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Original Communication

Research Recommendations for Applying Vitamin A-Labelled Isotope Dilution Techniques to Improve Human Vitamin A Nutrition

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Abstract: The current use of serum retinol concentrations as a measurement of subclinical vitamin A deficiency is unsatisfactory for many reasons. The best technique available for vitamin A status assessment in humans is the measurement of total body pool size. Pool size is measured by the administration of retinol labelled with stable isotopes of carbon or hydrogen that are safe for human subjects, with subsequent measurement of the dilution of the labelled retinol within the body pool. However, the isotope techniques are time-consuming, technically challenging, and relatively expensive. There is also a need to assess different types of tracers and doses, and to establish clear guidelines for the use and interpretation of this method in different populations. Field-friendly improvements are desirable to encourage the application of this technique in developing countries where the need is greatest for monitoring the risk of vitamin A deficiency, the effectiveness of public health interventions, and the potential of hypervitaminosis due to combined supplement and fortification programs. These techniques should be applied to validate other less technical methods of assessing vitamin A deficiency. Another area of public health relevance for this technique is to understand the bioconversion of β-carotene to vitamin A, and its relation to existing vitamin A status, for future dietary diversification programs.
Key words: Children, isotope dilution techniques, lactating women, pregnant women, public health monitoring, stable isotopes, vitamin A assessment

Introduction

The vitamin A-labelled isotope dilution (VALID) techniques are the only known methods that enable relatively non-invasive assessment of total body vitamin A reserves in humans across a continuum of vitamin A statuses, from deficiency to adequacy [1; 2] to hypervitaminosis [3–5]. The VALID techniques

employ retinol labelled with stable isotopes of carbon or hydrogen that can be safely and quantitatively administered to human subjects. Subsequent dilution of the labelled retinol with the endogenous retinol in the body is measured in blood samples to quantify the total exchangeable body retinol pool, providing an estimate of total body vitamin A reserves of groups of individuals [2]. The methods involve little to no risk, but are time-consuming, technically challenging, and relatively expensive. Field-friendly improvements are desirable to encourage application in developing countries where monitoring of vitamin A deficiency and the effectiveness of large-scale public health interventions are most needed.

The VALID techniques have been applied to assess vitamin A status, to evaluate the efficacy of vitamin A interventions (e. g. supplementation, food fortification, or dietary changes), to model human vitamin A requirements, and to determine the efficiency of the body's conversion of provitamin A carotenoids (from plant foods) to retinol [1]. Unlike methods such as the measurement of serum retinol, which is tightly regulated and can really only detect very low body stores [3], the VALID techniques provide the sensitivity needed to quantitatively evaluate the effectiveness of different vitamin A dosing and timing regimens for public health interventions [1].

This paper summarizes recommendations (Figure 1) resulting from a technical meeting on applying VALID techniques to improve human vitamin A nutrition, and includes topics related to the further development of the method as well as use of VALID techniques to meet public health needs for evaluating the safety, efficacy, and effectiveness of vitamin A intervention programs.

Summary of Recommendations

Further development of VALID techniques:

- **Make the method more field-friendly**
- **Increase accessibility and further verify usefulness of different tracers and doses**
- **Further establish guidelines for interpreting VALID techniques**
- **Further validate the method for different population groups, especially children and pregnant and lactating women**
- **Design studies with sufficient statistical power for specific conditions**
- **Determine how vitamin A status and other population characteristics influence vitamin A absorption, distribution, and metabolism**
- **Consider designs with paired comparisons and both positive and negative controls**

Further application of VALID techniques to benefit public health:

- **Monitor the safety and effectiveness of high dose supplementation to young children in developing countries**
- **Assess alternative vitamin A interventions**
- **Determine the effectiveness of routinely supplementing with or feeding β-carotene, rather than retinol**
- **Investigate the usefulness of VALID techniques to assess vitamin A status in populations affected by inflammation**
- **Improve understanding of the influence of iron or zinc deficiencies on vitamin A metabolism and status evaluation**

Figure 1: A summary of recommendations for future development and applications of the vitamin A-labelled isotope dilution (VALID) techniques.

Further development of the technique

Make the method more field-friendly and feasible in resource-poor settings

Challenges in applying the VALID techniques include the expense, technical expertise, and infrastructure required to operate mass spectrometry equipment in developing countries. The method requires extended participant involvement because of multiple blood collections and isotope mixing periods for intervention studies. Further, the method is complicated because the collected samples often need to be transported to distant laboratories under appropriate temperature and lighting conditions.

Various strategies can be applied to overcome some of these obstacles. For instance, a state-of-the-art mass spectrometry laboratory for nutrition research at St. John's Research Institute in Bangalore, India, has created a protected bank of relatively inexpensive automotive batteries that are continually recharged with electrical power to provide a source of uninterrupted power in case of an outage. The International Atomic Energy Agency has supported equipment and training to help build capacity, and also helps match investigators with collaborators at established analytical laboratories. Some sensitive equipment and techniques have been introduced that require considerably smaller sample volumes. Method modifications and further improved sensitivity have the potential to utilize finger pricks and blood spots, rather than venous blood draws for sampling and transport. This could leave important resources for population vitamin A assessment and evaluation using VALID techniques.

The strength of VALID techniques is the quantification of the body's exchangeable vitamin A pool and estimated liver reserves, but it comes with a requirement to allow a 2- to 3-week mixing period for the labelled vitamin A dose to equilibrate with vitamin A stores. Possible modification of the method to evaluate the isotope dilution in blood samples just 3 days after dosing has the potential, with further development and validation, to quantitatively assess body vitamin A status in a shorter timeframe; this would shorten the involvement of human subjects and, because of less catabolic loss of the labelled retinol, may reduce the required sample volumes [6].

Increase accessibility and further verify usefulness of different tracers and doses

Many tracers of retinol isotopically labelled with stable isotopes are currently commercially available. The two most commonly used are $^{2}H_{4}$-retinyl acetate and $^{2}H_{8}$-retinyl acetate, labelled with 4 and 8 deuterium atoms per molecule, respectively. Investigators have also used commercially synthesized $^{13}C_{10}$-labelled retinol [7] or privately synthesized $^{13}C_{2}$-retinyl acetate [8]. Increased commercial access to tracers is needed for greater application of the technique, preferably accompanied by reduced costs. An advantage of using two different tracers is that it can eliminate the need for another blood draw to establish a new baseline for a second application of the isotope dilution (VALID) technique to study post-intervention status. This is, however, dependent upon the type of mass spectrometer used for analysis.

Investigators must consider the dose size and predicted dilution of stable isotope that can be detected by their available instrumentation. Depending on the instrumentation and technique available, the doses of isotopically-labelled retinyl acetate administered orally in human studies have ranged from 1 to 35 μmol (0.3 to 10 mg). Doses need to be kept as low as possible to qualify as a tracer that does not influence vitamin A status and metabolism. With overt deficiency defined as liver retinol concentrations ≤0.07 μmol/g (≤118 μmol total in a 70 kg adult with the liver estimated as 2.4 % of body weight), a dose of 35 μmol (10 mg) that is 50 % retained could increase an adult's hepatic vitamin A pool by 15 % to 30 %, which cannot be regarded as a minimal "tracer" dose. Study hypotheses, designs, subjects, and doses must be selected appropriately to minimize this problem. Method development should continue to reduce the amount of tracer needed for adequate detection.

Improved sensitivity of instrumentation has revealed shifts in natural abundance of isotopes in biological samples. For instance, natural ratios of ^{13}C to ^{12}C are lower in vegetable sources of provitamin A carotenoids as compared to corn and corn-fed animal sources of meat and dairy foods [9]. Further development of the VALID method may benefit from testing to determine if results are influenced by the isotope mass of the deuterated or ^{13}C tracers, or by the proximity of the isotope to the portion of the retinol molecule involved in the active sites of metabolic reactions.

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Further establish guidelines for interpreting VALID techniques

Current guidelines for defining status are based on liver retinol concentrations rather than total body stores of vitamin A. The liver concentration to designate deficiency (<0.07 μmol/g liver) was based upon mathematical assumptions to prevent clinical ocular manifestations [10]. A newer cutoff for deficiency of 0.1 μmol/g liver was proposed and is based on biological response in the liver, including the up-regulation of storage enzymes [11] and the accumulation of retinol-binding protein [3]. Other categories of vitamin A status that have been suggested, such as excessive, sub-toxic, and toxic, need further data, potentially using VALID techniques, to better understand the relationship of high liver stores to health.

It may be useful to consider establishing guidelines for evaluating vitamin A status based on total body pool size to reduce the assumptions required when estimating liver concentration or content. Two assumptions used in translating from total body pool size to liver retinol concentrations are the liver size of the individual and the fraction of body retinol stored in the liver. This, of course, would differ by individual, and therefore adds variability to population estimates. For example, it is standard practice to estimate liver weight as 4, 3, and 2.4 % of body weight for infants, children and adults, respectively [2; 12]. However, what happens in the case of obesity? Is the excess body weight also accompanied by excess fat in the liver? And does extra liver fat reduce (by dilution) or elevate concentrations of (fat-soluble) vitamin A in the liver?

Assumptions about the fraction of total body retinol that is stored in the liver differ depending on the vitamin A status of the subject. For example, liver retinol was more accurate when estimated as 50 % of total body retinol in rats fed low dietary vitamin A, but as 80 % in rats fed moderate or high levels [13]. During acute deficiency in rats, retinol concentrations may be greater in the kidney than the liver [14]. During hypervitaminosis A, storage can rise to >90 % in the liver. This was demonstrated in rhesus monkeys with liver retinol concentrations of 18.8 ± 6.4 μmol/g and kidney concentrations of 0.010 ± 0.0032 μmol/g [15]. Development of guidelines for vitamin A status based on total body pool size rather than liver concentrations would reduce the number of assumptions required.

Further validate the method for different population groups, especially children and pregnant and lactating women

VALID techniques have been validated against liver reserves of vitamin A in rats, monkeys, and humans. In adult surgical patients in the USA, the range of liver vitamin A concentrations was 0.05 to 0.6 μmol/g liver [16]. In adult surgical patients in Bangladesh, total liver retinol stores were from 0.02 to 0.25 mmol/liver [17]. In animals, the VALID technique compared well with liver reserves in rats ($r = 0.98$) fed low, medium or high vitamin A orally, when assuming, respectively, that 50, 80, and 80 % of body vitamin A was hepatic [13], and in rhesus monkeys known to have hypervitaminosis A, with mean liver retinol concentration as high as 16.4 μmol/g [4].

To date, no validations involving liver biopsy have been performed in children. Some of the assumptions of the method may differ in children and therefore limitations must be considered. Nonetheless, as long as the same assumptions are used before and after an intervention or only population estimates are needed, the VALID techniques offer more information than any other current vitamin A method available to investigators.

To date, the VALID techniques have not been applied to pregnant and lactating women. While the methods are safe for use in these vulnerable groups, the research data interpretation is more complicated, and additional data collection and modelling are needed to estimate vitamin A pool size. During lactation, a significant amount of the tracer dose would be shunted into the breast milk, and this "loss" would need to be accounted for in a modified equation. During pregnancy, the increase in normal plasma volume, known as hemodilution, would influence the calculation of the amount of tracer in the blood circulation, but perhaps the interpretation of the VALID techniques could be modified to detect changes in status despite hemodilution. However, equations have not been modified to quantitatively estimate total body vitamin A pool size during pregnancy or lactation using typical oral administration of the tracer dose.

Finally, as the best methods available for vitamin A status assessment in humans, VALID techniques could be usefully applied to validate other less technically challenging methods of assessing vitamin A deficiency or hypervitaminosis A. Methods such as the dose response tests, do not have the same working dynamic range as VALID techniques [6]. Determining what response is similar to deficient, marginal, and adequate through excess vitamin A status assessed by VALID techniques would be useful for evaluation and monitoring programs

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at the population level. Another method that is understudied is circulating retinyl ester concentrations during a fasting state. Although retinyl ester concentrations >10 % of total serum vitamin A have been suggested as an indicator of excess vitamin A status, very little data have been generated with this biomarker in humans [18].

Design studies with sufficient statistical power for specific conditions

One of the key elements in clinical studies, including vitamin A studies, is an adequate sample size. Although sample sizes of 15–30 per group for the VALID technique were formerly estimated in a cost comparison of vitamin A assessment methods [19], such samples sizes may be insufficient for some conditions, and estimates of statistical power should be specific for the conditions of each study [2]. To calculate sample size, information is needed on the initial vitamin A pool size, the expected change in vitamin A pool size in response to an intervention, and the projected standard deviation of the change in vitamin A pool size. These can be estimated from results of previous studies or by estimating expected changes based on assumptions regarding retention and catabolism of vitamin A. To plan sufficient duration of vitamin A interventions, a starting assumption is that 50 % of supplemental vitamin A is retained in the body, with a body vitamin A catabolism rate of ~2.2 %/d in preschool-age children and ~0.5 %/d in adults or school-age children [20]. Factors that can affect the required sample size include the source (food or supplement), chemical form, dose and duration of the vitamin A intervention, the time allowed for dose equilibration, the baseline vitamin A status of the group studied, and potential effects of the physiological state (e.g. growth, pregnancy, lactation, inflammation or recent illness). Greater sample sizes may be needed to detect responses to an intervention when studying populations with higher baseline vitamin A reserves (and associated variation), compared with populations with lower reserves.

Compared with supplements, lower retinol availability can be expected from foods, and especially from foods containing vitamin A precursors, such as β-carotene. For instance, although 14 Bangladeshi men were sufficient to significantly confirm an estimated increase in body vitamin A reserves resulting after 60 d supplementation with 750 μg retinol equivalents from retinyl acetate or β-carotene, a smaller increase was observed with Indian spinach, and the smallest increase with sweet potatoes was not statistically significant [21]. From plant foods, human absorption of β-carotene can range from 5 to 65 %, and equivalency ratios for biological conversion of β-carotene to vitamin A can range from 3.8:1 to 28:1 by weight [22].

Determine how vitamin A status and other population characteristics influence vitamin A absorption, distribution, and metabolism

Less efficient bioconversion of β-carotene to vitamin A has been observed in animal studies as vitamin A status increases [23, 24]. More information is needed on how vitamin A status influences parameters, such as absorption, bioconversion, and catabolic rates. The VALID techniques have commonly used a calculation developed by Olson and colleagues [16] that estimates total liver retinol from the body exchangeable vitamin A pool size, based on rat studies indicating that 50 % of an orally administered dose is absorbed and stored in the liver [25]. As indicated above, vitamin A status may influence this factor. In addition, little is known about the efficiency of vitamin A absorption in humans, and how it may be influenced by the dose size or vitamin A status. The efficiency of vitamin A absorption can be measured with a dual isotope technique that determines relative retention of an isotopically labelled oral dose and a differently labelled intravenous dose in artificial chylomicra [26]. (While potentially useful for further development and interpretation of the VALID techniques, such application of intravenous doses would not be routinely practical in field situations.)

Haskell and colleagues [27] compared the 'equilibration time' after administering an oral dose of deuterium-labelled retinyl acetate to Bangladeshi and US adults ($n=4$/group). Although the pool size was much lower in Bangladeshi adults as compared with US adults, the mean 'equilibration times' were similar for US and Bangladeshi adults: 17.5 ± 4.4 and 16.3 ± 3.9 d, respectively. These data suggest that 'equilibration time' is not affected by vitamin A pool size. Similarly there was no change in the fractional catabolic rate. It remains to be investigated if the equilibration time and the fractional catabolic rate are influenced by vitamin A status in the higher range of liver reserves in these populations. Studies in rats demonstrated a higher catabolic rate with higher vitamin A intake, reserves, and serum concentrations [28]. Kinetic experiments are needed in various populations like schoolchildren and pregnant women to define life-stage influences on equilibration time and fractional catabolic rate.

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Consider designs with paired comparisons and both positive and negative controls

Inter- and intra-individual variability in vitamin A pool isotopic ratios are high; using paired comparisons by applying the VALID technique before and after an intervention in the same subjects improves the statistical power to detect differences due to the food or fortificant being tested. It can be helpful to design intervention studies with one or two control arms, that is, placebo and/or oral dose of retinyl ester, in order to improve the internal validity and subsequent interpretation of the study.

Further application to benefit public health

More research is needed in several areas related to vitamin A deficiency and body stores determined using stable isotopes. Central to these is the evaluation and monitoring of vitamin A interventions in populations at risk of deficiency, to assess effectiveness in addressing deficiency without adversely increasing the risk of hypervitaminosis A from supplementation and expanding fortification programs that have been applied for over a generation. In conjunction with stable isotopic estimates, functional rather than clinical indexes need to be established to define deficiency or excess. Comparing VALID methods with potential population-based assessments will be important to move programs forward.

Apply VALID techniques to monitor the safety and effectiveness of high dose supplementation of young children in developing countries

The cutoffs for excessive vitamin A levels in the total body or liver need to be better defined. Because VALID methods appear best suited to this application, it is important to standardize the method in children, and to perform these studies in locations where high dose vitamin A supplementation programs have been implemented for some time. Based on concerns about the efficacy and even toxicity of supplementation interventions, it is imperative to measure vitamin A status accurately in children. This can be done either by direct application of VALID techniques or by applying these methods to verify potentially simpler field methods, such as measurement of serum or urinary retinol metabolites, retinol-binding protein, or clinical indicators, such as nightblindness, Bitot's spots or xerophthalmia.

Apply the VALID method to assess alternative vitamin A interventions

Exploring alternatives to high dose vitamin A supplementation is an area needing further investigation. This includes interventions promoting locally available, culturally acceptable vitamin A-rich foods containing either preformed vitamin A esters, such as liver, milk, cheese, and eggs, or provitamin A carotenoid precursors, such as green leaves, carrots, ripe mangoes, and other orange-yellow vegetables and fruits. The efficacy of food-based approaches needs to be explored using appropriate indicators, such as VALID, which can assess vitamin A status across the continuum. Sensitive assessment of status can help policy makers decide the best strategies to promote adequate vitamin A intakes in ways that are cost-effective, appropriately distributed, and sustainable.

Determine the effectiveness of routinely supplementing with or feeding β-carotene, rather than retinol

A safe and sustainable solution to improve vitamin A status of pregnant and lactating mothers, and thereby exclusively breastfed infants, would be to use locally available, culturally acceptable green leafy vegetables, which are low in cost compared with other vegetables and fruits in many parts of the world [29]. Local food-based approaches should be evaluated by the VALID techniques to determine the efficacy of these strategies for improving vitamin A status of women and children [24]. Research is needed to determine the levels of ingested β-carotene that can avert vitamin A deficiency. Stable isotope-labelled β-carotene can be used to track its conversion to retinol. This is of interest under different states of vitamin A homeostasis, where deficiency could lead to increased bioconversion efficiencies, as well as in vegetarians whose intake of preformed retinol is low. Further, the storage, utilization, and total pool size of carotene in the body need to be better defined, with the goal of determining a serum β-carotene level that could assure adequate retinol status in people.

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Investigate the usefulness of the VALID method to assess vitamin A status in populations affected by inflammation

It is well known that one of the key areas of concern is the effect of other co-existing morbidities, such as infection and inflammation, on serum retinol concentrations, which fall because retinol-binding protein is a negative acute phase protein [30]. Serum retinol may not be responsive to supplementation efforts, and used by itself in children or populations that are at high risk of infection or living in unhygienic conditions, serum retinol may falsely exaggerate the frequency of vitamin A deficiency [3]. Stable isotope methods may be useful for determining how vitamin A is redistributed during infection/inflammation in humans.

Improve understanding of the influence of iron or zinc deficiencies on vitamin A metabolism and status evaluation

Iron and zinc supplementation increase serum retinol concentrations in malnourished children [31]. Deficiencies of these trace elements can negatively decrease the mobilization of vitamin A from liver storage [32]. Additional research is needed to accurately assess vitamin A status under conditions of multi-nutrient deficiencies.

Conclusions

The VALID techniques are the most sensitive methods available for vitamin A status assessment. Considering the extensive and worthwhile global health efforts to eradicate vitamin A deficiency with supplementation of preschool children and the introduction of fortified and biofortified foods, the accurate assessment of vitamin A status is essential for developing, assessing, and monitoring these public health measures.

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