Dried blood spot retinol and retinol-binding protein concentrations using enzyme immunoassay as surrogates of serum retinol concentrations

Sherry A. Tanumihardjo
William S. Blaner
Tianan Jiang

MOST Technical Report

June 2002
## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Foreword</td>
<td>5</td>
</tr>
<tr>
<td>Introduction</td>
<td>7</td>
</tr>
<tr>
<td>Major Points of Discussion</td>
<td></td>
</tr>
<tr>
<td>1. The quality and completeness of the process followed for development of each method and assessment of its performance across multiple panels of samples under laboratory conditions.</td>
<td>9</td>
</tr>
<tr>
<td>2. The outcome of such process in terms of method’s performance under laboratory conditions, e.g. accuracy, intra-assay variability, inter-assay precision, working range, quantification limit, and detection limit, compared with HPLC serum or plasma retinol RBP-EIA.</td>
<td>10</td>
</tr>
<tr>
<td>3. The quality, completeness, analytical approach, and results of the process followed for field validation of the method compared with HPLC serum or plasma retinol in children and/or adults with different levels of serum/plasma retinol, particularly within the low range commonly found in developing county populations at risk of vitamin A deficiency.</td>
<td>11</td>
</tr>
<tr>
<td>4. Eventually, identification of gaps in research and documentation of the method’s performance and analytical characteristics that would need to be filled in order to establish its potential as a surrogate for HPLC serum or plasma retinol.</td>
<td>12</td>
</tr>
<tr>
<td>5. Overall assessment of appropriateness and completeness of the available information as a basis for an IVACG formal technical group review.</td>
<td>14</td>
</tr>
<tr>
<td>References</td>
<td>17</td>
</tr>
</tbody>
</table>
Foreword

The need for simpler and more affordable laboratory methods for assessment of vitamin A status of population groups is widely recognized. The current high-performance liquid chromatography (HPLC) method for serum or plasma retinol is expensive, time consuming, and difficult to use in developing countries because of cumbersome handling, preservation, and transportation of specimens, complex laboratory instrumentation requiring specialized skills, and expensive equipment. Two alternative methods have apparently reached a sufficiently advanced stage of development and field validation to merit examination of their potential as surrogates for HPLC serum/plasma retinol: a dried blood spot (DBS) on filter paper HPLC method by Craft Technologies, and a retinol-binding protein (RBP) assay in serum using an enzyme-immunoassay (EIA) method and portable plate reader by the Program for Appropriate Technologies in Health (PATH).

MOST organized a rapid examination of the analytical performance of these two methods by a group of recognized experts in the field. The purpose of the review was to examine the available evidence on the analytical performance and field validation of the two methods as possible surrogates for HPLC retinol in order to establish the extent to which a further formal review by the International Vitamin A Consultative Group (IVACG) is warranted.

The review team examined the quality and completeness of the information on the methods’ performance in the laboratory and under field conditions. The team concluded that 1) both methods appear to have a good potential as surrogates to HPLC serum retinol, each with its own advantages and limitations for practical application; and 2) a formal review by IVACG could be undertaken if the review presented the methodologies as something to look forward to in the future, but not if such a review required presenting the procedures as a finished product.

Given the practical relevance of this matter, MOST would like to share the report of this review with the international community and interested groups in developing countries. As new and promising methods emerge with sufficient information to assess their potential as surrogates for HPLC serum/plasma retinol for assessment of sub-clinical vitamin A deficiency in population groups, MOST will continue to evaluate the new information and to share the results.

Jose O. Mora, MD
Policy Advisor
MOST Project
Introduction

The reviewers discussed the limitations of using serum retinol concentrations as a measure of vitamin A status. The major disadvantage as outlined by the World Health Organization (WHO) in 1996 is that retinol concentrations are decreased by acute and underlying infections. Seasonality of disease can shift serum retinol distributions regardless of dietary sources of vitamin A (1). Moreover, serum retinol does not always respond to treatment with vitamin A (2, 3). Poor iron status, which is more prevalent than vitamin A deficiency, also affects serum retinol concentrations (3, 4, 5). Nonetheless, the International Vitamin A Consultative Group (IVACG) recommends serum retinol as a legitimate measure of the vitamin A status of populations (6). Indeed, serum vitamin A concentrations are the most commonly used biochemical parameter for assessing the vitamin A status of populations. Simple, rapid, and inexpensive methods of determining serum retinol concentrations are still needed. Thus, the development of surrogate measures of serum retinol concentrations to help define the vitamin A status of populations that are field-friendly and cost-effective will certainly be worthwhile.

To this end, two different methodologies for use in assessing the vitamin A status of children at risk of vitamin A deficiency are being considered for further development. One of these methodologies is being proposed by Craft Technologies, Inc. (Craft) and makes use of dried blood spots (DBS) collected and processed uniformly in the field, followed by standard HPLC procedures for determining the concentration of retinol present in the DBS. The second methodology, being proposed by the Program for Appropriate Technologies in Health (PATH), makes use of an enzyme-immunoassay (EIA) to assess serum retinol-binding protein (RBP) concentrations. Because serum RBP concentrations are highly correlated with serum retinol concentrations, RBP serves in this assay as a surrogate measure for serum retinol.

Both Craft and PATH have strong track records in the development and use of methodologies for assessing micronutrient status and other health-related parameters (such as immunological parameters) in infants from at-risk populations. As might be expected of such excellent organizations, both proposals are solid and meritorious. The individual merits and potential for further investigation of each method are discussed below.
Major Points of Discussion

The consultation was centered around five major points of discussion. The consensus of the authors is systematically discussed.

1. **The quality and completeness of the process followed for development of each method and assessment of its performance across multiple panels of samples under laboratory conditions**

Craft: The most important advantage in the DBS method is eliminating the need for venous blood sampling, which is often a technical challenge in infants and young children. Another important advantage of using DBS is convenient and low cost transporting of specimens before analysis when comparing paper samples to larger volumes of plasma or serum. The disadvantages of this method include the increased work in the laboratory to elute the retinol from the filter paper and the increased additional variability of the resultant determination, compared with measurements of retinol in plasma samples. Also, accuracy is influenced by variables such as paper performance characteristics, blood spot size, blood hematocrit, and analyte distribution. However, Craft argues that these possible sources of error can be eliminated by including a simultaneous measure of sodium along with the retinol determination. Alternatively, the use of a precisely measured volume of blood would increase the accuracy and reproducibility of DBS.

The Craft method for analysis of retinol in dried blood spots has not been tested in the field from start to finish. All current information points to the fact that the samples were collected and brought back to the Craft laboratories. Experience with developing countries has suggested that countries would like to maintain control of sample analysis “in house.” Therefore, validation of the method for analysis within several geographically and culturally distinct regions of the world will be important for reaching a final conclusion about the usefulness of this method. Craft is currently working with investigators in Indonesia to accomplish this objective. The consultants feel that validation trials should be carried out in additional countries.

PATH: The RBP-EIA has been developed as a rapid, inexpensive test for quantification of RBP from individual serum samples. Compared with measuring retinol by HPLC, this method is relatively inexpensive and simpler. However, the method has only been tested with venous blood and a moderately expensive laboratory instrument such as a microplate reader may be required. PATH is currently developing an inexpensive portable unit for reading samples. The assay seems to be a practical alternative to measuring serum retinol in population surveys and provides a more cost-effective tool for assessing vitamin A status in compromised populations.

The PATH method has been done on a small number of samples. The method has not been tested at a field site from start to finish within a country. At the current time, PATH is setting up the appropriate collaborations for field-testing. As is the case for the DBS methodology, the consultants believe that the RBP-EIA also needs to be validated in three to five distinct settings.
2. The outcome of such process in terms of method’s performance under laboratory conditions, e.g. accuracy, intra-assay variability, inter-assay precision, working range, quantification limit, and detection limit, compared with HPLC serum or plasma retinol

A summary of analytical performance is outlined in the following table:

<table>
<thead>
<tr>
<th></th>
<th>Retinol in DBS</th>
<th>RBP-EIA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy</td>
<td>0.96 ± 0.04%</td>
<td>0.96 ± 0.04%</td>
</tr>
<tr>
<td>Detection limit</td>
<td>3.0 µg/dL (0.1 µmol/L)</td>
<td>1.1 µg RBP/ml (0.05 µmol/L)</td>
</tr>
<tr>
<td>Quantitation limit</td>
<td>4.3 µg/dL (0.15 µmol/L)</td>
<td>7.7 µg RBP/ml (0.37 µmol/L)</td>
</tr>
<tr>
<td>Linearity</td>
<td></td>
<td>0.997 ± 0.07</td>
</tr>
<tr>
<td>Range</td>
<td>4.3–70 µg/dL (0.15–2.5 µmol/L)</td>
<td>8.9–37.8 µg RBP/ml (0.4–1.8 µmol/L)</td>
</tr>
<tr>
<td>Analyte recovery</td>
<td>0.97 ± 0.04</td>
<td>1.02 ± 0.11</td>
</tr>
<tr>
<td>Intra-assay variability</td>
<td>&lt;6 %</td>
<td>8.9 % CV in the calibrated range</td>
</tr>
<tr>
<td>Inter-assay variability</td>
<td>&lt;6 %</td>
<td>6.25% CV</td>
</tr>
<tr>
<td>Validity (at 0.7 µmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sensitivity</td>
<td>73–93%</td>
<td>70% (83%, at 0.775 µmol/L)</td>
</tr>
<tr>
<td>Specificity</td>
<td>90–100%</td>
<td>93% (87%, at 0.775 µmol/L)</td>
</tr>
<tr>
<td>Comparison studies (r², compared to serum retinol by HPLC)</td>
<td>0.73–0.84 (0.95)</td>
<td>0.82–0.84</td>
</tr>
</tbody>
</table>

**Craft:** Currently, the dried blood spot analysis is equivalent to about 5 µL of serum. There was concern among the group that this level of detection cannot be achieved by most HPLC systems located in developing countries. This may limit the usefulness of this method. As suggested above in discussion point no.1, the DBS must be tested in several different countries under conditions common to that region in order for the methodology to be accepted as valid.

**PATH:** Currently, serum retinol concentrations by HPLC can easily go up to 200 µg/dL (7 µmol/L) while the PATH method will quantitate retinol levels up to 50 µg/dL (1.8 µmol/L),
equivalent to 38 µg RBP/ml. PATH explains that if exact values are needed, individual samples above 38 µg RBP/ml could be diluted so that the quantitation will fall within the reliable working range.

3. **The quality, completeness, analytical approach, and results of the process followed for field validation of the method compared with HPLC serum or plasma retinol in children and/or adults with different levels of serum/plasma retinol, particularly within the low range commonly found in developing county populations at risk of vitamin A deficiency**

**Craft:** The Craft manual is not a finished product to be used by countries to do the analysis. There are no data on the decomposition rate of blood spot retinol in children when their serum retinol concentrations are less than 30 µg/dL. Will the decomposition rate be the same in children who have multiple micronutrient deficiencies as those observed for healthy children? Will this rate be dependent on nutritional state or the concentrations of antioxidants in the blood? Has Craft tried adding an antioxidant coating to the blood spot to try to prevent the degradation that has been observed upon storage of the blood spots? Craft admits that the mechanism(s) responsible for retinol degradation is currently unknown. Because of the need for HPLC analysis, the DBS retinol determinations will need to be carried out in a centralized laboratory facility that will probably be located at a distance from the actual site of the vitamin A assessment campaign. One could argue that there would be merit in establishing a number of centralized laboratories for the determination of DBS retinol levels because this would make such determinations more standard. However, this also would be costly and would necessarily remove such determinations from the field where they may be most relevant and have the greatest direct impact on public health programs and their implementation.

Moreover, since DBS retinol levels must be normalized for blood sodium contents, the DBS approach requires that all samples undergo two determinations, one for retinol and the other for sodium. This adds extra cost and the possibility of additional measurement errors to the DBS methodology. Craft responded to this by stating that the instrumentation for the sodium determination is inexpensive and user-friendly.

The DBS method also requires that a subset of serum samples be analyzed separately for retinol concentrations using standard HPLC methodology. This will allow the calculation of the “adjustment factor” for degradation of retinol within the samples upon storage. The suggested serum subsample is ≥10% of the total number of dried blood spots analyzed.

**PATH:** The PATH method has given a workable “range” of 9 to 38 µgRBP/ml (0.4–1.8 µmol/L). While this will work for surveys this probably does not encompass all of the values one would see post-intervention, especially in some of the Central and South American countries that tend to have higher mean values of serum retinol concentration than the Asian countries. Is the method a yes/no category or is there a linear relationship? PATH addressed this issue by stating that they were given a working range of 10–50 µg/dL retinol (9–38 µg RBP/ml) and that is what they set out to accomplish. However, this working range could be increased as the method continues development and acceptability. PATH is also developing a method to categorize serum intervals. This probably is not useful for interventions but may be useful for broad surveys. Thus, for values obtained from the RBP-EIA methodology, it
will only be possible to determine the percentage of the population that is deficient below a “cut-off.” PATH has not done validation studies in a large number of samples, and this limits the consultants’ ability to have a definitive conclusion regarding this methodology.

4. Eventually, identification of gaps in research and documentation of the method’s performance and analytical characteristics that would need to be filled in order to establish its potential as a surrogate for HPLC serum or plasma retinol

**Gaps in research**

Both the CRAFT and PATH methods need to be evaluated by a third-party laboratory that has no financial interest in the outcome. This would best be accomplished in a laboratory of a developing country that has the capability to do both tests. As discussed at the consultation, this may be limited by the sensitivity of the HPLC system for blood spot retinol.

**Craft:** We question whether the analysis will be done in country or if the samples will be sent to Craft. Are the instruments needed for this analysis broadly available in countries or regions experiencing vitamin A deficiency? Who is going to analyze the samples and who is going to train the staff in country? Does Craft have the ability to do 15,000 samples in a timely manner? Typically in nutritional surveys thousands of samples are generated.

The most recent paper published by Craft in *Journal of Nutrition* suggests using tocol as an internal standard, yet the wavelength of detection for this standard is different for retinol. Many single wavelength detectors cannot easily change from one wavelength to another. This may create a complication in laboratories that only have a single wavelength detector available to them. Standardization with retinyl acetate is probably more relevant to global application. The DBS methodology needs to be made as friendly as possible for use in the developing world.

Ultimately, the DBS methodology must be tested in three to five distinct “real world” settings. Craft must establish that this methodology is universally applicable in settings where instrumentation, financial resources, and training are limited. This can only be established by field trials. Thus, further research into the utility and reproducibility of the DBS methodology in different countries is critically needed if this method is to be accepted as universally valid. Moreover, the intra-individual variation has not been assessed yet. It is important to determine how values from the same individual vary across many blood spots.

**PATH:** What is the standard that will be used in the RBP-EIA? Will it be purified human serum RBP? How will this vary from country to country? Or would this be part of a “kit” supplied with the instrumentation? RBP is stable as a lyophilized powder but not in solution unless stored at –80°C. How will storage of the standard influence assay results? These points need to be addressed in the manual and tested in the field. Typically, reagents are used until “gone” and this could confound the results if “old” RBP solutions are used.

There also is a question of whether monoclonal or polyclonal antibodies should be used in the EIA. Since a monoclonal has been selected by PATH for use, it is imperative to field test the monoclonal antibody in several different countries where the populations would be expected to have very distinct genetic backgrounds (to verify that the monoclonal antibody
recognizes equally well RBP present in the circulations of genetically distinct individuals. Field trials in several genetically and geographically diverse populations would establish validity of the RBP-EIA assay in a variety of settings. It was presumed that this would take care of many of the potential confounding variables that could result in misleading interpretations. It is important that these test populations have high prevalence of vitamin A deficiency. Also, there is a lack of knowledge about the ELISA and EIA methodologies in many developing settings and there may be a need to provide information on these methodologies as part of the kit manual.

The committee also expressed concern about the possible interference of transthyretin (TTR) in the RBP-EIA. There are hundreds of known polymorphisms in TTR, and TTR binds to many different substances that can be found in the circulation. Will this be a confounder? This has not yet been rigorously tested.

There have been a number of reports of apo-RBP in circulation. Wahed et al. (7) determined RBP saturation of 25 to 130 percent in Bangladeshi children. This point needs to be considered. What happens to RBP saturation in children with infections? It is known that serum retinol is depressed, but what about RBP? Is serum RBP depressed to the same extent as serum retinol or do increased levels of apo-RBP circulate? These questions need to be addressed by further research.

As is the case for the DBS methodology, the RBP-EIA needs to be validated in three to five “real world” settings. Moreover, since the RBP-EIA employs a monoclonal antibody, an attempt should be made to study its performance in genetically diverse populations to assure the assay’s universal applicability to all populations. These field trials must be carried out in order for conclusions to be made about the validity and usefulness of the RBP-EIA.

**Gaps in documentation**

**Blood spot manual:**

This manual is missing the actual detailed “cookbook” for the analysis of the dried blood spots. While great care is taken to explain how one goes about collecting the samples, the actual “how-to-analyze” is missing. This should be included so that the analysis can be done without reference to the publications.

The section on statistics and sampling could actually be left out. Typically, the sampling and statistics are determined by a different group of individuals, usually the principal investigator in consultation with a statistician. However, the field collection and analysis are typically performed by laboratory staff.

It is not useful to list prices of supplies as these will vary tremendously between countries and are constantly changing.

The blood spot manual needs to address the following questions in the manual: What are the ramifications of the “bad blood spots?” For instance, if there is a halo effect, how does that change the value? Are these spots still usable? What about a messy blood spot?
PATH manual:

Section 1: Please refer to serum retinol as “an” accepted biological indicator. This section seems to suggest that there are no other accepted indicators and that is not true. Serum retinol is one of many.

Section 2: The list of methods to assess vitamin A deficiency is not inclusive. Maybe this section should be titled: “Existing methods to assess serum vitamin A concentrations.”

What is the volume of serum that is needed for this analysis and how do freeze-thaw cycles affect the results?

The 1:1 relationship between RBP and retinol is assumed here, yet in some of the analyses presented 0.35 µmol/L was equivalent to 0.48 µmol/L RBP.

The manual also used “pre-albumin,” is not the preferred term “transthyretin” (also observe correct spelling)?

For the prevalence survey section, one would assume that you would only need 35 to 97 individuals based on the presentation on page 22. However, if you have a correlation of 0.55 between RBP and retinol with 200+ children in a survey, would these numbers truly be enough? Again, reiterating the inclusion of the statistical information, maybe the statistical information is more appropriate in the appendix and the current appendix material (the procedure) is more appropriate as a chapter.

Section 5: For the purposes of the manual is it necessary to devote an entire section to “interfering substances”? While it is important for the review of the methodology, the results suggest that these substances do not interfere and therefore a single sentence in the manual would be all that is necessary.

5. Overall assessment of appropriateness and completeness of the available information as a basis for an IVACG formal technical group review

Clearly, more work needs to be done before either of these methods can be recommended as surrogate measures for serum retinol. Therefore, if a formal review by IVACG would necessitate presenting the procedures as a finished product, the answer to this question is currently No. But if IVACG were to present the methodologies as something to look forward to in the future, then the answer is Yes.

Craft: The dried blood spot methodology is valuable because it can be used in remote locations that may lack electricity or sophisticated equipment. The samples can be taken back to a central laboratory for the analysis. The actual analysis of dried blood spots ($15–20 per test) is probably not cheaper than serum retinol analysis and may even be more expensive, considering the need to also measure sodium concentrations. Craft provided a great deal of data from the peer-reviewed literature regarding the characteristics of its DBS protocol and its use for the assay of blood retinol levels. This literature is strong and convincing. Using the DBS methodology proposed by Craft, it should be possible to obtain reproducible and accurate measures of blood retinol concentrations for a relatively large number of samples.
collected in the field. By coordinating and/or standardizing the HPLC analysis of DBS, a uniform and internationally accessible approach for assessing vitamin A status in at-risk populations could be established.

**PATH:** The cost of the RBP analysis is estimated to be $3.00/duplicate. This is very cost effective compared to serum retinol analyses by standard HPLC methodologies. Another strength of this procedure is that only 10 μL of serum is required, and this can be diluted for multiple analysis. It also maybe possible to use dried blood spots for RBP analysis. The use of the RBP-EIA procedure described by PATH should allow trained local public health workers to locally categorize the vitamin A status of their population. The potential for local assessment of vitamin A serum concentrations through RBP determination is promising. This would bring an accurate and simple vitamin A assessment methodology into the “toolbox” of local public health workers and this would likely have a positive and empowering impact on local vitamin A assessment/intervention programs.
References

1. Indicators for assessing vitamin a deficiency and their application in monitoring and evaluating intervention programmes. World Health Organization, 1996.


