Validity of the relative-dose-response test and the modified-relative-dose-response test as indicators of vitamin A stores in liver\textsuperscript{1–4}

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\textbf{ABSTRACT}

\textbf{Background:} Our group and many others have used the relative-dose-response (RDR) test and the modified-RDR (MRDR) test as proxy indicators of liver stores of vitamin A. However, we have become concerned about the validity of these indicators.

\textbf{Objective:} Simulation models were used to assess effects of random variations in serum retinol concentration on the RDR and to assess effects of group differences in serum retinol concentration on the distribution of RDR and MRDR values.

\textbf{Design:} Random and independent samples were drawn from normally distributed, computer-generated numbers whose distributions simulated serum concentrations of retinol and 3,4-didehydroretinol as obtained from published reports. The resulting data sets were used to compute surrogate RDR or MRDR values. In model 1, the relation between serum concentrations of retinol and RDR was examined within a fictitious population. In models 2 and 3, fictitious populations with different distributions of serum retinol concentration were compared with respect to their RDR and MRDR values.

\textbf{Results:} Simulated RDR values and serum retinol concentrations were negatively related. Models 2 and 3 showed that group differences in serum retinol concentrations necessarily produced group differences in mean RDR or MRDR values. A mathematical artifact may explain the negative relation reported between MRDR and serum retinol concentration, and it dictates that this relation will necessarily vary between populations with different degrees of vitamin A deficiency.


\textbf{KEY WORDS} Vitamin A, vitamin A deficiency, vitamin A metabolism, tissue distribution, statistical data interpretation, computer simulation

\textbf{INTRODUCTION}

Serum retinol concentration has been widely used and is recommended as the prime indicator for routine assessment of the occurrence and degree of vitamin A deficiency (1). However, it cannot be used to assess the degree of sufficiency because studies in animals fed a low-retinol diet showed that liver stores of retinol decline to marginal amounts before serum retinol concentrations decrease (2). Thus, serum retinol concentration is thought to be homeostatically set in persons, regardless of liver stores, provided that such stores are >20 \( \mu \text{g} / \text{g} \) (0.07 \( \mu \text{mol} / \text{g} \); 1). In most well-nourished populations with “adequate” stores, average serum retinol concentrations generally exceed 30 \( \mu \text{g} / \text{dL} \) (1.05 \( \mu \text{mol} / \text{L} \); 1). Within the homeostatically regulated range of >30 \( \mu \text{g} / \text{dL} \), concentrations of retinol in serum are considered to supply adequate vitamin A for tissue needs.

A search for suitable proxy biochemical indicators of liver stores of vitamin A has led to the development of 2 tests, the relative-dose-response (RDR) test and the modified–RDR (MRDR) test (3–5). These tests have been widely used as indicators that are complementary to serum retinol concentration for assessing vitamin A status in population surveys and for estimating the effects of interventions on vitamin A status. For example, on the basis of effects observed on the MRDR test, our group recently reported that iron supplementation might result in a redistribution of vitamin A from circulation to stores (6). However, we have become concerned that the formulas used to construct the RDR and MRDR tests produces mathematical artifacts that threaten the validity of the tests. Because of these concerns, the aim of this study was to review the validity of these indicators. Rather than using a formal mathematical approach, we decided to examine our concerns by using a series of simulations.

\textbf{METHODS}

\textbf{Background of RDR and MRDR tests}

Both the RDR and MRDR tests are based on the finding that retinol-binding protein accumulates in the liver when vitamin A intake is low. The design of the RDR test was based on evidence that any decrease in serum retinol concentrations resulting primarily from a deficiency in vitamin A stores is immediately and preferentially made up by a newly ingested supply of the vitamin (7). Thus, once vitamin A or provitamin A is supplied to a vitamin A–deficient person, retinol is released into the circulation, bound to retinol-binding protein, within a matter of hours. To measure the RDR, fasting persons are given an oral supplement of vitamin

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A as retinyl ester (450–1000 μg) in oily solution. The RDR is computed for each person by using the equation

$$RDR = \frac{(R_5 - R_0)}{R_5}$$  \hspace{1cm} (I)

where R₀ and R₅ are the serum retinol concentrations measured immediately before and 5 h after the ingestion of the supplement, respectively. Because the R₅ value is regarded as the serum concentration of retinol that is homeostatically regulated and normal to that person under vitamin A–supplemented conditions (7), it would appear that Rₐ and R₀ are not associated. Thus, the RDR has been conceived as the fraction by which predose serum retinol concentrations in a person have declined—as a consequence of inadequate dietary or liver stores—below homeostatic concentrations normal to that person under vitamin A–sufficient conditions (7). RDR values > 0.20 are considered as evidence of marginal or insufficient vitamin A stores (8).

The MRDR test has been considered to have an advantage over the RDR test, because the former requires only one blood sample, is reproducible in persons over time, and is highly responsive to therapeutic doses as assessed both in persons before and after receiving vitamin A and in intervention trials with vitamin A (5). In addition, it has been claimed that the MRDR is more responsive to changes in vitamin A status than was serum retinol concentration alone (9). The MRDR is ascertained 5 h after oral administration of 3,4-didehydroretinyl acetate, a synthesized metabolite of vitamin A that also occurs naturally, and is computed as the molar ratio of the serum concentration of 3,4-didehydroretinol to that of retinol. Neither 3,4-didehydroretinyl acetate nor its derivative is converted to retinol, and thus neither affects the steady-state serum concentrations of retinol (10). In conditions of marginal or insufficient liver stores of vitamin A, the serum 3,4-didehydroretinol concentration has been shown to be sharply increased. The ratio of the serum concentration of 3,4-didehydroretinol to that of retinol (i.e., the MRDR ratio) is inversely related to the liver stores of vitamin A because serum retinol concentration is a marker of these stores. Thus, the relation between liver stores of vitamin A and the MRDR can be approximated by using a hyperbola (10). On the basis of studies among healthy US volunteers and Indonesian children, MRDR values > 0.03 and > 0.06 have been provisionally recommended as evidence of insufficient vitamin A stores (4).

Computer simulations

Model 1

First, we assessed the effect of random variation of the serum retinol concentration on the RDR. For this purpose, we considered a fictitious population in whom the serum retinol concentration (R₀) is normally distributed at mean (±SD) 0.74 ± 0.26 μmol/L, a value that corresponds to values taken from the placebo group in the study by Wieringa et al (6). In addition, we arbitrarily assumed that, on average, ingestion of the fixed dose of retinyl ester produces a fixed effect on serum retinol concentration when measured after 5 h (R₅) but no effect on the variance. Thus, R₅ would have a value of 1.05 ± 0.26 μmol/L. Such averages correspond with the minimum values observed in well-nourished populations with “adequate” stores (1). We used PQRS software (version 3.2; 11) to draw 2 independent random samples (n = 300) from normally distributed data with means and SDs as described above, and we paired the resulting data to compute RDR values. The simulated RDR values thus obtained had an interquartile range of 0.02 to 0.47. By comparison, according to Olson (12), RDR values of 0.0, >0.2, and 1.0 indicate a good vitamin A status, a vitamin A–deficient state, and a very vitamin A–deficient state, respectively. Our simulated data also included negative values. Although negative values theoretically should not occur, they were reported by others in practice (12).

Model 2

For model 2, we used an approach similar to that in model 1 to assess the effect of differences in serum retinol concentration between 2 groups on the distribution of RDR values. For this purpose, we considered a fictitious trial in which 300 children randomly received a supplement of either retinol or its placebo, and vitamin A status was assessed at the end of the intervention period. In this model, we arbitrarily assumed that serum retinol concentrations in these 2 groups are 1.05 ± 0.26 and 0.51 ± 0.26 μmol/L, respectively. We further assumed that homeostatic serum concentrations of retinol within the normal range (R₅) would be the same in both groups—namely, 1.05 ± 0.26 μmol/L.

Model 3

In model 3, we used an approach similar to that in model 2 to assess the effect of differences in serum retinol concentration between 2 groups on the distribution of MRDR values. We drew random samples from normally distributed data simulating 2 groups of persons with serum retinol concentrations of 0.60 ± 0.26 and 1.05 ± 0.26 μmol/L, respectively. Because we wanted to examine the effect of group differences in serum retinol concentrations only, we assumed that the distribution of serum 3,4-didehydroretinol concentration was identical in both groups—namely, 0.05 ± 0.02 μmol/L—which is a range similar to that reported by Tanumihardjo et al (13). This assumption resulted in the vast majority of MRDR values being < 0.30, which appears realistic, considering that similar values were reported by many authors, eg, Wieringa et al (6).

In addition, we used these data to show that the mean of the MRDR or any indicator that incorporates the reciprocal of serum retinol concentration is a biased estimator of mean body stores of vitamin A. For each of the 2 groups, we calculated the mean serum retinol concentration and the mean of the reciprocal of serum retinol concentration. We then reexpressed the latter into its natural units by calculating its reciprocal (the reciprocal of the mean of the reciprocals), and we compared this value with the mean serum retinol concentration.

RESULTS

Model 1

The results of our first simulation (Figure 1) show that the RDR and serum retinol concentration are negatively related. Such a result is to be expected, because, if we have any 2 sets of random numbers, Rₐ and R₀, and if we plot (Rₐ−R₀)/R₀ on the y axis and R₀ on the x axis, a negative relation will be observed. This is because R₀ occurs in the y term and +Rₐ in the x term. In addition, the data show that variability in RDR values increases with increasing serum retinol concentrations.

Model 2

The results of the simulation are plotted in Figure 2. The placebo group had substantially higher mean RDR values than did the retinol group: 0.51 and −0.08, respectively.
As might be expected, the relation between MRDR and serum retinol concentration is a hyperbola (because the latter occurs in the denominator of the formula to compute the MRDR), so that MRDR values are higher in the group with relatively low serum retinol concentrations than in their peers who have relatively high serum retinol concentrations (Figure 3). The data also show that variability in MRDR values increases with low serum retinol concentrations.

In group 1, the mean values for the serum retinol concentration and the reciprocal of the mean of the reciprocal serum retinol concentration were 0.63 and 0.49, respectively. The corresponding values in group 2 were 1.1 and 1.0, respectively.

In persons with sufficient liver stores of vitamin A, serum concentrations of retinol and 3,4-didehydroretinol are homeostatically set and vary little (1). As a consequence, neither the RDR nor the MRDR can be used as a measure of the degree of vitamin A sufficiency. In that sense, the results of the RDR and MRDR tests do not complement serum retinol concentration as an indicator of vitamin A status. The question is whether they are useful as substitutes for serum retinol concentration in indicating the presence or the degree of vitamin A deficiency.

In all 3 models, we selected estimates of the variables somewhat arbitrarily to show that substantial bias may occur within ranges of serum retinol concentrations and RDR and MRDR values that have been obtained in various field studies (eg, 4–6, 8–10, 13).

As shown in Figure 1, the relation between RDR and serum retinol concentration is itself negative, because of random variation in serum retinol concentration. This variation also means that persons with high initial serum retinol concentrations ($R_0$) will, on average, have changes in serum retinol concentration ($R_5$) and have RDR values that are less than those in their counterparts with low initial serum retinol concentrations. Thus, for example, if random variation is due to measurement error, it might appear that persons with high serum retinol concentration have sufficient liver stores of vitamin A, whereas persons with low serum retinol concentration have marginal or depleted stores, regardless of the actual liver stores of vitamin A. This mathematical artifact is comparable to a regression-to-the-mean effect and will be greater in those with the greatest divergence from the group average of initial serum retinol concentrations. It can also explain, at least in part, why negative RDR values have been commonly observed. Selection of a cutoff of 0.20 for the RDR, as suggested by Olson (13), is not an appropriate method of remediying this problem of negative RDR values. Because of the mathematical artifact, observations of group differences in RDR values after supplementation (Figure 2) might be misinterpreted as evidence that vitamin A supplementation results in...
increased liver stores of vitamin A. Similarly, the relation between MRDR and serum retinol concentration may not accurately reflect vitamin A status.

In a study in Indonesian women, Tanumihardjo et al (4) found that only 1 of 13 subjects with serum retinol concentration < 0.70 μmol/L had an MRDR value < 0.06. In addition, MRDR values that were initially > 0.06 decreased to < 0.06 and, occasionally, to < 0.03 after generous supplementation with vitamin A. These observations were used to justify a provisional cutoff of 0.06 for an abnormal MRDR, so that higher values indicated low vitamin A status. A similar argument has been put forward to justify MRDR cutoffs for children (4, 13). However, as shown in Figure 3, these observations could be explained by the fact that an increase in serum retinol concentration will by definition result in lower MRDR values, regardless of actual liver stores of vitamin A. Tanumihardjo (9) also observed that MRDR is more responsive than is the serum retinol concentration alone to changes in vitamin A status. However, a small change in serum retinol concentration—particularly in the low range of serum retinol concentrations—will necessarily result in a relatively large change in MRDR, because serum retinol concentration constitutes the denominator in the formula for computing MRDR. More generally, if serum retinol concentration reflects vitamin A status, as is suggested by its close relation with liver stores of vitamin A when these are low (1, 14, 15), then estimators, such as the MRDR, that incorporate the reciprocal of serum retinol concentration will, by definition, be biased. The data in model 3 showed this because the reciprocal of the mean of reciprocal values of serum retinol concentration had a different value from the mean serum retinol concentration. This bias is particularly pronounced when serum retinol concentrations are close to zero, because such values result in outliers in considerations of the reciprocal values.

Several reports have explored group differences in the negative relation between the MRDR and serum retinol concentration. For example, Tanumihardjo et al (4) observed that the slope indicating the change in MRDR per unit of change in serum retinol concentration was higher in a control group of nonlactating Indonesian women with relatively high serum retinol concentrations than in a lactating group of Indonesian women with relatively low serum retinol concentrations. This was interpreted as evidence that the complex of retinol with retinol-binding protein is released more rapidly from the livers of lactating women than from those of control (i.e., nonlactating) women. Similarly, in a recent report of a randomized placebo-controlled trial assessing the effect of supplementation with various micronutrients in Indonesian infants, our group (6) observed that MRDR and serum retinol concentration were negatively correlated in children who had received iron placebo but not in their peers who had received iron supplements.

These analyses are wrong for several reasons. First, the use of linear regression in these analyses violates 2 of the basic conditions of linear regression: 1) MRDR is not normally distributed, and 2) its variance is not the same across the range of serum retinol concentrations found in the study (the latter condition is a violation of the assumption of homoscedasticity). A second, more pertinent point for the present discussion is that, as shown in Figure 3, the hyperbolic relation between MRDR and serum retinol concentration in itself dictates that a change in serum retinol concentration will produce a greater change in MRDR when serum retinol concentration is relatively low than when it is relatively high. The question that appears to be central to the study of Wieringa et al (6) has to do with the extent to which MRDR values are less than expected for the serum concentrations observed in the infant group receiving iron. Although visual inspection of Figure 3 in the article by Wieringa et al suggests that this may indeed have been the case—thus suggesting that supplemental iron may have inhibited the release of retinol-binding protein from the liver and resulted in a low response of serum 3,4-didehydroretinol concentration to the oral test dose of 3,4-didehydroretinol acetate—this question is not addressed by comparing correlation coefficients between groups receiving supplements with iron or iron placebo.

Because serum retinol concentrations are low in persons with vitamin A deficiency, it is conceivable that the RDR and MRDR also reflect a biologic response to vitamin A deficiency. However, they are inadequate as indicators of vitamin A deficiency because the extent to which they are affected by a mathematical artifact and that to which they reflect a biologic response cannot be ascertained. In addition, if a person’s serum retinol concentrations at 5 h (R5) reflect serum concentrations of retinol that are homeostatically regulated and normal to that person under conditions of vitamin A sufficiency (7), then the biologic response in RDR would be due entirely to the effects of vitamin A deficiency on pretest serum retinol concentrations (R0). In that case, an RDR value would not provide any information in addition to that provided by the pretest serum retinol concentration. Similarly, MRDR values would not provide any information in addition to that provided by serum retinol concentrations if serum 3,4-didehydroretinol concentrations were also assumed to reflect serum concentrations of retinol that are homeostatically regulated and normal to a person under conditions of vitamin A sufficiency.

In conclusion, results of the RDR and MRDR tests may provide indications of marginal or depleted liver stores of vitamin A similar to those provided by the serum retinol concentration. However, group differences in serum retinol concentration will necessarily result in differences in RDR or MRDR, regardless of actual liver stores of vitamin A. A mathematical artifact may explain the inverse relation that has been reported between MRDR and serum retinol concentration, and it dictates that this relation will necessarily vary between populations with different degrees of vitamin A deficiency. A variant of the RDR test, the 30-d serum dose-response test, has also been proposed (2, 14) but has not been discussed here, because our concerns about its validity apply equally to the RDR test. A continued search for alternative blood indicators of liver stores of vitamin A is needed. Such indicators should be independent of vitamin A concentrations in circulation and ideally should indicate the amount of vitamin A stores and thus the degree of vitamin A sufficiency, rather than the absence of liver stores.

HV conceived the idea for this study, carried out the mathematical analysis, and prepared a first draft of the manuscript; CEW contributed to the interpretation of the data and critically reviewed the manuscript. Neither of the authors had any personal or financial conflicts of interest.

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