Refinement of the modified relative dose response (MRDR) assay as a method to assess vitamin A status of humans

Sherry Ann Tanumihardjo
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Refinement of the modified relative dose response (MRDR) assay as a method to assess vitamin A status of humans

Tanumihardjo, Sherry Ann, Ph.D.

Iowa State University, 1993
Refinement of the modified relative dose response (MRDR) assay as a method to assess vitamin A status of humans

by

Sherry Ann Tanumihardjo

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition
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Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1993
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GENERAL INTRODUCTION

Vitamin A status can best be divided into five stages: deficient, marginal, satisfactory, excessive and toxic (Olson 1992). Liver retinol concentrations characteristic of the deficient, marginal and satisfactory states are <5 μg/g, 5-19 μg/g and ≥20 μg/g, respectively (Furr et al. 1989). While the deficient and the toxic states of vitamin A status have clear clinical signs, the marginal, satisfactory and excessive states are more difficult to assess without some sort of biochemical measure (Olson 1992).

Currently, several methods exist for the assessment of vitamin A status. These methods have been reviewed by Underwood (1990) and Olson (1992). To understand the manner in which the assessment techniques work, the basic metabolism of vitamin A is reviewed. This review is followed by a discussion of the current methods available in assessing vitamin A status with special emphasis on dose response tests for vitamin A status assessment.

Vitamin A Requirements and Metabolism

Vitamin A or provitamin A carotenoids are required in the diet of humans. The most important sources of vitamin A are preformed vitamin A, found in animal products, and the
biologically active carotenoids, obtained from plants (Underwood 1984). Because of the fat-soluble nature of vitamin A, it is treated in the gut much like a lipid. Dietary retinyl esters, retinol and provitamin A carotenoids (mainly $\beta$-carotene and $\beta$-cryptoxanthin in the human diet) are dispersed in the stomach. Then they pass into the duodenum, where the retinyl esters are hydrolyzed by a nonspecific pancreatic lipase (Lombardo and Guy 1980). The retinol and carotenoids are solubilized in mixed micelles, allowing them to reach the microvillus membrane. Absorption of retinol and the carotenoids takes place by passive diffusion (Hollander 1981).

After absorption into the mucosal cell, the $\beta$-carotene is cleaved by dioxygenase to retinaldehyde, which is subsequently reduced to retinol. The retinol is primarily reesterified with long-chain fatty acids, predominantly palmitic and stearic acid. Two microsomal enzymes are active in this process, lecithin:retinol acyltransferase (LRAT) and acyl CoA:retinol acyltransferase (ARAT). These retinyl esters are incorporated by mucosal cells into the core lipid of chylomicra. The chylomicra are transported through the lymphatic system via the thoracic duct into the plasma. In the plasma, the chylomicra acquire specific apolipoproteins (apoC and apoE) from plasma high-density lipoproteins. ApoC activates the enzyme lipoprotein lipase to hydrolyze the chylomicron triglycerides. Ultimately, this process leaves a
smaller particle called a chylomicron remnant. The retinyl esters are almost entirely retained in the chylomicron remnant. The apoE on the chylomicron remnant is responsible for its rapid hepatic uptake (Goodman and Blaner 1984).

Hepatic uptake of the chylomicron remnants most likely occurs through receptor-mediated endocytosis. Lysosomal degradation of the remnant occurs along with retinyl ester hydrolysis. After ester hydrolysis, the free retinol can be reesterified by LRAT or ARAT (Ross 1982), predominantly to palmitic acid; bound to retinol binding protein (RBP), which is secreted into the plasma; or converted to other metabolites, like retinyl β-glucuronide, 4-hydroxy-retinol, retinal and retinoic acid (Goodman and Blaner 1984).

Assessment of Vitamin A Status

Vitamin A deficiency continues to be a major public health problem in preschool-age children and in pregnant and lactating women, especially in underdeveloped nations. Of the five to six million children in the world who suffer from vitamin A deficiency, more than 250,000 become blind and half of these children die within two years (Sommer 1989).

The general term used for vitamin A-dependent ocular involvement is "xerophthalmia", meaning "dry eye" (Greek). The World Health Organization has classified xerophthalmia into primary and secondary signs. The primary signs
(classifications) include conjunctival xerosis (X1A), Bitot's spot with conjunctival xerosis (X1B), corneal xerosis (X2), corneal ulceration with xerosis (X3A) and keratomalacia (X3B). The secondary signs (classifications) include nightblindness (XN), xerophthalmia fundus (XF) and corneal scars (XS) (World Health Organization 1976).

Assessment of vitamin A status is not a straightforward process. The nutritional indicator for vitamin A status has been defined as the total body content of retinol and its esters, with the minimal acceptable reserve set at 20 μg (0.07 μmole)/g of liver. Under most circumstances, the liver has been found to contain 90% or more of the total body vitamin A (Arroyave et al. 1982). Plasma retinol concentrations alone are not a good indicator because they do not decrease significantly until liver reserves are very low (Underwood 1984). Techniques which detect marginal vitamin A deficiency before clinical signs are manifested are very useful in formulating public health policies.

Of course, the most accurate indicator of vitamin A status is the analysis of liver samples obtained at autopsy or by surgical and needle biopsies (Amedee-Manesme, Anderson and Olson 1984; Flores et al. 1983; Olson, Gunning and Tilton 1984; Tanumihardjo et al. 1990). However, this is only possible in special, justifiable situations in clinical or hospital studies.
Hatchell and Sommer developed a technique to determine the prevalence of goblet cells on the bulbar conjunctiva which is commonly called conjunctival impression cytology (CIC) (Hatchell and Sommer 1984). Goblet cells decrease in various tissues during vitamin A deficiency, while the epithelial cells become enlarged. CIC involves taking a cellulose acetate filter paper impression of the bulbar conjunctiva. The cells are histologically stained, and the impressions are examined by light microscopy to observe the goblet and enlarged epithelial cells. Hatchell and Sommer found that, before clinical signs of xerophthalmia appeared, the goblet cell population decreased and the number of enlarged epithelial cells increased. Thus, by using this relatively noninvasive technique, ocular surface abnormalities due to vitamin A deficiency can be detected before advanced xerophthalmia is evident. A training manual is available for CIC from the Dana Center for Preventive Ophthalmology (Wittpenn et al. 1988). A recent advancement in the technique using a disk applicator has improved the quality of the specimens obtained (Keenum et al. 1990).

Thornton developed a method to measure the rapid dark adaptation of individuals (Thornton 1977). The test is done under nighttime lighting conditions. The time that it takes for a subject to sort a pile of white, blue and red chips with 100% accuracy is measured. Vitamin A-deficient patients tend to take longer to sort the chips than do normal patients.
This method correlates well with the classical dark adaptation test (Vinton and Russell 1981). Recent development of a dark adaptometer may prove to be a useful, inexpensive method to evaluate vision restoration time (VRT) after bleaching the eye. VRT measurement may be useful in identifying populations at risk of vitamin A deficiency (Udomkesmalee 1992).

Isotope dilution techniques have been used with radioactive $^3$H-labeled vitamin A in rats, sheep and cattle (Rietz et al. 1973; Rietz, Wiss and Weber 1974; Hughes et al. 1976; Bausch and Rietz 1977). $^3$H-Vitamin A has also been used in humans (Bausch and Rietz 1977; Sauberlich et al. 1974), however, without verification by liver analysis. Furr and coworkers have used the non-radioactive deuterated analogs of vitamin A in place of the tritiated analogs in humans along with liver biopsy verification (Furr et al. 1989). The ratio of labeled to unlabeled (D/H ratio) in serum can be determined using capillary gas chromatography-mass spectrometry (GC-MS). Because of the highly specialized equipment, the use of isotope dilution techniques for large surveys in developing countries is unlikely in the near future.

The dose response tests:

The relative dose response (RDR) test, originally developed by Underwood and her colleagues in rats (Loerch, Underwood and Lewis 1979), is a good indicator of marginal
vitamin A status both in individuals and among populations. The RDR assay has been validated in humans using direct measures of liver vitamin A (Amedee-Manesme, Anderson and Olson 1984; Amedee-Manesme et al. 1987). During vitamin A deficiency and marginal status, apo-retinol-binding protein (RBP) accumulates in the liver (Soprano, Smith and Goodman 1982). After a small oral dose of retinyl esters is administered, the holo-RBP is released into the serum from the liver and transported to target tissues. The response of the individual to this dose of vitamin A is measured in the serum 5 hours after administration of the dose.

The RDR involves giving a standard oral dose of 450-1000 µg (1.6-3.5 µmole) of retinol equivalents dissolved in oil (Flores et al. 1984). The dose has also been given intravenously (1000 µg over a 30 min. interval) as a water-dispersed suspension to children with liver disease (Amedee-Manesme et al. 1987). Two blood samples are taken at time 0 and at 5 hours after the dose. Venous or capillary blood samples are taken depending on whether micro-analytical procedures are available. After lipid extraction of the serum samples, the serum vitamin A concentration is measured by use of high-performance liquid chromatography (HPLC) or of a suitable spectrophotometric or colorimetric method. The higher oral dosage level is recommended when the analytical procedure is HPLC because this method when routinely applied does not measure potential post-dosing, residual circulating
retinyl esters. The response is measured in the serum as a percentage \( (A_5 - A_0 / A_5 \times 100) \), where \( A_5 \) is the serum retinol concentration 5 hours after the dose and \( A_0 \) is the concentration at time 0. Theoretically, the value can be 0 to 100%. A response of 20% or higher is indicative of inadequate liver reserves (<0.07 μmole/g liver; <20 μg/g liver) (Underwood 1990; Olson 1992). Negative values often occur among individuals who are in adequate vitamin A status. These values are regarded as a 0% RDR and interpreted as reflecting an adequate liver store of vitamin A.

If the RDR value is ≥20%, the child is almost certainly in a marginal vitamin A status. To determine if a particular community is at risk, the prevalence of a marginal status can be assessed in a randomly sampled subpopulation. Thus, only a representative number of children needs to be studied in order to make suitable public health judgements about the vitamin A status in a population. Many more children are needed in surveys if serum retinol concentration alone is used as the indicator. After analysis, supplements can be made available to residents of communities who have a high incidence of positive RDR values. Typically, a dose of 50,000 IU-200,000 IU every six months, dependent on age, is enough to lower the mortality associated with marginal vitamin A status in preschool children (Sommer et al. 1986). Tentatively, a public health problem might be assumed to exist if >10% of a population of preschool children show abnormal RDR (>20%)
values (Barbara Underwood and Hernando Flores, personal communication).

The RDR is a good indicator of the individual's vitamin A status and has application for community assessment as well. The effects of confounding factors, such as infectious disease and moderate protein-energy malnutrition, on serum retinol concentrations are minimized by use of response tests. For analysis, investigators have a choice of using either HPLC, which is a fairly widespread technique, or any suitable colorimetric or photodegradative assay. The latter methods require much simpler instrumentation. However, because the latter methods measure total vitamin A (esters and RBP-bound retinol), care must be taken that the plasma retinyl esters, which are elevated transiently following the dose, are fully cleared by the liver within the 5-hour wait period. This concern becomes more important as the dosage is increased.

The major drawback of the RDR is that two blood samples are required. In some cultures, it is difficult to draw blood samples, particularly from children. Thus, the need to obtain two samples at a 5-hour interval can pose large logistic and cultural problems. Also, the need to analyze two blood samples to obtain a single human value increases the expense and time of each assay. Moreover, analytical accuracy and precision are extremely important when using two different samples for one calculation.
The RDR test has been used with success in several clinical and field studies, including France (Amedee-Manesme et al. 1987), Brazil (Flores et al. 1984; Campos, Flores and Underwood 1987), Thailand (Amatayakul et al. 1989; Sinawat et al. 1991) and Belize (Makdani 1990). Flores et al. (1984) used the RDR test at 4 different times over a 6-month interval to determine the duration of effectiveness of supplementing children with a single dose (200,000 IU) of the vitamin. The RDR value in an individual reverts to normal soon after supplementation, but then becomes abnormal again when liver stores become depleted. The RDR methodology has also been applied in Brazil to evaluate the effectiveness of a food-based intervention program (Mariath, Lima and Santos 1989).

The RDR test has also been applied to a 20% random subsample in a prevalence survey conducted over two seasons in north and northeast Thailand (Sinawat et al. 1991). No clinical signs were encountered in the survey, but 20% of the population were identified as marginally deficient by the RDR, 18% by CIC, and 9% by a serum retinol <20 μg/dL.

Thaitawee and Tosukhowong (1983) first suggested the use of 3,4-didehydroretinol (dehydroretinol, vitamin A₂, DR) as an indicator of vitamin A status. They observed that the ratio of dehydroretinol to retinol (DR/R) in the serum of rats 24 h after an appropriate dose of vitamin A₂ was inversely related to the amount of vitamin A stored in the liver. Based on this observation, dehydroretinyl acetate was used to assess the
vitamin A status of rats in which the response was found to vary inversely with the total liver reserves of vitamin A (Tanumihardjo, Barua and Olson 1987). Like R, DR binds to accumulated apo-RBP in the liver and is released into the serum as holo-RBP. Because dehydroretinol is found bound in the serum to RBP as is retinol (Wilson and Pitt 1986), an assay was developed to mimic the relative dose response using DR in place of R. The procedure was termed, "the modified relative dose response" (MRDR) (Tanumihardjo and Olson 1988; Tanumihardjo et al. 1990).

The MRDR assay involves first giving children a single oral dose (100 µg/kg body weight) of 3,4-didehydroretinyl acetate dissolved in an oil and then taking a single blood sample 4-6 hours later (Tanumihardjo, Koellner and Olson 1990; Tanumihardjo et al. 1990). Enough blood must be collected either by venapuncture or finger prick to yield approximately 200 µl or more of serum. After the serum (150-500 µl) is extracted with ethanol/hexane, the retinol and dehydroretinol are quantitated by HPLC. The monitoring wavelength of the detector is set at 350 nm, which optimizes the measurement of DR. The amount of serum needed is dependent upon the limits of detection of the HPLC system. Standards of both R and DR of known concentration are used to calibrate the HPLC system. Thereby, a molar ratio of DR to R can be calculated. MRDR values theoretically can range from 0 to ∞.
Children with DR/R ratios of $\geq 0.060$ are currently judged to be in a marginal vitamin A status. The suggested cutoff value of 0.060 was determined by comparative studies with the RDR and CIC techniques and requires further validation (Tanumihardjo et al. 1992). The MRDR has been applied to two groups of Iowan children (Tanumihardjo, Koellner and Olson 1990; Spannaus-Martin et al. 1992). In both studies, the MRDR molar ratio was always less than 0.06. A cutoff value of 0.030 was suggested earlier on the basis of ratios found in healthy American adults and children (Tanumihardjo et al. 1990; Tanumihardjo, Koellner and Olson 1990). Tentatively, a public health problem might be assumed to exist if $>10\%$ of a population subsample of preschool children or mothers show abnormal ($\geq 0.060$) MRDR ratios (Barbara Underwood, personal communication).

The MRDR offers several advantages over other procedures. DR is a naturally occurring form of vitamin A that is found predominantly in freshwater fish, but also to a small extent in mammalian tissues, including the human. DR has approximately 40\% of the biological activity of retinol (Shantz and Brinkman 1950). Only one blood sample is required, and the serum samples can be frozen and stored for analysis. The required HPLC instrumentation, although costly and sophisticated, is fairly widespread around the world. By employing the ratio of DR/R in a single sample, the effects of storage on vitamin A stability and of sample extraction
efficiency are minimized. The MRDR, like the RDR, gives a good indication of an individual's vitamin A status and can also be applied in community assessments. The MRDR can be used for evaluation of the success of intervention programs. Another advantage is that children can be dosed in the morning at their homes and brought to the clinic or the survey site several hours later for the sampling.

On the other hand, the MRDR assay has several constraints. Free DR is not stable once it is extracted from the serum. Therefore, one must exercise extreme care to protect the extracts from light and to analyze them as soon as possible in the HPLC system. Because DR-acetate currently is not available commercially, it must be synthesized or isolated from fish liver oils. Also, blood must be taken from the children, and the samples need to be analyzed using HPLC, which may pose serious limitations in some cultures and institutions.

The MRDR holds considerable promise as a minimally invasive technique by which a marginal vitamin A status can be detected in individual children before clinical manifestations appear. Correlation in individuals of MRDR values with their liver vitamin A concentration would be useful in defining the best ratio to be used as a cutoff between a marginal and satisfactory vitamin A status.
The research described in the following sections investigates the validity of the MRDR for use in both individuals and communities to assess vitamin A status.

Explanation of the Dissertation Format

This dissertation is divided into four major sections in addition to a general introduction and summary. In the introduction, the general metabolism of vitamin A has been reviewed along with the current methods available for evaluating vitamin A status. The literature cited in the general introduction is listed following the general summary and conclusions. PAPER I is published in the *European Journal of Clinical Nutrition*. This paper demonstrates the reproducibility of the MRDR value in healthy individuals and also compares the MRDR to CIC. Next, PAPER 2 is a manuscript to be submitted to the *American Journal of Clinical Nutrition*. This paper compares the MRDR, CIC and RDR techniques in two separate villages in Indonesia and helps to determine the best DR/R ratio to use for Indonesian children. PAPER 3 and PAPER 4 are also manuscripts to be submitted to the *American Journal of Clinical Nutrition*. The purpose of this work was: 1) to evaluate the vitamin A status of low-income Indonesian lactating and pregnant women, 2) to observe the DR/R ratio for several hours after administration of the DR-acetate, 3) to determine if the response to a standard dose is dependent upon
body weight and 4) to evaluate the effectiveness of an intervention trial. Finally, the dissertation ends with a general summary of the importance of dose response tests in the identification of subclinical vitamin A deficiency.
PAPER 1.

THE REPRODUCIBILITY OF THE MODIFIED RELATIVE DOSE RESPONSE (MRDR) ASSAY IN HEALTHY INDIVIDUALS OVER TIME AND ITS COMPARISON WITH CONJUNCTIVAL IMPRESSION CYTOLOGY (CIC)
The reproducibility of the modified relative dose response (MRDR) assay in healthy individuals over time and its comparison with conjunctival impression cytology (CIC)

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(Published in the European Journal of Clinical Nutrition)
ABSTRACT

The modified relative dose response (MRDR) assay is a minimally invasive method of detecting marginal vitamin A status. In the present study, the MRDR assay was performed four times in six well-nourished adults and in one male 5-y-old child over a 7-month period. In all instances, assay ratios of dehydroretinol (DR) to retinol (R) were <0.030, the tentative cutoff value of "normal" for the MRDR assay in humans. The mean ratio for all time points for all individuals was 0.015 ± 0.005, with a mean coefficient of variation of 27 ± 13%. In a related experiment, both MRDR and conjunctival impression cytology (CIC) tests were conducted on nine adults and the same male child. All DR/R ratios were <0.02, and all CIC specimens were normal. Thus, the DR/R ratio in well-nourished individuals, although showing some variation, does not oscillate between abnormal and normal responses. Furthermore, the DR/R ratios and CIC patterns were fully concordant.
INTRODUCTION

The relative dose response (RDR) test (Loerch, Underwood and Lewis, 1979; Flores et al., 1984) and the modified relative dose response (MRDR) assay (Tanumihardjo, Koellner and Olson, 1990; Tanumihardjo et al., 1990) are good indicators of an individual's marginal vitamin A status. During vitamin A deficiency and marginal status, apo-retinol-binding protein (RBP) accumulates in the liver. After the administration of a small oral dose in oil of either retinyl acetate in the RDR test or 3,4-didehydroretinyl acetate in the MRDR assay, holo-RBP is released into the serum from the liver. The plasma response of an individual is measured in the serum 5 h after administration of the dose.

The RDR test requires two blood samples taken at time 0 and 5 h after the dose. The response is measured in the serum as a percentage \( \frac{A_5 - A_0}{A_0} \times 100 \), where \( A_5 \) is the serum retinol concentration 5 h after the dose and \( A_0 \) is the concentration at time 0. A response of 20% or greater is indicative of inadequate liver reserves (<0.07 µmol/g liver; <20 µg/g liver) (Amedee-Manesme, Anderson and Olson, 1984; Amedee-Manesme et al., 1987).

Currently, the MRDR has been validated in rats of differing vitamin A status by directly measuring liver stores of vitamin A (Tanumihardjo, Barua and Olson, 1987; Tanumihardjo and Olson, 1988; Tanumihardjo et al., 1990). The
MRDR assay requires a single venous blood sample 4-6 h after an oral dose. A serum DR/R molar ratio of $>0.030$ is generally indicative of a marginal vitamin A status (Tanumihardjo et al., 1990).

Another indicator for measuring marginal vitamin A status is conjunctival impression cytology (CIC) (Hatchell and Sommer, 1984; Wittppenn and Sommer, 1984; Wittppenn, Tseng and Sommer, 1986; Wittppenn, Natadisastra et al., 1988). CIC is a noninvasive technique that does not require sophisticated equipment. The technique, however, does not seem to work as well in drier climates [i.e., Guatemala (Gadomski et al., 1989) and Senegal (Carlier and Amedee-Manesme, 1989)] as in humid tropical areas [i.e., Indonesia (Natadisastra et al., 1988)].

The reproducibility of the RDR in the same individual has recently been studied (Morrow et al., 1990; Solomons et al., 1990). Generally, more than one test may be needed to diagnose the vitamin A status of an individual. The study presented here is to show the variability of the MRDR ratio in seven healthy individuals over time and its comparison with another indicator of marginal status, conjunctival impression cytology.
MATERIALS AND METHODS

3,4-Didehydroretinyl acetate was synthesized from retinoic acid (Sigma Chem. Co., St. Louis, MO, USA) (Barua and Ghosh, 1972; Tanumihardjo et al., 1987).

The variability of the MRDR over time in the same individual:

Six adults and one male child (5-y-old) volunteered for the study. The adult volunteers (unfasted in most cases) received a single oral dose of 50 µg dehydroretinyl acetate (DR equivalents)/kg body weight dissolved in corn oil at four different times over 7 months. After dosing, the subjects were given a chocolate-coated ice milk bar. There was a 3-month interval between samples 1 and 2 and a 2-month interval between samples 2 and 3 and samples 3 and 4. The child received the same treatment, except that the dose level was 100 µg/kg body weight. The dose was administered in the morning; 5 h later, a single 5-mL blood sample was drawn from antecubital veins. The blood was wrapped in aluminum foil, brought back to the laboratory, allowed to clot, and then centrifuged. The serum was stored at -20°C under argon until analysis.

Duplicate 1-mL samples of thawed serum were treated with ethanol (1 mL) and extracted with hexane (1 mL) three times. Retinyl acetate was used as an internal standard. The
extracts were redissolved in 50 μl 4:1 isopropanol:diethylene chloride, of which 40 μl was injected onto the reversed-phase high-pressure liquid chromatography (HPLC) system, as described earlier (Tanumihardjo, Koellner and Olson, 1990).

The MRDR ratio in comparison with CIC:

Shortly after the MRDR assay was performed on nine adult subjects and the same 5-y-old male child, impressions of the bulbar conjunctiva were obtained by the procedure of Wittpenn, West et al., 1988. The cells were stained on the filter paper, mounted on slides, and read under a simple light microscope.
RESULTS

The MRDR ratio remained "normal" (<0.030) at four testing times in seven healthy individuals (Table 1). The mean DR/R ratio was 0.015 ± 0.005, which includes all individuals at all time points. The mean C.V. for the ratio was 27 ± 13% (Table 1).

Table 1. Variation of the molar ratio of 3,4-didehydroretinol to retinol over a 7-month period

<table>
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<th>No.</th>
<th>Sex</th>
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<th>3</th>
<th>4</th>
<th>Mean ± S.D.</th>
<th>C.V. (%)</th>
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<td>0.028</td>
<td>0.023</td>
<td>0.019 ± 0.010</td>
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<td>0.018</td>
<td>0.021</td>
<td>0.017 ± 0.003</td>
<td>18</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>0.018</td>
<td>0.015</td>
<td>0.016</td>
<td>0.020</td>
<td>0.017 ± 0.002</td>
<td>12</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>0.011</td>
<td>0.006</td>
<td>0.012</td>
<td>0.014</td>
<td>0.011 ± 0.003</td>
<td>27</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>0.012</td>
<td>0.011</td>
<td>0.013</td>
<td>0.020</td>
<td>0.014 ± 0.004</td>
<td>29</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>0.008</td>
<td>0.012</td>
<td>0.011</td>
<td>0.013</td>
<td>0.011 ± 0.002</td>
<td>18</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>0.016</td>
<td>0.008</td>
<td>0.013</td>
<td>0.016</td>
<td>0.013 ± 0.004</td>
<td>31</td>
</tr>
</tbody>
</table>

0.015 ± 0.005^b

27 ± 13%^c

^ Male child, age 5.
^ Mean of the DR/R ratios ± S.D. at all time points.
^ Mean of the C.V. ± S.D. for all subjects.
The ratio for a given individual remained fairly stable with the exception of the young boy, for whom the first ratio (0.004) was much lower than the other three values (Table 1). In this instance, however, vitamin supplements, including vitamin A, were given on a regular basis before the experiment but not after its initiation. Therefore, the increase in ratio is consistent with this information.

Serum retinol concentrations were normal, with a range of 29 to 71 \(\mu\text{g/dL}\) (Table 2). The mean C.V. was 8.7 ± 5.2\%. Subject 2, who had a viral infection during the first test, showed depressed serum retinol at that time that was 30\% below the other values. Infections are known to depress serum retinol values. If the C.V. for subject 2 is left out, the group CV value was 6.8 ± 1.7\%. Similar mean values for the C.V. of serum retinol have been found by other laboratories (Morrow et al., 1990).

In the second study of nine adults and one male child, both DR/R ratios (<0.02) and CIC staining patterns were all normal (Table 3). In the CIC, goblet cells and small rounded epithelial cells were abundant.
Table 2. Variation of the serum retinol concentration (μg/dL) over a 7-month period in one 5-y-old boy and six adults

<table>
<thead>
<tr>
<th>No.</th>
<th>Sex</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Mean ± S.D.</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>27</td>
<td>28</td>
<td>28</td>
<td>31</td>
<td>29 ± 1.7</td>
<td>5.9</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>33</td>
<td>54</td>
<td>47</td>
<td>46</td>
<td>45 ± 8.8</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>29</td>
<td>27</td>
<td>30</td>
<td>29</td>
<td>29 ± 1.3</td>
<td>4.5</td>
</tr>
<tr>
<td>4</td>
<td>F</td>
<td>63</td>
<td>55</td>
<td>61</td>
<td>58</td>
<td>59 ± 3.5</td>
<td>5.9</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>36</td>
<td>42</td>
<td>39</td>
<td>44</td>
<td>40 ± 3.5</td>
<td>8.8</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>79</td>
<td>71</td>
<td>67</td>
<td>65</td>
<td>71 ± 6.2</td>
<td>8.7</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>50</td>
<td>45</td>
<td>45</td>
<td>42</td>
<td>46 ± 3.3</td>
<td>7.2</td>
</tr>
</tbody>
</table>

8.7 ± 5.2%b

a Male child, age 5.
b Mean of the C.V. ± S.D. for all subjects.
Table 3. The DR/R compared with CIC in one child and nine adults

<table>
<thead>
<tr>
<th>Sex</th>
<th>DR/R</th>
<th>CIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>M(^a)</td>
<td>0.004</td>
<td>N(^b)</td>
</tr>
<tr>
<td>F</td>
<td>0.010</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.013</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.021</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.009</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.011</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.010</td>
<td>N</td>
</tr>
<tr>
<td>F</td>
<td>0.009</td>
<td>N</td>
</tr>
<tr>
<td>M</td>
<td>0.008</td>
<td>N</td>
</tr>
<tr>
<td>M</td>
<td>0.015</td>
<td>N</td>
</tr>
</tbody>
</table>

\(^a\) Male child, age 5.

\(^b\) N = Normal cytology test.
DISCUSSION

The relative dose response tests (i.e., the RDR and MRDR) are good indicators of an individual's vitamin A status. The effects of confounding factors, such as infectious disease and moderate protein-energy malnutrition, on serum retinol concentrations are minimized by their use. The MRDR has the advantage over the RDR of requiring only one blood sample. By employing the ratio of DR/R in a single sample, the effects both of storage on vitamin A stability and of sample extraction efficiency are also minimized. Furthermore, children can be dosed in the morning at their homes and brought to the clinic or the survey site several hours later for the single sampling, which is a significant logistic advantage.

The data presented here show that the DR/R ratio did not fluctuate between abnormal and normal responses within the same healthy individuals over the 7-month period. A similar observation has been made by using the RDR method (Morrow et al., 1990). In one instance, an unexpected change in the ratio could be attributed to prior supplementation with vitamin A (subject 1). Previously, we have shown that supplementation with vitamin A markedly decreases the ratio (Tanumihardjo, Koellner and Olson, 1990). In another instance, a marked change in the plasma retinol concentration (subject 2) caused by infection did not affect the ratio.
In all subjects, normal DR/R ratios were accompanied by normal CIC staining patterns. A comparison of the MRDR with the CIC test in children with a deficient or marginal vitamin A status should be valuable.

A DR/R ratio of $ \geq 0.030 $ seems to be characteristic of individuals in a marginal vitamin A status. A dose of 50,000 IU-200,000 IU every 6 months, dependent on age, seems to lower the morbidity and mortality associated with marginal vitamin A status in preschool children. Although public health criteria for the RDR and MRDR have not yet been set, we might tentatively suggest that, if $ >20\% $ of a population of preschool children show either abnormal RDR or MRDR values, a public health problem might well exist. Further studies are required to demonstrate the quantitative relationships among the MRDR, mortality, and morbidity.
ACKNOWLEDGEMENTS

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REFERENCES


PAPER 2.

COMPARISON OF ASSESSMENT TECHNIQUES FOR VITAMIN A STATUS IN
TWO INDONESIAN VILLAGES
Comparison of assessment techniques for vitamin A status in two Indonesian villages

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(To be submitted to the American Journal of Clinical Nutrition)
ABSTRACT

The vitamin A status of preschool-age children without clinical eye signs of vitamin A deficiency in two villages near Bogor, West Java, Indonesia, was studied by the modified relative dose response (MRDR) test, the relative dose response (RDR) test, and the conjunctival impression cytology (CIC) method. Of the children examined, 71% in the first village (Group I, n=75) and 36% in the second village (Group II, n=83) fell below the third percentile of the WHO reference standard of weight-for-age. The following cutoff values for an inadequate vitamin A status in Indonesia were used: MRDR (≥0.06), RDR (≥20%), CIC (an abnormal impression in one eye). The percent abnormal values were: Group I - MRDR, 48%; CIC, 51%; Group II - MRDR, 12%; RDR, 11%; CIC, 5%. Thus, the indicators gave concordant results for the two populations but did not necessarily identify the same individuals at risk. The consistency of the RDR assay was much improved by increasing the oral dose to 3.5 μmoles and by retesting only after a 3 week interval.
Marginal vitamin A status, also termed preclinical deficiency, continues to be a major public health problem in developing countries. A marginal vitamin A status is defined as a nutritional state whereby overt clinical eye signs are absent but in which the child is at an increased risk of infection or death because of depressed vitamin A stores. Mortality among preschool children has been shown to be significantly reduced by vitamin A supplementation in studies conducted in Indonesia, India and Nepal (Rahmathullah et al. 1990; Sommer, Katz and Tarwotjo 1984; Sommer et al. 1986; West 1991), but not in others carried out in India and in the Sudan (Vijayaraghavan et al. 1990; Herrera 1991). Nonetheless, increased vitamin A intake is regarded as an important public health issue to ensure the health of preschool children in many third-world countries. Thus, vitamin A status should be considered as one of many multivariates in childhood survival.

Populations at risk of a marginal vitamin A status must be appropriately assessed before public health policies are formulated. Suitable assessment methods for the marginal status include the relative dose response (RDR) (Amatayakul et al. 1989; Flores et al. 1984; Amedee-Manesme et al. 1987; Amedee-Manesme, Anderson and Olson 1984), the modified relative dose response (MRDR) (Tanumihardjo, Koellner and Olson 1990; Tanumihardjo et al. 1990; Tanumihardjo and Olson...

The purpose of this work was to compare three of these assessment methods, i.e., RDR, MRDR and CIC, in two separate population groups of Indonesian children with different nutritional statuses. Although each of these techniques has its advantages and disadvantages, they are good indicators of populations at risk. The dose response tests are also useful in determining the individual's vitamin A status.
METHODS

Materials:

3,4-Didehydroretinyl acetate was synthesized from retinoic acid (Tanumihardjo, Barua and Olson 1987; Barua and Ghosh 1972). The compound was purified twice on a 8%-water-deactivated alumina column and dissolved directly into 100% corn oil by sonication. An appropriate working dilution was made with corn oil to dose the children with a volume of 100 to 260 µl of oil. Retinyl acetate was obtained from Sigma Chemical Co. (St. Louis, MO).

Assay techniques:

The RDR assay was performed as described by Flores (1984), with the exception that 3.5 µmoles of retinyl acetate was employed with group II instead of 1.57 µmoles as originally recommended. The RDR assay involves taking two blood samples, one at 0 time and one at 5 h after dosing. The RDR value, expressed as a percentage, is calculated as follows: \[\frac{(A5-A0)}{A5} \times 100\]. Values of 20% or more were judged as abnormal. The modified relative dose response (MRDR) assay was conducted as described by Tanumihardjo (1990). The MRDR test was performed by administering a single oral dose of 3,4-didehydroretinyl-acetate at a dosage level of 0.35 µmoles (100 µg)/kg body weight. High-fat, low vitamin A-
containing snacks were provided after dosing. A single venous blood sample was taken 5 h after dosing. The CIC procedure was performed as described by Wittpenn (1988) with the modification described by Keenum (1990). The CIC specimens were fixed and stored for future staining. For group I, the specimens were stained in Indonesia as diagrammed in the ICEPO teaching manual (Wittpenn et al. 1988). Because stains that were purchased from a Indonesian local vendor did not stain the cells properly, the samples from group II were analyzed in the United States.

SAS statistical computer software (SAS 1989) was used in the data analysis.

Subjects:

Children (0.7-6.5 y) were enrolled through the Nutrition Research and Development Center in Bogor, Indonesia. All procedures were approved by the Institutional Review Board of Iowa State University and by Indonesian authorities with the same responsibilities.

Assessment of the vitamin A status of preschool Indonesian children in one village (Group I):

A total of 88 children, ages 1.9 through 6.5 y, were enrolled from a single village area. MRDR assays were
successfully performed on 75 of these children. A single blood sample was taken 5 h after administering the dose. At the time of blood drawing, the children were given general physical examinations at the local pos yandu (village health post) with the aid of local volunteers. After the blood samples were drawn, children were given gifts of food, clothing and toys.

Ten to 17 days after the MRDR test, the RDR test was performed on those subjects who agreed to further testing (n=56). Two blood samples were obtained from 50 children but not from the other 6. Serum was prepared and stored frozen until analysis. CIC specimens were taken on all cooperating subjects (n=55).

Assessment of the vitamin A status of preschool Indonesian children in a second village (Group II):

Children who had received a high-dose capsule within the last two months or who had eaten an egg or other foods with a high vitamin A content on the morning of the study were excluded. Thus, of a total of 129 selected children, baseline MRDR tests were performed on 85 of them, ages 0.7 to 5.6 y. These children were further subdivided into two subgroups, A and B. A RDR test was performed on the first group (n=47) three to four weeks after the MRDR.
The second group (n=21) received a single dose of vitamin A (approximately 157 μmoles (150,000 IU) delivered from a capsule) just after the first MRDR was conducted. A subsequent MRDR was performed 2 weeks later.

Thereafter, 13 children who showed high DR/R ratios in the first MRDR test from the two subgroups (10 from subgroup A, 3 from subgroup B) were given one or two large oral doses of vitamin A from capsules (approximately 157-315 μmoles). After 2 weeks, the MRDR test was repeated on all willing and available subjects (n=8).

CIC specimens were obtained from 80 of the children enrolled in the study. The specimens were fixed and stored for staining.

High performance liquid chromatography:

Group I: The serum samples were extracted and analyzed as reported previously (Tanumihardjo et al. 1990). Briefly, 500 μl of serum was treated with ethanol and extracted twice with hexane. The extracts were pooled, dried under nitrogen and redissolved in 50 μl isopropanol. All-trans retinyl acetate was used as an internal standard to determine extraction efficiencies. An aliquot of 40 μl was injected onto a 5-μm Waters 'Resolve' reversed-phase column via a Rheodyne 7125 injector. The methanol:water (87.5:12.5 V/V) solvent was pumped through the system with a 6000A Waters
delivery system at 1 ml/min. Absorbance was monitored at 350 nm by use of a Waters-λmax model 480 detector. A Shimadzu CR601 Chromatopac integrator was employed to calculate peak areas. DR and R values were quantitated against authentic standards, and the ratio was determined.

Group II: The HPLC system described above was used, except that the solvent was changed to 90:10 methanol:water and the detector was replaced by a Shimadzu SPD-6AV UV-VIS absorbance detector.
RESULTS

The weights of the two groups of children are categorized in Table 1 as a percentile of the criteria set by the World Health Organization (1983). No child in Group I showed a weight-for-age above the 40th percentile, whereas 12% of those in Group II did. Although both groups were small for age by these criteria, Group I was affected more by malnutrition, with 71% of the children below the third percentile compared with 36% in Group II.

Table 1. Weight-for-age data for both groups of Indonesian children with percentile range rankings of the WHO reference standards

<table>
<thead>
<tr>
<th>Percentile range</th>
<th>Group I n</th>
<th>% of total</th>
<th>Group II n</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;3</td>
<td>53</td>
<td>70.7</td>
<td>30</td>
<td>36.1</td>
</tr>
<tr>
<td>3-5</td>
<td>12</td>
<td>16.0</td>
<td>10</td>
<td>12.0</td>
</tr>
<tr>
<td>5-10</td>
<td>7</td>
<td>9.3</td>
<td>9</td>
<td>10.8</td>
</tr>
<tr>
<td>10-20</td>
<td>1</td>
<td>1.3</td>
<td>12</td>
<td>14.5</td>
</tr>
<tr>
<td>20-30</td>
<td>1</td>
<td>1.3</td>
<td>8</td>
<td>9.6</td>
</tr>
<tr>
<td>30-40</td>
<td>1</td>
<td>1.3</td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td>40-50</td>
<td></td>
<td></td>
<td>2</td>
<td>2.4</td>
</tr>
<tr>
<td>50-60</td>
<td></td>
<td></td>
<td>4</td>
<td>4.8</td>
</tr>
<tr>
<td>60-70</td>
<td></td>
<td></td>
<td>3</td>
<td>3.6</td>
</tr>
<tr>
<td>70-80</td>
<td></td>
<td></td>
<td>1</td>
<td>1.2</td>
</tr>
</tbody>
</table>
The distributions of serum retinol concentrations in Groups I and II are shown in Figure 1. The mean serum retinol concentrations (± S.D.) of Groups I and II were 0.684 ± 0.240 and 0.904 ± 0.253 μmoles/L, respectively. The distribution of values in Group I was biphasic, while that of Group II was more normally distributed.

RDR and MRDR assays:

The distributions of the MRDR ratios in Groups I (n=75) and II (n=85) are shown in Figure 2. A significant difference was noted between the MRDR responses of the two groups (F=15.73, p<0.0001). Difficulty was encountered in applying the RDR assay to children in Group I. When the specified oral dose of 1.57 μmoles (450 μg as retinol) retinyl acetate was administered, the mean RDR value was 2.5 ± 12.9%. This value is not significantly different from 0 (p>0.10). Although 10% of the children had RDR values ≥20%, the variation was excessively large. Even children who were diagnostically deficient, i.e., with serum retinol concentrations ≤0.350 μmoles/L (≤10 μg/dL), did not show a response to the 1.57 μmole (450 μg) retinol dose. For example, the RDR values of two children with retinol concentrations of 0.33 and 0.15 μmole/L (9.5 and 4.4 μg/dL) were 3% and 2%, respectively. In contrast, 48% of the
Figure 1. The serum retinol (μmoles/L) distribution curves for both Group I and Group II
Figure 2. The 3,4-didehydroretinol to retinol ratio distributions for both Group I (bottom) and Group II (top)
children in Group I showed DR/R ratios $\geq 0.06$, which is considered an abnormal response.

In Group II, 12% and 11% of the children tested by the MRDR method (cutoff value of 0.06) and by the RDR procedure (cutoff value of 20%), respectively, gave abnormal values (Table 2). Of the 47 children in Group II who were examined by both the MRDR and the RDR tests, the two tests were concordant in 3 of the abnormal cases and 39 of the normal cases and discordant in the remaining 5 children.

In two of the five discordant cases, (#7 and #8), *Ascaris* infections were initially present (Table 3). These children were treated for roundworms at the time of the MRDR test and would have been cleared of the infection before the RDR test. Thus, the initial DR/R ratios may have been low because of the slower intestinal absorption of the dose of DR in the presence of roundworms. This point warrants further investigation.

**Conjunctival impression cytology (CIC):**

Of the 55 CIC specimens that were collected from Group I, 10 (18%) were abnormal in both eyes (AA), 18 (33%) were abnormal (NA) in one eye and 27 (49%) were normal (NN) in both eyes. In Group II, 80 CIC specimens were collected: none (0%) was AA, 4 (5%) were NA, and 76 (95%) were NN (Table 4).
Table 2. Group comparisons for the modified relative dose response (MRDR), conjunctival impression cytology (CIC) and the relative dose response (RDR)

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>n</th>
<th>Abnormal</th>
<th>% Abnormal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRDR</td>
<td>75</td>
<td>36</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>RDR</td>
<td>50</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIC</td>
<td>55</td>
<td>28</td>
<td>51</td>
</tr>
<tr>
<td>Group II:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MRDR</td>
<td>85</td>
<td>10</td>
<td></td>
<td>12</td>
</tr>
<tr>
<td>RDR</td>
<td>47</td>
<td>5</td>
<td></td>
<td>11</td>
</tr>
<tr>
<td>CIC</td>
<td>80</td>
<td>4</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

aValues not reported.
bAbnormality in one or both eyes is considered as an abnormal response.

Table 2 also demonstrates the population correlation between the MRDR and CIC tests in Group I and the MRDR, RDR and CIC tests in Group II. When NA is classified as an abnormal response, the degree of marginal vitamin A status agreed better with the MRDR results in both groups and with
the RDR results in Group II than when AA was considered as the only abnormal response.

Table 3. Comparison between the modified relative dose response (MRDR) and relative dose response (RDR) tests in individuals showing abnormal responses in one or both tests

<table>
<thead>
<tr>
<th>Subject</th>
<th>MRDR</th>
<th>RDR</th>
<th>Observed</th>
<th>MRDR after megadose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>DR/R</td>
<td>%</td>
<td>Illness</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>0.186</td>
<td>30</td>
<td>---</td>
<td>0.050</td>
</tr>
<tr>
<td>2</td>
<td>0.088</td>
<td>0</td>
<td>---</td>
<td>0.020</td>
</tr>
<tr>
<td>3</td>
<td>0.076</td>
<td>8</td>
<td>---</td>
<td>0.067</td>
</tr>
<tr>
<td>4</td>
<td>0.069</td>
<td>24</td>
<td>---</td>
<td>0.045</td>
</tr>
<tr>
<td>5</td>
<td>0.066</td>
<td>10</td>
<td>---</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>0.064</td>
<td>29</td>
<td>URTI&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.020</td>
</tr>
<tr>
<td>7</td>
<td>0.045</td>
<td>20</td>
<td>Ascaris&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.051</td>
</tr>
<tr>
<td>8</td>
<td>0.042</td>
<td>23</td>
<td>Ascaris&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.030</td>
</tr>
</tbody>
</table>

<sup>a</sup> Upper respiratory tract infection.

<sup>b</sup> Treatment given at time of first MRDR for roundworms.

<sup>c</sup> Not determined.
<table>
<thead>
<tr>
<th></th>
<th>NN</th>
<th>NA</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I (n=55)</td>
<td>49%</td>
<td>33%</td>
<td>18%</td>
</tr>
<tr>
<td>Group II (n=80)</td>
<td>95%</td>
<td>5%</td>
<td>0%</td>
</tr>
</tbody>
</table>

Where N is a normal reading and A is abnormal.
DISCUSSION

The MRDR has been tested and validated in rats of varying vitamin A status by directly measuring liver stores of retinol and its esters (Tanumihardjo, Barua and Olson 1987; Tanumihardjo and Olson 1988; Tanumihardjo et al. 1990). At present the MRDR has been applied to children and adults in several population groups around the world. In this investigation, the MRDR and CIC tests have been compared with each other as well as with the RDR assay, which has been validated against liver reserves in humans (Amedee-Manesme, Anderson and Olson 1984).

On the basis of these comparisons, Indonesian children with DR/R ratios ≥0.06 were judged to be in a marginal vitamin A status. A cutoff of 0.03 was suggested earlier on the basis of ratios found in healthy American adults and well-nourished children. On the other hand, approximately 30% of American children in a lower socioeconomic status have shown ratios ≥0.03 (Tanumihardjo, Koellner and Olson 1990, Spannaus-Martin et al. 1992), although no American child has thus far yielded a ratio of ≥0.06. Children with ratios between 0.03 and 0.06 fall in an intermediate category in which an increased intake of vitamin A may be beneficial.

Similar distributions of the retinol concentrations found in the two groups have been observed in studies in Guatemala (Arroyave 1986) and in Brazil (Flores 1991). The relationship
was observed in a Guatemalan group of preschool children before and after a sugar fortification intervention with vitamin A and in Brazil before and after supplementation with a large dose of vitamin A.

The CIC test identified the same group as being at an increased risk of vitamin A deficiency as the MRDR. Moreover, when an abnormal impression in a single eye rather than in two was considered as a positive CIC response, a similar percentage of the groups were designated as abnormal (Table 2) by using either MRDR or CIC. Thus, Group I had 48% with MRDR responses ≥0.06 and CIC was 51% abnormal when both abnormal-normal (AN) and abnormal-abnormal (AA) were considered as abnormal. However, when AA was used, only 18% of the group was considered abnormal. A similar observation was found in Group II, where 12% were abnormal by using the MRDR assay, 5% by using AN in the CIC test and 0% by using AA in the CIC test.

In considering solely the transport of holo-RBP in the plasma and its uptake by retinal pigment epithelial cells, one would expect that both eyes would be affected more or less equally by vitamin A deficiency. However, Bitot’s spots are commonly found in only one eye in field surveys of very young children, and contralateral blindness frequently results from vitamin A deficiency. Therefore, classifying an abnormal impression in only one eye as an indicator of an inadequate vitamin A status accords well with clinical observations.
Nonetheless, the possibility exists that the abnormal CIC specimen may have been inappropriately taken or incorrectly interpreted, which would provide an overestimation of an inadequate status.

By using AN as an abnormal indicator in a group of Indonesian lactating women (Stolzfus et al. 1993), a significant decreasing trend in abnormal CIC was noted in the vitamin A treated group but not in the placebo group over a 6 month period. Also relative to the use of AA, sensitivity was increased nearly two-fold when AN was used as an indicator of risk of low milk retinol concentration.

The negative RDR results obtained in Group I with a dose of 1.57 μmoles retinyl acetate was at first unsettling, especially since 8% of the children tested had serum retinol values ≤0.35 μmoles/L. However, similar results were found in a group of Thai children when 2.1 μmoles retinyl palmitate was administered (Udomkesmalee et al. 1992). In that study only 2 of the children (n=133) tested gave positive RDR values. In fact, more of the children must have been at risk of deficiency because both CIC and serum retinol concentrations improved after supplementation with vitamin A. In two other studies in Indonesia, one in children (Jean Humphrey, personal communication) and the other in lactating women (Stoltzfus et al. 1993), the RDR test also gave inconsistent results when an oral dose of 1.57 μmoles was administered. The mean responses were close to zero with very high standard deviations. Thus,
the dose must not have been large enough to elicit an appropriate liver response in these Indonesian children and women.

Dose response tests should detect vitamin A depletion before histological changes occur on the surface of the eye (Olson 1992). Therefore, when properly applied, the MRDR and RDR should be positive before abnormal cytology is observed in an individual. Similarly, recovery from an inadequate status should be detected more rapidly by biochemical than by histological procedures.

Time between testing and retesting with the dose response tests seems to be critical. Our experience with group I led us to believe that one to two weeks was too soon to re-administer a dose and expect a reproducible response. Perhaps in children where protein status is not satisfactory, a longer time period is needed to reaccumulate the apo-retinol binding protein (RBP) in the liver. When the RDR test was performed 1 week apart in a group of Guatemalan adults, agreement between the two tests was rather poor (Bulux et al. 1992). Furthermore, the dose of retinol used in this study (1.68 μmoles) was similar to that (1.57 μmoles) which gave poor results in our study.

In a group of Guatemalan children, in which the RDR and the MRDR assays were compared (De Paz 1991), the correlation between the two tests was only fair. In this case as well, confounding factors were: 1) that the RDR test was performed
only one week before the MRDR, and 2) that low doses [1.57 μmoles vitamin A (RDR) and 0.18 μmole 3,4-didehydroretinyl acetate/kg body weight (MRDR)] were used in both tests.

By using the RDR assay, the vitamin A statuses of breastfed Indonesian infants and their mothers have been compared; positive RDR values were found in 23% of the infants tested but only in 1.5% of the unsupplemented mothers (Stoltzfus et al. 1993). The same low dose (1.57 μmoles retinyl acetate) was used for both the infants and their mothers. If the dose administered to the mothers had been increased, a higher proportion of positive RDR values might have been found.

Thus, by increasing the dose to 3.5 μmoles and increasing the time between the two dose response tests to 3 weeks in our studies, good agreement was found between the RDR and MRDR tests (Table 2, Group II).

The MRDR is a minimally invasive technique by which a marginal vitamin A status can be detected in individual children before clinical manifestations appear. Correlation in individuals of MRDR values with their liver vitamin A concentration would be the most useful in defining the best ratio to be used as a cutoff between a marginal and satisfactory vitamin A status. Liver biopsy samples cannot be obtained in field studies. Therefore, by correlating the MRDR to the RDR, which has been validated against liver reserves of vitamin A, a ratio of ≥0.06 seems to be more predictive of
vitamin A depletion in less industrialized countries than the previously suggested ratio of 0.03, which was based on well-nourished American adults and children (Tanumihardjo, Koellner and Olson 1990; Tanumihardjo et al. 1990; Tanumihardjo and Olson 1991).

To improve the effectiveness of these response assays, we are currently recommending doses of: 1) 3.5 μmoles for the RDR (when HPLC methods are used), 2) 0.35 μmoles/kg for the MRDR test in children <25 kg in body weight, and 3) 8.8 μmoles for children ≥25 kg and for adults.
REFERENCES


PAPER 3.

ASSESSMENT OF THE VITAMIN A STATUS IN LACTATING AND IN NON-LACTATING, NON-PREGNANT INDONESIAN WOMEN BY USE OF THE MODIFIED RELATIVE DOSE RESPONSE (MRDR) ASSAY
Assessment of the vitamin A status in lactating and in non-lactating, non-pregnant Indonesian women by use of the modified relative dose response (MRDR) assay


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ABSTRACT

By use of the modified relative dose response (MRDR) assay, the vitamin A statuses of two groups of Indonesian women were compared: 1) lactating, non-pregnant women of lower socioeconomic status (n=64) and 2) better educated, premenopausal, non-pregnant, non-lactating women (n=14). At times from 3 to 6 h after dosing, the mean ratio of dehydroretinol to retinol (DR/R) in the serum was approximately 3-fold higher in the lactating women than in the control group, e.g., 0.109 ± 0.073 and 0.034 ± 0.015, respectively at 5 h. By using a DR/R cutoff ratio of 0.06, the vitamin A status of 70% of the lactating women and of 7% of the control women was inadequate. Only 7% of the variability in abnormal MRDR responses could be attributed to body weight. Both abnormal and normal responses were highly reproducible when performed 0.5-3.5 months after the first test.
INTRODUCTION

The modified relative dose response (MRDR) assay has been used in both children (Tanumihardjo, Koellner and Olson 1990; Tanumihardjo et al. 1990; Spannaus-Martin et al. 1992) and healthy adults (Tanumihardjo et al. 1990; Tanumihardjo and Olson 1991) to assess vitamin A status. The MRDR assay has been validated in rats against liver reserves of vitamin A (Tanumihardjo, Barua and Olson 1987; Tanumihardjo and Olson 1988; Tanumihardjo et al. 1990). The MRDR assay is similar in principle to the relative dose response (RDR) assay for assessing vitamin A status, which has been validated in humans and used as a survey technique in several contexts (Amatayakul et al. 1989; Amedee-Manesme, Anderson and Olson 1984; Amedee-Manesme et al. 1987, Flores et al. 1984). Briefly, as vitamin A depletion worsens, apo-retinol binding protein (RBP) accumulates in the liver (Soprano, Smith and Goodman 1982). When a moderate dose of either retinyl acetate or 3,4-didehydroretinyl acetate is orally administered, the retinol or dehydroretinol binds to liver apo-RBP, which is rapidly secreted into the plasma as the holo-RBP complex. Two blood samples are required in the RDR test. The percentage change is calculated by the formula, \([(A5-A0)/A5] \times 100\), where A5 and A0 are the serum retinol concentrations at 5 hours after dosing and at time 0, respectively. Values of \(\geq 20\%\) are considered abnormal. Because circulating concentrations of
dehydroretinol (DR, vitamin A2) are typically very low (<3.5 nmoles/L, or 1 ng/mL), only one blood sample is required in the MRDR test. Currently, a molar DR/R ratio of ≥0.06 is considered abnormal in third world countries (Tanumihardjo et al. 1992).

Lactating Indonesian women show a significantly higher serum retinol concentration after supplementation with vitamin A as compared to a placebo-treated control group (Stoltzfus et al. 1993). Furthermore, by using the RDR as an indicator, the vitamin A status of breast-fed infants was significantly better in the supplemented than in the unsupplemented mothers (Stoltzfus et al. 1993). Pregnant Indonesian women are also at an increased risk of deficiency (Suharno et al. 1992). In a cross-sectional study of West Javanese women, 34% of the sample (n=318) had serum retinol concentrations <0.70 µmoles/L and 9% of a subsample gave positive responses in RDR tests (n=45). Suharno et al. (1992) concluded that these women would benefit from a vitamin A supplementation program during pregnancy.

The consequences of vitamin A deficiency during pregnancy and lactation have not been fully realized. Although spontaneous abortion and congenital defects can result from severe vitamin A deficiency in animals, the effects of vitamin A deficiency in women are not as clear (Wallingford and Underwood 1986). However, the probability for survival of young children with suboptimal vitamin A status, at least in
some geographical areas, is significantly decreased (Rahmathullah et al. 1990; Sommer, Katz and Tarwotjo, 1984; Sommer et al. 1986; West 1991).

In this study, we have investigated the responses at various times after administering a standard oral dose (8.8 μmoles) of 3,4-didehydroretinyl acetate to groups of lactating and of non-pregnant, non-lactating Indonesian women. Furthermore, we have assessed the reproducibility of abnormal and normal responses in lactating women at various times after the first test.
SUBJECTS AND METHODS

The MRDR response over time:

Subjects were recruited from the suburban areas surrounding Bogor in West Java, Indonesia. The sample consisted of 64 lactating mothers aged 17 to 37 years with 1 to 10 children. The age of their breastfed infants was between 2 and 4 months. The control group (n=14) was recruited from the staff at the Nutrition Research and Development Center in Bogor. The control women were premenopausal, non-pregnant women aged 29-41 with 0 to 4 children. Informed consent procedures were employed that were in full accord with the guidelines established by the University Committee on the Use of Human Subjects in Research of Iowa State University and by Indonesian authorities with the same responsibilities.

On the day of the study, each mother was given a dose of 8.8 \( \mu \)moles (2.5 mg) 3,4-didehydroretinyl acetate (DR-acetate) dissolved in corn oil in the morning, usually at her home. The dose was followed by a high-fat, low vitamin A snack. The DR-acetate, which was synthesized from retinoic acid (Barua and Ghosh 1972; Tanumihardjo, Barua and Olson 1987), was carefully purified by column chromatography, and then was dissolved in corn oil by using sonication.
Later in the morning, the mothers were brought to a volunteer's home. Height and weight were measured and questions were asked regarding parity, age and any recent or current illnesses. At this time, venous blood samples were drawn from antecubital veins at 3 and 5 h (n=31) or at 4 and 6 h (n=33). Of the 78 women in the study only one subject went home before the second blood sampling. Blood samples were placed on ice inside a closed cooler while in the field, were carried to the laboratory and then were immediately centrifuged. Serum was stored frozen at -20°C until analysis.

For the control women, the dose was administered upon their arrival at the clinic of the Nutrition Research and Development Center. Blood samples were drawn in a central location close to the laboratory. High-fat snacks and a low-vitamin A containing lunch were provided for the participants.

The reproducibility of the MRDR:

At various times (0.5 to 3.25 months) after the first test, the MRDR assay was repeated on 14 of the same lactating women. The women were again dosed with 8.8 μmoles of DR-acetate and a single blood sample was drawn 5 h after dosing.
Extraction and the high-pressure liquid chromatography (HPLC) system:

In a dimly-lit room, 500 \( \mu l \) aliquots of thawed serum were treated with an equal volume of ethanol to precipitate proteins and then were extracted twice with hexanes. Retinyl acetate dissolved in ethanol was used as an internal standard to determine extraction efficiencies. The hexane layers were pooled and evaporated to dryness with nitrogen. The sample was redissolved in 50 \( \mu l \) of a 3:1 ratio (v/v) of methanol to methylene dichloride; then 40 \( \mu l \) was injected onto the HPLC system via a Rheodyne 7125 manual injector. A Shimadzu SPD-AV UV-VIS absorbance detector monitored the wavelength at 350 nm, which optimizes detection of DR. A Waters 6000A pump delivered 90:10 methanol:water at a flow rate of 1 mL/min to the 5-\( \mu m \) Waters "Resolve" 15 cm reversed-phase column. A Shimadzu CR601 Chromatopac integrator calculated peak areas. DR and R were quantitated against authentic standards (Tanumihardjo et al. 1990).
Anthropometric and social characteristics:

The mean body weight of the lactating women was 47.0 ± 5.2 (± S.D.) kg, while that of the controls was 53.2 ± 6.0 kg. The mean number of children was 2.7 ± 1.9 and 2.2 ± 1.4 for the lactating and control women, respectively. The mean ages of the groups were 24.8 ± 5.0 and 35.6 ± 4.0 y for the lactating and control groups, respectively. The two groups differed significantly in weight (p<0.001) and age (p<0.001) but not in parity. The non-pregnant, non-lactating control group consisted of women who were better educated and had a general knowledge of vitamin A and nutrition.

MRDR ratio versus time:

At all times studied the mean responses of the lactating women were approximately 3 times higher than those of the control group (Table 1, Figure 1). The mean slopes of the individual responses between the groups showed a highly significant difference (p<0.001, t=6.08, df=75).
Table 1. The modified relative dose response (MRDR) in lactating and control women at various times after dosing

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>n</th>
<th>DR/R ratio (Mean ± S.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lactating</td>
</tr>
<tr>
<td>3</td>
<td>31</td>
<td>0.053 ± 0.037</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>0.076 ± 0.043</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>0.114 ± 0.091</td>
</tr>
<tr>
<td>6</td>
<td>33</td>
<td>0.136 ± 0.066</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0.016 ± 0.007</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>0.028 ± 0.011</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>0.032 ± 0.014</td>
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<tr>
<td>6</td>
<td>6</td>
<td>0.047 ± 0.025</td>
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</tbody>
</table>

*Time points 3 and 5 h and 4 and 6 h are from the same individuals.

A 5-h-predicted value for half of the subjects was calculated by taking the average of the 4 and 6 h points for both the lactating and control groups. The mean DR/R ratios obtained by combining 5-h and 5-h-predicted values for lactating and control groups, were 0.109 ± 0.073 and 0.034 ± 0.015, respectively. These two values also showed a highly significant difference (p<0.001, t=7.46, df=75).
Figure 1. The change in the ratio of 3,4-didehydroretinol to retinol (DR/R) over time in lactating (●) and control (○) Indonesian women.
Response versus body weight:

Figure 2 shows the 5-h and 5-h-predicted responses versus body weight. When data from all lactating women are included, there is a significant negative correlation (p<0.005). Even when the extremely malnourished woman (body weight = 32.5 kg, age 21 y) is excluded from the analysis, the relationship is still significant (p<0.05). When all subjects or all subjects except for this malnourished woman are considered, however, only 15% and 7%, respectively, of the variability in MRDR responses can be accounted for by body weight.

The MRDR values of the control group also show a significant negative correlation to body weight (p<0.01). However, the two lines are completely different, with DR/R ratio intercepts at 0 kg body weight of 0.37 (0.27 for n-1) and 0.11 for lactating and control women, respectively. The slopes of the lines are close to zero, with values of -0.0055 (-0.036 for n-1) and -0.0015 for the lactating and control groups, respectively.

Serum retinol concentrations:

The mean serum retinol concentrations were 0.99 ± 0.35 and 1.47 ± 0.31 μmoles/L for the lactating and control women, respectively. These means are significantly different (p<0.001). Nonetheless, the ranges of values for the two
Figure 2. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to body weight in lactating (●) and control (○) Indonesian women.
groups showed considerable overlap, with a range of 0.43 to 1.83 μmoles/L for the lactating women and 1.05 to 2.0 μmoles/L for the control women. The relationship between the 5-h-predicted and actual 5 h DR/R responses and serum retinol concentrations is shown in Figure 3. Using 0.06 as a cutoff for an abnormal DR/R ratio, all but one woman with a serum retinol concentration <0.70 μmoles/L tested positively. Moreover, when serum retinol concentrations were >1.4 μmoles/L, all but one woman tested normally. Similar relationships have been found in children, except that the lower abnormal cutoff value of serum retinol for children is 0.35 μmoles/L (Tanumihardjo et al. 1990).

The DR/R response for the lactating women is negatively correlated to serum retinol with r=-0.61, (p<0.001). One would expect some correlation between these indicators, in as much as R is the denominator of the DR/R ratio. In the control group alone, the serum DR/R ratio is also correlated (r=-0.59) with serum retinol. However, the slopes and intercepts are again completely different. The slopes were -0.13 and -0.028, and the DR/R ratio intercepts were 0.24 and 0.076 for the lactating and control women, respectively.
Figure 3. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to serum retinol concentrations in lactating (●) and control (○) Indonesian women. Abnormal DR/R ratios are ≥0.06. Serum retinol concentrations of <0.7 and >1.4 μmoles/L are abnormal and normal, respectively.
Reproducibility of the DR/R ratio:

In seven of the 14 retested lactating women, the second MRDR assay was performed 2 to 3.25 months after the first test (subjects A through G). In the other 7 women, retesting was performed within one month (subjects H through N). Figures 4 and 5 illustrate the changes in ratios. In subjects A through G, all the women tested abnormally at both times. In subjects H through N, 5 women tested abnormally both times, subject L tested normally both times, and subject K's response changed from a borderline normal value of 0.048 to a borderline abnormal reading of 0.060. The mean absolute change of the DR/R ratio with time was more variable in the group that was retested \( \geq 2 \) months after the first test than in that retested \( \leq 1 \) month, e.g., 0.083 ± 0.071 for subjects A through G, and 0.029 ± 0.017 for subjects H through N. This difference approaches significance (p<0.10). The DR/R ratio also became more positive from the first to the second assay in 4 of 7 of the first group; e.g., subjects A, B, D and F. All of these women continued to breastfeed during the interval, and retesting was performed at least 3 months after the first MRDR.

One of the control women tested positively to the MRDR, i.e., her DR/R 5-h-predicted ratio was 0.070. One week later a repeat 5-h DR/R ratio was 0.077. An informal dietary questionnaire revealed that her diet was indeed low in both
Figure 4. The change in the ratio of 3,4-didehydroretinol to retinol (DR/R) in 7 individuals (subjects A through G) who were retested ≥2 months after the first DR/R ratio determination.
Figure 5. The change in the ratio of 3,4-didehydroretinol to retinol (DR/R) in 7 individuals (subjects H through N) who were retested within 1 month after the first DR/R ratio determination.
preformed vitamin A and provitamin A-containing foods. She was not supplemented with vitamin A but instead was encouraged to incorporate more green leafy vegetables into her daily meals. Her mean serum retinol concentration was $1.07 \pm 0.11 \mu\text{moles/L}$ from 4 determinations. This subject’s body weight was 43.6 kg, which was on the lower end of the control group. However, another control subject with a body weight of 42.3 kg tested normally with a 5-h predicted DR/R ratio of 0.025. The informal dietary questionnaire showed that this control subject consumed vitamin A-fortified milk daily.
DISCUSSION

The vitamin A status of a group of Indonesian lactating women from the suburban areas surrounding Bogor, West Java was assessed using the MRDR. Abnormal MRDR responses (DR/R ≥0.06) were observed in 70% of these women, which indicates that their nursing infants are at potential risk of developing vitamin A deficiency. By using serum retinol concentrations alone, only 20% of the sample would have been considered in a marginal vitamin A status using a cutoff value for serum retinol of ≤0.7 μmoles/L. Thus, the MRDR classified more women with a marginal status than serum retinol values alone.

Within the time frame studied, 3 to 6 h, the DR/R ratio continued to rise. Previous experience has shown us that the ratio begins to fall in normal individuals between 8 and 12 h (Tanumihardjo et al. 1990). At all times studied, the DR/R ratio was approximately 3-times greater in the lactating women than in the control women. Thus, the standard 5-h sampling time still seems best for field surveys, although samples taken 4-h to 6-h after dosing still yield useful results. Our field conditions did not allow us to take samples beyond a 6-h interval. In a clinical setting, longer time intervals, i.e., 8-h and 10-h, may be selected to define the plateau of the DR/R ratio in a vitamin A-depleted population. In vitamin A depleted rats, for example, the DR/R ratio was still inversely related to liver reserves 30-36 h after a large dose of DR-
acetate (1.76 μmoles/rat) (Tanumihardjo, Barua and Olson 1987).

The response to the dose was very different between the two groups as analyzed by the mean slopes of the individual responses. The holo-RBP complex was apparently released more rapidly from the livers of lactating women than from those of the controls.

Although a significant relationship was found in the analysis of body weight versus DR/R ratio when a standard dose was administered, only 7% of the variability in the MRDR responses, excluding one malnourished women, could be attributed to differences in body weight. Thus, the response clearly is much more dependent on vitamin A status than on body weight. To simplify field procedures, a standard dose of 8.8 μmoles of 3,4-didehydroretinyl acetate was employed in our current studies in adult women. Use of a standard dose will help to simplify field procedures. Although this dose of DR-acetate worked well for individuals ≥25 kg, further testing is needed to determine whether this dose or a smaller standard dose should be used in future MRDR surveys of children.

In past studies with children, the dose of DR-acetate has been based upon body weight (0.35 μmoles/kg). Previously, we found that the DR/R ratio in a single child was increased more than 2-fold with a 5-fold increase in dosage level (Tanumihardjo et al. 1990). Thus, cutoff values may be affected by the dosage that is selected.
Because of the extensive overlap of serum retinol concentrations between abnormal and normal responders to dose response tests, the lack of utility of serum retinol as an indicator of an individual's vitamin A status is again demonstrated. However, serum retinol concentrations <0.7 μmoles/L in lactating Indonesian women were associated with a high percentage of abnormal MRDR responses and those above 1.4 μmoles with normal MRDR responses. The one subject whose MRDR was normal (DR/R = 0.048) but whose serum retinol was 0.69 μmoles/L was, at the time of testing, suffering from an infection resulting in a fever that persisted for more than three days. Infections are known to depress serum retinol values. In another case, a normal MRDR response was found in a woman whose serum retinol was reduced 30% by infection (Tanumihardjo and Olson 1991).

Normal serum reference curves for children (Flores et al. 1991) and for adults clearly are different (Pilch 1987). In the NHANES I and SW HHANES surveys in the United States, only 0.2% and 1.1% of adult women had serum concentrations <0.7 μmoles/L, respectively (Pilch 1987). In contrast, 92% of the lactating women in our study with serum retinol concentrations <0.7 μmoles/L had abnormal DR/R ratios (>0.06). Thus, the vitamin A status of groups of women in surveys where only serum retinol data are available might be estimated. Nonetheless, confounding factors, such as infections, protein
nutriture and zinc status, should restrain such generalization.

In normal, healthy individuals, the DR/R ratio is very reproducible over time (Tanumihardjo and Olson 1991). At all time points the mean ± S.D. was 0.015 ± 0.005 for 7 American individuals having 4 MRDR determinations during a 7 month period. The MRDR values for a given individual in this group never changed from normal to abnormal.

When the MRDR test was repeated in 14 lactating Indonesian women at varying time intervals from 0.5 to 3.25 months, the MRDR values in 13 of the women were concordant. The other individual showed borderline DR/R ratios, first normal and then abnormal. The mean absolute change in the DR/R ratio of the lactating group was more variable when retesting was done ≥2 months after the first test (0.083 ± 0.071) than when retesting was ≤0.75 months (0.029 ± 0.017). During the longer period, the vitamin A status, of course, may have changed. Four of the 7 women who were retested 3 to 3.25 months after the first assay had a net increase in ratio. These women, who continued to breastfeed, may well have become further depleted during this time. Moreover, the only control subject with an abnormal response (DR/R ratio of 0.070), gave a similar value (0.077) upon retesting.

When the RDR assay was first developed, a standard dose of 1.57 μmoles of retinyl acetate in corn oil was recommended for adults and children (Flores et al. 1984). In subsequent
studies, in which 1.57 and 2.11 μmoles of retinyl acetate were used in less industrialized countries, RDR responses were unexpectedly found to be normal in both adults and children who otherwise showed signs of inadequate vitamin A status (Tanumihardjo et al. 1992; Udomkesmalee et al. 1992; Stoltzfus et al. 1993). In studies using a dose of 1.68 μmoles, the reproducibility of the RDR test in a population of Guatemalan adults has been questioned (Solomons et al. 1990; Morrow et al. 1990; Bulux et al. 1992). We have also encountered some inconsistencies in the RDR test results at this dosage level (S. A. Tanumihardjo, unpublished observations). If the administered dose of retinyl acetate is increased to 3.5 μmoles, however, RDR values are both reproducible and generally concordant with MRDR values in individuals (S. A. Tanumihardjo, unpublished observations). Whether doses of retinyl acetate higher than 3.5 μmoles will yield even more consistent RDR values should be explored.

In this study, the MRDR test clearly identified lactating Indonesian women as having a marginal vitamin A status, thereby potentially placing their nursing infants at risk of vitamin A deficiency. Although vitamin A supplementation is available to children through local health clinics in developing countries, less attention has been paid to the vitamin A status of nursing and pregnant women. Because of possible teratogenic effects of large doses of vitamin A on fetal development, supplementing these groups with vitamin A
poses some special problems. Currently, the World Health Organization recommends supplementing lactating mothers with a 200,000 IU capsule (0.21 mmole) of retinyl palmitate only within the first month post-partum (World Health Organization 1988). After this time, the risk of another pregnancy increases. Thus, if the logistics are feasible, safe, low dose oral supplements of vitamin A (<8000 IU) should be made available to lactating and pregnant mothers in at-risk populations. Indeed, provitamin A carotenoids, which show no toxicity, might be preferable as supplements.
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APPLICATION OF THE MODIFIED RELATIVE DOSE RESPONSE (MRDR)
ASSAY TO PREGNANT INDONESIAN WOMEN FOR
ASSESSING VITAMIN A STATUS
Application of the modified relative dose response (MRDR) assay to pregnant Indonesian women for assessing vitamin A status

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ABSTRACT

The modified relative dose response (MRDR) assay for vitamin A status has been applied to a group of pregnant Indonesian women (n=144) from West Java. A standard dose of 8.8 μmoles (2.5 mg) 3,4-didehydroretinyl acetate was administered orally in corn oil and a single blood sample was drawn 5 h post-dosing. The mean dehydroretinol/retinol (DR/R) ratio was 0.039 ± 0.031. Of women tested, DR/R ratios ≥0.06 were found in 17%, between 0.03 and 0.06 in 35%, and ≤0.03 in 48%. A subgroup of these women (n=27) was randomized into four supplemental groups, and MRDR assays were repeated 8 weeks after the beginning of supplementation. The daily supplements given were placebo (n=7), 8.4 μmoles (8000 IU) vitamin A (n=7), 1.07 mmoles (60 mg) iron (n=5), and 8.4 μmoles vitamin A plus 1.07 mmoles iron (n=8). The MRDR ratio was reduced more markedly by the combined supplement of vitamin A and iron than by either nutrient alone. The mechanism of the enhancing effect of iron on the vitamin A-induced reduction in MRDR values is not known. If a prevalence of >10% of DR/R ratios of ≥0.06 is used a public health cutoff value, the vitamin A status of pregnant Indonesian women clearly merits attention.
INTRODUCTION

Vitamin A deficiency during pregnancy affects both the prospective mother and the fetus. While the manifestations of vitamin A deficiency during pregnancy have been clearly shown in animals, evidence for adverse effects in humans is indirect (Wallingford and Underwood 1986). Several recent studies in Indonesia have shown that indeed Indonesian pregnant and lactating women do not have a satisfactory vitamin A status (Suharno et al. 1992; Stoltzfus et al. 1993; Tanumihardjo et al. 1993).

Based on serum retinol <0.70 μmoles/L, 34% of a sample (n=318) of Indonesian women were considered to be in a marginal vitamin A status (Suharno et al. 1992). By applying the relative dose response test (Flores et al. 1984) to a subgroup of these women (n=45), 9% showed positive (abnormal) responses. Serum retinol alone is not a good indicator of status because it is homeostatically controlled. Moreover, hemodilution during pregnancy may also falsely put women into the marginal category. In a longitudinal study (Kelner et al. 1969), a 30% reduction was observed in serum retinol concentration from the first to the third trimester of pregnancy. Because the plasma volume expands approximately 50% during pregnancy, the concentrations of both vitamin A and its carrier protein are correspondingly reduced (King and Weininger 1990).
Before appropriate public health measures can be applied to a community, nutrient needs must be assessed. The modified relative dose response (MRDR) has been used in children (Tanumihardjo, Koellner and Olson 1990; Tanumihardjo et al. 1990; Tanumihardjo et al. 1992), low-income, pregnant American women (Duitsman et al. 1993) and lactating Indonesian women (Tanumihardjo et al. 1993) to assess vitamin A status. We have now applied the MRDR to a group of pregnant women (n=144) from ten different villages in West Java. Because of the prevalence of both vitamin A and iron depletion in developing countries, the effect on the MRDR with supplementation of both vitamin A and iron was investigated.
METHODS

Subjects:

Pregnant women (n=144), in the second or early third trimester, were recruited from ten different villages near Bogor in West Java, Indonesia. Ages ranged from 15 to 38 y and parity from 0 to 5 children. Informed consent guidelines were used as established by the University Committee on the Use of Human Subjects in Research of Iowa State University and by Indonesian authorities with the same responsibilities.

The study was done in conjunction with the local health posts ('posyandu'). Approximately 70% of the pregnant women used the Mother and Child Health Services available in the villages (West Java Health Office 1989). Anthropometric measurements included weight and height. Information on age and parity (both live children and miscarriages) was obtained. Gynecological exams were performed by a medical doctor.

MRDR assay:

The 3,4-didehydroretinyl acetate (DRA) was synthesized from retinoic acid (Barua and Ghosh 1972; Tanumihardjo, Barua and Olson 1987), purified and dissolved directly in corn oil using sonication. All of the women received a single dose of 8.8 μmoles DRA dissolved in corn oil in the morning. Women
were dosed either in their homes, at the health post or at the clinic. The dose was followed by high-fat, low-vitamin A snacks. Five h after dosing, a single blood sample was drawn from the antecubital vein. The blood was stored on ice in a light-protected cooler until transported to the laboratory. Clotted blood was centrifuged, and the serum was stored at -20°C until analysis.

Supplementation trial:

After the initial MRDR determination, the women were randomized into 4 supplementation groups: 1) a placebo, 2) 8.4 μmoles (8000 IU) vitamin A, 3) 1.07 mmol (60 mg) ferrous sulfate and 4) vitamin A plus iron. The daily supplementation was monitored by using a control card and check list by the cader (village volunteer) who was responsible for the administration of the doses. After eight weeks of supplementation, the MRDR assay was repeated on 27 of the pregnant women.

Extraction and the high-pressure liquid chromatography system:

Serum was thawed, and 500 μl aliquots were extracted and analyzed for dehydroretinol (DR) and retinol (R) by using HPLC. All extractions were done in a dimly-lit room. Retinyl acetate dissolved in ethanol was used as an internal standard.
to determine extraction efficiencies. The serum was treated with ethanol and then extracted twice with hexane. The hexane layers were pooled and dried under nitrogen. The samples were redissolved in 50 μl of 3:1 methanol:methylene dichloride, aliquots of which 40 μl were injected onto a 5-μm Waters "Resolve" 15-cm reversed-phase column. The wavelength of detection was set at 350 nm to optimize for DR. The flow rate was 1 mL/min of 90:10 methanol:water. HPLC purified standards were used to quantitate the DR and R.
RESULTS

Anthropometric and social characteristics:

The body weights of the women, at the time of the MRDR, ranged from 31.0 to 69.8 kg with a mean of 48.7 ± 7.0 kg. Figure 1 shows the relationship of the DR/R ratio to body weight. Although the DR/R ratio is significantly related to body weight (r=-0.224) in this sample, only 5% of the variability can be attributed to weight. The mean number of children was 1.5 ± 1.4 with a range of 0 to 5. As illustrated in Figure 2, the DR/R ratio is not correlated with parity. The mean age of the women was 23.1 ± 4.7 y. Similarly, the DR/R ratio is not significantly correlated with age (Figure 3).

When the women are analyzed by the village in which they live (Figure 4), the mean DR/R ratio ranges from 0.032 ± 0.020 (village five) to 0.058 ± 0.052 (village seven). The number of women from each village studied ranged from 5 in village five to 29 in village three. The DR/R ratio of the 144 women ranged from 0.005 to 0.222, with a mean of 0.039 ± 0.031.

Serum retinol concentration:

The mean serum retinol concentration was 0.89 ± 0.26 μmoles/L with a range of 0.33 to 2.09 μmoles/L. Figure 5
Figure 1. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to body weight in pregnant Indonesian women.
Figure 2. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to parity.
Figure 3. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to the age of the mother.
Figure 4. The mean ratios of the 3,4-didehydroretinol to retinol (DR/R) in 10 Indonesian villages
Figure 5. The relationship of the ratio of 3,4-didehydroretinol to retinol (DR/R) to serum retinol concentration.
shows the relationship of the DR/R ratio to serum retinol concentration. Using the 0.06 cutoff for subclinical deficiency, 17% of the women would be classified as having inadequate liver reserves. Thirty-five percent of the women (n=51) fell into the intermediate zone of 0.03 to 0.06 and 48% of the women tested normally with a DR/R <0.03. All but one woman with a serum retinol ≤0.53 µmoles (n=11) had a DR/R ratio ≥0.03. Moreover, all the women (n=4) with a serum retinol >1.4 µmoles/L showed DR/R ratios ≤0.03.

The serum retinol concentration was significantly correlated to the DR/R ratio (r=-0.465), but only 22% of the variability can be explained by serum retinol.

The MRDR after the supplementation trial:

Of the women retested with the MRDR after supplementation, only two had previous DR/R ratios >0.06. Nonetheless, the DR/R ratios of many of the women decreased after supplementation with vitamin A (Figure 6). In the placebo (P1) group (n=7, subjects A through G), DR/R ratios increased in 3 cases and decreased in 4 others. In the vitamin A (A) treated group (n=7, subjects H through N), DR/R ratios fell in 5 cases and increased in 2 subjects. One of these latter women (subject K) who clearly was still vitamin A depleted, probably did not take the provided supplement. Her DR/R ratio increased from 0.113 to 0.138 and her serum
Figure 6. The change in the ratio of 3,4-didehydroretinol to retinol (DR/R) after 8 wk of daily supplementation with either placebo (Pl), 8.4 μmoles vitamin A (A), 1.07 mmoles iron (Fe) or vitamin A plus iron (A+Fe). Black bars are pretreatment and shaded bars are posttreatment DR/R ratios.
retinol fell from 0.53 μmoles/L to 0.34 μmoles/L. In the iron (Fe) supplemented group (n=5, subjects O through S), the DR/R ratios decreased in 2 subjects and increased in 3 others. In these three groups, i.e., PI, A and Fe, no significant effect in the DR/R ratio was found due to treatment.

The most dramatic changes were observed in the vitamin A plus iron (A+Fe) supplemented group (n=8, subjects T through AA). In 6 subjects, the ratios decreased drastically and in the other two increased only slightly. The mean decrease in the DR/R ratio was highly significant in the A+Fe treated group (p<0.01). The A+Fe treatment response was also significantly different from all of the other treatment groups. PI versus A+Fe yielded a p<0.025; A versus A+Fe yielded a p<0.05; Fe versus A+Fe yielded a p<0.005. All of the other comparisons were not significantly different from one another.

The mean serum retinol remained fairly constant except in the PI group, where it decreased by 0.16 ± 0.22 μmoles/L. The other changes were as follows: vitamin A group, 0.022 ± 0.15 μmoles/L; iron group, 0.024 ± 0.27 μmoles/L; A+Fe group, 0.093 ± 0.29 μmoles/L. The difference between the PI group and both the A and A+Fe groups approached significance (p<0.10).
The vitamin A status of pregnant Indonesian women was evaluated using the modified relative dose response (MRDR) assay. Seventeen percent of the women were judged to be in a marginal status with a DR/R ≥0.06. Another 35% of the women tested in the intermediate zone of 0.03 to 0.06. In low-income, pregnant American women tested to date (Duitsman et al. 1993), 11% have fallen into the 0.03 to 0.06 range and no woman has tested ≥0.06.

The hemodilution of vitamin A during pregnancy is a major confounding factor in the interpretation of serum retinol concentrations. Kelner et al. (1969) noted a 30% decrease in serum retinol from the first to the third trimester. In lactating Indonesian women, where hemodilution is not a factor, serum retinol concentrations below 0.7 μmoles/L were almost invariably associated with positive MRDR ratios (DR/R ≥0.06) (Tanumihardjo and Olson, unpublished observations). Similarly, serum retinol values ≤0.53 μmoles/L in pregnant Indonesian women were usually associated with positive MRDR values (Figure 5). Thus, the serum retinol concentration below which abnormal MRDR values prevailed in pregnant Indonesian women is 25% lower than in lactating women, which accords with the expected hemodilution.

Baker et al. (1975) found that 17% of American, low-income pregnant women who were taking prenatal vitamin
supplements had serum retinol concentrations <0.7 μmoles/L. Therefore, a certain percentage of vitamin A-sufficient women might well have a serum retinol concentration <0.7 μmoles/L during pregnancy. By using the MRDR, one can better assess the severity of the vitamin A problem than with serum retinol concentrations alone. The DR/R ratio is less affected by hemodilution and more dependent on vitamin A status.

Of the 15 pregnant women who were in either the vitamin A or vitamin A plus iron supplemented groups, 11 (73%) had a net decrease in the DR/R ratio. The 3 women who had net increases in DR/R ratios (subjects K, L and W), also showed an average decrease in serum retinol concentration of 27% between testing points. Although the decrease in serum retinol values may have been due to hemodilution, the increases in DR/R ratios indicate that they were probably not taking the supplements provided to them. In a recent evaluation of the iron-supplementation program in Jakarta, Indonesia (Schultink et al. 1993), 64% of the women given supplements claimed that they took the pills. However, only 36% of the women showed a positive stool test for iron. In the present study, we relied upon the village caders to administer the supplements and to monitor the project. It is likely, therefore, that compliance was not universal.

Two of the villages had mean DR/R ratios >0.05. While these means are not significantly different from those found in other villages, the vitamin A status of various villages
may well be different. Clustering of clinical signs of vitamin A deficiency in Indonesia has previously been shown (Sommer 1982). Thus, by using the MRDR test, villages most in need of vitamin A may be identified for subsequent intervention programs (World Health Organization et al. 1988).

Currently, a public health problem is presumed to exist if >10% of a given population have positive MRDR or RDR responses (Barbara Underwood and Hernando Flores, personal communication). By this criterion, pregnant Indonesian women clearly are in a marginal status that merits the attention of public health authorities.
REFERENCES


In the past decade, much effort has been devoted to the determination of a marginal vitamin A status. Although the ocular manifestations of vitamin A deficiency are easy to recognize, a marginal status or subclinical deficiency is much more difficult to diagnose.

Vitamin A supplementation programs have shown significant reduction in the mortality rates of children. For example, in two controlled field studies in Indonesia, reductions of 34% and 46% were noted in the mortality rate of preschool-age children given semiannual vitamin A supplements (Sommer et al. 1986) and fortified distributed monosodium glutamate (Muhilal et al. 1988). In India (Rahmathullah et al. 1990) and in Nepal (West et al. 1991), mortality rates were reduced with small weekly doses and periodic large doses of vitamin A, respectively. Supplements of vitamin A reduced mortality in yet other, but not in all studied localities.

The main purpose of dose response tests, i.e., the RDR and MRDR, is to identify groups of individuals who are subclinically deficient but who have not developed ocular lesions. Because dose response tests are a more sensitive indicator of vitamin A status than serum retinol concentrations alone, sample sizes do not have to be as large.
The main drawback of the RDR is that two blood samples are required at a five-hour interval. By substituting dehydroretinol in the assay, the need for two blood samples is eliminated. The purpose of the current work was to refine the MRDR assay technique and to apply it to Indonesian subjects who might suffer from a marginal vitamin A status.
CONCLUSIONS

1. The DR/R ratio is reproducible in healthy well-nourished individuals. The abnormal DR/R ratio is also reproducible in Indonesian lactating women.

2. The MRDR is concordant with CIC in well-nourished individuals. At the village level, the MRDR and CIC identify the same village as being at an increased risk of deficiency.

3. RDR values based on an oral dose of 1.57 μmole (450 μg) retinyl acetate gave poor results. When a higher dose (3.5 μmole) of retinyl acetate was used, the RDR and MRDR results were comparable.

4. A DR/R ratio of 0.06 is indicative of low liver reserves based on comparisons to the CIC and RDR tests. Individuals having a DR/R ratio between 0.03 and 0.06 are in an intermediate range, and those with ratios <0.03 are considered to be in a satisfactory vitamin A status.

5. Suburban, low income, Indonesian lactating women are at a high risk of vitamin A deficiency and should be a target group for intervention programs. Pregnant women and their unborn children also would benefit from intervention programs which do not compromise their health.
6. Only 5-7% of the variability in the DR/R ratio can be attributed to the women’s body weight when a standard dose of 8.8 μmoles (2.5 mg) is used.

7. Abnormal MRDR ratios are commonly found in lactating women with serum retinol concentrations <0.70 μmoles/L and in pregnant women with serum retinol levels <0.53 μmoles/L. In both groups, serum retinol concentrations >1.4 μmoles/L are associated with normal MRDR values.

8. The DR/R ratio of the lactating women was about 3-fold higher than the control group at all times studied. Therefore, the time of drawing the blood samples is not critical and a range of 4-6 h seems ideal.
LITERATURE CITED


Wilson, T. C. M. and Pitt, G. A. 1986. 3,4-Didehydroretinol (vitamin A₂) has vitamin A activity in the rat without conversion to retinol. *Biochem. Soc. Trans.* 14: 950-951.


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