules to tracee molecules in the body tissues versus plasma are not identical.[13,50,57] Generally, after a dose of labeled VA has mixed in the body, there is a higher proportion of tracer to tracee in the storage pool compared with plasma. The ratio of plasma retinol-specific activity to storage VA-specific activity after adequate time for mixing (7 days) was estimated from rat experiments to be 0.65 by comparing specific activity in serum to specific activity in liver.[50] This value appears to be fairly consistent when liver VA stores are adequate; however, at very low liver VA concentrations[50] and 3 days after administration,[30] this value appears to be closer to 1 (ie, retinol-specific activities in plasma and stores are about the same). This will also be the case if dietary VA intake is eliminated after the tracer is given.[36] In this scenario, the system goes into a quasi-isotopic equilibrium because the specific activity in all compartments will become equal, despite the fact that loss is still occurring. However, complete elimination of dietary VA intake for the period between isotope administration and blood collection to determine the tracer to tracee ratio cannot be achieved under field conditions.

Although these approaches are useful for providing an estimate of VA status under usual conditions,[12,13,19,27,42] several modifications of the methods would enhance their utility and applicability in other physiological states, such as hypervitaminosis A. The fractional catabolic rate of VA is currently used to adjust for irreversible VA loss, and this adjustment is made with a constant fractional loss of 0.5%/day based on the data of Sauberlich et al.[56] However, VA utilization rate depends on VA status,[58-61] iron status,[62] inflammatory state,[63] and life stage.[42] Thus, to be more broadly applicable, the correction for

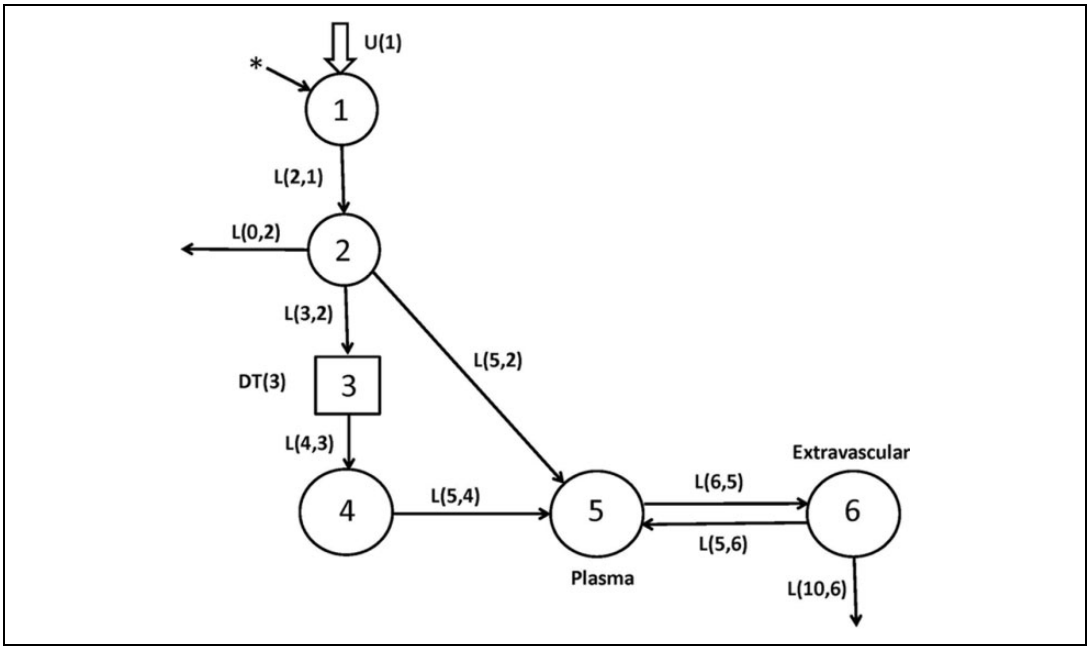

Figure 3. Six-component model for vitamin A (VA) kinetics in humans. Circles represent compartments; component 3, shown as a rectangle, is a delay element, and interconnectivities between compartments (L[I, J]s) are fractional transfer coefficients or the fraction of retinol in compartment J that is transferred to compartment I each day. Compartments 1 to 4 (including component 3) correspond to VA digestion and absorption, chylomicron production and metabolism, liver uptake of chylomicron remnant retinyl esters, and hepatic processing of retinol. Compartment 5 represents plasma retinol bound to retinol-binding protein and transthyretin. This retinol exchanges with VA in one extravascular pool (compartment 6), which includes liver VA stores. The asterisk represents the site of input of an orally administered stable isotope of VA, and U(1) represents dietary VA input. Adapted from Cifelli et al.[65]

irreversible loss should be situation dependent. Alternatively, if a shorter time period can be used, the influence of catabolic loss on the determination of total body VA status will be reduced. Finally, it will be important to be assured that both the Olson equation and the mass balance method provide similar estimates of total body stores of VA. Direct comparisons between these methods are currently lacking.

An alternative to using prediction equations for estimating VA status is compartmental modeling, which provides kinetic information in addition to VA pool size. Compartmental modeling also allows for substantial improvements in the various correction factors needed to transform the basic principles of isotope dilution to equations that can handle the complexities of VAD and possibly toxicity in different stages of life and in different disease states. However, this technique requires the collection of multiple blood samples at defined time points over a relatively long duration, combined with the need for expertise in analyzing and modeling the data.[58,61-64] Since the 1980s, model-based compartmental analysis has been used to describe and quantitate VA metabolism in rodent models (for reviews, see[65] and[66]) and, more recently, humans.[30,38,58] Quantification of exchangeable total body VA stores using model-based compartmental analysis has been performed in humans after oral administration of stable isotopes of deuterium[58] or ^{13}C[30,38] over a duration of 52 and 14 days, respectively. After converting plasma tracer response data to fraction of administered dose versus time, modeling using the Simulation, Analysis and Modelling software,[67] and fitting to a 6-component model (Figure 3), the total amount of VA stores (mean $\pm$ SD) was

calculated as 233 ± 109 μmol for 14 Chinese participants,[58] 892 ± 637 μmol for 12 Americans,[58] and 114 ± 72 μmol for 33 UK participants.[30,38] An expedient approach in the future may be to strategically design isotope/compartmental modeling experiments to determine key pieces of information. These studies could inform development of simpler calculation methods that require fewer blood samples for field situations when multiple blood collections combined with compartmental modeling are not feasible.

Current Limitations of the Isotope Dilution Technique

The RID technique has been validated in adults on the basis of liver VA concentration and modified for use in preschool- and school-aged children.[12,28,42] The validation studies in adults indicated that the RID technique provides an accurate estimate of liver VA stores for groups of individuals, but it does not provide a precise estimate of liver VA stores for individual participants.[12,13] Plasma retinol kinetics of an oral dose of stable isotope-labeled VA has not yet been described in infants <12 months of age or in pregnant and lactating women.[1,8] More information is needed on VA turnover rates in these population subgroups to modify the RID technique and prediction equations. This is important because infants and pregnant and lactating women are at risk of VAD and its consequences in low-income countries, and many interventions are targeted to these subgroups.[1,8] The RID technique could be a useful tool for testing the efficacy of interventions in these subgroups.

As mentioned earlier, the RID technique is dependent on absorption, distribution, mixing, and disposal of a labeled VA dose. It is not known whether the technique provides an accurate estimate of total body VA stores in populations with high rates of infection and micronutrient deficiencies that may affect VA metabolism. Absorption and retention of labeled VA are primary concerns during acute infection, particularly as more sensitive analytical techniques enable investigators to use smaller tracer doses. Diarrhea, parasitic infections, and febrile illnesses are all known to reduce VA absorption.[55,68-71] This effect does not appear to be limited to acute infection but rather extends into the period following recovery. Data from Zambia, for example, illustrate a significant reduction in absorption of a tracer dose in the case of recently reported fever.[41] Systemic febrile infections also increase VA utilization and excretion.[55,72] The association between hyporetinolemia and inflammation[73] can be explained by decreased synthesis of retinol-binding protein (RBP) in the liver[74] and, consequently, reduced hepatic mobilization of retinol.[63,75] Modeling revealed an effect of inflammation on both the mobilization of VA and on the tracer to tracee ratio in the plasma compartment.[63] This inflammation-induced change in specific activity could lead to overestimation of VA pool size by the RID technique.[66] The magnitude of this effect is likely to depend on the timing of the specific activity measurement and, potentially, the nature of the stressor. Much of the research in this area focuses on the normalization of plasma retinol concentration following resolution of the inflammatory insult,[76-78] similar to what was modeled by Gieng and colleagues.[63] Thus, the extent to which chronic, low-grade inflammation influences retinol mobilization, or potential consequences for prolonged sequestration in the liver, requires further investigation.

Vitamin A metabolism is also known to be affected by iron deficiency. Specifically, studies in pregnant women have shown greater improvements in the relative dose–response test and in dark adaptation when VA was coadministered with iron or iron plus riboflavin, compared with VA supplementation alone.[79,80] Reduced plasma retinol concentrations have been observed in anemic rats,[81] even when animals were fed a VA-rich diet,[82] and are associated with VA accumulation in the liver and a higher molar ratio of liver retinyl esters/retinol.[82,83] These relationships have also been investigated by model-based compartmental analysis, which showed decreased absorption of VA and inhibited mobilization of VA stores in diet-induced iron deficiency in rats.[62] Furthermore, the activity of the key enzyme in provitamin A conversion, β,β-carotene 15,15′-dioxygenase, is iron dependent.[84,85] These results underscore the importance of considering iron status in RID studies to calculate

total body VA stores because iron deficiency with or without anemia may influence the amount of tracer in the plasma, leading to an incorrect estimation of pool size.

Interactions between zinc and VA have long been recognized; however, research regarding the impact of zinc deficiency on VA metabolism is inconclusive.[86] Animal research suggests a role for zinc in intestinal absorption of VA, mobilization from the liver, and cellular uptake, all of which may be compromised during zinc deficiency.[87-90] Observational studies have also highlighted a direct association between zinc status and plasma retinol concentration.[91,92] However, it is unclear whether these associations were the result of zinc deficiency or other associated factors. Of 4 randomized, controlled trials to consider this question, 2 reported a significant increase in serum retinol concentrations with zinc supplementation alone.[93,94] This discrepancy from the other 2 trials[95,96] may be related to baseline nutritional status, as the effect of zinc supplementation was greater in children deficient in zinc or VA, and previous research supports an effect primarily among children with moderate to severe protein energy malnutrition and/or low baseline zinc status.[93,97]

Detection and Consequences of VA Toxicity

Hypervitaminosis A is an issue of potential concern in low- and middle-income countries, where children may be exposed to multiple VA interventions, including high-dose supplements, fortified foods, and, to a lesser extent, MNP.[2,4,5] Chronic excessive VA intake can create liver abnormalities, including perisinusoidal fibrosis and hypertrophy and hyperplasia of stellate cells, which are key effector cells in the evolution of fibrosis and cirrhosis.[4,98,99] These changes result in obstruction of blood flow through the liver. Because clinical signs and symptoms occur late in the course of VA intoxication and the consequences are severe, it is important to identify early and reliable biomarkers that indicate when VA overload is occurring but before any clinical sequelae have developed.

Circulating retinyl esters have, to date, been the most widely used biomarker to indicate VA intoxication. Normal fasting retinyl ester concentrations are <70 to 100 μg/L, and the ratio of retinyl esters to total retinol plus retinyl esters is <0.08.[100-102] In addition to VA intoxication, higher than normal circulating concentrations of retinyl esters may be seen transiently in the postprandial state after a VA-rich meal or may indicate underlying liver disease (ie, failure of the liver to take up newly absorbed retinyl esters from the circulation and/or inappropriate release of retinyl esters from the liver's storage cells, known as stellate or Ito cells). High levels of circulating retinyl esters may also be seen in hypertriglyceridemia because newly absorbed retinyl esters are carried in the circulation on chylomicron remnants.[103] In VA intoxication, retinyl esters may represent as much as 70% of total circulating VA.[104] However, a quandary arises in interpreting retinyl ester values in geographic localities where there is a high prevalence of underlying liver disease (particularly hepatitis). In this situation, high levels of retinyl esters in plasma may indicate VA intoxication, but they may also reflect an inappropriate release of retinyl esters into the circulation due to liver inflammation (from hepatitis) or impaired hepatic uptake of retinyl esters after a meal by an inflamed liver.[105] Also, because high retinyl ester values may correlate with abnormal liver function (ie, transaminase levels in blood), even in populations with low hepatitis prevalence, the reliability of retinyl ester levels as a biomarker for excessive intake is questionable. As mentioned in paper 1 of this series, when investigating the retinol to RBP ratio as a biomarker for VA intoxication, synthesis of RBP in the liver may become impaired in an inflamed liver, potentially influencing this ratio.

Although there are concerns about an increased risk of bone fracture, even when preformed VA intake is less than the upper intake level of 3000 μg/d,[5] the link between a higher incidence of fractures, lower bone mineral density, and higher VA intakes remains speculative due to methodological issues related to the

accurate assessment of VA intake and status. Furthermore, high VA intakes were only associated with a modest increase in total fracture risk in women with low vitamin D intake (<11 μg/d).[106] Even though exposure to increasing doses of VA in animal models induced a progressive calcification of the epiphyseal-resting zone, followed by bony tissue replacement and then complete disappearance of the growth plate,[107-109] it is still not clear whether these detrimental effects occur at usual intakes of <3000 μg/d.

Although the RID technique has been applied to measure the full range of status in populations, there were discrepancies between measured and predicted total body stores in rhesus monkeys with hypervitaminosis A.[35] Both the RID test and compartmental modeling underestimated total body VA stores. This was largely due to limited exchange of extravascular VA with the tracer, indicating that most of the measured VA was in pools that did not exchange with plasma.[35] When VA status is high, lipid droplet size increases and morphology changes in the liver stellate cells[110]; thus, it is not surprising that all of these droplets do not readily exchange with plasma retinol. The enlargement of stellate cells due to high VA stores may protect the liver from fibrogenesis.[111] High VA concentrations induce perisinusoidal fibrosis and hypertrophy and hyperplasia of stellate cells. Because of these quandaries, additional biomarker development for VA intoxication is needed. Identifying biomarkers that would change as VA overload is approached but before the occurrence of organ damage is essential.

Important Considerations for Population-Based Assessments of VA Status

The RID technique is currently being proposed for use in population-level VA status assessments as well as being used to monitor and evaluate interventions, justified by its utility across the full range of VA status.[9] To date, the primary goal of population status assessments has been to identify groups at risk of deficiency. Guidelines are available for the design, implementation, and analysis of assessments using plasma retinol or RBP,[112] as are several decades of data that enable program managers and policy makers to track progress toward VAD control[1] that can be used to guide interpretation.[8,73]

Population assessments require choosing a representative sample in order to accurately describe status and/or draw inferences about burden, such as the proportion of children with excessive liver stores. In RID studies undertaken to date, researchers have screened out participants with febrile illness or diarrhea in the past day or week,[3,13,21,26-29,41,42,43] liver disease,[13,27,28,43,113,114] other chronic diseases,[3,26,41,44,113,115] and helminthiasis[113] because these conditions are likely to affect the absorption and/or retention of the tracer dose or otherwise alter the VA metabolism.[8] However, in the context of a population status assessment, removing certain individuals as a result of screening is likely to introduce bias. Efforts can be made to control some conditions, such as through the presumptive treatment of intestinal helminthiasis 1 week prior to tracer dosing.[7,13,28] Data on other factors, such as recent infections and chronic disease history, should be collected and considered in analyses. Measurement of inflammatory proteins such as C-reactive protein or α_1-acid glycoprotein, as well as both iron and zinc status indicators, is also recommended to test for potential effect modification.[8] Previous RID studies have considered chronic liver conditions in their study design.[13,27,28,43,113,114] Although the etiology of liver disease may differ in low- and middle-income countries, viral hepatitis,[116,117] malaria,[118-120] and aflatoxin intoxication[121] are all characterized by hepatic dysfunction. Further research is clearly needed to guide the application and interpretation of RID methods for population status assessments in cases of acute infection, chronic inflammation, other micronutrient deficiencies, and exposure to hepatic insults. Researchers should consider the potential influence of highly prevalent liver conditions[122-124] on VA metabolism, including potential confounding effects on VA status assessment.

Conclusions and Future Directions

The use of stable isotopes in various aspects of VA biology is powerful, and stable isotope methods have been successfully applied in healthy adults and children to assess the efficacy of various VA and provitamin A food-based interventions in both clinical and community settings in low-income countries. In addition, the technique has been successful in estimating VA requirements. However, a number of factors must be considered before recommending the RID technique for use in population status assessments. We currently lack a standardized methodology with regard to isotope selection, appropriate doses for various target groups, the timing of blood sampling(s) relative to dosing, and calculation of total body stores. This limits our ability to compare results across studies or to follow trends over time. Future work in this area should encourage methods requiring only one blood collection, which would significantly ease logistics and likely increase compliance in the field.

Uncertainty currently exists in the way to calculate total body stores of VA. Factors such as absorption of the tracer, its metabolism, and the different distribution between the plasma and storage compartments need to be taken into consideration. Inflammation and specific micronutrient deficiencies reduce absorption efficiency of the isotope dose; thus, it is currently recommended to exclude participants with identified inflammatory responses. Future studies are needed to address this uncertainty of tracer absorption by measuring the tracer concentration in feces. However, to enable better monitoring of these confounding factors, on-site evaluation of micronutrient deficiencies and inflammation combined with early blood sampling (eg, 3 days after administration of the tracer dose) may be recommended. Although physiological levels of tracers are recommended and currently used, it is not clear what amount should be administered in various age-groups and to what extent the amount of administered isotope influences the determination of total body stores. Improvements in mass spectrometers may lead to detection methods that can be performed on small blood volumes, which will be especially important for studies in young children.

Chronic excessive VA intake has been cited as a potential concern in communities exposed to multiple VA interventions, including high-dose supplements, fortified foods, and MNP. Early biomarkers to predict VA overload before any organ damage occurs are urgently needed. Although the RID technique has been proposed to detect VA status from deficiency through toxicity, there are concerns that the method may be compromised at high VA liver concentrations due to increased storage of tracee in lipid droplets in liver stellate cells, resulting in reduced exchange between the liver and plasma tracer pools. Further developments of new potential biomarkers of hypervitaminosis A, such as bone or liver fibrosis markers, are therefore required.

Published RID studies have generally relied on highly skilled individuals to prepare doses and administer the isotope to research participants. In order to implement a population-level status survey, clear guidelines on staffing requirements, training, and supervision are needed. Thus, although RID methodology is becoming more widely applied, remaining questions related to its implementation and interpretation in population-based field studies need to be addressed, especially in settings where infections and micronutrient deficiencies are common.

Authors' Note

This is paper 3 in a series of meeting reports from an International Atomic Energy Agency Technical Meeting (TM-48778) held in Vienna, Austria, March 24-25, 2014, entitled "Assessing Vitamin A Safety in Large-Scale Nutrition Intervention Programmes: Setting the Research Agenda." Sherry A. Tanumihardjo acted as guest editor for this series.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Financial support for this manuscript was provided by the International Atomic Energy Agency, the Bill & Melinda Gates Foundation,

and Global Health funds at the University of Wisconsin-Madison (S.A.T.).

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