

Retinol-Binding Protein Stability in Dried Blood Spots, Masako Fujita,^{1,2*} Eleanor Brindle,² Jane Shofer,² Philip Ndemwa,³ Yeri Kombe,³ Bettina Shell-Duncan,^{1,2} and Kathleen A. O'Connor^{1,2} (¹ Department of Anthropology, University of Washington, Seattle, WA; ² Center for Studies in Demography and Ecology, University of Washington, Seattle, WA; ³ Center for Public Health Research, Kenya Medical Research Institute, Nairobi, Kenya; * address correspondence to this author at: Department of Anthropology, Box 353100, University of Washington, Seattle, WA 98195-3100; fax 206-543-3285, e-mail masakof@u.washington.edu)

Background: Retinol-binding protein (RBP) is accepted as a surrogate biochemical marker for retinol to determine vitamin A (VA) status. A recently developed enzyme immunoassay for RBP uses serum or whole blood stored as dried blood spots (DBS). However, the stability of RBP in DBS has not been examined.

Methods: RBP stability was studied in a laboratory and in field conditions in northern Kenya. For the laboratory study, 63 DBS collected by finger prick and stored sealed in a plastic bag with desiccant were exposed to 1 of 5 time/storage-temperature treatments: (a) baseline, (b) 30 °C/7 days, (c) 30 °C/14 days, (d) 30 °C/28 days, and (e) 4 °C/38 days. Baseline RBP concentrations were compared to those obtained after the storage treatments. For the field study, 50 paired DBS and serum specimens were prepared from venous blood obtained in northern Kenya. DBS were stored in a sealed plastic bag with desiccant at ambient temperature (12 °C–28 °C) for 13–42 days, and sera were stored at –20 °C to –70 °C. Recovered RBP concentrations were compared with serum retinol for stability, correlation, sensitivity, and specificity.

Results: RBP in DBS stored in the laboratory at 30 °C remained stable for 2–4 weeks, but specimens stored at 4 °C for 38 days produced values below baseline ($P = 0.001$). DBS stored under field conditions remained stable for 2–6 weeks, as demonstrated by good correlation with serum retinol, a result that suggests that RBP in DBS will have good sensitivity and specificity for predicting VA deficiency.

Conclusion: RBP in DBS can withstand storage at a relatively high ambient temperature and thus facilitate accurate VA assessments in populations in locations where serum collection and storage are unfeasible.

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Vitamin A (VA) deficiency (VAD) is a serious public health problem in developing countries where dietary intake of VA is low (1–4). VA is essential for vision, growth, cellular differentiation, immune function, and reproduction (4,5). Vision-related symptoms, such as night blindness, can be used to identify clinical VAD, but only biochemical markers can detect subclinical VAD (4). Both clinical and subclinical VAD are associated with

increased morbidity and mortality (4,6,7). Furthermore, in populations facing the dual challenges of VAD and HIV, maternal VAD is associated with increased mother-to-child transmission of HIV (8). For these reasons, accurate and efficient assessment of subclinical VA status is crucial in many developing nations.

The relative instability of serum retinol (9), a biochemical marker of VA status, precludes its use in field research, although recent studies demonstrate stability of retinol in whole blood stored as dried blood spots (DBS) (10,11). Serum retinol-binding protein (RBP), the carrier protein of retinol, is accepted as a surrogate marker (10,12). A recently developed enzyme immunoassay for RBP that uses serum or DBS is commercially available (Scimedx Scanlisa RBP enzyme immunoassay). The use of DBS eliminates the need for sample vials and simplifies sample transportation (14). Although RBP is believed to be more stable than retinol (15), the stability profile of RBP in DBS is not well described except for a recent abstract (16) reporting tolerance of DBS-RBP to dry heat for several days. We investigated the effects of storage temperature and duration on RBP concentration in DBS in 2 studies, a laboratory study in which DBS were subjected to a range of storage conditions and durations simulating those likely to be encountered in field research settings and a field study in a VAD endemic area of northern Kenya, where we compared RBP concentration in DBS collected and stored at ambient temperatures to retinol concentrations in matched sera stored at –20 °C to –70 °C. Research procedures were approved by the Institutional Review Boards of the University of Washington and the Kenya Medical Research Institute (KEMRI).

For the laboratory study, 4 female volunteers in North America donated blood spots collected from free-flowing capillary blood obtained by finger prick with sterile lancets. DBS were collected on filter paper (Schleicher and Schuell 903), dried completely (4 h) at ambient laboratory temperature, and placed in 7 separate sealed plastic bags, each containing desiccant and 3 DBS from each individual. Each bag was exposed to 1 of 5 conditions in a freezer, incubator, or refrigerator: (i) –20 °C immediately after drying (baseline), (ii) 30 °C for 7 days, (iii) 30 °C for 14 days, (iv) 30 °C for 28 days, and (v) 4 °C for 38 days (1 bag/person for conditions 1–4, and 3 bags/person for 4 °C). After the respective treatments, all specimens were frozen (–20 °C) until assay analysis. The Scimedx RBP enzyme immunoassay has been described in detail elsewhere (13). One-quarter inch center disk, containing approximately 6.1 μL of serum, was punched from each DBS for elution in assay buffer. All specimens were assayed in a single batch 42 days after collection.

In the laboratory study result, specimens from all 4 volunteers had RBP concentrations above the cutoff for subclinical VAD (0.96 $\mu\text{mol/L}$). For 1 study participant all data were excluded from analysis because baseline RBP was above the linear range (1.92 $\mu\text{mol/L}$). The remaining

63 observations, shown in Figure 1A, were used for statistical analyses. The within- and between-assay CVs were 3.7% and 1.8%, respectively, for 9 replicates for the 4 °C 38-day conditions for DBS from each of 3 volunteers run on 2 assay plates. Linear mixed effects models of RBP on storage treatment were used to determine if different storage treatments were associated with differences in RBP concentrations. RBP concentrations in samples stored

at 30 °C for 7, 14, or 28 days did not differ significantly from baseline RBP at the group level ($P = 0.551, 0.624,$ and $0.083,$ respectively), although by day 28 there was significant variation in RBP across sample donors ($P = 0.0001$; range of differences between 28 days and baseline was -0.27 to -0.10 $\mu\text{mol/L}$). In contrast, specimens stored at 4 °C for 38 days showed RBP concentrations significantly lower than baseline (mean difference between 38 days

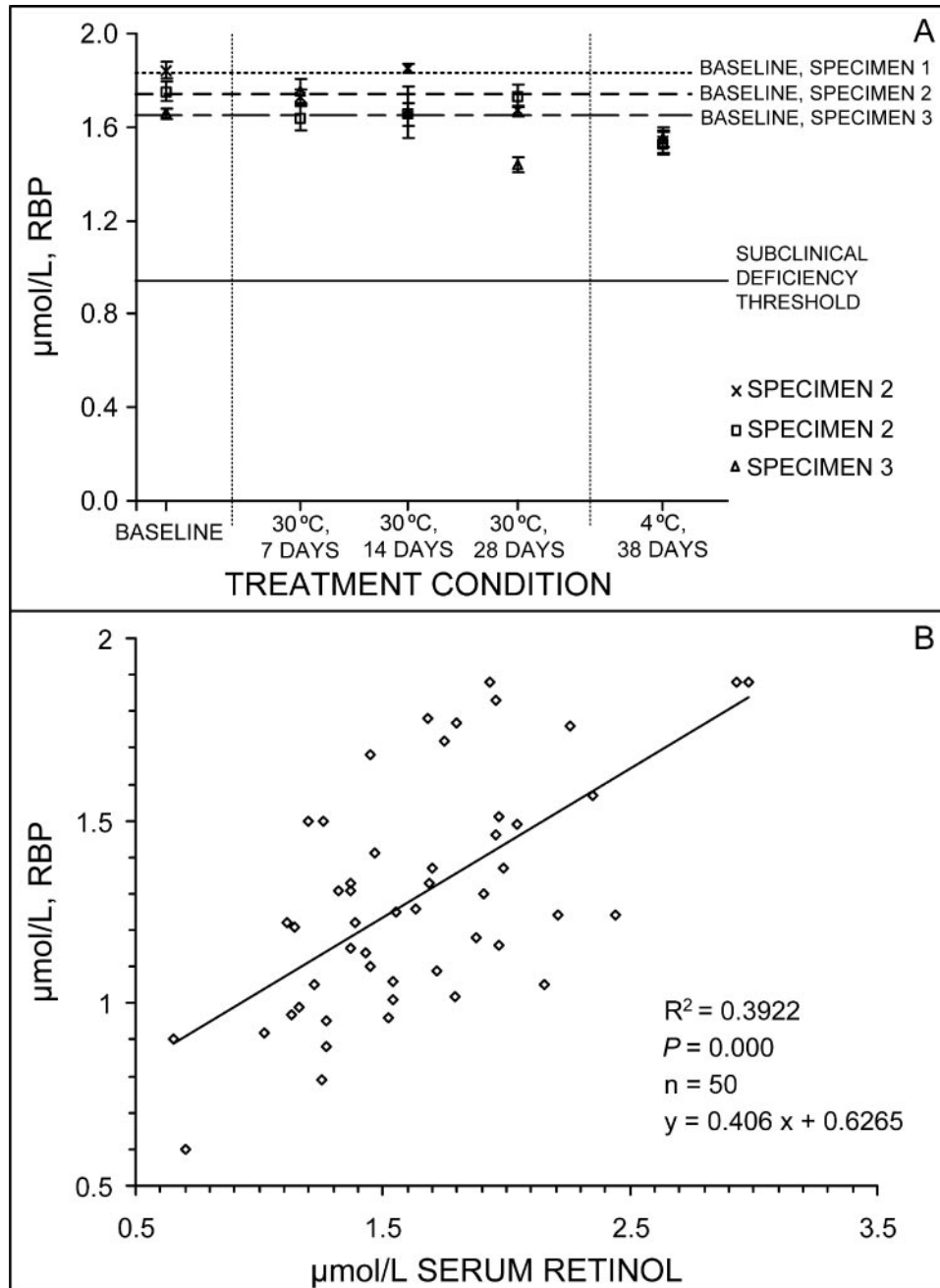


Fig. 1. (A), stability of DBS in the laboratory study.

In the laboratory study of DBS collected from US study participants, RBP stored at 30 °C for 7, 14, and 28 days did not differ significantly from baseline RBP, whereas RBP stored at 4 °C for 38 days produced values significantly lower than baseline. (B), stability of DBS-RBP in a field setting compared with the serum-retinol gold standard. Kenyan DBS stored at ambient temperature (12 °C–28 °C) of northern Kenya for 13–42 days and matched sera stored at -20 °C to -70 °C showed a strong linear relationship (linear regression, $P = 0.000$, $R^2 = 0.392$, $n = 50$).

and baseline was $-0.20 \mu\text{mol/L}$, 95% CI, -0.301 to -0.104 , $P < 0.001$). Statistical calculations were done using the software package, R 2.5.0 (17).

This study demonstrated high stability of RBP stored at ambient temperature in DBS collected from VA-sufficient individuals, but could not address the stability of RBP in DBS of VA-deficient individuals or how DBS-RBP may correlate with serum retinol. To address these questions, a study was conducted in northern Kenya.

For the field study, DBS-RBP and serum retinol were measured in 50 matched DBS and serum specimens, including morning and afternoon venous blood samples from 25 individuals randomly selected from a larger study conducted in 241 lactating Ariaal women of Marsabit District, northern Kenya. DBS were prepared by pipetting drops of blood collected by venous blood draw and stored in a sealed plastic bag with desiccant at ambient field temperatures ($12\text{--}28^\circ\text{C}$) for 13–42 days. Remaining blood specimens were kept away from light and allowed to clot, and serum was separated after field centrifugation. Sera were frozen within 3 h of venipuncture, stored in liquid nitrogen for 13–42 days, and then transported to KEMRI laboratory in Nairobi. All samples were then shipped via air express on dry ice to the University of Washington and stored at -20°C until analysis. Two DBS specimens were lost during the transport. Serum retinol was determined by reversed-phase HPLC in 2 batches on 2 consecutive days (177–205 days after collection). Assay controls were within the laboratory's acceptable range. The RBP assay protocol was the same as above, with a slight modification to conserve samples: from each DBS, 4 one-eighth inch peripheral disks containing approximately $6.1 \mu\text{L}$ of serum were punched out for elution. DBS specimens were assayed in a single batch 256–285 days after collection. Assays of 2 DBS specimens with original results above the assay range were repeated after dilution 1 week later.

The Kenyan samples had a mean (SD) serum retinol of 1.64 (0.48) $\mu\text{mol/L}$ ($n = 50$), ranging from 0.65 to $2.98 \mu\text{mol}$. Two samples from 1 study participant indicated subclinical VAD ($<0.7 \mu\text{mol/L}$). Stability of DBS-RBP across storage time was assessed with a linear mixed effects model (using R 2.5.0) of DBS-RBP on storage time with serum retinol as the gold standard, by including serum retinol as a covariate in the model and adjusting for time of blood collection. Storage time, serum retinol concentration, and time of day were considered fixed effects, and study participant was considered random. The results indicated no duration-dependent variation in DBS-RBP between 13 and 42 days ($P = 0.5$, $n = 50$, result not shown).

DBS-RBP as a surrogate measure of serum retinol was also assessed. Linear regression of DBS-RBP against serum retinol showed that the 2 measures are significantly linearly correlated ($P = 0.000$, $R^2 = 0.392$, $n = 50$, Figure 1B). The accuracy of DBS-RBP for identifying low serum

Table 1. Accuracy of DBS-RBP identifying low serum retinol (VAD).

Serum retinol	DBS-RBP		Total
	$\geq 0.96 \mu\text{mol/L}$	$< 0.96 \mu\text{mol/L}$	
$\geq 0.7 \mu\text{mol/L}$	43	5	48
$< 0.7 \mu\text{mol/L}$ (VAD)	0	2	2
Total	43	7	50

retinol was analyzed with a 2-by-2 table (Table 1). Specificity of DBS-RBP ($<0.96 \mu\text{mol/L}$) identifying no VAD among individuals without low retinol ($<0.7 \mu\text{mol/L}$) was 89.6% [CI, 80.9%–98.2%, statistical calculation performed with the software package, Stata/SE 9.2, (18)] and sensitivity of DBS-RBP finding VAD among those with low retinol was 100% in this small sample. However, because only 1 individual in this cohort was VAD, these results must be considered preliminary estimates of DBS-RBP performance, and with 5 specimens giving positive results in patients without VAD, further studies will help clarify the positive predictive value. These results suggest that despite the layers of differences—specimen types, storage conditions, and assay methods—DBS-RBP is a useful surrogate measure of serum retinol to assess VAD prevalence.

Overall, these studies suggest that RBP in DBS can withstand storage at relatively high ambient temperatures in dry conditions. In the laboratory, RBP in DBS collected by finger prick from VA-sufficient North American individuals and stored in a sealed plastic bag with desiccant were stable at 30°C for 2–4 weeks. In a field setting of VAD-endemic northern Kenya, RBP in DBS prepared from venous blood samples of individuals with a wider range of VA status, including subclinical deficiency, also appeared to withstand storage at ambient temperatures for 6 weeks in a sealed plastic bag with desiccant and produced results that correlated well with serum-retinol values. Furthermore, our results for RBP in DBS stored at ambient temperatures suggested good sensitivity and specificity in predicting low serum retinol.

Our laboratory and field studies differed in blood collection (finger-prick capillary vs venous draw) and stability assessment methods (DBS-baseline vs serum-retinol gold standard). These differences may have affected the observed storage stability of DBS-RBP. However, other work indicates that recovery of RBP from DBS does not vary with different blood collection methods (19).

We conclude that RBP in DBS collected by finger prick can withstand storage at a relatively high ambient temperature (30°C) for at least 2 weeks, thus facilitating accurate VA status assessment in population locations where serum collection and cold storage are unfeasible. The minimally invasive and field-friendly medium of DBS obtained from finger pricks and stored at ambient tem-

peratures can serve as a powerful tool for assessment of VAD prevalence, providing impetus for researchers in public health, human biology, and social science to incorporate estimates of biochemical VA status in their field research.

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