Vitamin A: biomarkers of nutrition for development

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ABSTRACT
Vitamin A is essential for multiple functions in mammals. Without vitamin A, mammals cannot grow, reproduce, or fight off disease. Because of its numerous functions in humans, biomarkers of vitamin A status are quite diverse. Assessment of liver reserves of vitamin A is considered the gold standard because the liver is the major storage organ. However, this measure is not feasible in human studies. Alternative biomarkers of status can be classified as biological, functional, histologic, and biochemical. Historically, signs of xerophthalmia were used to determine vitamin A deficiency. Before overt clinical damage to the eye, individuals who suffer from vitamin A deficiency are plagued by night blindness and longer vision-restoration times. These types of assessments require large population-based evaluations. Therefore, surrogate biochemical measures of vitamin A status, as defined by liver reserves, have been developed. Serum retinol concentrations are a common method used to evaluate vitamin A deficiency. Serum retinol concentrations are homeostatically controlled until liver reserves are dangerously low. Therefore, other biochemical methods that respond to liver reserves in the marginal category were developed. These included dose-response tests and isotope dilution assays. Dose-response tests work on the principle that apo-retinol–binding protein builds up in the liver as liver reserves become depleted. A challenge dose of vitamin A binds to this protein, and serum concentrations increase within a few hours if liver vitamin A concentrations are low. Isotope dilution assays use stable isotopes as tracers of total body reserves of vitamin A and evaluate a wide range of liver reserves. Resources available and study objectives often dictate the choice of a biomarker.

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INTRODUCTION
The most common forms of preformed vitamin A in the human diet are retinol and retinyl esters. Retinol has 20 carbons, and the functional group is located on carbon 15 (Figure 1). Vitamin A has many functions in the human body, including growth, vision, epithelial differentiation, immune function, and reproduction (1). The storage form is retinol esterified to fatty acids predominantly as retinyl palmitate. Retinal is the form involved in vision, whereas retinoic acid is involved in growth and cellular functions. The enzymatic formation of retinal and retinyl esters from retinol is reversible; however, the oxidation of retinal to retinoic acid is not reversible (Figure 1).

Unfortunately, many women and children suffer from vitamin A deficiency, which leads to vision loss, increased morbidity, and mortality. According to the World Health Organization (2), 45 countries have a public health problem at the clinical level, which includes overt signs of deficiency, and 122 countries have subclinical levels of vitamin A depletion with marginal liver reserves. Although progress has been made globally to alleviate overt clinical signs of vitamin A deficiency, marginal vitamin A status is still prevalent and difficult to diagnose.

Because of concerns related to marginal vitamin A status, multiple indicators have been developed to diagnose different degrees of vitamin A status. In 2001, these indicators were reviewed (3) and ranked against a continuum of liver reserves (Figure 2). Vitamin A biomarkers can be grouped into 2 broad categories as follows: 1) biological, functional, and histologic indicators and 2) qualitative and quantitative biochemical indicators. This brief review will cover both of these categories and relate indicators to predicted liver stores of vitamin A.

BIOLICAL, FUNCTIONAL, AND HISTOLOGIC INDICATORS

Biological: clinical signs of deficiency
The first group of biological indicators is clinical and involves the eye. If an individual presents with visual or ocular histologic indicators, they should be considered severely vitamin A deficient and treated with high-dose supplements. Xerophthalmia has different degrees of severity from Bitot’s spots, which are reversible with vitamin A treatment, to irreversible blindness because of scarring of the cornea. Xerophthalmia is a population indicator, and a minimum prevalence of 0.5% in preschool children signifies a public health problem (4).

Functional
Night blindness results when the vitamin A pool in the eye becomes depleted, and the concentration in the rod cells is lowered. Many countries have a specific term in local languages for this symptom of deficiency. Night blindness that is due to vitamin A...
deficiency is reversible with increased vitamin A intake or supplementation. In countries where marginal vitamin A status is prevalent, night blindness may occur during pregnancy. Whether this is due to increased demands during pregnancy or lowered serum retinol concentrations because of plasma volume increases is not entirely known.

Although night blindness is typically used as a population indicator of vitamin A status, impaired dark adaptation has been used to evaluate intervention studies (5, 6). Specifically, dark adaptation measured by the pupillary threshold in Nepali women with night blindness improved when liver, fortified rice, amaranth leaves, carrots, or retinyl palmitate were consumed for 6 wk (5). Such improvement may be further enhanced with iron and riboflavin supplementation (6). The vision restoration time or adaptation under dark conditions has also been used to evaluate vitamin A depletion in groups. Zinc supplementation sometimes synergistically improves the vision restoration time (7).

Histologic

Conjunctival impression cytology evaluates the morphology of the surface of the eye. During vitamin A deficiency, the presence of mucin-containing goblet cells decreases, and epithelial cells become enlarged and distorted. Cells can be counted and evaluated by taking a filter paper sample from the surface of the eye and staining with hematoxylin and eosinophil. Conjunctival impression cytology was used in the 1990s (7, 8), but because of concerns of its lack of utility in groups with trachoma (9) or subclinical deficiency in young children (10), the method has not been widely adopted.

QUALITATIVE BIOCHEMICAL INDICATORS

Serum retinol concentrations

Serum retinol concentrations are the most common population indicator used. In addition to analysis with HPLC, surrogate analyses for the carrier protein retinol-binding protein (RBP) have been developed by using either serum (11) or blood spots (12). The ratio of retinol to RBP may be influenced by vitamin A deficiency (13) or obesity (14), which may negatively affect prevalence rates of vitamin A deficiency when expressed as RBP concentrations. Both serum retinol and RBP concentrations are static measures and do not always change in response to an intervention. For example, in a study in 309 Indonesian children (15), the initial and final serum retinol concentrations did not differ between groups that received 210 μmol vitamin A and groups that did not 3–4 wk after supplementation. In groups of children who received vitamin A, the after-to-before ratio was 0.97–1.03, and in groups that did not receive vitamin A, the ratio was 0.96–0.99 (15).

FIGURE 2. Biomarkers of vitamin A status relative to qualitative liver reserves of vitamin A. These relations were proposed as part of the James Allen Memorial symposium in 2001 (adapted from reference 3). RAG, retinoyl β-glucuronide; RBP, retinol-binding protein; RDR, relative dose response.
On the other hand, distribution curves of serum retinol concentrations have shown distinct differences (8). When used as an evaluation tool, the serum retinol distribution differed in children between two areas in Indonesia. However, in the study, the degree of infection was not assessed. Therefore, it was unknown what effect correction for inflammatory markers would have had on the distribution curves (16). Infection and inflammation have a negative effect on serum retinol concentrations because RBP is an acute phase protein.

In women, serum retinol concentrations have responded to vitamin A supplementation if values are initially low, such as in Indonesian women given low-dose supplements for 35 d (17). However, in some groups, serum retinol concentrations may not respond even to high-dose supplements, such as in Ghanaian women given 210 or 420 µmol retinyl ester (18) or fed indigenous green leafy vegetables for 3 mo (19). The lack of response for serum retinol concentrations is partly due to retinol’s homeostatic control over a wide range of liver reserves. For example, in rats that were on 3 different amounts of daily vitamin A supplements, serum retinol concentrations did not differ despite a 6-fold difference in liver reserves of vitamin A (20).

Retinoyl β-glucuronide hydrolysis test and RBP: prealbumin measurements

Two biochemical methods that have not been widely applied include the retinoyl β-glucuronide (RAG) hydrolysis test (21, 22) and RBP:prealbumin measurements (23). Both of these methods were established in the rat model (21–23) and applied to humans (22–25). The RAG hydrolysis test is a response test in which a dose of RAG is given, and the change in the retinoid acid concentration is measured in the serum. The retinoid acid response is expected to be greater in individuals with poorer vitamin A status. Doses of 25, 50, and 75 mg RAG have been piloted in Indian children aged 3–18 y old (25). Elevated retinoid acid concentrations were observed in children whose serum retinol concentrations were <0.85 µmol/L. The advantages of this test compared with serum retinol concentrations corrected for the degree of infection or inflammation will need to be further investigated. The RBP:prealbumin method is a static measure and may have utility in groups of humans with a high degree of infection or inflammation. Measurements have been done in Zambian children with measles (23), children from Papua New Guinea, and Bangladeshi surgical patients (24). In the Bangladeshi surgical patients, a cutoff ≤0.36 for RBP:prealbumin was associated with vitamin A deficiency defined as <0.07 µmol retinol/g liver (24).

Breast-milk retinol concentrations

Breast-milk retinol concentrations are a unique indicator and an evaluation tool for lactating women (26) with potential extrapolation to the nursing infant. However, the response to supplementation was modest in Kenyan mothers (27). As a biomarker, breast-milk retinol concentrations may reflect recent dietary intakes and not necessarily be a reflection of vitamin A status as shown in rats (28) and swine (29). A comparison among vitamin A indicators suggested that breast-milk retinol determined from a casual sampling, compared with full breast-milk expression, may have performed better when corrected for the fat content (30).

**QUANTITATIVE BIOCHEMICAL INDICATORS**

These described methods are largely qualitative. Methods that are semiquantitative and quantitative include the dose-response tests, isotope dilution assays, and direct measurement through biopsy or autopsy samples of the liver. In 2004, the Vitamin A Tracer Task Force (31) assigned liver-reserve concentration values to a variety of vitamin A biomarkers (Figure 3). The dose-response tests have utility from deficiency through the adequate range of vitamin A liver reserves. However, the tests likely do not quantitatively reflect a status above the adequate range. Isotope

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**Figure 3.** Top: Recommended biomarkers of vitamin A (VA) status by the Vitamin A Tracer Task Force in 2004 (adapted from reference 31) in relation to liver-reserve concentrations (µmol VA/g liver). Bottom: Replacing the “?” in 2010 regarding the utility of isotope dilution testing in the hypervitaminotic state.
Dose-response tests

The dose-response tests work on the principle that RBP accumulates as vitamin A liver reserves become low. In rats that were fed a vitamin A–deficient diet, apo-RBP accumulated in the liver before serum retinol concentrations decreased, and the liver was depleted (32). Thus, when a challenge dose of retinyl or 3,4-didehydroretinyl ester is administered, the retinol or 3,4-didehydroretinol binds to this accumulated RBP and is rapidly released into the serum. The relative-dose-response (RDR) test was originally applied to a rat model (33). When applied in the field today, 1 mg retinyl ester is administered dissolved in oil (8). Two blood samples are collected (ie, one sample at baseline and another sample 5 h after dosing). The RDR value, which is expressed as a percentage, is calculated as

$$\left( \frac{A_5 - A_0}{A_5} \right) \times 100 \quad (I)$$

where \(A_5\) is the serum retinol concentration at 5 h postdosing, and \(A_0\) is the serum retinol concentration at baseline. If the percentage difference is >20%, the individual likely has deficient liver reserves.

Although the RDR test is a useful indicator, the test is somewhat invasive because it requires 2 blood samples from the same individual 5 h apart. An accurate RDR value is dependent on correct analysis and consistent retinol recovery from both serum samples. Therefore, the modified RDR (MRDR) test was developed in rats (34–36) and applied to humans in the United States (36, 37) and Indonesia (38). The test was further refined to make it more field friendly by establishing standard test doses and suggesting a range of response times to obtain the blood sample (39). The test works on the same principle as the RDR test, but because the 3,4-didehydroretinol analog is administered instead of retinol, a single blood sample can be taken. HPLC easily separates 3,4-didehydroretinol from retinol in the same sample. Endogenous concentrations of 3,4-didehydroretinol are quite low in humans and therefore, the baseline blood sample is not needed. A distinguishing response between depleted and sufficient liver reserves can be measured in ≥4 h and has been validated in rats and piglets against liver vitamin A reserves (34–36, 40) and compared with the RDR test in children (8). After the serum sample is analyzed, the molar ratio of 3,4-didehydroretinol to retinol is calculated, which is sometimes referred to as the MRDR value. A cutoff of 3,4-didehydroretinol to retinol ≥0.060 usually signifies low liver reserves of retinol that can be corrected with vitamin A supplements (15, 41).

The MRDR test has been applied in several countries to evaluate interventions and the prevalence of low liver reserves in surveys. One of the first comparisons was done in preschool-age children in Indonesia (8). In the first group, the distribution of values approached a normal distribution, and most children were vitamin A adequate. A child in the first group who had an MRDR value of 0.18 had just moved into the area. In the second group of children, MRDR values were quite disparate and reached a value of 0.83 in one female subject. An informal dietary interview in the community revealed that the first group of children ate a significant amount of eggs. Eggs provide a highly bioavailable source of preformed retinol and, thus, can make a measurable difference in the vitamin A status of children if consumed regularly.

The application of the MRDR test in a study of the combined treatment of vitamin A supplements and albendazole for deworming children infected with Ascaris lumbricoides showed a significant improvement in the mean (±SD) value of 0.055 ± 0.042 before supplementation to a value of 0.033 ± 0.017 after supplementation (P < 0.0001) (15). This magnitude of difference was not seen with serum retinol concentrations. In another evaluative study of children with helminthic infections, the MRDR test correctly identified children that had received a vitamin A supplement from the local health post (41). MRDR values were 0.021 ± 0.012 and 0.054 ± 0.038 in the children who had and had not received the supplement, respectively. Serum retinol concentrations did not differ between children who had received the supplement and children who had not, and the serum retinol concentrations did not respond to treatment.

The MRDR test has been used in a variety of countries to evaluate population status. It gives more information than serum retinol concentrations alone. For example, in a group of rural lactating women in Ghana, baseline serum retinol concentrations and MRDR values were 1.4 ± 0.5 μmol/L and 0.048 ± 0.037, respectively (18). After treatment with either 210 or 420 μmol retinyl ester, a significant improvement in vitamin A status occurred as assessed by the MRDR test (P < 0.0001), but serum retinol concentrations did not differ (P = 0.87). Furthermore, in an urban group of Ghanaian lactating women, the baseline serum retinol concentration was 1.5 ± 0.6 μmol/L, and the MRDR value was 0.09 ± 0.05 (19), which indicated a much poorer vitamin A status than in the rural women even though serum retinol concentrations were identical. After an intervention with indigenous African green leaves, serum retinol concentrations did not change or differ during the study (P ≥ 0.41), but the MRDR test improved within the intervention group (P = 0.0001). These results contradicted a simulated analysis in which the difference shown in MRDR values was proposed to occur because of underlying different serum retinol concentrations between groups (42).

Even in the United States, vitamin A status can be poor especially in low-income groups. Specifically, in children who qualified for the Special Supplemental Nutrition Program for Women, Infants, and Children, 32% of children were in the uncertain area for MRDR values, which was defined as 0.030–0.060 (43). This result was in contrast to that in children with a generally higher economic status in the United States where the mean MRDR value was 0.019 ± 0.010 in 22 children tested 2–10 h after an oral dose of 3,4-didehydroretinyl acetate (37). Only 2 children tested >0.030 at 4 and 6 h after the dose, which was within the recommended time interval for the test sample to be taken (39). Furthermore, an assessment of low-income pregnant women showed that an alarming 9% were above the international MRDR value cutoff of 0.060 (44). Serum carotenoid concentrations were also analyzed in these low-income women and children. In some cases, serum β-carotene concentrations were not detectable, which indicated that vegetable consumption was likely very low (43, 44).

Although the MRDR test is very useful to evaluate a deficient through normal vitamin A status, as currently applied, it does not have utility in defining the subtoxic and toxic range of liver reserves. Nonetheless, the magnitude of the ratio is related to liver
reserves. When data from several piglet studies were combined (40, 45–47), liver reserves <17 μg vitamin A/g liver (0.06 μmol vitamin A/g liver) were exclusively associated with an MRDR value ≥0.060 (Figure 4). Liver reserves from 0.06 to 0.1 μmol vitamin A/g liver were scattered above and below the MRDR cutoff, and liver values >0.1 μmol vitamin A/g liver were almost invariably associated with MRDR values <0.060. From these data by using the MRDR test and data obtained in rats in which the down-regulation of lecithin:retinol acyltransferase, which is responsible for retinol esterification, occurred at the same liver concentration (48), we propose that 0.1 μmol vitamin A/g liver should signify vitamin A deficiency instead of the current cutoff of 0.07 μmol vitamin A/g liver. If liver reserves illicit a biological response to a vitamin A challenge dose, the vitamin A status is not in equilibrium, and the individual should be considered at risk of vitamin A deficiency.

Retinol isotope dilution

The most sensitive biomarker of vitamin A status to liver reserves of vitamin A is isotope dilution testing (31, 49). The most widely used isotope test uses deuterated retinyl acetate as the tracer. 13C-retinyl acetate has been used as the tracer in rats (20), rhesus monkeys (50), and humans for the assessment of vitamin A status (51). The difference in practice is the type of equipment used for the analysis. The deuterated retinol test uses conventional gas chromatography–mass spectrometry (GC-MS), whereas the 13C-retinol test uses gas chromatography–combustion-isotope ratio mass spectrometry (GCCIRMS). GC-MS analysis requires the formation of the tert-butylidimethylsilyl derivative before injection into the gas chromatograph, whereas the GCCIRMS does not. GC-MS with electron-capture negative chemical ionization detection increased the sensitivity of the method (52, 53), but 13C-retinol with GCCIRMS requires a smaller dose to trace total body stores (50, 54).

All methods that calculate total body stores of vitamin A use the same fundamental mass-balance equation (55) as follows:

\[ (F_a \times a) + (F_b \times b) = (F_c \times c) \]  

where \( a \) refers to the amount of dose absorbed and stored (experimentally determined to be 0.5–0.8 of the dose administered), \( b \) represents baseline reserves of vitamin A, \( c = a + b = \) total posdose reserves of vitamin A, and \( F = R \div (R + 1) \), where \( R \) is 13C:12C. Various adaptations of this equation have been used to reflect the unique metabolism of vitamin A (49, 50).

Paired-isotope dilution tests to evaluate baseline and follow-up liver reserves have been used to determine changes in total-body vitamin A reserves in response to an intervention (56, 57). Examples of this included the estimation of relative vitamin A equivalency factors (58) and assessment of the effect of different intakes of vitamin A on calculations of total body reserves (59) and effects of supplementation on liver stores (53, 60). Vitamin A supplementation and abrupt changes in dietary intakes may result in the exaggeration of liver reserves or affect value estimates with the test (53, 59). With the use of a graded supplementation strategy, paired-isotope dilution testing predicted that higher intakes of vitamin A were needed to maintain a higher total-body vitamin A pool size (60). Thus, vitamin A utilization and requirements vary based on the amount of vitamin A contained in the body.

The 13C-retinol dilution test has been validated in rats against liver reserves with an \( r = 0.98 \) (20). When the atom percentage excess was evaluated at 3 different intakes of vitamin A supplementation at 4 time intervals after isotope dosage, none of the relations overlapped, which showed the robustness of the test to assess differences in liver vitamin A reserves. This test required very small doses of the isotope tracer, and thus, vitamin A trafficking was not perturbed. After the rat study, the test was applied to rhesus monkeys (50) that were known to have hypervitaminosis A based on liver-reserve analysis in monkeys from the same colony (61). Measured compared with predicted liver reserves in these monkeys revealed a linear relation, and all monkeys were diagnosed as having hypervitaminosis A by the predicted values (50). The 13C-retinol dilution test has been applied to humans.

A recent application of 13C-retinol analysis by GCCIRMS was to evaluate the compliance to a high vegetable intake (62). Plant foods have different enrichments of 13C depending on whether they are C4 or C3 plants, the former which contains higher amounts of 13C (63). Most vegetables consumed in the United States are C3 plants and therefore have less 13C than C4 plants such as maize. The shift in natural abundance of 13C in retinol was used as a biomarker of the formation of retinol from the β-carotene consumed in the diet. The provided diet was high in pumpkin, carrots, and spinach, which are all C3 plants. Indeed, the group that received more vegetables during the intervention had a lower enrichment of 13C in the purified retinol from their serum (62).

Although isotope dilution testing is usually too expensive to consider as a means to evaluate a program, an evaluation of a sugar-fortification program was performed in a small group of Nicaraguan children (64). The baseline mean liver retinol concentration was 0.57 μmol retinol/g liver, which was well above what is currently considered deficient (0.07 μmol retinol/g liver). All children had serum retinol concentrations between 0.74 and 1.31 μmol/L. One year after the sugar fortification was implemented, the estimated liver reserve concentration increased to
an average of 1.2 μmol retinol/g liver. In 9 of 21 children, liver vitamin A concentrations were calculated to be >1.05 μmol vitamin A/g liver after fortification, which was defined as toxic in 1990 (65). Because many foods are being considered for fortification, this sensitive methodology may have to be used because no other method except a liver biopsy is able to diagnose hypervitaminosis A.

Considering the validation in monkeys and these results in children, an isotope methodology can be useful in defining the hypervitaminotic range of liver reserves. Specifically, liver reserves >10 μmol vitamin A/g were quantified (50). Thus, a question mark that vitamin A experts published in 2004 in relation to the upper useful limit for isotope dilution can now be replaced with the value of 10 μmol vitamin A/g liver (Figure 3) in 2010. However, a paucity of data exists in humans regarding the ramifications of a subtoxic or toxic vitamin A status. In 1990, excessive liver reserves were defined as 0.70–1.05 μmol vitamin A/g liver, and toxic status was defined as >1.05 μmol vitamin A/g in humans (65). However, after fortification of sugar with vitamin A in Nicaragua, many of the children had liver reserves greater than this range. The liver vitamin A concentration at which ill health in humans occurs needs to be examined more carefully. Are there ramifications of having a liver reserve that hovers at ~1 μmol vitamin A/g liver, or is the human body able to sequester this concentration in the liver? Considering the degree of fortification in some developing countries, the improvements in the stability of fortificants used in formulations and the high consumption of some of these fortified foods, an additional examination of toxicity or hypervitaminosis A needs to occur. For example, in the study performed in hypervitaminotic rhesus monkeys, lactate dehydrogenase, aspartate aminotransferase, and serum albumin were elevated in 50%, 19%, and 44% of the monkeys, respectively (50). Elevations of these enzymes are markers of liver disease or malfunction (66) and may be a vitamin A–toxicity outcome (67, 68). The key questions are as follows: Should we be concerned about vitamin A toxicity in countries with a high degree of fortification? Or is the alleviation of vitamin A deficiency more important than the potential ramifications from hypervitaminosis A?

Liver biopsy or autopsy

The liver is the main storage organ for vitamin A. Liver biopsies have been done under surgical conditions in the United States and Bangladesh, in part to validate the isotope dilution assay (69, 70). These studies showed very good agreement between the estimated values for the liver vitamin A concentration from the isotope dilution assay and the measured liver vitamin A concentration of the group. Samples from autopsy and liver-transplant patients have also been analyzed under special circumstances (71). The distribution of vitamin A across the liver is not uniform (72), but biopsy samples likely reflect the vitamin A status of the individual better than any other biochemical method. However, for obvious reasons, a liver biopsy cannot be widely used.

CONCLUSIONS

If we could teach communities to garden and consume the produce or have a wide adoption of staple crops biofortified with provitamin A carotenoids (73, 74), markers of vitamin A status would not be needed. However, this ideal will likely not occur until poverty is alleviated (75). Therefore, biomarkers of vitamin A status are still needed for the near future to more specifically identify populations at risk of vitamin A deficiency and to evaluate the effectiveness of different interventions or programs. Obtaining vitamin A from provitamin A sources is ultimately safer than fortification with preformed vitamin A considering the potential risk of hypervitaminosis A (74). However, this obtaining requires an adequate distribution in the poor and often a behavior change, which are not easily accomplished.

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