Adjacments to the Modified Relative Dose Response (MRDR) Test for Assessment of Vitamin A Status Minimize the Blood Volume Used in Piglets

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ABSTRACT The modified relative dose response (MRDR) test is widely used in public health research to assess vitamin A (VA) status of populations and individuals. However, method adjustments intended to make the test more useful in large field studies and/or less invasive have not been systematically verified. To compare the similarity between modified tests and the standard MRDR test, and validate both modified and standard tests against liver reserves of VA, we used a piglet model. Following the typical MRDR procedure, piglets (n = 10) were dosed with 5.3 μmol 3,4-didehydroretinyl acetate. Method adjustments were made to the postdose blood sample collection time to decrease both the amount of serum analyzed and sample throughput time. We collected 3 blood samples/piglet at 3, 5, and 7 h or 4, 6, and 8 h postdose. Postdose blood samples obtained between 4 and 7 h gave MRDR values that did not differ. Serum volumes as small as 200 μL, half the volume of the standard method, yielded accurate MRDR values. Method adjustments to reduce sample throughput time require further investigation. In conclusion, because 200 μL of serum can be used in the test, only 0.5 mL, as opposed to 1 mL of blood has to be collected from an individual. This adjustment allows for easier application of the test to individuals, especially infants, from whom it is difficult to obtain a large venous blood sample, thus increasing the utility of the test for researchers.

KEY WORDS: • didehydroretinol • didehydroretinyl acetate • retinol • vitamin A-2 • modified relative dose response

Vitamin A deficiency (VAD) is one of the most prevalent micronutrient deficiencies in developing countries. Because of the negative effects that VAD can have on human health, assessing VAD at the individual and population level is of extreme importance. Accurate assessment of VAD, ranging from subclinical to clinical, can allow for better evaluation of the effect of vitamin A (VA) interventions, give insight into the effect of subclinical VAD on human health, and justify the allocation of government funds for food or supplementation programs.

Several techniques exist to assess VA status of individuals and populations. One of the most widely used techniques is the modified relative dose response (MRDR) test. This method has been employed in the United States and Canada, and in numerous countries throughout South America, Europe, Asia, and Africa (1). The MRDR test, based on the relative dose response (RDR) test (2,3) and developed by Tanumihardjo et al. (4–7), takes advantage of the VAD-dependent accumulation of apo-retinol binding protein (RBP) in the liver (8). For the MRDR test, individuals are given an oral dose of 3,4-didehydroretinyl acetate (DRA) which appears, a few hours later, in the serum bound to RBP as 3,4-didehydroretinol (DR). DR, commonly called vitamin A-2, is a naturally occurring analog of retinol (R) found in low concentrations in mammalian tissues. At 4 to 6 h postdose, a blood sample is drawn from the individual and the ratio of DR to R in the serum, or the MRDR value, is determined through HPLC analysis. Currently, an MRDR value ≥ 0.060 is considered indicative of subclinical VAD.

The MRDR test has the advantage of requiring only 1 blood sample/person, making it less invasive and more feasible to use in large field studies than the RDR test, which requires a baseline and 5-h blood sample postdose. The MRDR test was refined (7) and validated after VA supplementation in children (9) and women (10) using 400–500 μL of serum (~1 mL of blood) per analysis. Some investigators have performed the test using a serum volume as small as 100 μL (11,12); however, this alteration to the method has not been validated against the standard method.

Clearly, application of the MRDR test depends in part on the ability to obtain a blood sample of sufficient volume. Obtaining 1 mL of blood from certain individuals may be...
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Difficult due to community acceptance or anatomy, i.e., inaccessible veins. The number and age of subjects to be assessed may influence the method of blood collection, making small blood samples obtained by heel stick or finger prick most desirable due to ease and speed of collection. Obtaining ample blood samples from infants and young children is possible but can be painful (13,14). Heel stick and finger prick typically yield volumes of ~400 to 800 µL per puncture (15,16). For example, collection of 1.2 mL blood in infants by skin puncture took 3.6 punctures and 13.1 min (17). To reduce pain, venepuncture may be preferred over heel sticks for drawing blood from infants (13,18–20). However, venepuncture requires a well-trained phlebotomist, whereas heel stick can be performed by general health workers. Additionally, venepuncture causes higher anxiety among mothers than heel stick in some populations (13), making skin puncture necessary.

Performing the MRDR test on infants and children would be less invasive (i.e., fewer skin punctures) and easier (i.e., shorter duration of blood collection and child restraint) if a smaller blood sample could be taken. Smaller blood samples would also make large field studies using the MRDR more feasible. Additionally, if sample analysis time could be reduced, the test would be less costly in large field studies. Finally, if the postdose blood samples could be collected over a wider time range than currently recommended (4–6 h postdose), more individuals could be evaluated, or more flexibility could be allowed during dosing and blood collection.

To evaluate the feasibility of using smaller blood samples, decreasing sample analysis time, and widening the time range for postdose blood collection without affecting the MRDR value, we applied the test to weaning piglets. We rigorously compared MRDR values obtained from serum volumes <400 µL to the 400-µL standard method. Finally, we attempted to decrease HPLC analysis time by using a shorter column and alternative solvents to those used currently. We sought to make all method changes under the condition that the current cutoff value for subclinical deficiency remained at ≥0.060 to ensure continuity and clarity between previous and future studies using the MRDR test.

MATERIALS AND METHODS

Animals and diet. The piglet was chosen as the model for this study due to the physiologic and anatomical similarity to human infants (21–23) and because of additional similarities in form and function of the gastrointestinal tract and digestive physiology between swine and humans (23–25). Additionally, swine were used previously as a model for humans in vitamin A research (26,27). Furthermore, the results of the MRDR test could be validated against liver reserves, which would not be ethical in children. Last, we could ethically modify the piglet diet to resemble the diets many people consume in developing countries, giving us a more accurate model of the population to which the MRDR test is generally applied.

Male piglets (n = 10) were purchased from the UW-Madison Swine Research and Teaching Center and were housed in a single pen at the UW-Madison Livestock Laboratory. All piglets were the same crossbreed (White Duroc, Landrace, and Large White) but were from litters of 3 different sows. Piglets were allowed to nurse from their mothers until they were brought to the UW-Madison campus, at which time they were weaned. Due to piglet availability at the time of purchase, 8 piglets were 14 d old and 2 were 24 d old. Upon arrival at the UW-Madison campus, piglets consumed a rice and oat gruel containing no vitamin A (Table 1) ad libitum for 4 d with unlimited access to water. The gruel was intended to mimic a high-starch, low-protein, vitamin- and mineral- and mineral-insufficient diet often fed to young children of poor families in developing countries. Because piglets at weaning prefer to eat as a group, as they do when nursing from the sow (28,29), food was served in several group feeders that allowed the piglets to see and interact with each other during feeding. Water was provided by a snout-level waterer of the same design that the piglets had been exposed to in the farrowing pen with the sow. Food was checked 4 times/d, and when necessary, fresh food was added to keep the feeders full. Every morning, food remaining from the evening before was discarded and the feeders were filled with fresh food. The University of Wisconsin Research Animal Resources Center approved all procedures.

Dose preparation. 3,4-Dihydroxymetacrylic acid was synthesized using previously published procedures (4,30), dissolved in corn oil, and stored at −30°C. The stock solution was diluted so that each dose would contain 1.5 mg (5.3 µmol) DRA in ~0.6 mL corn oil. To determine the exact concentration of the dosing solution, 25 µL of the dosing solution was diluted in 25 mL of hexanes and an absorbance was obtained using UV-visible spectroscopy. Using the E1%1cm of DRA (1455 at 350 nm), the concentration was 2.46 g DRA/L oil; thus, a dose of 0.61 mL contained 1.5 mg DRA. This is the standard dose used for infants and children <6 y old. The dose of DRA/kg body weight accounts for only 5–7% of the variability in the MRDR value in children and adults (7,31,32), and this same standard dose (5.3 µmol DRA) has been applied to infants (11,33). Therefore, we chose this dose size because the piglet body weights at 2–3 wk of age are similar to those of human infants. The mean body weight of the piglets at the time of dosing was 5.1 ± 1.3 kg (range: 3.2–6.9 kg).

Dose administration and tissue collection. On the morning of d 5 postweaning, piglets were randomly divided into 2 groups so that each group consisted of 3 piglets and neither group contained >3 piglets from the same litter. Dividing piglets into 2 groups allowed us to minimize the number of blood collections per piglet for the time analysis of the MRDR test. Every piglet was dosed with 1.5 mg DRA via oral gavage at time 0. Food was withheld until all piglets in the pen were dosed. Blood samples were taken via jugular venepuncture from group 1 at 3 and 5 h postdose and from group 2 at 4 and 6 h postdose. Blood was collected in Vacutainer brand tubes containing a serum separator and clot activator. If possible, 1 tube of blood (up to 7 mL) was collected per piglet at each blood draw. Groups 1 and 2 were killed via electrical stunning and exsanguination by jugular puncture at 7 and 8 h, respectively, at which time final blood samples (15 mL of whole blood per piglet from jugular blood flow) and livers were collected. Each piglet was weighed before it was killed. A total of 30 blood samples (3/piglet) was collected.

Blood samples were kept in the dark and allowed to clot at room temperature for ~30 min before they were centrifuged at 1250 × g for 20 min. Samples were then transported in the dark to the laboratory where the serum was stored in cryogenic vials at −30°C until analysis. Livers were blotted upon excision, weighed, stored in amber glass jars, and placed on dry ice in the dark until transported to the laboratory, where they were stored at −30°C.

Serum preparation and HPLC analysis. All samples were prepared and analyzed under yellow lights. Serum was analyzed for DR and R using the standard method and test method 1,2, 3, and/or 4 (Table 2). For the standard method, which will hereafter be called the 400-µL method, we followed previously published methods (9,31). An internal standard (40 µL; retinyl acetate 3.8 mmol/L ethanol) was added to 400 µL thawed serum in a glass test tube. Proteins were denatured with 500 µL pure ethanol, followed by brief

<table>
<thead>
<tr>
<th>Component</th>
<th>g/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long grain jasmine rice, enriched, cooked</td>
<td>622</td>
</tr>
<tr>
<td>Raw oat groats</td>
<td>310</td>
</tr>
<tr>
<td>Corn oil</td>
<td>55</td>
</tr>
<tr>
<td>NaCl</td>
<td>13</td>
</tr>
</tbody>
</table>

1 Piglets nursed from their mothers for either 14 or 24 d before being weaned to this diet.
2 13% crude protein, 5% crude fat, 2.5% crude fiber.

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mixing on a vortex (15 s); then the sample was extracted twice with 500 µL of hexanes. The organic layers were pooled and dried under argon. The dried sample was reconstituted with 40 µL of 75:25 (v/v) methanol: dichloroethane (MeOH:DCE), vortexed, and centrifuged briefly at 1380 X g for 30 s before 35 µL was injected into an HPLC system. A Waters Resolve C18, 5-µm, 3.9 × 150 mm column was used, absorbance was monitored at 350 nm to maximize detection of DR, and a mobile phase of 89:11 methanol:water (MeOH:H2O) with 0.73 g/L triethylamine (TEA) was run at a flow rate of 1 mL/min.

In test methods 1 and 2, we used either 100 or 200 µL of serum, respectively, and adjusted the volumes of the internal standard and solvents accordingly (Table 2). In test methods 3 and 4, we used either 100 or 200 µL of serum, respectively, adjusted the volumes of internal standard and solvents (Table 2), and used a shorter column (Waters Symmetry C18, 3.5-µm, 4.6 × 75 mm) and an alternative mobile phase (75:20:5 methanol:acetonitrile:water, v:v:v with 0.73 g/L TEA) at a flow rate of 0.7 mL/min.

We analyzed all samples using the same isocratic HPLC system (injector, Rheodyne; detector, Shimadzu SPD-10A UV-VIS; pump, Beckman; data processor, Shimadzu C-R7A Chromatopac). HPLC purified standards of DR and R were assayed on the same system and used to quantify the DR and R in the samples.

Liver preparation and HPLC analysis. A 1-g piece of liver was randomly taken from the whole liver and ground with sodium sulfate (2–3 g) by mortar and pestle. An internal standard (1 mL; 14 µmol retinyl butyrate) was added to determine extraction efficiency. The mixture was repeatedly extracted to a total volume of 50 mL using dichloromethane. The extract (1 mL) was dried under argon, reconstituted in 100 µL of 50:50 MeOH:DCE (v/v), and 50 µL was injected into a dual-wavelength gradient HPLC system (detector, Waters 2487 Dual A Absorbance Detector; pump, Waters 600E Multisolvent Delivery System; data processor, Shimadzu C-R7A Chromatopac). HPLC purified standards of DR and R were assayed on the same system and used to quantify the DR and R in the samples.

TABLE 2
Specifications for the standard 400-µL modified relative dose response test and test methods 1–4

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>400-µL method</th>
<th>Test method 1</th>
<th>Test method 2</th>
<th>Test method 3</th>
<th>Test method 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum, µL</td>
<td>400</td>
<td>100</td>
<td>200</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Retinyl acetate, µL (3.8 mmol/L ethanol)</td>
<td>40</td>
<td>20</td>
<td>40</td>
<td>20</td>
<td>40</td>
</tr>
<tr>
<td>Ethanol, µL</td>
<td>500</td>
<td>100</td>
<td>250</td>
<td>100</td>
<td>250</td>
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<tr>
<td>Hexanec, µL (×2)</td>
<td>500</td>
<td>100</td>
<td>300</td>
<td>200</td>
<td>300</td>
</tr>
<tr>
<td>75:25 MeOH:DCE</td>
<td>40 µL added after the sample was dried under inert gas</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volume injected, µL</td>
<td>35</td>
<td>40</td>
<td>35</td>
<td>40</td>
<td>35</td>
</tr>
<tr>
<td>Column used</td>
<td>Waters Resolve C18, 5-µm, 3.9 × 150 mm</td>
<td>Waters Symmetry C18, 3.5-µm 4.6 × 75 mm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobile phase</td>
<td>89:11 MeOH:H2O (v:v) (0.73 g/L TEA)</td>
<td>75:20:5 MeOH:ACN:H2O (by vol) (0.73 g/L TEA)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate, mL/min</td>
<td>1.0</td>
<td>350</td>
<td>350</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wavelength, nm</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

To examine the change in MRDR value over time, samples were analyzed twice using the standard 400-µL method. Mean MRDR values of the replicates were used in the statistical analysis and groups 1 and 2 were examined separately. A SAS PROC MIXED with repeated-measures of TYPE AR program was used to account for correlations between MRDR values obtained from the same piglet over time.

To compare the test methods with the standard 400-µL method, samples were analyzed once using the 400-µL method and once using a given test method. The MRDR value obtained by the test method was plotted vs. that obtained by the 400-µL method for each sample, and a line obtained. The line equation was then tested to determine whether it was significantly different from the line of equality (y = x), and R² was evaluated to determine how tightly the data points fell around the line. Because it was our goal to determine whether any test method could be used interchangeably with the 400-µL method, it was decided before the experiment that a mean difference between methods ≥ 0.005 U would be too large for the test method to be a direct substitute. A maximum difference of 0.005 U was chosen because this was the magnitude of the SD seen in individuals (n = 7) of normal vitamin A status on whom the MRDR test was repeated 4 times (34). The following scenario also aided in the decision to use 0.005 as a maximum difference: if an alternative method consistently gave MRDR values 0.005 U smaller than those from the 400-µL method, MRDR values near 0.05 would be indicative of sufficient liver reserves (by standard cut-off values); however, these same individuals would have MRDR values of 0.065 by the 400-µL method and would be considered to have insufficient liver reserves. The opposite situation would occur for alternative methods consistently giving MRDR values 0.005 U greater than the 400-µL method.

Moreover, the decision was made that test methods should be simple replacements for the 400-µL method, and therefore test methods requiring mathematical manipulations, such as transformations to satisfy the assumptions for statistical analysis, were not considered as substitutes.

If a test method differed from the 400-µL method by <0.005 U and did not require mathematical manipulation, it was decided that agreement would be further assessed using statistical techniques suggested by Bland et al. (35,36). These included plotting the difference between the new and 400-µL method against the mean of the 2 methods, and a repeatability assessment of both the new and 400-µL method.

Plotting the difference vs. the mean of the 2 methods indicates the nature of the relation between 2 methods over the range of values tested, i.e., it shows whether the new method is consistent with the old at all values. The repeatability gives an idea of the precision of multiple measurements taken from an individual under identical conditions, using a given assessment method (37). In this experiment, repeatability gives an idea of how precisely each method measures the MRDR value for a given serum sample analyzed multiple times by 1 method.

To estimate serum retinol concentration for each piglet, a mean retinol concentration was calculated from the retinol values obtained from all replicates of the 400-µL method and test method 2 at all time points for the given piglet. After calculating the mean serum retinol concentration for each piglet, the group means were deter-
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RESULTS

Examination of MRDR values over time. In group 1, we did not obtain enough serum from 1 piglet at the 3-h time point to analyze the serum using the 400-μL method. Therefore, this point was not included in our data analysis. The 3-h MRDR value was lower than both the 5-h (P = 0.024) and 7-h (P = 0.009) MRDR values (Fig. 1). The 5-h MRDR value did not differ from the 7-h value (P = 0.20) (Fig. 1).

Only 4 piglets were included in the group 2 time analysis because the 6-h blood sample for 1 piglet was collected late due to a failed first attempt, and there was inadequate serum to perform replicates using the 4-h sample. The 4- and 8-h MRDR values differed (P = 0.037) as did the 6- and 8-h values (P = 0.012) (Fig. 1). The 4- and 6-h MRDR values did not differ (P = 0.47) (Fig. 1). The same time analysis was performed using test method 2 (200 μL), and the same relations were seen among the various time points (data not shown).

Comparison of MRDR values obtained between test methods vs. the standard 400-μL method. All piglets were included in the MRDR method comparison data and at least 4 piglets at any given time point provided MRDR data for method comparisons. Because it was difficult to obtain a large blood sample from some of the piglets at certain time points, serum was a limiting factor. Consequently, the priority of analysis was as follows: each sample was analyzed once by the 400-μL method, test method 2 (200 μL), test method 3 (100 μL), test method 1 (100 μL), and test method 4 (200 μL), in that order; then replicates were performed on the 400-μL method and any test method that was a potential substitute. Therefore, not all blood samples were analyzed using every method. Hence, the total n within a comparison of any 2 methods may be different from the n of another comparison.

Test method 1. The MRDR value was obtained for 20 serum samples prepared using test method 1 (100 μL serum, 15-cm column). These values were plotted against the MRDR values obtained for the same 20 samples prepared using the 400-μL method (Fig. 2A). The equation of the regression line was y = 1.06x + 0.0026, where y = test method 1 and x = the 400-μL method. The slope did not differ from 1 and the intercept was not different from 0. The adjusted R² was 96.6%. The MRDR values using the 400-μL method and test method 1 were 0.057 ± 0.035 and 0.063 ± 0.038, respectively. The difference between the 2 methods was −0.006 ± 0.007 (P < 0.001).

Test method 2. The MRDR values obtained using test method 2 (200 μL serum, 15-cm column) were plotted vs. the MRDR values obtained using the 400-μL method for 27 samples (Fig. 2B). The equation of the regression line was y = 0.96x + 0.0016, where y = test method 2 and x = the 400-μL method. The slope differed from 1 (P = 0.007) and the intercept did not differ from 0 (P = 0.15). The adjusted R² was 99.3%. The MRDR values using the 400-μL method and test method 2 were 0.058 ± 0.036 and 0.057 ± 0.035, respectively. The difference between the 2 methods was 0.001 ± 0.003 and was not significant (P = 0.11). The limits of agreement between the methods (difference ± 2 SD) were −0.0056 and 0.0076, and the 95% CI for the difference was (−0.00026, 0.0023). The difference between the 2 measurements vs. the mean of the 2 measurements for each sample was plotted (Fig. 3). No obvious relation between the difference and mean was apparent from these data.

Test method 3. The MRDR values obtained from 27 samples using test method 3 (100 μL serum, 7.5-cm column) were plotted against those obtained using the 400-μL method (Fig. 2C). The equation of the regression line was y = 1.25x + 0.0056, where y = test method 3 and x = the 400-μL method. The slope was different from 1 (P < 0.001) and the intercept was different from 0 (P < 0.001). Although the regression line obtained differed from y = x, the data fell tightly along the regression line, with an adjusted R² of 99.5%. The MRDR values obtained from the 400-μL method and test method 3 were 0.058 ± 0.036 and 0.078 ± 0.045, respectively. The difference between the 2 methods was −0.020 ± 0.009 (P < 0.001).

Test method 4. The MRDR values obtained for 20 samples using test method 4 (200 μL serum, 7.5-cm column) were plotted vs. those obtained using the 400-μL method (Fig. 2D). The equation of the regression line was y = 1.20x + 0.0055, where y = test method 4 and x = the 400-μL method. The slope was different from 1 (P < 0.001) and the intercept was different from 0 (P = 0.002). However, the adjusted R² was 99.5%. The MRDR values obtained by the 400-μL method and test method 4 were 0.067 ± 0.035 and 0.086 ± 0.042, respectively. The difference between the 2 methods was −0.019 ± 0.008 (P < 0.001).

Assessing the repeatability of the 400-μL method and test method 2. To assess the repeatability of the 400-μL method, 2 MRDR values for each of 25 samples were obtained. The difference between replicates was not significantly different from zero. The repeatability coefficient (2 SD) of the 400-μL method was equal to 0.004. The repeatability of test method 2 was also determined. Samples (n = 29) were measured twice for an MRDR value using test method 2. The difference between the measurements was not different from zero. The repeatability coefficient was equal to 0.008, twice the repeatability coefficient of the 400-μL method.

Serum retinol concentrations. Serum retinol concentration for group 1 was 0.80 ± 0.11 μmol/L (range: 0.63–0.92 μmol/L).
μmol/L) and for group 2 was 0.60 ± 0.21 μmol/L (range: 0.41–0.95) (P = 0.10).

**Quantification of liver reserves.** The liver reserve for group 1 was 27.1 ± 9.0 RE/g liver and for group 2 was 17.9 ± 8.1 RE/g liver (P = 0.13). The total liver reserve [RE/(g liver * total weight of liver)] was also calculated for each piglet. The total liver reserves for group 1 and 2 were 4210 ± 1910 and 2780 ± 1520 RE/liver, respectively, which did not differ.

**DISCUSSION**

The intent of this study was to simplify the MRDR analytical method and to further validate the most appropriate time frame in which the postdose blood sample should be collected. Although a simplified MRDR analytical method was published (12), it was not rigorously compared with the standard method from which the MRDR test was developed and validated after VA supplementation.

We examined 4 test methods, all modified versions of the 400-µL method, and compared them to the 400-µL method. Of the 4 test methods, our results supported only test method 2 as an adequate replacement for the 400-µL method. Test method 2 yielded MRDR values nearly identical to the 400-µL method. Although the slope of the regression line differed significantly from the line of equality (y = x), it was not biologically different. Evidence for the similarities between the 2 methods is further supported by the fact that the difference between the 2 was only 0.001, which was not significant. A difference of 0.001 between methods is small given the fact that the MRDR value is rounded to the nearest thousandth and the current cutoff value indicating subclinical VAD is 0.060. Additionally, there was no evidence of a relation between the difference and mean between test method 2 and the 400-µL method, indicating that test method 2 gave measurements similar to the 400-µL method at all ranges of MRDR values in this study. Finally, the repeatability coefficient obtained by this laboratory of test method 2 was relatively low. This indicates that 95% of all differences between replicates of the same serum using test method 2 should fall within ±0.008 U of each other. However, there did appear to be an outlier (Fig. 3) and elimination of the outlier from the data set gave a slightly reduced repeatability coefficient of 0.006 U. This is comparable to the repeatability coefficient of 0.004 U obtained for the 400-µL method.

Given the rigorous examination of test method 2 compared with the 400-µL method, we feel that it is sound to recommend using the 2 methods interchangeably. Test method 2, which we will hereafter call the 200-µL method, uses half the serum required for the 400-µL method; therefore a smaller blood sample can be taken from an individual. It uses smaller
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The other test methods did not show an accurate and precise correlation with the 400-μL method. Test method 1, designed to reduce the amount of serum used in the MRDR test to 100 μL, appeared to yield MRDR values very similar to those given by the 400-μL method but the means differed by 0.006 U. We consider this too great a difference to be used interchangeably with the 400-μL method, especially when evaluating the effect of an intervention. Test methods 3 and 4 were designed to reduce the size of the blood sample required, decrease sample analysis time, and thus increase sample throughput. However, both gave MRDR values that differed quite noticeably from the 400-μL method (Fig. 2C and 2D). Further analysis of the discrepancy in the ratio showed that R concentration was significantly lower (P < 0.0001) and DR concentration was significantly higher (P = 0.008) on the shorter column (7.5 cm) compared with the standard column (15 cm). Although liver reserve times of R and DR were reduced on the short column, the resolution of cis/trans isomers may have been enhanced. Therefore, it could be that co-elution of a minor cis isomer of R with the DR caused this discrepancy. Finally, differences between methods 3 and 4 and the 400-μL method appeared to become larger as MRDR values increased (Fig. 2C and 2D). For these reasons, test methods 3 and 4 were not considered as alternatives.

Without further validation and field testing, we cannot recommend analyzing serum volumes of 100 μL or less to determine an MRDR value, nor can we recommend using a 3.5-μm, 7.5-cm, C18 column with a mobile phase of 75:20:5 methanol:acetonitrile:water (by vol) with 0.73 g/L TEA, until further testing can be done.

The change in MRDR value over time was also examined in this study. The results obtained from our time analysis showed that in group 1, there was no significant change in MRDR value between 5 and 7 h, and in group 2, there was no significant change in MRDR value between 4 and 6 h. Because we had 2 separate groups of piglets, we cannot determine from these data at what point between 3 and 4 h would be the earliest acceptable time to take the postdose blood sample, nor can we determine at what point between 7 and 8 h would be the latest acceptable time to take the postdose blood sample. Given the results of this study, we recommend that blood samples be taken between 4 and 7 h after administering the DRA dose. A similar range of sampling time was observed previously in children (7,38,39) and women (32).

Of final interest are the liver reserves of the piglets. Group 1 tended (P = 0.13) to have markedly higher liver reserves (27.1 RE/g liver) than did group 2 (17.9 RE/g liver), and there was a clear biological difference in the VA status of the 2 groups. Currently, a liver reserve of <20 RE/g liver indicates VAD (40). Given this indicator value, group 2 was deficient, whereas group 1 was not. This was an unintentional and surprising result because piglets were randomly assigned to groups and all piglets were fed the same VA-free diet for the duration of the study. Using the 200-μL method, the MRDR value for group 1 at 5 h was 0.038 and for group 2 at 4 h was 0.085 (P = 0.013). Using a value of ≥0.060 to indicate VAD, these MRDR values indicate that group 1 had sufficient VA status, whereas group 2 was deficient, which is in agreement with the tendency for a higher liver vitamin A concentration in group 1 compared with group 2.

In summary, we propose that the 200-μL method, with a blood sample collected between 4 and 7 h postdose, is an acceptable substitute for the 400-μL method. The 200-μL method uses the same DRA dose size per individual as the 400-μL method (i.e., 5.3 μmol for children <6 y old, 7.0 μmol for children aged 6–12 y, and 8.8 μmol for adults and children >12 y old), but only 0.5 mL, as opposed to 1 mL, of blood has to be collected. Sample preparation must be carried out in glass because we found unacceptable results with extractions performed in plastic (data not shown). To determine extraction efficiency, we recommend that an internal standard (e.g., retinyl acetate) be added to the serum before any solvents are added. Serum proteins should be denatured with 250 μL pure ethanol followed by 2 extractions using 300 μL of hexanes, pooling organic layers. The organic layer should be dried under inert gas and reconstituted in 40 μL of 75:25 MeOH:DCE. For HPLC analysis, a C18, 5-μm, 15-cm reversed-phase column is recommended. We found that a mobile phase of 89:11 methanol:water with 0.73 g/L TEA at a flow rate of 1 mL/min separated DR from B satisfactorily in our laboratory. A wavelength of 350 nm should always be used to optimize detection of DR.

Other investigators have used 100 μL serum, different columns, and different solvents in large survey studies of VAD in which sample throughput is of utmost importance (12). Although greater discrepancy from the 400-μL method may be acceptable for survey studies in which only the prevalence of marginal deficiency is of interest, for intervention studies in which the main outcome is change in VA status, we strongly recommend the validated standard method of 200 or 400 μL serum to be able to make a comparison with existing MRDR data.

We recommend that the 200-μL method be used when evaluating individuals, such as infants and children, from whom blood samples are obtained by finger stick or heel prick, or when performing large studies. Collecting less blood from these sensitive areas will reduce the duration of any pain felt from the procedure and may make parents feel more comfortable about having their infants and children tested for VAD. The collection of smaller blood volumes will also allow for easier and speedier blood collection in large-scale studies and allow for more efficient transport and storage. Having a quick, accurate, and less invasive test will allow researchers to have improved success evaluating the vitamin A status of individuals and populations at risk for VAD and related health problems.

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LITERATURE CITED

7. Tanumihardjo, S. A., Cheng, J. C., Permaesih, D., Muherdiantiningr,


28. Kansas State University Agricultural Experiment Station and Cooperative Extension Service (1997) Starter Pig Recommendations. Publication no. MF2300. Kansas State University, Manhattan, KS.


