A HealthTech Report

Validation and Stability of Retinol-Binding Protein—Evidence From Tanzania

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1. Validation study

A study to assess the correspondence between retinol and retinol binding protein (RBP) was undertaken through the collaboration of PATH under the HealthTech program with the International Centre for Eye Health/Clinical Research Unit at the London School of Hygiene and Tropical Medicine. The study took place in Misungwi District of Mwanza Region, Tanzania, in January 2003.[1] Misungwi District neighbors Mwanza City, Magu District, Kwimba District, and the Mwanza Gulf of Lake Victoria. Children between 6 and 71 months of age living in two rural inland wards in Misungwi District were eligible to participate in the study.

Sera were collected from a total of 522 children, from which retinol and RBP were determined. Children had 2 mL of blood taken by venipuncture using Sarstedt Monovettes (SARSTEDT AG & Co., Newton, NC, USA). The blood was left to clot for at least half an hour, then centrifuged and separated into three aliquots of sera which were wrapped in aluminum foil to protect them from light and transported on ice to the laboratories of the National Institute for Medical Research (NIMR) in Mwanza City later the same day.

One aliquot of serum (50 µL) was refrigerated at 2ºC–4°C overnight and analysed by the retinol binding protein enzyme immunoassay test (RBP-EIA) the following day. To perform the assay, the specimens, calibrator sera, and anti-RBP antibody were diluted in assay buffer and added to the individual wells. The assay was read on a standard ELISA plate reader (LabSystems Multiskan Plus, Version 2.03, Type 314) using a 450 nm filter, and the results were calculated based on the values obtained from the calibrator sera. All tests were done in duplicate, and if the results differed by more than 10%, the test was repeated on a new plate. Test results were available 40 minutes after the start of the assay. It was possible for one laboratory technician to process 250 to 300 specimens in 8 hours. A second aliquot of serum (250 µL) was frozen at –20ºC until sent on dry ice to the DSM Nutritional Products laboratory in Basel, Switzerland, where the serum retinol concentration was measured by standard high performance liquid chromatography (HPLC) within three months. The third serum aliquot (250 µL) was stored frozen at –20°C at NIMR as a backup.

The proficiency of RBP to estimate vitamin A deficiency (VAD) at different serum retinol cut-off points is presented in Table 1. Using the same cut-off points for RBP as for retinol, the sensitivity was highest at a cut-off point of ≤1.05 µmol/L, at 96.1% with a specificity of 91.3%. When using a cut-off point of ≤0.7 µmol/L, the sensitivity of RBP in estimating VAD relative to retinol was 72.3% with a specificity of 92.9%.

As has been done in several other studies, a receiver operating curve analysis was conducted to identify the most appropriate RBP cut-off points to increase screening proficiency against retinol at < 0.70 µmol/L and < 1.05 µmol/L. Two cut-off points were identified; < 0.831 µmol/L to correspond with < 0.70 µmol/L retinol and < 1.309 µmol/L RBP to correspond with < 1.05 µmol/L retinol. The results of the screening analysis with these modified cut-off points are summarized in Table 1.

Table 1: Validity of RBP-EIA Test at Different Serum Retinol Concentrations, n=487

Serum retinol cut-off point (µmol/L)	Prevalence		RBP							
			Sens[y] (%)		Spec[y] (%)		PPV (%)		NPV (%)	
	n	%	n	%	n	%	n	%	n	%
	RBP (Using same cut-off points as retinol)									
≤0.35	62	12.7	19	30.7	416	97.9	19	67.9	416	90.6
≤0.70	332	68.2	240	72.3	144	92.9	240	95.6	144	61.0
≤1.05	464	95.3	446	96.1	21	91.3	446	99.6	21	53.9
	RBP <0.352 µmol/L									
≤0.35	62	12.7	19	30.7	416	97.8	19	67.9	416	90.7
	RBP <0.831 µmol/L									
≤0.70	332	68.2	307	92.5	101	65.2	307	85.04	101	80.2
	RBP <1.309 µmol/L									
≤1.05	464	95.3	459	96.4	6	54.6	459	98.9	6	26.1

Using a serum RBP cut-off point of ≤0.83 µmol/L to classify VAD as estimated by serum retinol concentration of ≤0.7 µmol/L, the sensitivity was high (92.5%) and the specificity was fair at 65.2%. The sensitivity to identify children with VAD (serum retinol concentration ≤ 0.7 µmol/L) did not differ whether children were diagnosed as ill or healthy (92.7% and 92.2% respectively; p=0.747), as was the specificity (66.2% vs. 64.3%, p=0.730).

2. Stability studies

In addition to the validation study, the Tanzania researchers collected a subsample of sera which were used in three stability studies to assess the stability of RBP subjected to varying temperature conditions over time, as well as to assess the effect of light and the feasibility of using dried blood spots (DBS).

Exposure to different temperatures

The stability of serum RBP was tested at four different storage temperatures: –20°C, 4°C, ambient temperature, and 37°C on days 3, 5, 7, 10, and 14 as depicted in Table 2.

Table 2: Characteristics of Different Stability Conditions Tested—Temperature

	Temperature			
Time point	**–20°C**	**4°C**	**Ambient**	**37°C**
1 day	Baseline			
3 days	1	2	3	4
5 days	5	6	7	8
7 days	9	10	11	12
10 days	13	14	15	16
14 days	17	18	19	20

Exposure to light

Fifteen aliquots of sera were used in a study of light exposure. Of these, 5 aliquots per participant were stored in the dark, 5 at room light, and 5 in direct sunlight (Table 3). One aliquot per storage condition was withdrawn and stored in the refrigerator after half an hour, 1 hour, 4 hours, at the end of day one (average of 8 hours), and at the end of day two. Serum RBP-EIA was determined on all the 15 aliquots per participant at the end of day two.

Table 3: Characteristics of Different Stability Conditions Tested—Light Exposure

	Light exposure		
Time point	**Dark**	**Room**	**Sunlight**
Half hour	1	2	3
1 hour	4	5	6
4 hours	7	8	9
8 hours	10	11	12
2 days	13	14	15

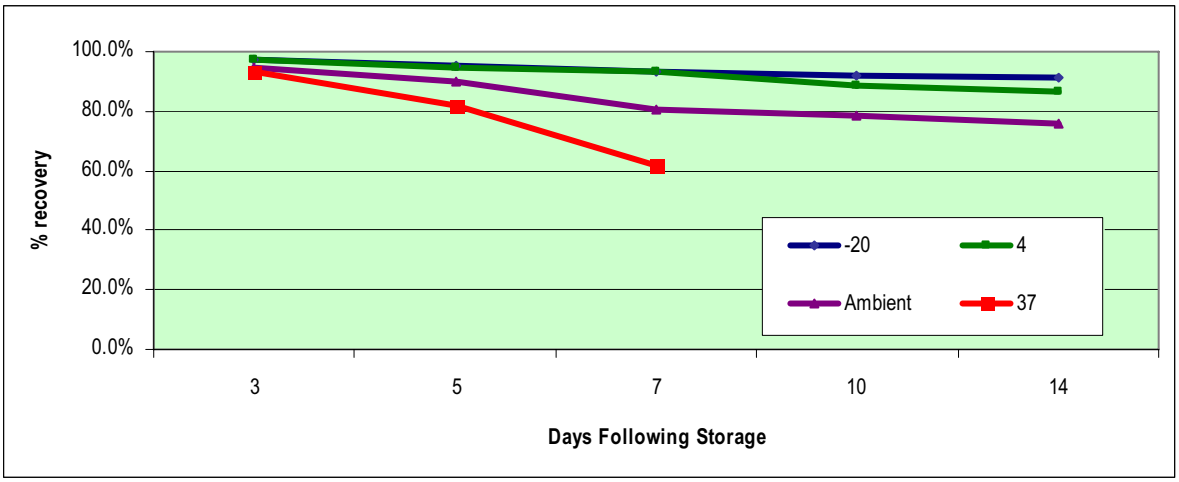
Feasibility of using DBS

Finally, the Tanzania study included a small experiment to establish the feasibility of using filter paper matrices for the storage of whole blood through the preparation of a small number of samples of DBS from a subsample of whole blood following collection in the field. However, while the stability study could be carried out on the sera samples and the results were important in establishing the optimal minimum conditions under which sera would need to be stored in order to maintain specimen integrity, there were many problems with the DBS and that component of the study could not be completed.

Results

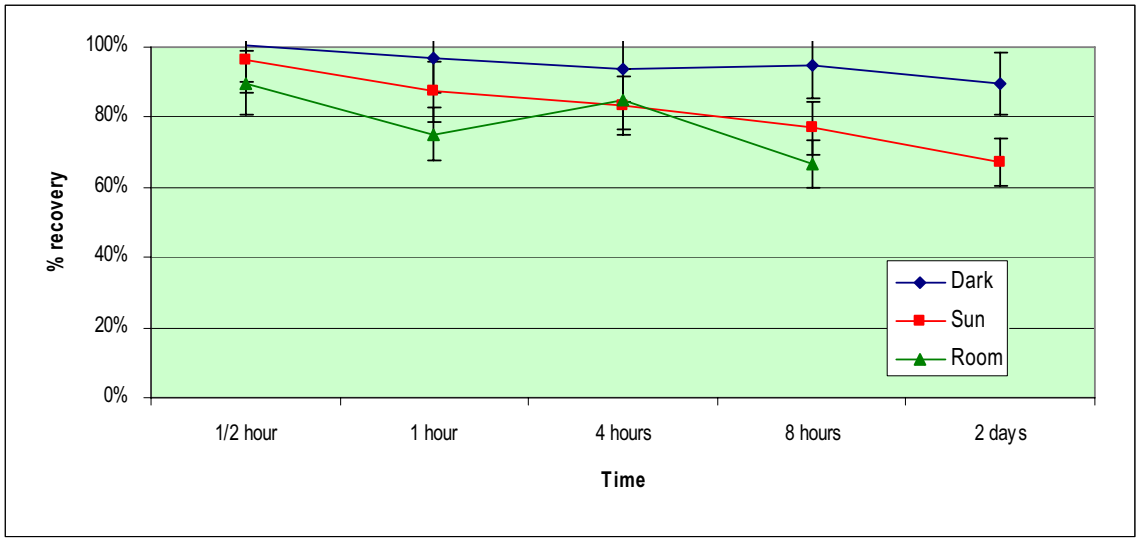
In the temperature study (Figure 1), specimens stored at 20°C and at 4°C showed good stability through one full week, while only those at the lower temperature maintained > 90% retention through a second full week. In contrast to this, the specimens stored at ambient temperature (average of 20ºC to 22°C) showed rapid decline in the first 3 to 5 days and by one week had lost 20% of the original RBP concentration. The samples that were exposed to the highest temperature had an even more precipitous deterioration of RBP, showing the vulnerability of the molecule to extreme temperature.

Figure 1. Serum Stability (Temperature)



In the study that examined the effect of light (Figure 2), the samples maintained under controlled dark conditions at an average temperature of 4°C showed the greatest stability, while the other two subsamples exposed to room light (and an average temperature of 20°C) and sunlight (average temperature (30ºC–32°C) saw much more dramatic degradation of the RBP, as soon as 1 hour following exposure, with increasing declines over time.

Figure 2. Serum Stability (Light)



As above, the complete set of DBS specimens prepared from the sera was not able to be used due to technical problems in the field, and consequently results are not available on the RBP concentration from these samples. Still, the effort in the operational study of DBS provided valuable insight regarding the proper collection and handling requirements of DBS.

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[1] Wedner SH, Ross DH, Gorstein J, Hix J. Validation of the retinol binding protein enzyme immunoassay to assess vitamin A deficiency. Report to Task Force Sight and Life. London, UK; March 2005.

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