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Handbook on Vitamin A Tracer Dilution Methods to Assess Status and Evaluate Intervention Programs

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and the Vitamin A Tracer Task Force

HarvestPlus

The vitamin A Tracer Task Force was initiated by IAEA, USAID, and HarvestPlus to prepare a series of three documents. The first on the appropriate use of vitamin A tracer (stable isotope) methods was published in 2004 by USAID/ILSI through the Micronutrient Global Leadership(MGL) project: http://www-naweb.iaea.org/nahu/nahres/pdf_files/vitatracer.pdf. This handbook us the second Tracer Force Handbook and is being published as a HarvestPlus Technical Monograph. The third handbook will be on model-based estimates of absorption efficiency and will be published by the IAEA in 2005.

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Marjorie J. Haskell, Judy D. Ribaya-Mercado, and the **Vitamin A Tracer Task Force**, 2005.
Handbook on Vitamin A Tracer Dilution Methods to Assess Status and Evaluate Intervention Programs
HarvestPlus Technical Monograph 5.

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Introduction

Vitamin A deficiency is a serious public health problem affecting ~140 million children and ~20 million pregnant women annually in many developing nations worldwide. Vitamin A deficiency can result in anemia, reduced resistance to infection, impaired cellular differentiation, xerophthalmia, and ultimately blindness and death. Because of the detrimental effects of vitamin A deficiency on human health, accurate assessment of vitamin A status is necessary to make informed decisions regarding intervention programs.

Vitamin A is stored primarily in the liver, and thus liver vitamin A concentration is considered to be the best indicator of vitamin A status. However, because obtaining liver specimens is difficult and usually not justified, indirect assessment techniques such as serum retinol concentration and the relative dose response tests are commonly used to assess vitamin A status. Another indirect assessment method is the stable isotope dilution technique, which provides a quantitative estimate of the size of the exchangeable body pool of vitamin A. The 'exchangeable body pool of vitamin A' refers to the vitamin A in the body that is in a dynamic state. Hereafter, the exchangeable body pool will be referred to as the total body vitamin A pool. By using assumptions regarding the liver to body weight ratio, estimates of liver concentrations of vitamin A can also be obtained. The stable isotope dilution technique has the advantage that it is the only indirect assessment method that provides a quantitative estimate of vitamin A status across the continuum of status, from deficient to excessive vitamin A pool sizes. Thus, the technique can also be used for assessing vitamin A pool size in populations at risk of excessive status because of exposure to too much vitamin A either through the use of dietary vitamin A supplements and/or excessive consumption of vitamin A-rich or fortified foods. The analogs of vitamin A that are used for assessing vitamin A pool size are labeled with stable isotopes (deuterium or ^{13}C) and are not radioactive. There is no known health risk associated with ingestion of stable isotope labeled vitamin A. The stable isotope dilution technique has been used successfully to assess total body vitamin A pool size in children and/or adults in the United States, Bangladesh, Guatemala, China, Philippines, Peru, and Nicaragua, and to evaluate the efficacy and effectiveness of vitamin A interventions.

The purpose of these guidelines is to assist researchers in designing and carrying out studies using the stable isotope dilution technique to assess the efficacy and effectiveness of vitamin A interventions aimed at improving vitamin A status in populations at risk of deficiency. Although other stable and radiolabeled isotopic techniques are available for studying vitamin A and carotenoid metabolism, these guidelines focus on the stable isotope dilution technique for assessment of vitamin A status. This technique has been validated against liver vitamin A concentrations in surgical patients with low to adequate liver vitamin A stores (Furr et al. 1989, Haskell et al. 1997). Results of those studies indicate that the technique provides an accurate quantitative estimate of mean total body vitamin A pool size for groups of subjects. It is important to note that the technique does not provide precise estimates of vitamin A pool size for individual subjects, and hence it is not intended for use as a diagnostic method for assessing vitamin A status in individuals.

The paired stable isotope dilution technique consists of estimating total body vitamin A pool size before and after an intervention to assess the change in vitamin A pool size in response to supplementation with vitamin A or provitamin A carotenoids. The paired stable isotope dilution technique has been used to assess the efficacy and effectiveness of vitamin A interventions for vitamin A status in populations at risk of deficiency. These guidelines describe the theoretical basis of the stable isotope dilution technique and include study protocols for using the paired isotope dilution technique in field settings. Information is also provided on the technical aspects of working with stable isotopes of vitamin A, recognizing that specific technical procedures will vary depending on the choice of labeled vitamin A analog and analytical instrumentation. For this reason, it is important for field and laboratory researchers to work closely together during the planning phase of a study to ensure that the choice of labeled vitamin A analog(s) and analytical methods are appropriate to address the specified research question.

1 | Description of the Stable Isotope Dilution Technique

1.1 Requirements for working with stable isotope labeled analogs of vitamin A

1.1.1 Facilities, equipment, and support

A relatively sophisticated biochemistry laboratory is required for working with stable isotope labeled analogs of vitamin A. Specifically the laboratory must be clean, dust-free, and well-ventilated, with temperature and lighting control. It must be equipped with a biosafety cabinet and a chemical fume hood for safe handling of biological and chemical materials; a digital balance (0.0000 g); a UV/VIS spectrophotometer (preferably a scanning UV/VIS spectrophotometer to assess degradation of compounds); an HPLC with a UV/VIS detector; and freezers (–20°C for retinoids and/or –80°C for carotenoids and retinoids). A suitable mass spectrometer is required for stable isotope analyses. The type of mass spectrometry (i.e., gas chromatography-mass spectrometry [GC-MS] or gas chromatography-combustion-isotope ratio mass spectrometry [GC-C-IRMS]) will vary depending on the type of labeled compound used. Other necessary supplies include positive displacement pipettes and general laboratory supplies such as a centrifuge, vortex mixer, sonicator, glassware, vacuum flasks and nitrogen or argon gas. It is important for the laboratory to have maintenance contracts for the analytical instruments (HPLC and MS) and easy access to replacement parts.

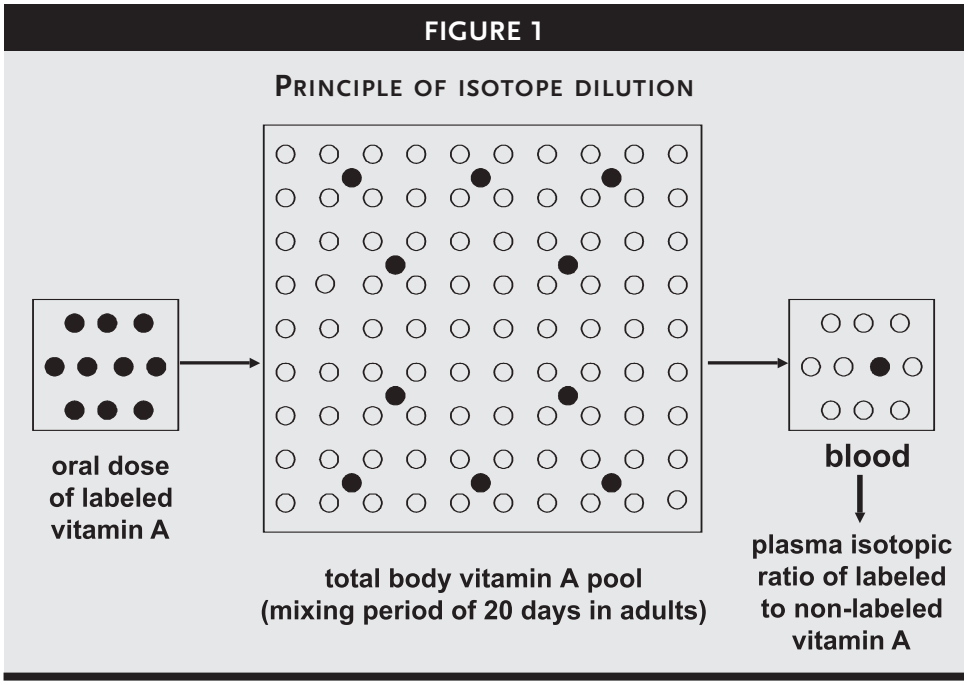
1.1.2 Personnel

Laboratory personnel must have extensive experience with basic biochemical and analytical chemistry techniques and training in the safe handling of chemicals and human biological samples (blood, breast milk, urine, stool) as well as the safe disposal of hazardous chemical and biological wastes. Additionally, laboratory personnel must be able to quantitatively analyze human plasma or serum for retinol and carotenoids by HPLC, and be familiar with HPLC methods for analyzing food samples for vitamin A and carotenoid contents. Experience with GC-MS or GC-C-IRMS is desirable, although initially the MS analyses can be done in collaboration with researchers and institutions with the necessary expertise and instrumentation.

1.2 Theoretical basis of the stable isotope dilution technique

The stable isotope dilution technique for estimating the total body vitamin A pool is based on the principle of isotope dilution, as shown in Figure 1. A known dose of stable isotope labeled vitamin A is administered orally. After a mixing period (~20 days in adults), during which the labeled vitamin A mixes with the endogenous vitamin A pool, a blood sample is obtained for measurement of the plasma or serum isotopic ratio of labeled to non-labeled retinol. (Either plasma or serum can be used; for simplicity, "plasma" will be used hereafter.) Total body vitamin A pool size (mmol retinol) is estimated quantitatively using the measured plasma isotopic ratio of labeled to non-labeled retinol and the equation developed by Olson

FIGURE 1



and coworkers (referred to hereafter as the Olson equation) (Furr et al. 1989):

$$\text{Total body vitamin A pool size (mmol)} = F \times dose \times (S \times a \times \{(1/\text{D:H}) - 1\})$$

where F is a factor for the efficiency of storage of an orally administered dose, and is assumed to be 0.5 based on the work of Bausch and Rietz (1977); *dose* is the amount of labeled vitamin A administered in mmol retinol equivalents; S corrects for the inequality between the plasma and liver ratios of labeled to non-labeled retinol; a is used to correct for irreversible loss of labeled vitamin A during the mixing period; D:H is the isotopic ratio of labeled to non-labeled retinol in plasma; and finally, the value –1 corrects for the contribution of the mass of the labeled dose to the total body vitamin A pool. The oral dose of labeled vitamin A does not truly equilibrate with the endogenous vitamin A pool because of continuous intake of unlabeled dietary vitamin A and catabolism of the dose of labeled vitamin A during the 20-day mixing period. Newly absorbed dietary vitamin A is preferentially secreted into the blood stream (Loerch et al. 1979), and thus labeled vitamin A in the plasma is diluted to a greater extent than labeled vitamin A in the liver. The factor S in the Olson equation corrects for the inequality of the isotopic ratios of labeled to non-labeled vitamin A in plasma versus liver. A value of 0.65 is used for the factor S, which is based on the mean observed plasma to liver ratio of the specific activities of radiolabeled vitamin A in rats with varying levels of dietary vitamin A intake and liver vitamin A stores (Hicks et al. 1984). The factor a is included to adjust for catabolism of the dose of labeled vitamin A during the mixing period. This factor is calculated based on the half-life of vitamin A turnover, which is estimated as 140 days in adults, and is assumed to be independent of the size of the liver reserves of vitamin A, and to be time-invariant ($a = e^{-kt}$, where k = ln 2/140 days and t = time in days since dosing).

The stable isotope dilution technique has been validated against hepatic vitamin A concentrations in surgical patients in the US and Bangladesh with low to adequate vitamin A pool sizes (Furr et al. 1989, Haskell et al. 1997). In addition to its use in estimating total body vitamin A pool size, the stable isotope dilution technique can be used to detect quantitative changes in total body vitamin A pool size in response to supplementation or dietary intervention. In Bangladeshi men, the technique detected increases in total body vitamin A pool size in response to three different levels of vitamin A supplementation, and the increases in pool size were similar to the expected theoretical increases based on the amounts of vitamin A provided (Haskell et al. 1999).

The Olson equation provides a quantitative estimate of total body vitamin A pool size for non-pregnant and non-lactating adults and school-age children. For pregnant and/or lactating women, estimates of retention of the dose of labeled vitamin A and the system catabolic rate have yet to be defined; thus the Olson equation is not recommended for these groups. To date, no equation has been developed to quantitatively estimate total body vitamin A pool size during pregnancy and lactation. However, the stable isotope dilution technique can be used to obtain qualitative estimates of vitamin A status in these population groups, as described below.

1.3 Three-day stable isotope dilution procedure

The conventional stable isotope dilution technique provides a quantitative estimate of vitamin A pool size based on the plasma isotopic ratio of labeled to non-labeled retinol after the labeled vitamin A dose has mixed with endogenous vitamin A. However, studies in rats indicate that the fraction of the dose of labeled vitamin A in plasma at 3 days after dosing is highly correlated with liver vitamin A stores, and that this fraction of the labeled dose in plasma accurately predicts vitamin A pool size in rats with low to high liver vitamin A stores (Adams and Green 1994). Similarly, preliminary studies in adults and children indicate that serum or plasma isotopic ratios of labeled to non-labeled retinol measured at 3 days after dosing are highly correlated with estimated vitamin A pool size, and thus that predictive equations can be developed to estimate total body vitamin A pool size using day-3 plasma isotopic ratios. Strong linear correlations have been reported in adults between serum isotopic ratios of labeled to non-labeled retinol measured at 3 and 20 days

after dosing, with correlation coefficients (*r*) of 0.94 and 0.90 in two separate populations (Ribaya-Mercado et al. 2003). Similar observations regarding the potential usefulness of isotopic ratios measured at 3 days after dosing have been made in children aged 7–13 years (Ribaya-Mercado et al. 2000, Tang et al. 2002) and in children aged 1-2 years (Haskell et al. 2003). The 3-day stable isotope dilution procedure is advantageous because of the shorter blood sampling time. However, the predictive equations for quantitative estimation of total body vitamin A pool size using day-3 serum or plasma isotopic ratios have not yet been validated against direct measurements of liver vitamin A concentrations in humans. Nevertheless, because the plasma isotopic ratios of labeled to non-labeled retinol measured at 3 days after dosing are highly correlated with estimated vitamin A pool size, the ratios provide a qualitative estimate of vitamin A pool size and can be used to assess qualitative changes in vitamin A pool size in response to an intervention. This has been demonstrated among Filipino children who were fed controlled diets containing carotene-rich fruits and vegetables (Ribaya-Mercado et al. 2000), and among elderly Guatemalans who were fed controlled diets plus vitamin A supplements (Ribaya-Mercado et al. 1999).

1.4 Procedures for estimating total body vitamin A pool size

1.4.1 Choice of stable isotope labeled analog of vitamin A

The choice of labeled analog(s) of vitamin A will depend on the target group, available instrumentation, and cost. Deuterium labeled vitamin A or ^{13}C-labeled vitamin A can be used to estimate total body vitamin A pool size using the stable isotope dilution technique. Deuterium labeled vitamin A compounds are commercially available (Cambridge Isotopes, Andover, MA), and ^{13}C-labeled vitamin A compounds can be special-ordered as a custom synthesis from commercial suppliers of isotopically labeled pharmaceuticals. GC-MS is used to analyze deuterium labeled vitamin A (Handelman et al. 1993, Tang et al. 1998); GC-C-IRMS can be used to analyze ^{13}C-labeled vitamin A (Tanumihardjo 2000). The doses of deuterium labeled vitamin A that are used to estimate vitamin A pool size are generally larger than doses of ^{13}C-labeled vitamin A because of differences in the sensitivities of the analytical instruments. Thus, doses of deuterium labeled vitamin A are more likely to perturb the endogenous vitamin A pool than doses of ^{13}C-labeled vitamin A. In addition, the minimum number of blood samples required for the paired stable isotope dilution technique will differ depending on which labeled compound is used. An additional blood sample is required when GC-C-IRMS is used to correct for residual ^{13}C in plasma from the first dose of ^{13}C-labeled vitamin A (this is described in detail below). Because differently substituted analogs of deuterium vitamin A compounds are available (i.e., $^{2}H_4$, $^{2}H_8$), pre- and post-intervention pool sizes can be estimated using different deuterium labeled analogs, obviating the need to collect a blood sample prior to administration of the second dose of deuterium labeled vitamin A. In target groups in which it is desirable to minimize the number of blood samples collected (i.e., children), this may be an important consideration when selecting the labeled vitamin A compound. Finally, availability of instrumentation and cost will also affect the choice of labeled compound. Thus, field and laboratory researchers should carefully consider the target group, instrumentation, and costs before deciding on which labeled vitamin A compound to use.

1.4.2 Dose of labeled vitamin A

The dose of labeled vitamin A will vary depending on the labeled compound used, the target group, and the detection limit of the mass spectrometer. Oral doses of deuterium labeled retinyl acetate are typically 5–10 mg retinol equivalents (RE) for adults and 2.5–5 mg RE for children. The use of ^{13}C-labeled vitamin A is relatively new. In the one study performed to date, an oral dose of 5.0 mg RE of ^{13}C-retinyl acetate was used for an adult subject (Tanumihardjo 2001). However, because the ^{13}C was still detectable at 4 years after dosing, smaller doses of ^{13}C-retinyl acetate can be used in future studies (Tanumihardjo 2003). If plasma isotopic ratios are measured only at 3 days after dosing, the deuterium labeled doses can be reduced to 2.5–5 mg for adults and to 1.25–2.5 mg for children. The specified doses are appropriate for the GC-MS and GC-C-IRMS methods that are currently used

(Handelman et al. 1993, Tang et al. 1998, Tanumihardjo 2000). However, it is likely that the sensitivity of mass spectrometers will improve with time; thus it may be possible to administer lower doses in the near future. Because the sensitivity of the instrument impacts the size of the dose required, it is important to know the capabilities of the mass spectrometer when deciding on the dose.

1.4.3 Preparation of labeled vitamin A doses

Commercially available analogs of vitamin A labeled with stable isotopes are usually provided in powder form. For administration to human subjects, doses of labeled vitamin A are prepared in vegetable oil. The following is a brief description of procedures for preparing deuterium labeled retinyl acetate in corn oil. Because vitamin A is light-sensitive, it is very important that all procedures be conducted in dim light or under gold fluorescent lighting (Barua and Furr 1998). As an example, a known amount of [$^2H_4$]-retinyl acetate is carefully dissolved in ethanol in a volumetric flask so that the concentration is ~40 mg/mL (e.g., 1.0 g in 25 mL). A careful dilution of the stock solution is prepared in ethanol to achieve an optical density (OD) reading between 0.5 and 1.0 absorption units using UV/VIS spectrophotometry at 325 nm. The concentration of the stock solution of [$^2H_4$]-retinyl acetate is determined using the molar extinction coefficient for retinol in ethanol of 52,770 M^{-1} cm^{-1} and the molecular weight of [$^2H_4$]-retinol of 290, and adjusting for the dilution factor. The concentration of the stock solution is based on the [$^2H_4$]-retinol content and is expressed as mg retinol equivalents (mg RE), rather than mg retinyl acetate. The chemical purity of the labeled compound can be assessed by reverse-phase HPLC by injecting an aliquot of the solution of labeled vitamin A (~1–2 µg RE of [$^2H_4$]-retinyl acetate), monitoring the absorbance at 325 nm, and collecting spectra from 200–800 nm using a photodiode array detector. The isotopic purity can be checked by MS. After checking the isotopic and chemical purity, α-tocopherol (~2 µg per mg of vitamin A acetate) is added as an antioxidant to the stock solution of [$^2H_4$]-retinyl acetate in ethanol.

To prepare the labeled vitamin A in corn oil, a known amount of [$^2H_4$]-retinyl acetate in ethanol is carefully transferred to a new, clean vial and ethanol is evaporated in the dark under nitrogen or argon gas. An appropriate amount of corn oil is added to the residue to achieve the desired concentration, and the vial is shaken vigorously using a vortex mixer or sonicator to dissolve the labeled vitamin A thoroughly in the corn oil. For example, if the desired dose of labeled vitamin A is 5 mg RE in 200 µL of corn oil, a solution of 25 mg RE/mL is needed. To determine the concentration, a dilution of the solution of labeled vitamin A in corn oil is carefully prepared in hexane to achieve an OD reading of between 0.5 and 1.0 absorption units, and the concentration is determined by UV/VIS spectrophotometry as described above, that is, by using the molar extinction coefficient for retinol in hexane of 51,770 M^{-1} cm^{-1}, the molecular weight of 290 for [$^2H_4$]-retinol, and adjusting for the dilution factor. The volume required for a dose is determined based on the concentration of the labeled vitamin A in corn oil. A positive displacement pipette is used to carefully place the desired volume of labeled vitamin A into opaque gelatin capsules. Alternatively, a positive displacement pipette can be used to administer the desired volume of the solution of labeled vitamin A directly into the mouths of subjects, as described below. The vial containing the corn oil solution of labeled vitamin A is flushed with nitrogen or argon gas, protected from light, and stored at ≤ –20°C. Under these conditions, the labeled vitamin A is stable for at least 2 years.

1.4.4 Administration of labeled vitamin A doses

For adults and school-age children, an oral dose of labeled vitamin A dissolved in vegetable oil is administered in a gelatin capsule; for infants and preschool-age children, doses are administered directly into the mouth using a positive displacement pipette with a disposable tip. In both cases, a low vitamin A/high-fat snack is provided immediately following administration of the labeled vitamin A to enhance absorption. Examples of snacks for adults and school-age children are peanut butter on a cracker, deep-fried curried potato pastries, or other low vitamin A deep-fried pastries. Infants should be breastfed after administration of the dose, even though breast milk contains vitamin A. For non-breastfed infants, animal milk or infant formula should be provided after administration.

1.4.5 Blood collection, processing, and transport procedures

Venous blood samples are collected into evacuated, foil-wrapped, blood collection tubes specifically designed for the collection of serum (no additive) or plasma (containing EDTA as an anticoagulant). The amount of blood required will vary by age group and sensitivity of the analytical method. Generally, 3–5 mL of blood is collected from infants and preschool-age children, and 7–10 mL of blood is collected from school-age children and adults. It is important to protect the blood from light to minimize photodegradation (by using foil-wrapped tubes and working in dim or gold fluorescent light), to keep the samples cool, and to handle the samples gently during all procedures to prevent hemolysis. If serum is used, the blood is allowed to clot prior to centrifugation. If plasma is used, the tubes are inverted gently to mix the blood with the anticoagulant. The foil is removed and the tubes are centrifuged at 2800 x g for 15 minutes to separate serum or plasma. Equal portions of serum or plasma are transferred to two screw-cap vials, and flushed with nitrogen or argon gas. The vials are wrapped in foil to protect the samples from light and are stored in opaque boxes at ≤ –20°C. Two aliquots of serum or plasma are prepared so that there is a back-up sample in case one sample is damaged or lost. It is very important to clearly label vials and boxes with waterproof markers. If the samples are to be shipped to another institution for analysis, they must be kept frozen during shipment. The samples can be packed in dry ice or shipped on liquid nitrogen in specially designed containers for biological materials. It is important to check with the local shipping company to determine specific regulations for shipping biological samples on dry ice or liquid nitrogen.

Biological waste that is generated during the blood collection and processing procedures must be handled and disposed of according to regulations for safe handling of biological materials and safe disposal of biological waste.

1.5 Rationale for blood sampling times for measurement of plasma isotopic ratios of labeled to non-labeled retinol

Blood samples are collected at 24 hours, 3 days, and 20 days after dosing. The 24 hour sample is collected to provide qualitative information on absorption and/or mobilization of the dose of labeled vitamin A from the liver. Plasma kinetic studies indicate that the plasma isotopic ratio of labeled to non-labeled retinol peaks at approximately 24 hours after dosing (Haskell et al. 1998). Thus, if the plasma ratio of labeled to non-labeled vitamin A is low at this time point, it can be interpreted as an indication of reduced absorption and/or reduced mobilization of labeled vitamin A from the liver. This information is useful in analyzing the data and interpreting the results. Specifically, the 24-hour plasma isotopic ratios are examined using descriptive statistics to identify outliers, and the 24-hour ratios are compared with estimates of vitamin A pool size to determine whether there is an association between the two variables.

The day-3 sample is collected because the plasma isotopic ratio of labeled to non-labeled retinol measured on day 3 is highly correlated with estimates of vitamin A pool size and provides a qualitative estimate of the total body pool of vitamin A, as described in Section 1.3. Pre- and post-supplementation day-3 plasma isotopic ratios of labeled to non-labeled vitamin A can be used to assess whether vitamin A status has changed in response to an intervention. Because the Olson equation has not yet been verified in pregnant and lactating women, it is currently recommended that changes in vitamin A status be assessed in these population groups using plasma isotopic ratios of labeled to non-labeled retinol measured 3 days after dosing. Also, in population groups that are known to have high infection rates, it may be desirable to use the day-3 plasma isotopic ratios in addition to the day-20 ratios to assess vitamin A status, because infection is less likely to occur within a 3-day sampling period than within a 20-day sampling period. The day-20 plasma isotopic ratio provides a quantitative estimate of the total body vitamin A pool size using the Olson equation in school-age children and adults, as described in Section 1.2. A shorter sampling time of 14 days is

recommended for preschool-age children (Haskell et al. 2003); the optimal blood sampling time for infants aged 0–12 months is not known.

1.6 Measurement of plasma isotopic ratios of labeled to non-labeled retinol

Plasma isotopic ratios of deuterium labeled retinol to non-labeled retinol are measured by GC-MS using electron ionization (Handelman et al. 1993) or electron capture negative chemical ionization (Tang et al 1998) as previously described; $^{13}C/^{12}C$ in retinol is measured by GC-C-IRMS (Tanumihardjo 2000). The referenced analytical methods provide detailed information on the procedures for carrying out the analyses. The following is a brief description of the procedures for measurement of plasma isotopic ratios of [$^2H_4$]-labeled retinol to unlabeled retinol using GC-MS and electron ionization (Handelman et al. 1993). Retinol is extracted from plasma (1.0 mL) and purified by HPLC, and the *tert*-butyldimethylsilyl (tBDMS) derivative of retinol is formed. An advantage of transforming retinol to its tBDMS derivative is that this derivative is stable for at least one year; thus the GC-MS analyses do not need to be performed immediately after isolating the retinol from the plasma. Nevertheless, it is recommended that the derivatized samples be analyzed by GC-MS as soon as possible. Plasma isotopic ratios of [$^2H_4$]-retinol to retinol are determined by GC-MS using a quadrupole mass spectrometer and 70 eV electron ionization. Selected ion monitoring is carried out for fragment ions of the tBDMS derivatives at m/z 255 (retinol) and m/z 259 ([$^2H_4$]-retinol). A set of calibration standards with [$^2H_4$]-retinol/retinol weight ratios of 0.00, 0.0167, 0.050, 0.167 and 0.50 is analyzed with each set of plasma samples. A linear regression equation is calculated between the weight ratios of the calibration standards and the integrated areas for m/z 259 and m/z 255. The area ratios for retinol from the plasma samples are substituted into the regression equation to solve for weight ratios. The within-run precision of the isotopic ratio measurements is estimated by periodically analyzing standards with each set of plasma samples. The coefficient of variation for the mean isotopic ratio measurements for the standards is typically <5%. Plasma isotopic ratios of [$^2H_8$]-retinol/retinol are measured using the same method, with selected ion monitoring carried out for fragment ions of the tBDMS derivatives at m/z 255 (retinol) and m/z 263 ([$^2H_8$]-retinol).

1.7 Factors that may affect plasma isotopic ratios of labeled to non-labeled retinol

Factors that may potentially affect the plasma isotopic ratio of labeled to non-labeled vitamin A, as well as estimates of vitamin A pool size, are dietary vitamin A intake during the mixing period, intestinal parasites, infection, inflammation, iron and zinc status, and intestinal malabsorption. Because newly absorbed dietary vitamin A is preferentially secreted into plasma, it is desirable to maintain dietary vitamin A intake at a low and constant level during the 20-day mixing period after administration of the labeled dose to minimize the effect of dietary vitamin A on the plasma isotopic ratio of labeled to non-labeled vitamin A. In community settings, dietary vitamin A intake cannot usually be carefully controlled; however, it is assumed that intake is low and relatively constant in populations in which vitamin A deficiency is endemic, and that the effect of dietary vitamin A intake on the plasma isotopic ratio is minimal. Nevertheless, assessment of dietary vitamin A intake during the 20-day mixing period is recommended.

Intestinal parasites may reduce absorption of the labeled vitamin A dose. For efficacy studies, subjects are treated with antihelminthics 1 to 2 weeks prior to beginning the study procedures. For effectiveness studies, subjects are not treated for intestinal helminths.

Subjects with clinical symptoms of infections are excluded from participating in a study. However, it is possible that subclinical infection or inflammation may affect the plasma isotopic ratio of labeled to non-labeled vitamin A. At present, there is no known association between plasma concentrations of C-reactive protein (CRP) and plasma isotopic ratios of labeled to non-labeled retinol. Nevertheless, it is recommended that plasma concentrations of CRP and α1-acid glycoprotein (AGP) be measured to examine whether there is an association between subclinical infection or inflammation and plasma isotopic ratios of labeled to non-labeled vitamin A. When possible, iron and zinc status should

also be assessed because absorption and/or mobilization of vitamin A from the liver may be reduced in iron- and/or zinc-deficient subjects.

Because intestinal malabsorption affects plasma isotopic ratios of labeled to non-labeled vitamin A, an indicator of intestinal absorption is useful. Intestinal permeability tests or fat absorption tests can be performed to obtain information on intestinal absorption. However, these tests will not provide specific information on the absorption efficiency of the dose of labeled vitamin A. Absorption of the dose of labeled vitamin A can be assessed qualitatively by collecting a blood sample 24 hours after the labeled vitamin A is administered, as described in Section 1.5. More sophisticated tracer techniques exist for assessing absorption quantitatively (Green and Green 1997, Dueker et al. 2000), but these methods are not feasible for routine use in community settings.

1.8 Assessing adequacy of vitamin A status using the stable isotope dilution technique

Vitamin A deficiency is defined as a liver vitamin A concentration <0.07 µmol/g, and excessive vitamin A status is defined as >1.05 µmol/g (Olson 1987a). Estimates of total body vitamin A pool size obtained by the stable isotope dilution method can be converted to liver vitamin A concentrations by assuming that liver weight is 2.4% of body weight in adults, 3% of body weight in children, and 4.2% of body weight in infants (Olson 1987b). It is also assumed that the liver contains 90% of total body vitamin A, although the proportion of total body vitamin A in the liver varies with vitamin A status. In well-nourished individuals, it is estimated that the liver contains ≥ 90% of total body vitamin A; in poorly or marginally nourished persons, the kidneys and other tissues contain an appreciable amount (10-50%) of total body vitamin A, thus the amount in liver can range from 50-90% (Olson 1987b). Thus, this method for converting total body pool size to liver vitamin A concentration is less than ideal, but until a cut-off value is determined for assessing the adequacy of vitamin A status based on total body vitamin A pool size, this method is recommended. Additional calculations can be done to estimate liver vitamin A concentrations in deficient and marginally deficient populations by making the assumption that <90% (for example 50% or 70%) of total body vitamin A is in the liver (Ribaya-Mercado et al. 2004a).

2 | Study Protocols for Using the Paired Stable Isotope Dilution Technique

The paired stable isotope dilution technique consists of estimating total body vitamin A pool size before and after a vitamin A intervention in order to assess the impact of the intervention on vitamin A status. Briefly, the mean change in vitamin A pool size (final pool size minus initial pool size) is calculated, and the mean change in vitamin A pool size of the vitamin A supplemented group(s) is compared with the mean change in vitamin A pool size of a negative control group. Procedures for carrying out a study using the paired stable isotope dilution technique are described below.

2.1 Site selection

When choosing a study site, it is important to consider factors that can affect the feasibility of successfully carrying out the study procedures in the study population. Such factors include, 1) religious and/or cultural practices that affect food intake and access to the study population, 2) the political climate and how that may affect study logistics and safety of study personnel and participants, and 3) seasonal differences that may affect food availability and logistics, such as transportation difficulties due to increased probability of flooding and landslides.

2.2 Selection of study participants

The population groups that are considered to be at high risk of vitamin A deficiency are infants (6–12 months of age), young children, and pregnant and lactating women in low-income communities in developing countries. A recent study in the Philippines showed that the elderly are also at risk of vitamin A deficiency (Ribaya-Mercado et al. 2004a). The study population should be well defined, and subjects should be similar with respect to age, socio-economic status, and physiological status.

2.3 Subject inclusion and exclusion criteria

Potential study subjects are recruited from communities in which vitamin A deficiency is known to be a public health problem. Subjects are examined by a physician to assess their current state of health and to obtain their medical histories. A finger prick blood sample is obtained for measurement of hemoglobin concentration. Subjects with fever, chronic disease, diarrhea within the past week, malaria within the past 2 weeks, severe anemia, or signs or symptoms of xerophthalmia are excluded from the study. Participants with severe anemia are treated with iron and those with xerophthalmia are treated with vitamin A according to WHO guidelines (WHO 1989, WHO/UNICEF/IVACG Task Force 1988). The study protocol must be approved by the research and ethical review boards of the participating institutions, and informed consent to participate in the study must be obtained from the study participants or their legal guardians.

2.4 Full study protocols for using the paired stable isotope dilution technique

The study protocol will vary depending on the research question(s) and the target group. The full study protocol is presented first, recognizing that it may not be feasible or appropriate to use this protocol in certain situations. Partial study protocols appropriate for certain target groups are presented later. The advantage of the full protocol is that it provides qualitative information on apparent absorption and/or mobilization of the dose of labeled vitamin A, and both qualitative and quantitative estimates of vitamin A pool size.

As shown in Figure 2, the full protocol for non-pregnant, non-lactating adults and school-age children consists of using the paired isotope dilution technique to assess total body vitamin A pool size before and after a vitamin A intervention. Subjects are randomly assigned to the intervention and control groups. An oral dose of labeled vitamin A is administered to participants on study day 0 and blood samples are collected at 24 hours, and 3 and 20 days after dosing for qualitative and quantitative estimation of initial vitamin A pool size. During the 20-day mixing period, subjects consume their usual diets. The intervention is implemented on study day 21, and, in this example, ends on study day 81. Thereafter subjects resume their usual diets. After a 10-day stabilization period, a second dose of labeled vitamin A is administered to participants (study day 92); blood samples are obtained at 24 hours, and 3 and 20 days after dosing (study days 93, 95, and 112) for qualitative and quantitative estimation of final vitamin A pool size.

The full study protocol for preschool-age children is similar (Figure 3). However, because the dose of labeled vitamin A mixes with endogenous vitamin A within 8–12 days after dosing in this age group, a blood sample is collected at 14 days after dosing for quantitative estimation of vitamin A pool size. For preschool-age children, a shorter stabilization period of 7 days is recommended prior to administration of the second dose of labeled vitamin A.

The number of blood samples collected will differ depending on which analog of labeled vitamin A is used. If different forms of labeled vitamin A are used to assess initial and final vitamin A pool sizes (e.g., $[^2H_4]$-retinyl acetate and $[^2H_8]$-retinyl acetate), it is not necessary to collect an additional blood sample prior to administering the second dose of labeled vitamin A. However, if the same form of labeled vitamin A is used to estimate initial and final pool sizes (e.g., ^{13}C-retinyl acetate or $[^2H_4]$-retinyl acetate), it is necessary to collect an additional blood sample prior to administering the second dose to correct for residual labeled vitamin A in plasma from the first dose (study day 92, Figure 2; study day 83, Figure 3).

Various approaches can be used to assess the plasma retinol response to an intervention. The first approach is to measure plasma retinol concentrations on study days 20 and 82, immediately before and after an intervention consisting of daily administration of a source of vitamin A. The mean change in plasma retinol concentration of the vitamin A supplemented group(s) is compared with that of the negative control group to assess the effect of the intervention on plasma retinol concentration. However, if subjects have marginal

vitamin A status initially, it is possible that the first dose of labeled vitamin A will increase their plasma retinol concentrations. In that case, the plasma retinol concentration measured on day 20 may be higher than the initial plasma retinol concentration at baseline. If the plasma retinol concentration increases to within the normal homeostatic range on day 20, the mean change in plasma retinol concentration between study days 20 and 82 in response to the intervention may be lower than expected. The second approach is to measure plasma retinol concentration at baseline (study day 0) before the first dose of labeled vitamin A is administered, and immediately following the intervention on study day 82. In this case, the change in plasma retinol concentration reflects the effect of the first dose of labeled vitamin A plus the vitamin A provided during the intervention. The third approach is to estimate the effect of the intervention on plasma retinol concentration by measuring plasma retinol concentrations immediately before and after the intervention, on study days 20 and 82, in a separate group of subjects who are not administered labeled vitamin A but are exposed to the same intervention.

If small amounts of vitamin A are provided daily during the intervention (study days 21-81), the plasma retinol concentration on study day 82 may reflect recent intake of supplemental vitamin A to some extent. Thus, the plasma retinol concentration can also be measured on study day 92, after the 10-day stabilization period.

FIGURE 2

Full protocol for assessing impact of a vitamin A intervention in non-pregnant, non-lactating adults and school-age children

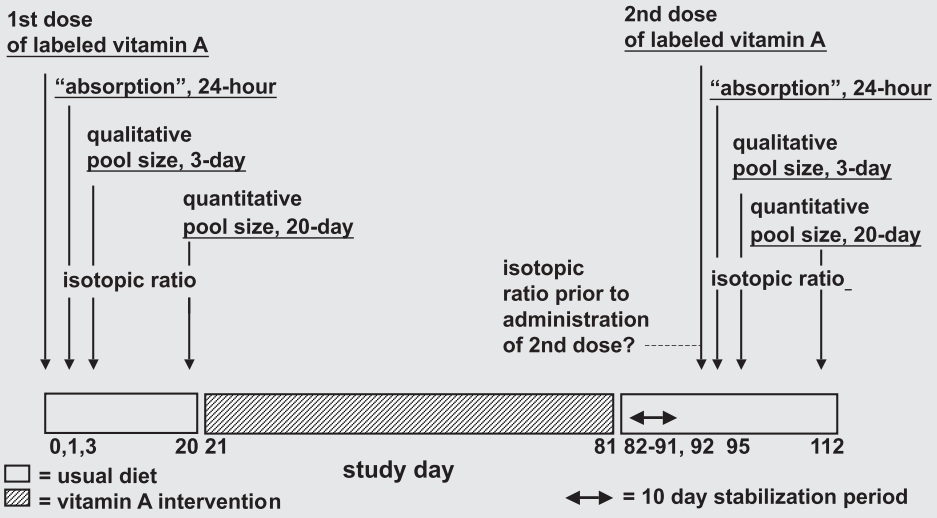
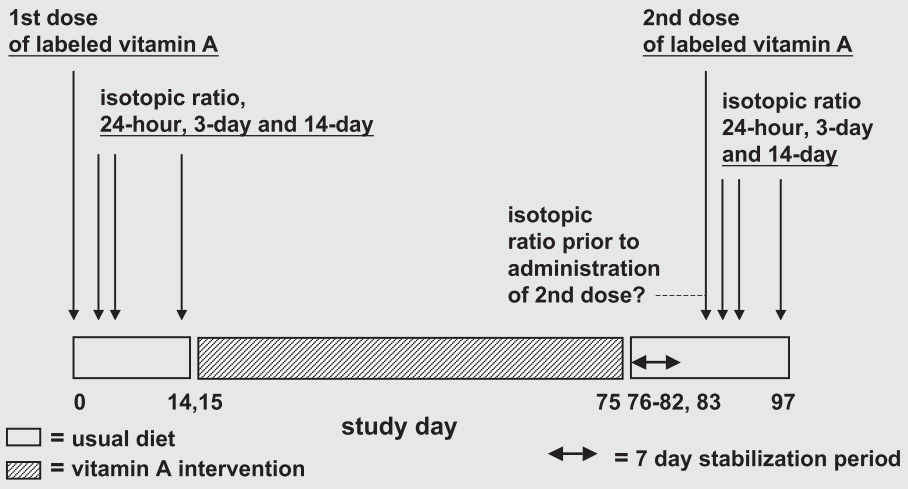


FIGURE 3

Full protocol for assessing impact of a vitamin A intervention in preschool-age children



The plasma retinol concentration measured on study day 92 may be a better indicator of the effect of the intervention on vitamin A status because at that time point the supplemental vitamin A that was provided during the 60-day intervention had 10 days to mix with the exchangeable vitamin A pool.

Plasma carotenoid concentrations reflect dietary intake; therefore plasma carotenoid concentrations are measured on study days 20 and 82, immediately before and after the intervention. If possible, plasma concentrations of ferritin and zinc are also measured because iron and zinc status may affect vitamin A absorption and/or mobilization. It is important to measure plasma concentrations of CRP and AGP to evaluate the effect of subclinical infection or inflammation on plasma concentrations of retinol, carotenoids, ferritin and zinc, and on the isotopic ratios of labeled to non-labeled retinol.

In preschool-age children, approaches the same as those described above can be used to assess the effect of an intervention on plasma retinol concentration. Briefly, plasma retinol concentrations can be measured immediately before and after the intervention (study days 14 and 76), or at baseline (study day 0) and immediately after the intervention (study day 76). Alternatively, the initial plasma retinol concentration can be measured at baseline (study day 0), or immediately before the intervention (study day 14), and the final plasma retinol concentration can be measured following the intervention after the 7-day stabilization period (study day 83). Alternatively, plasma retinol concentrations can be measured before and after the intervention in a separate group of children who are exposed to the same intervention but are not given labeled vitamin A.

2.5 Partial study protocols for using the paired stable isotope dilution technique

In certain situations, it may be necessary to use a partial version of the full study protocol. It is recognized that in some cultures it may not be possible to collect blood at more than one time point after dosing with labeled vitamin A. In these situations, partial study protocols that require fewer blood samples are recommended. Because the Olson equation has not been verified to estimate vitamin A pool size in lactating and pregnant women, it is more appropriate to use the day-3 isotope dilution procedure for qualitative assessment of vitamin A pool size in these population groups.

For non-pregnant, non-lactating adults and school-age children, a partial protocol that consists of collecting blood samples at 24-hours and 20 days after dosing is recommended (Figure 4A) for quantitative estimation of vitamin A pool size. However, if blood can be obtained at only one time point, the blood sample should be collected at 3 or 20 days after dosing with labeled vitamin A for qualitative or quantitative estimation of vitamin A pool size, respectively (Figure 4B).

For pregnant and lactating women, a 3-day isotope dilution procedure that consists of collecting blood samples at 24 hours and 3 days after dosing (Figure 4C, 4D, pg 16) is recommended (These protocols can also be used for non-pregnant, non-lactating adults and school-age children.) However, if blood can be obtained at only one time point, the blood sample should be collected at 3 days after dosing with labeled vitamin A for qualitative estimation of vitamin A pool size.

For preschool-age children, a partial protocol that consists of collecting blood samples at 24 hours and 14 days after dosing is recommended (Figure 5A, pg 17) for quantitative estimation of vitamin A pool size. However, if blood can be obtained at only one time point, the blood sample should be collected at 3 or 14 days after dosing with labeled vitamin A for qualitative or quantitative estimation of vitamin A pool size, respectively (Figure 5B, pg 17). Partial protocols using the 3-day isotope dilution procedure can also be used for preschool-age children (Figures 5C, 5D, pg 18).

Whenever possible, the full protocol is recommended (except for pregnant and lactating women). A partial protocol that includes a blood sample at 24 hours after dosing with labeled vitamin A is preferable to a protocol that consists of only a single blood sample at 3, 14, or 20 days after dosing.

When a 3-day isotope dilution protocol is used for adults and school-age children (Figures 4C-D, pg 16), the effect of the intervention on plasma retinol concentrations can be assessed using several approaches. If the initial dose of labeled vitamin A is relatively large (5–10 mg RE) and the subjects have marginal vitamin A status at baseline, it is likely that the plasma retinol concentration on study day 3 will increase transiently in response to the dose of labeled vitamin A. Because this transient increase will depend on an individual's initial vitamin A status, it cannot be assumed that the mean increase in plasma retinol on day 3 in response to the labeled dose will be similar in all treatment groups. For this reason, when the intervention begins on study day 4 (Figure 4C, pg 16), measurement of plasma retinol concentration at baseline (study day 0) is preferable to measurement at 3 days after dosing. The final plasma retinol concentration can be measured immediately following the intervention (study day 65), or after the 10-day stabilization period (study day 75). If the intervention begins on study day 21 (Figure 4D, pg 16), the pre-intervention plasma retinol concentration can be measured at baseline (study day 0) or on day 20. Although the initial dose of labeled vitamin A may still affect the plasma retinol concentration on study day 20, the effect is likely to be less than on day 3 because by 20 days after dosing the labeled vitamin A will have mixed to a greater extent with the endogenous vitamin A pool. The final plasma retinol concentration can be measured immediately following the intervention (study day 82),

FIGURE 4A

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION IN NON-PREGNANT, NON-LACTATING ADULTS AND SCHOOL-AGE CHILDREN USING 24-HOUR AND 20-DAY ISOTOPIC RATIOS

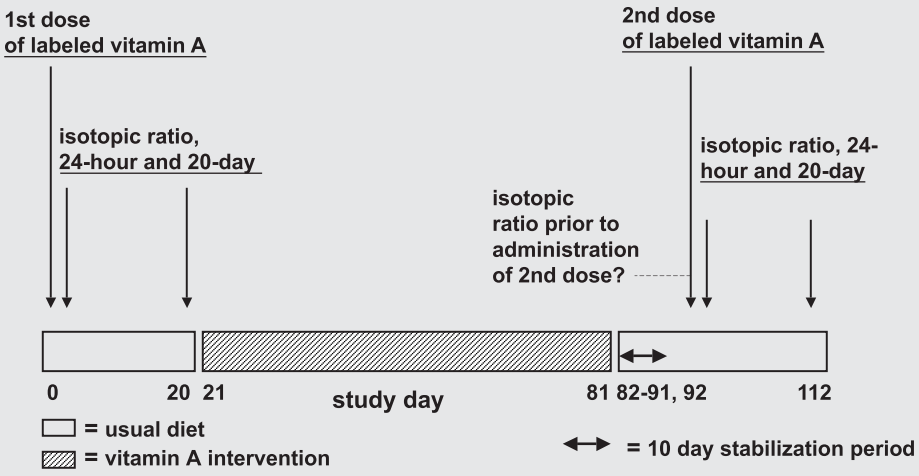
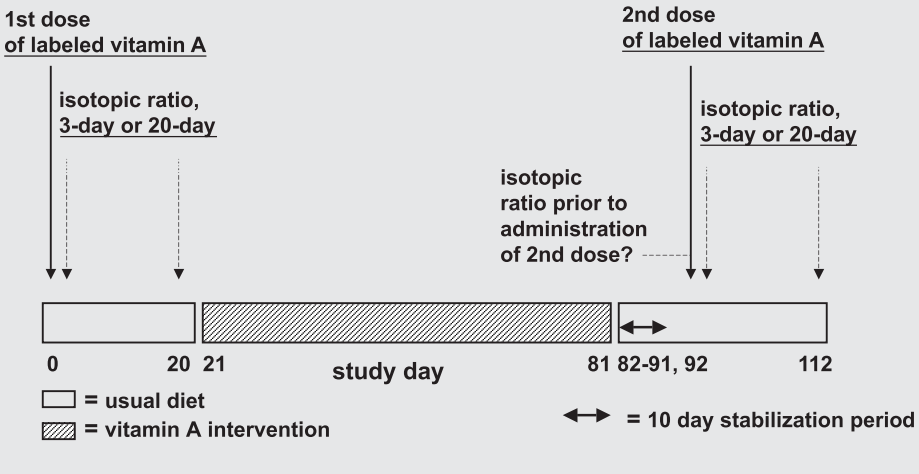


FIGURE 4B

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION IN NON-PREGNANT, NON-LACTATING ADULTS AND SCHOOL-AGE CHILDREN USING 3-DAY OR 20-DAY ISOTOPIC RATIOS



or after the 10-day stabilization period (study day 92; Figure 4D). The rationale for the different approaches is the same as that described for the full study protocol. The choice of a day-3 protocol will depend on whether a protocol of shorter duration is desirable, and which method is considered optimal for assessing the plasma retinol response to a particular intervention based on the advantages and disadvantages of the different approaches, as described above.

For preschool-age children, the approaches are similar for assessing the effect of the intervention on plasma retinol concentrations when a 3-day isotope dilution protocol is used. When the intervention begins on study day 4 (Figure 5C, pg 18), it is preferable to measure the initial plasma retinol concentration at baseline (study day 0). The final plasma retinol concentration can be measured immediately following the intervention (study day 65), or after the 7-day stabilization period (study day 72). When the intervention begins on study 15 (Figure 5D, pg 18), the initial plasma retinol concentration can be measured at baseline (study day 0) or the day before the intervention begins (study day 14). The final plasma retinol concentration can be measured immediately following the intervention (study day 76) or after the 7-day stabilization period (study day 83).

In addition to the biochemical data, information on anthropometry, dietary intake, and morbidity is collected. Measurements of weight, height (or length) and mid-upper arm circumference are recommended. Dietary intake can be assessed weekly by food frequency questionnaires; morbidity can be assessed weekly by questionnaire.

FIGURE 4C

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION USING 3-DAY ISOTOPE DILUTION PROCEDURE IN ALL ADULTS AND SCHOOL-AGE CHILDREN (INTERVENTION BEGINS ON STUDY DAY 4)

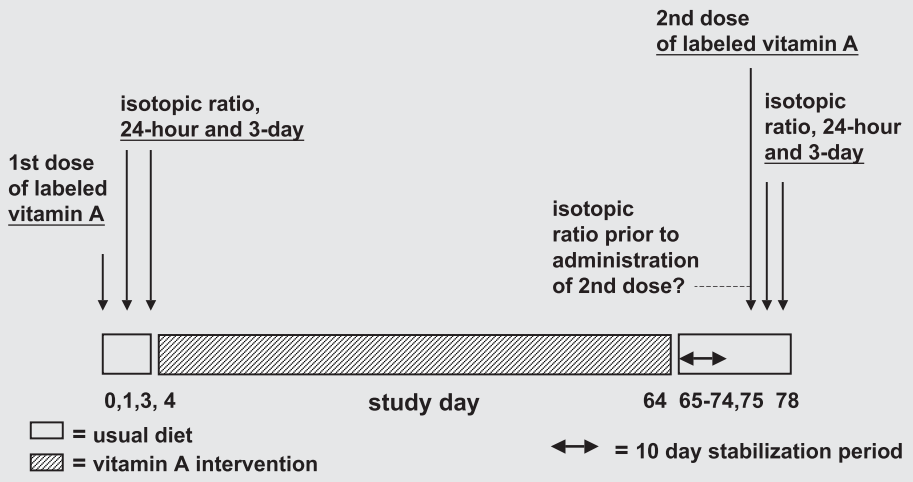
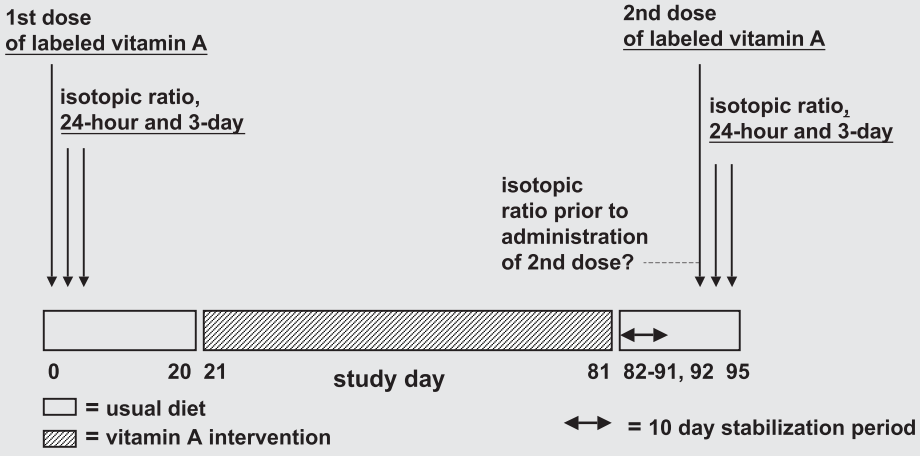


FIGURE 4D

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION USING 3-DAY ISOTOPE DILUTION PROCEDURE IN ALL ADULTS AND SCHOOL-AGE CHILDREN (INTERVENTION BEGINS ON STUDY DAY 21)



2.6 Estimation of duration of intervention

The duration of an intervention will vary depending on the amount of vitamin A provided and the frequency with which the vitamin A is administered. The expected effect of supplementation with vitamin A on vitamin A pool size in adults and school-age children can be estimated by assuming that 50% of supplemental vitamin A is retained in the body and ~0.5%/d of the vitamin A pool is catabolized. For preschool-age children, it can be assumed that ~50% of supplemental vitamin A is retained and ~2.2%/d of the vitamin A pool is catabolized (Haskell et al. 2003). These assumptions are used to estimate the expected effect on vitamin A pool size of administering a particular amount of supplemental vitamin A for a particular period of time. Initial estimates of vitamin A pool size can be obtained from the scientific literature for different age groups with low or high socio-economic status (Olson et al. 1984, Olson 1979, Flores and de Araujo 1984, Dahro et al. 1983, Dorea et al. 1984, Suthutvoravoot and Olson 1974). Most of the published data on liver vitamin A concentrations are based on direct measurement of liver vitamin A in specimens obtained at autopsy. Total body vitamin A pool size can be estimated by using liver vitamin A concentrations and estimates of liver weight as a proportion of body weight, as described in Section 1.8. In general, interventions that provide close to the RDA (FAO/WHO 2002) of vitamin A, 5–7 days/week, are 8–12 weeks in duration.

FIGURE 5A

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION IN PRESCHOOL-AGE CHILDREN USING 24-HOUR AND 14-DAY ISOTOPIC RATIOS

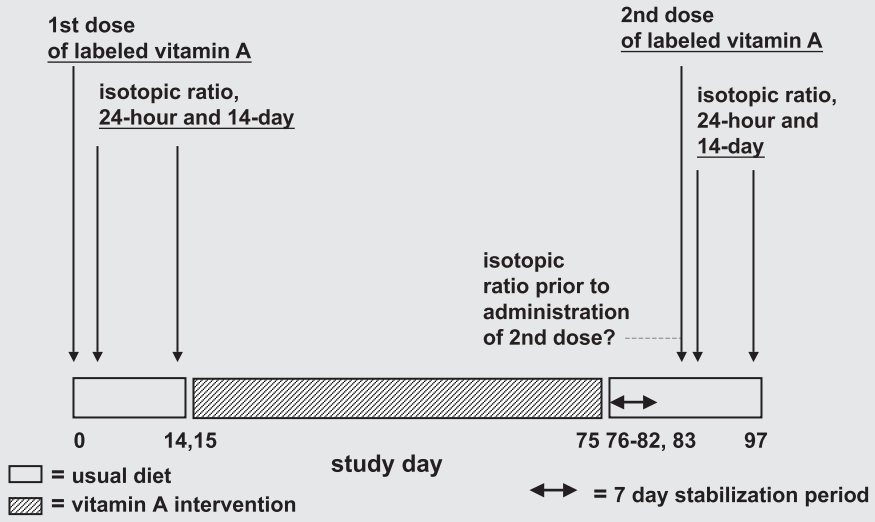
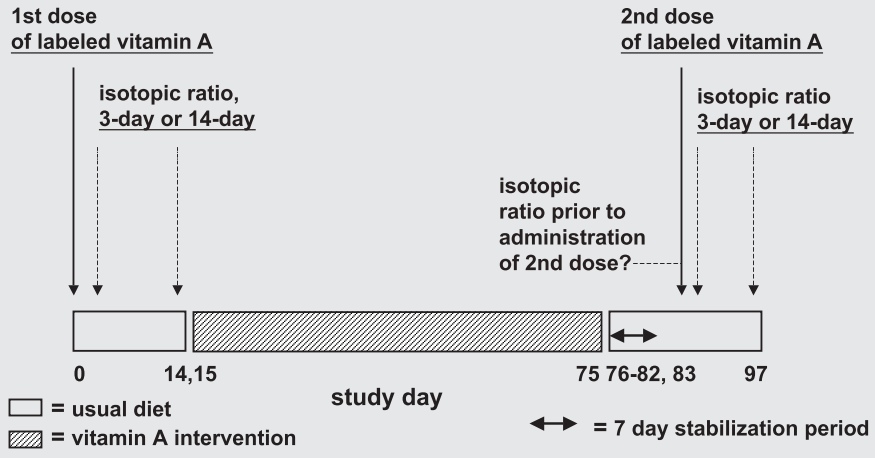


FIGURE 5B

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION IN PRESCHOOL-AGE CHILDREN USING 3-DAY OR 14-DAY ISOTOPIC RATIOS



2.7 Sample size calculation

The sample size calculation and plan for data analysis are performed in consultation with a statistician. To calculate the sample size, information is needed on initial vitamin A pool size, the expected change in vitamin A pool size, and the standard deviation of the change in vitamin A pool size. Estimates of initial vitamin A pool size can be obtained from the scientific literature. The expected change in vitamin A pool size can be estimated from results of previous studies or by estimating the expected changes, based on assumptions regarding retention and catabolism of vitamin A, as described above. An estimate of the standard deviation of the change in vitamin A pool size can be obtained from previous studies that report mean changes in vitamin A pool size in response to an intervention. The estimated sample size should be increased to adjust for potential drop-outs and possible loss of data due to field or laboratory errors, or other causes during the course of the study.

FIGURE 5C

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION USING 3-DAY ISOTOPE DILUTION PROCEDURE IN PRESCHOOL-AGE CHILDREN (INTERVENTION BEGINS ON DAY 4)

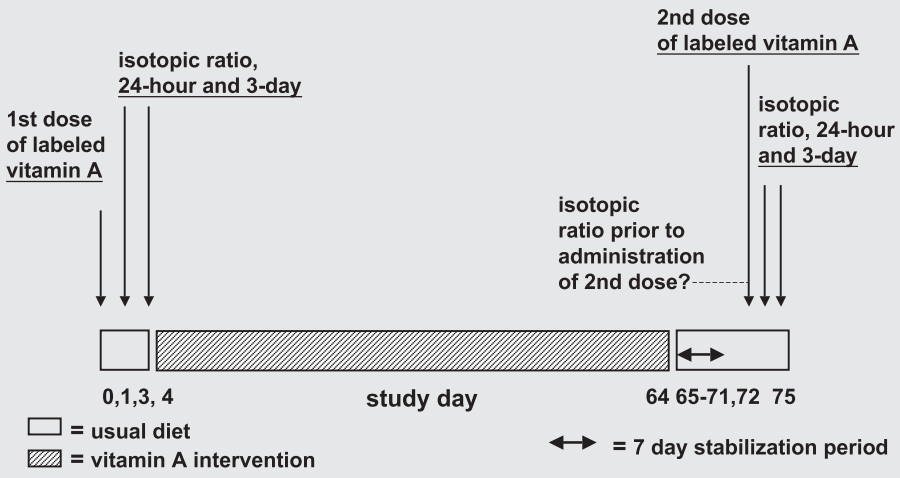
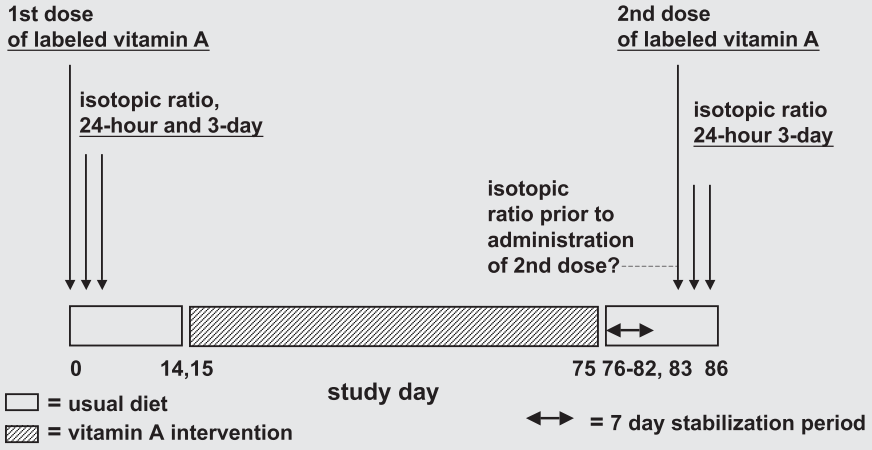


FIGURE 5D

PARTIAL PROTOCOL FOR ASSESSING IMPACT OF A VITAMIN A INTERVENTION USING 3-DAY ISOTOPE DILUTION PROCEDURE IN PRESCHOOL-AGE CHILDREN (INTERVENTION BEGINS ON DAY 15)



2.8 Statistical analysis

Descriptive statistics are calculated for all variables to check for outliers. Variables that are not normally distributed are appropriately transformed to approximate a normal distribution. Change in vitamin A pool size and change in plasma retinol concentration are examined in relation to plasma concentrations of CRP and AGP to determine whether there is an association between the indicators of vitamin A status and indicators of subclinical infection and inflammation.

Correlation analysis is used to determine whether there is an association between 24-hour plasma isotopic ratios and day-3 plasma isotopic ratios and/or estimates of total body vitamin A pool size. Final mean vitamin A pool sizes or final mean day-3 plasma isotopic ratios between the supplemented and non-supplemented groups are compared using analysis of covariance with initial values and any other variables as covariates. Final mean plasma retinol and carotenoid concentrations between the supplemented and non-supplemented groups are compared using analysis of covariance with initial values and any other variables as covariates. The relationship between change in plasma retinol concentration and change in vitamin A pool size is examined using regression analysis.

3 | Paired Stable Isotope Dilution Technique for Estimating Relative Vitamin A Equivalency Factors for β-carotene

The paired isotope dilution technique can be used to estimate the relative vitamin A equivalency of β-carotene from plant source foods (Haskell et al. 2004). The study design consists of an intervention group(s) (high β-carotene vegetables or fruit), a negative control group (low vitamin A vegetables or fruits), and a positive control group (retinyl palmitate). Vitamin A pool size is estimated before and after approximately 60 days of intervention. The mean change in vitamin A pool size is estimated for each treatment group (final pool size minus initial pool size). The relative vitamin A equivalency of β-carotene from plant sources of vitamin A is estimated by comparing the mean change in vitamin A pool size of the plant group(s) with that of the retinyl palmitate group, as described below. It is also possible to assess the efficacy of supplementation with plant sources of vitamin A for improving vitamin A status by comparing the mean change in vitamin A pool size of the plant group(s) with that of the negative control group. To obtain accurate estimates of the relative vitamin A equivalency of β-carotene from plant sources, the diet is carefully controlled throughout the entire study period. This requires that the study be carried out in a controlled setting with access to dieticians and a research kitchen where foods can be carefully prepared using standardized recipes.

3.1 Site selection

The considerations for selection of the study site are the same as those described in Section 2.1. In addition, adequate facilities and trained personnel must be available for careful preparation of controlled diets and careful monitoring of food consumption. Access to clinical staff is also required to monitor the health status of the participants throughout the study period.

3.2 Selection of study participants

Because the study diets must be carefully controlled and food consumption must be monitored, access to the study participants is necessary throughout the day for the entire study period. Thus, a study group should be selected that is willing to consume their daily meals and snacks under supervision at the study facility (i.e., metabolic wards/out-patient facilities, boarding schools, etc.).

3.3 Subject inclusion and exclusion criteria

Relative vitamin A equivalency factors may vary with vitamin A status. Thus, the initial vitamin A status of the subjects selected for the study should be similar. If the purpose of the study is to estimate vitamin A equivalency factors in populations at risk of vitamin A deficiency, study subjects are recruited from communities in which vitamin A deficiency is known to be a public health problem. The subjects are screened for plasma concentrations of retinol, CRP and AGP, and for hemoglobin concentration. The subjects are examined by a physician to assess their current state of health and to obtain their medical histories; exclusion criteria are the same as those described in Section 2.3. Eligible subjects with the lowest plasma retinol concentrations and normal CRP and AGP concentrations are selected for the study.

3.4 Study design and protocol

The study design consists of a group(s) that receives high β-carotene vegetables or fruit and a corn oil capsule, a negative control group that receives low β-carotene vegetables or fruit and a corn oil capsule, and a positive control group that receives low β-carotene vegetables or fruit and a capsule containing corn oil in which is dissolved an amount of retinyl palmitate equivalent to the amount of vitamin A in the high β-carotene vegetables or fruit, as estimated by assuming 1 μg retinol = 12 μg β-carotene (Institute of Medicine 2001); food and capsule supplements are provided daily:

Intervention Group	Capsule	Food supplement
Plant source food(s)		
	corn oil	High β-carotene vegetable or fruit
Negative control		
	corn oil	Low vitamin A vegetable or fruit
Positive control		
	retinyl palmitate in corn oil	Low vitamin A vegetable or fruit

The full study protocol described above is the preferred study design; however, a partial version of the protocol can be used if it is not feasible to collect all of the blood samples required in the full protocol. Note that this study design differs from that of the full protocol for community-based studies in that the diet is controlled. As shown in Figure 6, subjects consume a controlled low vitamin A diet during the 20-day mixing periods after administration of the initial and final doses of labeled vitamin A and during the 10-day stabilization period following the intervention. During the intervention period, subjects consume the low vitamin A diet in addition to their assigned food and capsule supplements.

In addition to the biochemical data, anthropometric data are collected and subjects are monitored for morbidity weekly. Because the diet is carefully controlled, it is not necessary to obtain information on dietary intake.

3.5 Selection of high β-carotene and low vitamin A foods

A low vitamin A diet is developed in consultation with a dietician. The diet is based on foods typically consumed by the study population and is replete in all nutrients except vitamin A, which is present in low amounts (~25% of the RDA). The plant source food(s) is carefully selected based on the β-carotene content, and availability and acceptability to the study population. To determine which high β-carotene foods (dark green leafy vegetables, dark orange vegetables and fruits) to include in the study, focus group discussions are conducted to determine which foods are locally available and acceptable to the study population, and to obtain information on usual food preparation techniques.

The food preparation techniques that are used in the study will depend on the research question. If the purpose of the study is to assess the vitamin A equivalency of β-carotene from plant sources of vitamin A as the foods are typically consumed, the food is prepared according to the traditional food preparation techniques. If the purpose of the study is to assess the effect of food preparation techniques on estimates of vitamin A equivalency, a variety of preparation methods are used. In either case, the foods are prepared in the form they will be consumed by the study participants, and samples of each food are analyzed by HPLC to determine the carotenoid content. The portion size required to supply ~80–100+ % of the RDA for the study population is determined based on the all-*trans* β-carotene content of the food and the currently recommended vitamin A equivalency factor of 12:1. *Cis*-β-carotenes and the other provitamin A carotenoids are quantified, but because of uncertainty regarding the bioavailability and vitamin A equivalency of these carotenoids, they are not included in the determination of the portion size. An acceptability trial is conducted to determine whether the study population is willing to consume the specified portion size of the selected food(s). It may be necessary to provide the food(s) more than once daily if the portion size is too large. The selected plant source food(s) is obtained from the same supplier throughout the study period to minimize variability in the β-carotene content. Samples of the prepared foods are collected weekly for analysis of the carotenoid content.

The low vitamin A vegetables or fruits should also be locally available and acceptable to the study population. Procedures the same as those described above can be used to select the low vitamin A plant source foods. The types and amounts of fiber in the portion of low vitamin A vegetables or fruits should be similar to that of the high β-carotene vegetables or fruits. It may be necessary to adjust the amounts of low vitamin A containing foods in the diet to achieve similar types and amounts of dietary fiber in all treatment groups.

3.6 Estimation of duration of intervention

The duration of the intervention can be estimated as described in Section 2.6.

3.7 Procedures for estimating relative vitamin A equivalency factors

The relative vitamin A equivalency of β-carotene from plant source foods is estimated by comparing the mean changes in vitamin A pool size in the groups that receive plant sources of vitamin A with that in the group that receives retinyl palmitate. First, the mean change in vitamin A pool size is determined for each treatment group, controlling for initial values. Second, the mean change in vitamin A pool size in the negative control group is subtracted from the mean change in vitamin A pool size in each of the groups that received a vitamin A source to obtain the net mean change in vitamin A pool size in relation to the negative control group. Third, the net mean changes in vitamin A pool size in the groups that receive plant sources of vitamin A are compared with the net mean change in vitamin A pool size in the retinyl palmitate group to estimate the relative vitamin A equivalency of the plant sources of vitamin A, using the following equation:

(μg β-carotene consumed per day in the vegetable or fruit groups/μg RE consumed per day in the retinyl palmitate group) * (mean Δ in net vitamin A pool size [relative to the negative control group] of the retinyl palmitate group/mean Δ in net vitamin A pool size [relative to the negative control group] of the vegetable or fruit groups)

The relative vitamin A equivalency factor provides an estimate of the equivalency of β-carotene from a particular food source in relation to retinyl palmitate. Thus, an estimated relative vitamin A equivalency factor of 10:1 for a particular plant source food means that 10 μg of β-carotene from that food is equivalent to 1 μg of retinol as retinyl palmitate.

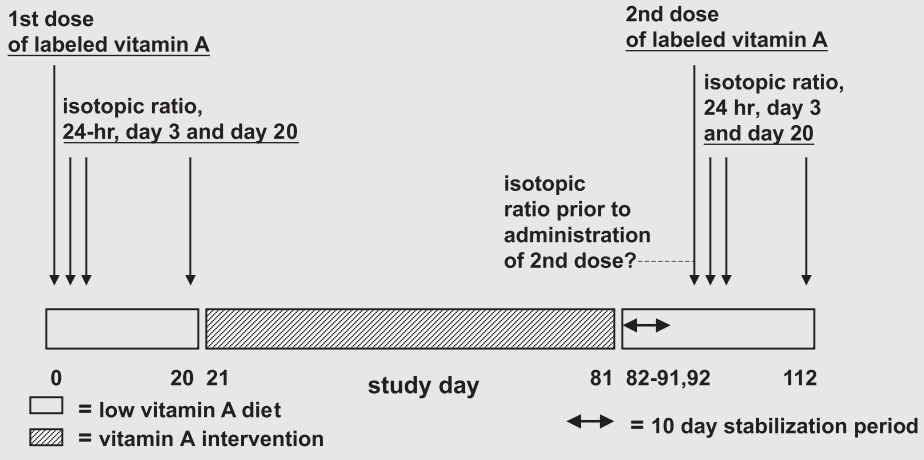
The impact of supplementation with plant sources of vitamin A on vitamin A status is assessed by comparing the mean change in vitamin A pool size in the supplemented group(s) with the mean change in vitamin A pool size in the negative control group.

3.8 Sample size calculation

The sample size is calculated using procedures the same as those described in Section 2.7.

FIGURE 6

PAIRED ISOTOPE DILUTION TECHNIQUE: ESTIMATING THE RELATIVE VITAMIN A EQUIVALENCY OF β-CAROTENE AND IMPACT OF AN INTERVENTION IN NON-PREGNANT, NON-LACTATING ADULTS AND SCHOOL-AGE CHILDREN (RESEARCH SETTING)



4 | Monitoring the Effectiveness of Vitamin A Intervention Programs

The stable isotope dilution technique has been shown to be very useful for monitoring the effectiveness of programs designed to improve the vitamin A status of populations. For example, when the Republic of Nicaragua initiated a national program of vitamin A fortification of table sugar for domestic consumption, the effectiveness of the program was monitored in 21 school-age children by measuring their total body vitamin A pool sizes using the stable isotope dilution technique at baseline and 1 year after the start of the availability of fortified sugar in their local markets (Ribaya-Mercado et al. 2004b). To avoid seasonal variability in carotene-rich foods, the study was conducted during the same calendar month, albeit 1 year apart. No attempt was made by the investigators to influence the subjects' consumption of sugar or other aspects of their diet. An oral dose of [$^2H_4$]-retinyl acetate was provided at baseline, and [$^2H_8$]-retinyl acetate after 1 year, in order to distinguish [$^2H_8$]-retinol from any residual [$^2H_4$]-retinol. In this study, the minimum of 1 blood draw was obtained at baseline and at the repeat test 1 year later. The blood was obtained 21 days after dosing with the labeled vitamin A and was used for estimation of total body vitamin A pool size using the Olson equation and for determination of plasma retinol. Total body vitamin A increased in all of the 21 study participants during the year, with a median increase of 112%. In contrast, plasma retinol increased in 16 subjects and decreased in 5 subjects; the median change was an increase of 18.6%. Thus, plasma retinol changes were small and inconsistent, unlike the changes in total body vitamin A pool size obtained using stable isotope dilution methodology, making the latter the method of choice for monitoring the effectiveness of vitamin A programs.

4.1 Selection of study participants

In monitoring program effectiveness, it is important to choose subjects who are representative of the program's intended target groups. In vitamin A intervention programs, the target groups are those who are at risk for vitamin A deficiency, i.e., children and pregnant or lactating women, especially those residing in poor areas. In addition, the elderly are also at risk of vitamin A deficiency, as has been documented in a recent study in rural Philippines, where 14.5% of men and women between 60 and 88 years old had low liver vitamin A concentrations (<0.07 μmol/g) as assessed by stable isotope dilution methods (Ribaya-Mercado et al. 2004a).

4.2 Sample size calculation.

The sample size is calculated using the procedures described in Section 2.7.

4.3 Subject inclusion and exclusion criteria for studies monitoring program effectiveness

The subject inclusion and exclusion criteria are similar to those of other labeled vitamin A studies mentioned in Section 2.3. That is, the participants should be in general good health, with no major chronic diseases, acute illnesses, or gastrointestinal problems. Because the goal of the study in Nicaragua was to document whether the new national program of sugar fortification with vitamin A had an impact on the vitamin A status of school-age children during the inaugural year of the program, it was important that the results were not confounded by other existing vitamin A intervention programs such as the Nicaraguan National Health Campaign, which administers high-dose vitamin A (200,000 IU or 60,060 μg vitamin A) twice a year to children <5 years of age. Thus, in that study (Ribaya-Mercado et al. 2004b), only children who did not receive a high-dose vitamin A supplement during the previous year were included as study participants.

4.4 Importance of periodic monitoring of vitamin A status in intervention programs

Periodic monitoring of the vitamin A status of target groups is desirable to document the continued effectiveness of intervention programs in maintaining an adequate vitamin A status in vulnerable groups. In addition, periodic monitoring is done to ensure that liver vitamin A stores remain at safe levels. Although the upper safe limit of liver vitamin A stores is unknown, a physiologic range of liver vitamin A concentration is between 0.07 and 1.05 μmol/g liver (between 20 and 300 μg/g liver) (Olson 1984). It would be prudent to periodically monitor liver vitamin A concentrations in

a small number of subjects, especially when multiple vitamin A intervention programs are in place, to confirm that continued increased intakes of vitamin A from all sources pose no risk of adverse health effects. Stable isotope dilution methods are the only indirect methods available for the quantitative estimation of liver vitamin A stores. The fortified food used in vitamin A programs should be continuously monitored for quality control to ensure that vitamin A levels do not shift outside the desired range of safe and efficacious levels.

4.5 Subject inclusion and exclusion criteria for periodic monitoring of vitamin A status in intervention programs

Because in a national vitamin A fortification program everyone is exposed to the nutrition intervention, periodic monitoring of vitamin A status by stable isotope dilution methods is recommended in a small cross-sectional sample of subjects not only from poor communities, but also from higher-income communities. The study participants should include children, women of reproductive age, and the elderly. They should be generally healthy with no major chronic diseases, acute illnesses, or gastrointestinal problems.

5 | Studies on Vitamin A Requirements

The stable isotope dilution methodology has the potential to provide useful information regarding the dietary vitamin A requirements of various age groups. For example, the dietary vitamin A intakes (retinol activity equivalents, RAE) of ≥60-year-old Filipinos with adequate (≥0.07 µmol/g or ≥20 µg/g) or low (<0.07 µmol/g) liver vitamin A concentrations have been studied (Ribaya-Mercado et al. 2004a). An acceptable or sufficient dietary vitamin A intake value (in µg RAE/kg body wt) associated with adequate liver vitamin A was estimated by taking the mean RAE ± 2 SDs consumed by subjects whose liver vitamin A concentrations were adequate and dividing this value by their mean body weight. Adding 2 SDs to the mean RAE gives a margin of safety to cover the needs of most healthy elders. The acceptable daily dietary vitamin A intake associated with adequate liver vitamin A reserves was estimated to be 6.4 µg RAE/kg body wt. For a reference 76-kg man and a 61-kg woman aged ≥60 years, acceptable vitamin A intakes were estimated to be 490 and 393 µg RAE/d, respectively. These numbers may be rounded to 500 and 400 µg RAE/d, respectively. On the basis of these results, the 1989 Philippine recommendations of 525 and 450 µg RE/d, and the 2002 FAO/WHO recommendations of 600 and 500 µg RE/d for elderly men and women appear adequate, whereas the current US recommendations of 900 and 700 µg RAE/d for elderly men and women are high. The procedure of estimating vitamin A requirements described above is possible only in populations with minimally adequate vitamin A status. It will not be valid in populations with liver vitamin A stores that greatly exceed the minimum adequate value of 0.07 µmol/g because their vitamin A intakes are presumably well above the vitamin A requirements. It is of interest to note that total body vitamin A and/or liver vitamin A (both estimated using stable isotope dilution techniques), correlated with dietary RAE, preformed vitamin A, β-carotene, fat, and protein; in contrast, serum retinol was unrelated to any of these dietary intakes (Ribaya-Mercado et al. 2004a). The above technique of obtaining information regarding dietary vitamin A requirements relied primarily on dietary intake data and assessment of liver vitamin A stores by stable isotope dilution. Other more sophisticated isotope techniques (e.g., model-based compartmental analysis) (Green and Green 1990) are available which can be used for obtaining information about dietary vitamin A requirements based on kinetic methods for studying vitamin A dynamics in vivo.

Summary

Vitamin A tracer dilution methodology is a powerful tool for assessing vitamin A status and for evaluating the efficacy and effectiveness of vitamin A intervention programs. The method can be applied in both clinical and field settings. It is useful for estimating relative vitamin A equivalency factors for β-carotene, and could be used to obtain information on vitamin A requirements. It is useful for assessing a wide range of human vitamin A status, from deficiency to sub-toxicity. It is the only indirect measure of vitamin A status that provides a quantitative estimate of total body vitamin A pool size and liver vitamin A concentration.

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