Retinol concentrations in capillary dried blood spots from healthy volunteers: method validation¹⁻³

Neal E Craft, Jesús Bulux, Carlos Valdez, Yukang Li, and Noel W Solomons

ABSTRACT

Background: Vitamin A deficiency (VAD) is a major public health problem in the developing world, leading to >3 million eye-related problems in preschool children. Nearly 250 million children have subclinical VAD, resulting in a 23% increase in childhood mortality. Difficulties in obtaining samples to assess VAD have hampered the detection, intervention, and surveillance of VAD. The use of dried blood spots (DBS) could ameliorate many problems of vitamin A assessment.

Objective: The objective of this study was to validate the use of retinol in DBS for vitamin A assessment by comparing it with venous and capillary serum retinol.

Design: Venous and capillary blood specimens were obtained simultaneously from 20 healthy adult volunteers. From each blood specimen, both DBS and liquid serum were prepared (a total of 80 samples). All specimens were maintained at −70°C until HPLC analysis.

Results: The mean retinol concentrations in the 4 sample types were as follows: venous serum (2.02 ± 0.42 μmol/L, or 58 ± 12 μg/dL), capillary serum (2.06 ± 0.42 μmol/L, or 59 ± 12 μg/dL), venous DBS (2.06 ± 0.49 μmol/L, or 59 ± 14 μg/dL), and capillary DBS (2.09 ± 0.45 μmol/L, or 60 ± 13 μg/dL). Of the 6 possible 2-way combinations, the $R^2$ values ranged from 0.77 for capillary DBS versus venous DBS to 0.95 for venous serum versus capillary serum.

Conclusions: DBS retinol measured by HPLC is comparable with serum retinol. Thus, it is possible to compare and combine blood retinol concentration data obtained from DBS with current and historic measurements in serum. Am J Clin Nutr 2000;72:450–4.

KEY WORDS Vitamin A deficiency, retinol, dried blood spots, filter paper, capillary blood, venous blood, HPLC

INTRODUCTION

In recent years, renewed interest has developed in detecting and redressing endemic micronutrient deficiencies. The principal nutrients of interest have been vitamin A, iron, and iodine (1, 2). The most widely accepted approach to nutritional assessment has been to assay blood serum for the specific analytes or related enzymatic indicators. Both the World Health Organization and the United Nations Children’s Fund (3) have proposed the use of serum retinol as a key indicator of vitamin A deficiency (VAD). In developing countries, several barriers exist to the use of venous blood in field studies. First, venous blood collection requires trained phlebotomists to obtain the samples. Second, because of local taboos, cultural beliefs, and concerns about disease transmission, there is a pervasive resistance to having blood drawn through hypodermic needles. This is particularly true in parents of young children. A third barrier to the collection of venous blood is the increasing risk of acquiring or transmitting blood-borne viral diseases, such as chronic hepatitis and AIDS. Additionally, to isolate the serum component, blood samples require centrifugation, which is impractical in the absence of electricity. Finally, a “cold-chain” of low-temperature storage and transportation must be maintained from the time of sample collection until analysis.

Most of these obstacles can be avoided by analyzing dried blood spots (DBS) rather than venous serum. Recently, Craft Technologies (Wilson, NC) developed a procedure to measure blood retinol concentrations in DBS by using HPLC (4, 5). The application of this technique to assess vitamin A status in public health surveys could improve subject participation and reduce logistic problems.

Currently, little information is available regarding the comparability between retinol concentrations of venous or capillary serum and venous or capillary DBS. The method was developed and the initial comparison was made by using DBS prepared from venous blood. To further validate this technique, we present the results of a comparison of retinol concentrations measured in liquid serum and in DBS specimens obtained by venipuncture and finger prick from healthy adult volunteers in Guatemala.

¹From Craft Technologies, Inc, Wilson, NC, and the Center for Studies of Sensory Impairment, Aging and Metabolism (CeSSIAM), Guatemala City.
²Supported by Task Force Sight & Life, Basel, Switzerland; Latin American and France-Benelux divisions of the Kellogg Company; and Roche Interamericana for Central America, San José, Costa Rica. Tocol was donated by Hoffmann-La Roche, Basel, Switzerland.
³Reprints not available. Address correspondence to NE Craft, Craft Technologies, Inc, 109 Park Avenue, Wilson, NC 27893. E-mail: ncraft@crafttechnologies.com.

Received September 2, 1999.
Accepted for publication January 1, 2000.
SUBJECTS AND METHODS

Subjects

Twenty healthy volunteers (3 males and 17 females) aged 14–75 y (median: 37 y) were recruited in Guatemala City for the study. All subjects were informed about the experimental protocol before providing informed consent. Four blood specimens, 2 serum [venous (VS) and capillary (CS)] and 2 dried blood spots (DBS) [venous (VDBS) and capillary (CDBS)], were obtained virtually simultaneously from the 20 subjects to generate 80 samples for retinol analysis; 2 of the subjects were pregnant at the time of sampling. The study was performed in accord with the 1983 revision of the Helsinki Declaration.

Sample collection and processing

Subjects were sampled in either the fasting or the postprandial state. For the capillary samples, isopropyl alcohol was applied to the voral surface of the distal phalanx of a finger and then a puncture was made into the subcutaneous capillary bed with a lancet (Microtainer; Becton Dickinson Vacutainer Systems, Franklin Lakes, NJ). Approximately 2 drops of whole blood directly from the finger prick were used to fill each of 5 inscribed circles on the surface of the blood-spot collection card (no. 903; Schleicher & Schuell, Keene, NH). Additional capillary blood from the finger was collected into a microsample tube without anticoagulant (Microtainer) to supply capillary serum. These samples were centrifuged at 1200 × g for 10 min and centrifuged at 10000 × g for a maximum of 2 wk until transported on ice. The serum was transferred to a plastic screw-top vial (V anguard; Sumitomo Bakelite Co, Osaka, Japan). The total volume of capillary blood collected was ≈400 μL; however, only 50–100 μL is necessary for DBS retinol analysis.

For the venous samples, 5 mL blood was drawn from an antecubital vein into a syringe and transferred to an evacuated tube (Becton Dickinson Vacutainer Systems) without anticoagulant; immediately, 1–2 drops of blood were placed with a plastic pipette onto each of the 5 inscribed circles on another blood collection card. The remaining whole blood was allowed to coagulate at ambient temperature for ≈10 min and centrifuged at 1200 × g at ≈25°C and the serum was transferred to a plastic screw-top vial. Care was taken to protect the samples from light during all procedures. All samples were placed in a freezer within 20 min of collection and maintained at -20°C for a maximum of 2 wk until transported on dry ice to the analytic laboratory (Craft Technologies).

Chemical analyses

On receipt at the analytic laboratory, samples were stored at -70°C until analyzed. Serum and DBS retinol were measured with HPLC by using modifications of the procedures described previously (5, 6). All chemicals used were reagent grade or better.

Serum retinol

The HPLC consisted of a vacuum solvent degasser, quaternary gradient pump, programmable ultraviolet-visible detector fitted with deuterium and tungsten lamps, and an autosampler with a refrigerated sample compartment and column oven (Thermo Separation Products, San Jose, CA). After being thawed, 150–μL aliquots of serum were diluted with 150 μL water. Samples were deproteinized by vortex mixing with 300 μL ethanol containing tocol (6.2 μmol/L; Hoffmann-La Roche, Basel, Switzerland) as an internal standard and butylated hydroxytoluene (136 mmol/L; Sigma Chemical Co, St Louis) as an antioxidant. The samples were extracted twice with 1 mL hexane and the combined supernate was evaporated under a steam of nitrogen. The residue was dissolved in 35 μL ethyl acetate and mixed for 10 s. The sample was then diluted with 100 μL of the mobile phase and was ultrasonically agitated for 10 s before being transferred into an autosampler vial; the injected volume was 15 μL. The HPLC separation of retinol was performed isocratically by using a mobile phase of acetonitrile:dioxane:methanol (83:13:4, by vol) containing 100 mmol ammonium acetate/L and triethylamine (0.1%, by vol; Sigma Chemical Co) at a flow rate of 1.5 mL/min. A Spherisorb column (ODS 2; 3 μm, 150 × 4.6 mm) with a matching guard column (Keystone Scientific, Bellefonte, PA) was used while the temperature was maintained at 30°C. Retinol was monitored at 325 nm and the internal standard was monitored (tocol) at 300 nm.

DBS retinol

A 0.635-cm (0.25 inch) disk was punched from the center of a blood spot on the collection card. A standard hole punch was used to ensure that the same quantity of sample was removed from each subject’s card. The punched spot was placed in a 12 × 75 mm borosilicate test tube with 1 mL of 150 mmol phosphate buffer/L containing 57 mmol (1%, wt:vol) ascorbic acid/L and 2 mmol diethylentriamine pentaacetic acid/L (Sigma Chemical Co). Tubes were sonicated for 15 min and then 100 μL retinyl acetate (1.4 μmol/L, internal standard) in ethanol and 900 μL ethanol containing 100 mmol butylated hydroxytoluene/L and 50 mmol hydroquinone/L as antioxidants were added and vortex mixed for 20 s. A 2-mL portion of hexane was added and vortex mixed for 1 min. This mixture was centrifuged at 500 × g for 1 min at ≈22°C to separate the phases; the hexane layer was removed and the extraction was repeated. The combined hexane layers were dried under a nitrogen stream and dissolved in 60 μL of mobile phase facilitated by sonication for 10 s followed by mixing for 30 s. The reconstructed analytes were placed in a conical insert before HPLC analysis. The column used was a Hypersil octadeylsilane (3 μm, 4.6 × 75 mm), protected with a 3-μm guard column (Javelin; Keystone Scientific, Bellefonte, PA). The mobile phase was methanol:acetonitrile:water (65:34:1, by vol) at a flow rate of 1.0 mL/min. Retinol and the internal standard were monitored at 325 nm. An injection volume of 25 μL was used.

Calibration

For both types of analysis, an internal standard calibration was prepared by using peak areas to quantify retinol. Linear calibration curves were prepared consisting of 3 concentrations of retinol that spanned the physiologic concentrations in serum. Because the volume of serum in the DBS punches was not known, it was necessary to generate a “recovery/volume adjustment” factor (serum retinol/DBS retinol) to convert measured DBS retinol values to values equivalent to serum retinol. This was accomplished by dividing the serum retinol concentration by the measured retinol concentration in matching DBS to determine the ratio between the 2 sample types. The median factor (26.3) was then applied to all DBS retinol values to convert them to serum retinol equivalents. For populations examined thus far (in Nepal, Liberia, and Guatemala), this factor has varied by ≤10%.
RESULTS

With use of the venous retinol concentration as the gold-standard reference specimen, the values for our population ranged from 1.25 ± 1.25 g/dL (58 ± 85 μmol/L), to 2.96 ± 2.96 g/dL (85 ± 58 μmol/L). The mean (±SD) retinol concentrations in the 4 sample types were as follows: VS (2.01 ± 0.49 μmol/L, or 59 ± 14 μg/dL), VDBS (2.05 ± 0.49 μmol/L, or 59 ± 14 μg/dL), and CDBS (2.08 ± 0.45 μmol/L, or 60 ± 13 μg/dL). There were no significant differences. A chromatographic overlay of VS and CDBS retinol concentrations from the same subject is illustrated in Figure 1.

The $R^2$ values for the 6 bivariate combinations are given in Table 1. Values ranged from a low of 0.77 for CDBS retinol versus VDBS retinol to a high of 0.95 for VS retinol versus CS retinol. Lin’s concordance correlation coefficient for the same combinations ranged from 0.97 for CS retinol versus VS retinol to 0.87 for VDBS retinol versus CDBS retinol (Table 1). Scattergrams for 3 of the more practically important bivariate combinations are illustrated in Figure 2. Across the range of retinol concentrations represented by this population, the Bland-Altman relation of intermethod differences and means indicated that there was no significant bias in any of the bivariate combinations. The relation between differences in retinol concentrations obtained from VS and capillary DBS and the average of the values from these 2 methods are illustrated in Figure 3.

DISCUSSION

The biochemical assessment of vitamin A status in populations at risk of deficiency continues to be a challenging task. Many large trials of vitamin A intervention were conducted without any attempt to biochemically assess vitamin A status (10). A study in Tamil Nadu found it possible or prudent to take blood from only 280 (1.8%) of its 15 419 child participants (11). Those who have worked in developing countries with rural populations that refuse to provide venous blood will appreciate the tremendous strides that capillary blood sampling entails. Although the analysis of DBS retinol is not a panacea for all obstacles of vitamin A assessment, DBS samples are easily obtained and offer direct comparisons with serum retinol.

The purpose of this study was to provide further validation for the use of DBS retinol as an indicator of vitamin A status comparable with serum retinol. Of the various correlations examined during this validation exercise, 3 are worthy of special comment. First, there was a high correspondence between the retinol concentration in VS and CS samples (Figure 2). This was also observed by others (KP West Jr and S Yammini, unpublished observations, 1997). This means that laboratories able to measure retinol in serum microsamples (50–100 μL) can justify obtaining capillary blood to assess an individual’s vitamin status. This is significant because, even today, some laboratories use 500–1000 μL serum to measure retinol. The next comparison of most practical interest is that of CDBS retinol and VS retinol (Figure 2). The concordance here ($R^2 = 0.88$) indicates that, at least in healthy subjects, comparative statements between CDBS retinol surveys and archival, historical data of plasma or serum retinol are as valid as comparisons among studies using venous retinol samples. This observation appears to also hold true in subjects with marginal vitamin A status (NE Craft, unpublished observation, 1998).

Finally, inclusion of DBS made from venous blood and those made from capillary blood into a common data pool is justified (Figure 2, bottom panel). This situation arises in surveys when a subsample of a population undergoes extensive analysis (venous sample), whereas most of the population undergoes a basic screen-

<table>
<thead>
<tr>
<th>Variable</th>
<th>y Variable</th>
<th>$R^2$</th>
<th>$r$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I Venous serum</td>
<td>Capillary serum</td>
<td>0.95</td>
<td>0.97</td>
</tr>
<tr>
<td>II Venous serum</td>
<td>Capillary DBS</td>
<td>0.88</td>
<td>0.93</td>
</tr>
<tr>
<td>III Venous serum</td>
<td>Venous DBS</td>
<td>0.85</td>
<td>0.91</td>
</tr>
<tr>
<td>IV Capillary serum</td>
<td>Capillary DBS</td>
<td>0.85</td>
<td>0.92</td>
</tr>
<tr>
<td>V Capillary serum</td>
<td>Venous DBS</td>
<td>0.81</td>
<td>0.89</td>
</tr>
<tr>
<td>VI Capillary DBS</td>
<td>Venous DBS</td>
<td>0.77</td>
<td>0.87</td>
</tr>
</tbody>
</table>

*DBS, dried blood spots.*
ing (finger prick). An illustration of this would be subjects from whom both serum and DBS retinol are measured to determine a recovery/volume adjustment factor.

Our intent was to validate the DBS method across a range of retinol concentrations (deficient to adequate). However, the correspondence among methods for retinol concentrations in the low and deficient range was not established in this exercise because none of the subjects had serum retinol concentrations < 1.05 μmol/L (30 μg/dL). It is not surprising that all circulating retinol concentrations were > 1.05 μmol/L (30 μg/dL), the cutoff for adequacy in adults (12), because our subjects were healthy adults living in a country with vitamin A–fortified table sugar. However, in a previous trial with paired venous serum and DBS samples from Nepalese mothers, samples with serum retinol concentrations < 0.70 μmol/L (20 μg/dL) correlated well ($R^2 = 0.79$) with DBS retinol values, despite an incomplete cold chain (13).

It is apparent from Figure 1 that the peak area of retinol is much smaller and the demands on the HPLC system are much greater for the analysis of DBS retinol than for serum retinol. The concentration of retinol in the unadjusted DBS extracts ranged from ~0.017 to 0.12 μmol/L (0.5 to 3.5 μg/dL). Even so, the adjusted lower limit of retinol quantification in DBS is 0.1 μmol/L (3 μg/dL) and the short-term day-to-day variability of a standard blood spot sample was found to be <2%. These values fall well below the range indicating deficiency in young children, for whom this method is particularly suited. As described, some of the limitations of the method include retinol elution too close to polar extractants and occasional coelution of retinol or the internal standard with strongly retained components from prior injections. It was necessary to flush the HPLC system routinely with a high percentage of tetrahydrofuran to remove strongly retained blood components. Recently, a modified chromatographic method for analyzing DBS retinol was published, which minimizes these limitations (5). This method can be used for both DBS and serum retinol measurements.

Although the measurement of DBS retinol is a great stride forward, several authors (14), including those associated with the present study (13, 15), have pointed out the limitations of using

**FIGURE 2.** Correlations between capillary serum (CS) and venous serum (VS) retinol concentrations, between capillary dried blood spot (CDBS) and VS retinol concentrations, and between venous dried blood spot (VDBS) and CDBS retinol concentrations in 20 healthy adults.

**FIGURE 3.** Bland-Altman relation between the difference in retinol concentrations obtained from venous serum (VS) and from capillary dried blood spots (CDBS) and the average of values from the 2 methods.
circulating retinol concentrations as an indicator of individual vitamin A status. However, within these accepted limits, we showed that in healthy subjects DBS retinol is comparable with serum retinol. Furthermore, the use of capillary blood is much less problematic in terms of acceptance, safety, and logistics. At present, the DBS retinol samples can tolerate limited exposure to ambient temperatures but must be frozen for longer storage. Preliminary data indicate that measured retinol concentrations in DBS samples, at several storage temperatures, decrease until homeostasis is reached, at which time the concentration remains stable for weeks (5). A project is under way to validate this finding in a population with moderate-to-deficient vitamin A status and to closely examine the effects of sample temperature on retinol concentrations. In addition, efforts are under way to further simplify methods for the determination of retinol status, eg, to minimize sample refrigeration time and to develop a portable instrument for on-site analysis.

We gratefully acknowledge the assistance of Ivania Mena in recruiting subjects.

REFERENCES