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Biochemical Indicators of Vitamin A Deficiency: Serum Retinol and Serum Retinol Binding Protein[1]

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ABSTRACT Two biochemical indicators are currently recommended for determining whether vitamin A deficiency (VAD) is a public health problem: serum retinol and serum retinol-binding protein (RBP). After consideration of 40 data sets and the original rationale for previously proposed cut-offs, a cut-off for serum retinol concentration was proposed at <0.70 μmol/L (20 μg/dL) in $\geq$15% of the sampled population. This cut-off should be applied to a representative group of preschool age children (6–71 mo). Because measurement of low serum retinol concentrations requires high precision, analysis should be done by HPLC. For serum RBP, a cut-off cannot be reliably specified, because available data are too few and too variable. However, because serum RBP concentration correlates well with serum retinol concentration, it can be used to determine whether VAD is a public health problem in those populations for which the relationship between serum concentrations of retinol and RBP have been established. More efforts to establish a reliable cut-off for RBP is warranted, because analysis, in particular radial immunodiffusion (RID), is relatively simple and inexpensive. Whereas HPLC and RID analyses must be done in a laboratory, methods are being developed for assessing serum retinol and RBP under more remote conditions. J. Nutr. 132: 2895S–2901S, 2002.

KEY WORDS: • *serum retinol* • *serum retinol-binding protein* • *vitamin A deficiency*

This paper addresses biochemical indicators of vitamin A deficiency (VAD)[3]: serum retinol concentration and serum retinol-binding protein (RBP) concentration. We discuss for each indicator why it was selected and what is proposed for determining whether VAD is a public health problem. We conclude with a brief overview of laboratory and field methods available for analyzing these indicators.

SERUM RETINOL

Why serum retinol

Serum retinol concentration reflects an individual's vitamin A status, particularly when the body's reserves of vitamin A are limited, because serum retinol concentration is homeostatically controlled and will not drop until body stores are significantly compromised (1).

However, serum retinol concentration is also affected by factors that affect release of holo-RBP from the liver, infection, protein status, adequacy of other nutrients and organ disease. In general, these factors lower serum retinol concentration (1–3). Details about the effect of infection on serum retinol concentration are discussed by Thurnham (3). However, populations with a high prevalence of infection are also more likely to suffer from VAD. The cut-off for serum retinol concentration has been chosen irrespective of the prevalence of infection in a particular population.

Serum retinol is usually assessed by high-performance liquid chromatography (HPLC) or spectrophotometry (4). Although spectrophotometry is much simpler and less costly, it is also much less accurate; therefore, HPLC analysis is preferred. Although many other biochemical indicators of vitamin A status can be assessed (5), serum retinol is the preferred indi-

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[3] Abbreviations used: EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography; IVACG, International Vitamin A Consultative Group; PATH, Program for Appropriate Technology in Health; RBP, retinol-binding protein; RIA, radioimmunoassy; RID, radial immunodiffusion; VAD, vitamin A deficiency; WHO, World Health Organization.

cator for population level assessment of VAD because many laboratories can analyze it, and it is the best established biochemical indicator of vitamin A status.

Cut-offs previously proposed

Several different cut-offs previously have been proposed for the prevalence of a low serum retinol concentration among preschool age children that would indicate that VAD is a public health problem in the population surveyed. Initially, the World Health Organization (WHO) recommended a prevalence of ≥5% with a serum retinol concentration of <0.35 μmol/L (10 μg/dL) (6). This was based on its association with the presence of eye signs of VAD. Because accurate determination at this very low concentration requires high analytical precision, and because detecting a prevalence of 5% with reasonable precision requires a large sample size, the International Vitamin A Consultative Group (IVACG) later changed this cut-off to a prevalence of ≥15% with a concentration of <0.70 μmol/L (20 μg/dL) (7).

In 1992 an informal WHO working group suggested a more complex classification scheme for serum retinol concentrations of <0.70 μmol/L: mild, 2 to <10% prevalence; moderate, 10 to <20% prevalence; severe public health problem, ≥20% prevalence (5). Although this classification might be useful for comparing prevalence rates, it is confusing when the question is whether there is a VAD problem that needs to be combated. Therefore, one cut-off for determining whether VAD is a public health problem will best serve global needs.

Distributions of serum retinol concentration and cut-off chosen

Because a serum retinol concentration of 0.70 μmol/L is a well-recognized cut-off, but 1.05 μmol/L is sometimes proposed as an alternative, prevalence rates below both values were compared in 40 data sets, which were generously shared by groups from around the world (see **Fig. 1**). Details about the individual data points and their references are provided in **Tables 1** and **2**, respectively. All but two of the data sets were from preschool age children; some encompassed the entire age range from 0 to 71 mo, but most were limited to a narrower range. The smallest data set consisted of 60 children and the largest included 1566 children.

Because the previous cut-off recommended by IVACG was for a serum retinol concentration of <0.70 μmol/L in ≥15% (7) and because these same parameters represented the midpoint suggested by the WHO working group for moderate VAD (5), this was considered a potentially appropriate standard.

HPLC analysis can detect a concentration of <0.70 μmol/L with adequate precision (although this is not the case for spectrophotometric analysis). Because a higher prevalence criterion requires a smaller sample size for similar precision, we assessed whether we could choose instead a higher prevalence rate of a higher serum retinol concentration (<1.05 μmol/L). Figure 1 shows the relationship between the prevalence of serum retinol at <0.70 and <1.05 μmol/L in the same 40 populations.

Despite the obvious correlation between these two cri-

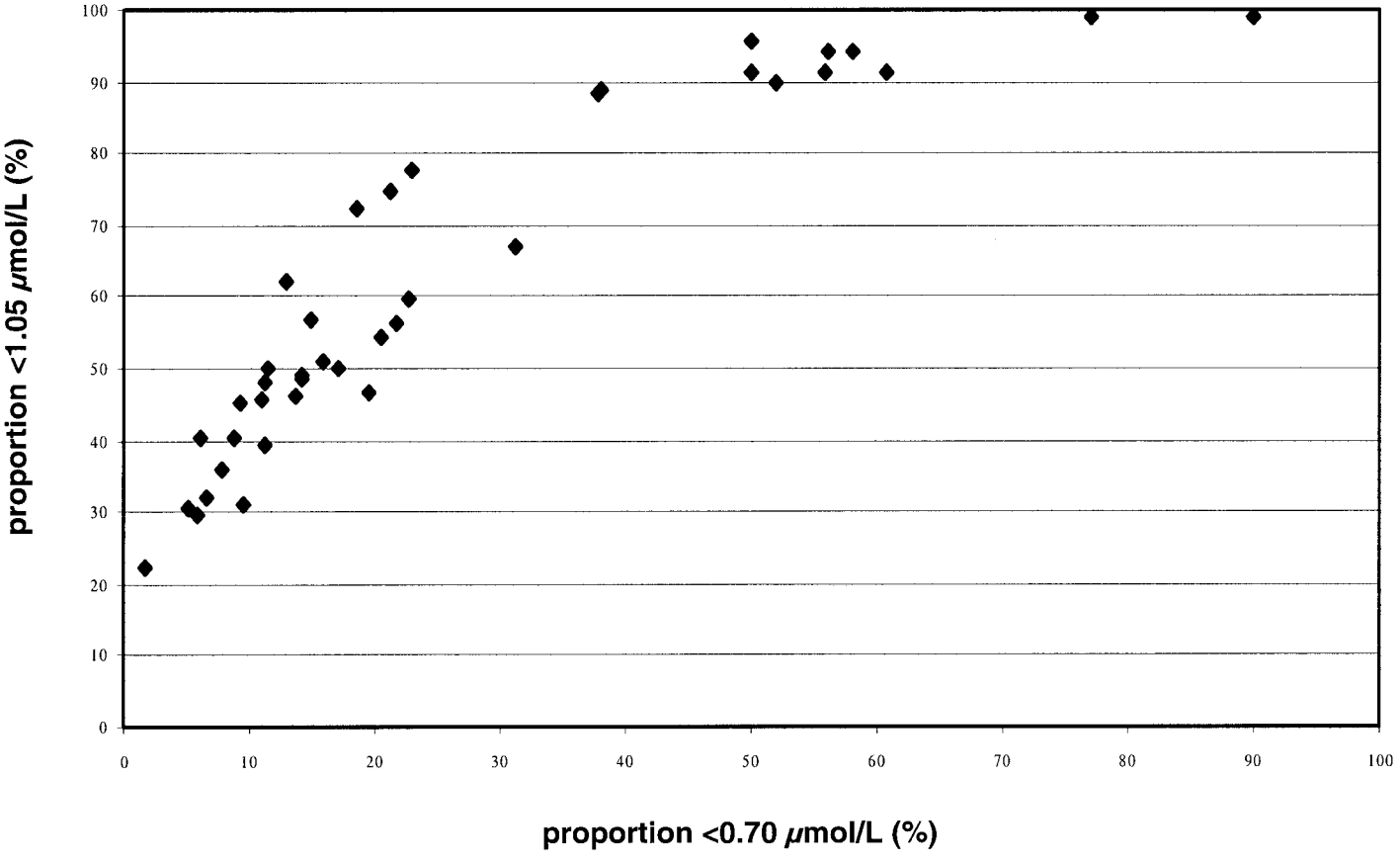

FIGURE 1 Proportions of children in different populations with serum retinol concentrations of <0.70 μmol/L and <1.05 μmol/L (n = 40). For details on data points, see Tables 1 and 2.

TABLE 1

Proportions with serum retinol concentrations <0.70 and <1.05 μmol/L in different populations and method of analysis used to determine serum retinol concentration, in order of increasing proportion <0.70 μmol/L

Population (*n*) [source; see Table 2]	Age range of population	Proportion <0.70 μmol/L	Proportion <1.05 μmol/L	Method used for retinol analysis[1]
		%	%	
USA (*n* = 1592) [1]	4–5 y	0.6	26.4	HPLC
Chieng Mai, Thailand (*n* = 245) [2]	2–6 y	1.6	22.4	HPLC
Guatemala (*n* = 644) [3]	1–4 y	5.1	30.7	Spectro
Panama (*n* = 1103) [4]	1–4 y	5.8	29.4	Spectro
Payao, Thailand (*n* = 212) [5]	2–6 y	6.1	40.1	HPLC
Southern Thailand (*n* = 362) [33]	2–6 y	6.6	32	HPLC
Northern and northeastern Thailand, rainy season (*n* = 485) [14]	2–6 y	7.8	35.8	HPLC
Costa Rica (*n* = 573) [6]	1–4 y	8.7	40.1	HPLC
Guatemala (*n* = 721) [3]	1–5 y	9.2	45.2	Spectro
Panama (*n* = 924) [7]	1–4 y	9.4	30.9	Spectro + HPLC
Southern Thailand (*n* = 373) [8]	2–6 y	11	45.6	HPLC
Guatemala (*n* = 676) [3]	1–4 y	11.1	39.1	Spectro
Bolivia, poor regions (*n* = 891) [9]	1–4 y	11.3	48.3	Spectro
Central Java, Indonesia (*n* = 748) [10]	0–2 y	11.5	50	HPLC
England and Wales, UK (*n* = 816) [11]	1.5–4.5 y	13	62	HPLC
Honduras (*n* = 1518) [12]	1–4 y	13.6	46.1	Spectro
Colombia (*n* = 786) [13]	1–4 y	14.2	48.4	Spectro + HPLC
Guatemala (*n* = 585) [3]	1–4 y	14.2	49.2	Spectro
Northern and northeastern Thailand, dry season (*n* = 485) [14]	2–6 y	14.9	56.7	HPLC
Guatemala (*n* = 772) [15]	1–4 y	15.8	51	Spectro + HPLC
Ecuador, poor regions (*n* = 1232) [16]	1–4 y	17	50	HPLC
Central Java, Indonesia (*n* = 689) [17]	0–2 y	18.6	72.3	HPLC
Dominican Republic, two regions (*n* = 505) [18]	1–4 y	19.6	46.5	Spectro
Central Java, Indonesia (*n* = 1025) [19]	0–2 y	20.5	54.6	HPLC
Bangladesh (*n* = 1111) [20]	6 mo to 4 y	21.3	74.7	HPLC
Guatemala (*n* = 543) [3]	1–4 y	21.7	56.5	Spectro
Dominican Republic (*n* = 1566) [21]	1–4 y	22.7	59.8	Spectro
Western Cape, poor areas, South Africa (*n* = 60) [22]	6–12 mo	23	77.5	HPLC
Nicaragua (*n* = 1436) [23]	1–4 y	31.3	67.2	Spectro
KwaZulu-Natal, poor areas, South Africa (*n* = 115) [24]	4–24 mo	37.7	88.2	HPLC
Yap, Federated States of Micronesia (FSM) (*n* = 216) [30]	2–4 y	38	89	HPLC
West Java, Indonesia (*n* = 188) [25]	6–13 y	50	95.7	HPLC
KwaZulu-Natal, poor areas, South Africa (*n* = 164) [26]	1–5 y	50	91.5	HPLC
Pohnpei, FSM (*n* = 363) [31]	2–4 y	52	90	HPLC
KwaZulu-Natal, poor areas, South Africa (*n* = 400) [27]	5–11 y	55.75	91.25	HPLC
Chuuk, FSM (*n* = 223) [32]	18–24 mo	56	94	HPLC
Yap and Kosrae, FSM (*n* = 267) [30]	2–4 y	58	94	HPLC
Marshall Islands (*n* = 919) [28]	1–5 y	60.7	91.3	HPLC
Chuuk, FSM (*n* = 137) [32]	3–6 y	77	99	HPLC
Embu province, Kenya (*n* = 414) [29]	6–8 y	89.9	99	HPLC

[1] HPLC, high-performance liquid chromatography; Spectro, spectrophotometry; Spectro + HPLC, all samples analyzed by spectrophotometry, when <25 μg/dL, sample also analyzed by HPLC.

teria, it was decided that a serum concentration of <1.05 μmol/L had three potential limitations. First, there was concern that there might be a larger proportion of false positives (that is, low serum retinol concentration but adequate liver stores) at this higher level. Second, two data sets, one of English and Welsh children under 5, showed a prevalence of >50% for concentrations of <1.05 μmol/L but <15% for concentrations of <0.70 μmol/L; these presumably well-nourished populations would have been classified as having VAD at the <1.05 criterion but not at the <0.70 criterion. Third, using two different criteria for serum retinol would be confusing. Because levels of <0.70 μmol/L were considered the better cut-off but since this is a relatively low concentration, serum retinol concentrations should be measured by HPLC analysis.

There is some concern about the use of a single cut-off across the entire age range of 6–71 mo. Therefore, we compared the prevalences of low serum retinol concentrations among subgroups of children under 5 from a number of different populations. **Figure 2** shows that the prevalence of concentrations of <0.70 μmol/L tended to be lower for children aged 48–59 mo than for those aged 12–23 mo but that there was no consistency about the exact age when the prevalence was lower. Gregory et al. reported that there was no difference in serum retinol concentration among different age groups of English and Welsh 1.5- to 4.5-y-olds (8). Because there is evidence that infants <6 mo old have a lower serum retinol concentration than older infants and young children (5,9), the cut-off should not be applied to infants <6 mo of age. To facilitate future comparisons, it is recommended that data on serum retinol concentrations be presented as the proportion of the total population that is below the cut-off but also provide the distribution of serum retinol concentrations.

TABLE 2

References for data in Table 1

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SERUM RBP

Why serum RBP

Serum RBP occurs in a 1:1:1 M complex with retinol and transthyretin (10). Because of the 1:1 complex, serum RBP concentration should reflect serum retinol concentration and therefore might be substituted for it as an indicator of vitamin A status. Assessment of RBP is easier than assessment of serum retinol. First, because RBP is a protein, it can be detected with an immunologic assay, which is simpler and less expensive than HPLC analysis of serum retinol. Second, serum handling is easier because RBP is more stable than retinol with respect to light and temperature. Third, RBP analysis requires a very small amount of serum, 10–20 μL, which can be obtained from a finger prick, whereas the amount required for retinol analysis by HPLC is at least 100 μL, which generally requires venous blood.

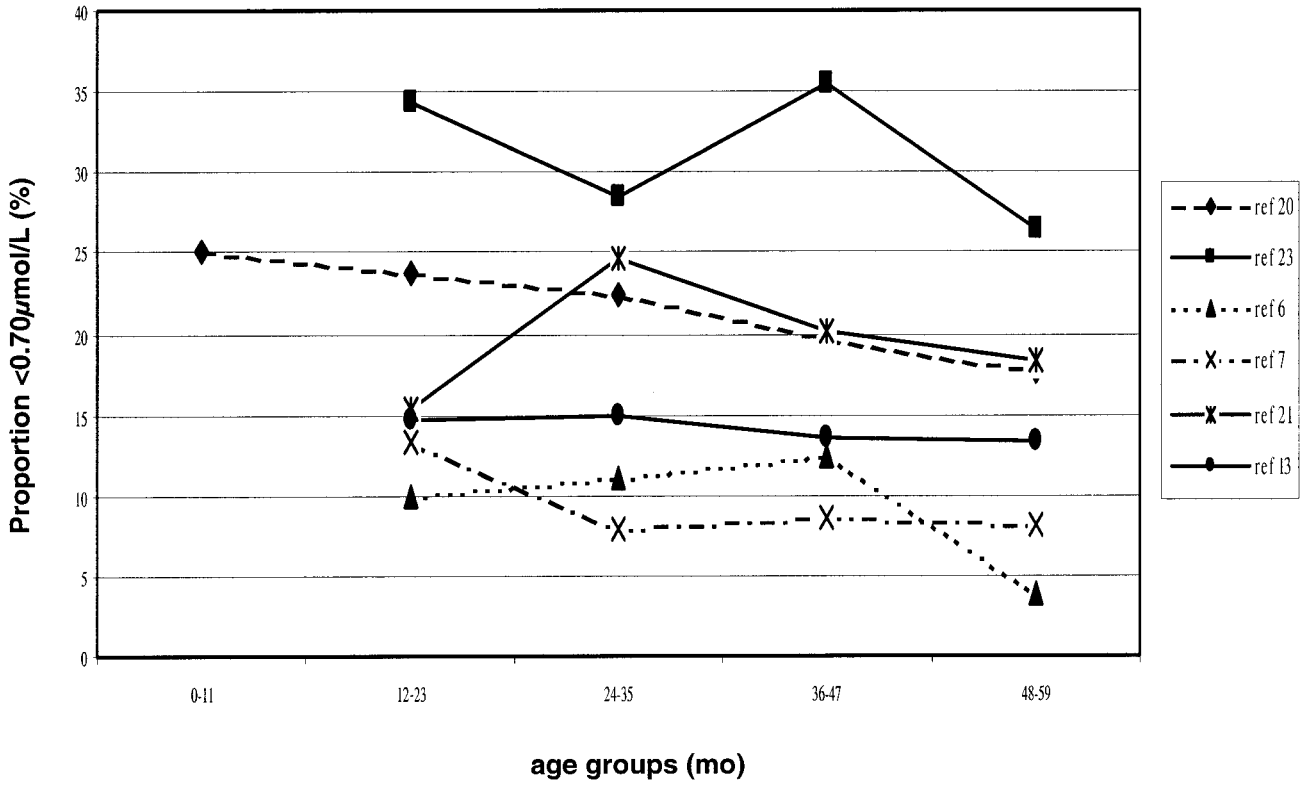

FIGURE 2 Prevalence of serum retinol concentration of <0.70 μmol/L by child age among different populations. For reference details, see Tables 1 and 2.

Despite the theoretical 1:1 M ratio, a cut-off for serum RBP concentration has not been accepted. Not all RBP found in serum is complexed with retinol (holo-RBP), and the proportion that is not (apo-RBP) varies under a range of concentrations. Immunologic assays cannot distinguish between holo- and apo-RBP. Second, the binding of RBP to retinol is influenced by a number of factors such as the presence and degree of acute-phase response, protein energy malnutrition, liver disease, chronic renal failure and acutely stressful situations (for example, just before delivering a baby). Many of these factors also affect serum retinol concentration and therefore may not affect the relationship between serum concentrations of retinol and RBP in a particular population; predicting those in which it does and in which it does not is problematic.

Serum concentrations of RBP and retinol

Two very different cut-offs have been proposed in the past for serum RBP concentrations that would be comparable to a serum retinol concentration of <0.70 μmol/L. Gibson (11) proposed a cut-off of 1.14 μmol/L (a serum RBP concentration of 1 μmol/L is equivalent to 21 mg/dL), and Bakerman suggested a cut-off of 0.70 μmol/L (12). The latter assumed 100% saturation of RBP with retinol. Earlier studies, mainly from the early 1970s, that looked at the relationship between serum concentrations of RBP and retinol found correlations between 0.62 and 0.93 (13–15). However, the method used to measure serum retinol concentration in those studies, fluorometry, was less precise and specific than the currently used HPLC method. Further, cut-offs that would reflect a serum retinol concentration of <0.70 μmol/L were not proposed.

Recently, several groups have proposed cut-offs for serum RBP concentration expected to reflect a serum retinol concentration of <0.70 μmol/L (16–19). **Table 3** presents these findings. The proposed cut-offs cover a wide range from 0.69 μmol/L in 3- to 6-y-old Indonesian children (19) to 1.29 μmol/L in breast-feeding Indonesian women (16).

A study among children of the Marshall Islands found that the saturation of RBP with retinol was lowest among those children with a relatively low serum retinol concentration: 79% with a serum retinol concentration of <0.35 μmol/L compared with 96% at >0.70 μmol/L (18). A similar finding among infants 2–10 mo of age was recently reported from Indonesia (20). The relationship between serum concentrations of RBP and retinol appears to depend on the serum retinol concentration of the population. The authors of the study from the Marshall Islands recommended a cut-off for a serum RBP concentration of 0.70 μmol/L (18) because that was previously suggested (12) and gave good sensitivity and specificity for their population. However, a cut-off derived from their regression equation, 0.77 μmol/L, would be even better, because it takes into account the average saturation of RBP with retinol in their population (89%).

Neither Gamble et al. (18) nor Semba et al. (19) found evidence for an altered relationship between RBP and retinol related to an acute-phase response.

Cut-off for serum RBP concentration

Because potential and proposed cut-offs varied too greatly (Table 3), it is not yet possible to suggest a cut-off for the prevalence of serum RBP concentration that would validly and reproducibly reflect a serum retinol concentration of <0.70 μmol/L. To propose a cut-off in the future, the relationship between serum concentrations of RBP and retinol needs to be determined in more populations with widely varying serum

TABLE 3

Cut-offs for serum RBP[1] concentration that reflect a serum retinol concentration of 0.70 μmol/L as determined in various populations

Population	Serum RBP concentration, method used and level in population surveyed (*n*)	Serum retinol concentration, level in population surveyed	Cut-off determined for serum RBP concentration	Sensitivity	Specificity
				%	%
Breast-feeding women, Indonesia (*n* = 173) [16]	Nephelometry, mean: 1.48–1.73 μmol/L (groups by age of breast-fed child)	Mean: 0.72–0.90 μmol/L (groups by age of breast-fed child)	1.29 μmol/L (27.1 mg/L)	72.1	70.5
Pregnant women, Malawi (*n* = 872) [17]	RID[2], range: 0.2–2.9 μmol/L 42.0% at <1.00 μmol/L	Range: 0–2.10, 44.5% at <0.70 μmol/L	1.00 μmol/L (21.1 mg/L)	87.6	94.6
Children (1–5 y), Marshall Islands (*n* = 239) [18]	RIA[3], range: 0.05–1.4 μmol/L	27% at <0.35 μmol/L, 39% at 0.35–0.70 μmol/L, 33% at >0.70 μmol/L	According to regression equation: 0.77 μmol/L (16.2 mg/L)	96	88
			0.70 μmol/L (14.7 mg/L), suggested by authors because mentioned in literature	87	98
Children (3–6 y), Indonesia (*n* = 206) [19]	RID, range: 0.2–1.5 μmol/L 57% at <0.69 μmol/L	Range: 0.2–1.5, 52.4% at <0.70 μmol/L	0.69 μmol/L (14.5 mg/L)	75	63.2

[1]RBP, retinol binding protein; [2]RID, radial immunodiffusion; [3]RIA, radioimmunoassay.

retinol concentrations. At present however, serum RBP concentration, which correlates well with serum retinol concentration, can be used for determining whether VAD is a public health problem when the relationship between serum concentrations of RBP and retinol have been determined in a subsample of the particular population.

ANALYSIS OF SERUM RETINOL AND SERUM RBP

To complement these recommendations, it is worth discussing how these compounds can be analyzed and likely future developments.

Laboratory methods: serum retinol

Serum retinol can be assessed by fluorometry, spectrophotometry, and HPLC. Fluorometry is rarely used because its specificity is poor. However, the Centers for Disease Control and Prevention (Atlanta, GA) is working on refining the Futterman laboratory method, which measures fluorescence of the retinol-RBP complex. The main disadvantage to spectrophotometry is similar to that of fluorometry: it has poor specificity, is not very accurate and is difficult to validate especially when frozen serum is used. HPLC analysis is preferred, but it requires well-skilled and well-trained technicians and benefits from interlaboratory comparisons. The need for the latter is illustrated by a recent proficiency test of 16 laboratories in developing countries (12 in Africa, 4 elsewhere), 14 of which used HPLC. Only 7 of these laboratories produced acceptable results for serum retinol concentration (21). Because a minimum of 100 μL of serum is required for HPLC analysis of serum retinol, blood should be obtained by venipuncture.

Laboratory methods: serum RBP

RBP can be analyzed by immunoassay, which binds the protein to antibodies that can then be detected in a variety of ways. A method that is used only in research laboratories is the radioimmunoassay (RIA), which radioactively labels the antibodies. Another assay, probably the least expensive, is the enzyme-linked immunosorbent assay, in which an enzyme is attached to an antibody that changes the color of a substrate. The intensity of the color change depends on the amount of RBP bound. However, commercial kits are not available for this analysis, and the method requires an optical density reader. Nephelometry is another relatively easy-to-use method, but the machine is expensive. The radial immunodiffusion (RID) test is the easiest for laboratories with limited equipment, and a commercial kit is available. With a micropipette, serum is put into a well on a plate and left to diffuse at room temperature for about 3 d, after which a ring can be observed around the well. The diameter of the ring can be measured using a jewelers' eyepiece with a micrometer. A calibration line derived from standards is then used to determine the RBP concentration in the serum tested. Compared with laboratory assessment of serum retinol, the RID test is much simpler, requires a much smaller volume of serum and costs less. The measurement of the diameter makes the method vulnerable to inter-observer error, but that can be minimized when one person determines the diameter of all samples, including the standards.

Blood spot/field methods

Under certain circumstances, collecting blood by venipuncture can be difficult. For example, the community may not accept this practice. Furthermore, a lack of electricity will prevent centrifuging blood to obtain serum and producing ice to keep samples cool/frozen during transport. Therefore, collecting blood by finger prick and either storing it on filter paper for subsequent analysis in a distant laboratory or analyzing it immediately in the field would be much preferred. At present, no such method is available for general use, but efforts to develop such methods are ongoing.

Craft Technologies is developing a method that measures retinol concentration in blood preserved on filter paper, but this requires an optimized HPLC system with a highly sensitive detector because the blood spot contains only 10–12 μL of serum (22,23). At present, the cost of this analysis is US $15 per sample. Program for Appropriate Technology in Health

(PATH, Seattle, Washington) and collaborators are developing a method for measuring RBP concentration in blood preserved on filter paper. First results of this enzyme immunoassay (EIA) method were presented at the XX IVACG meeting (24). The costs of this determination will be approximately USD 0.25 per sample. But, the success of filter paper methods appears to be highly dependent on the kind of filter paper used (25,26). Therefore, both groups are also working on a method that could give results immediately in the field.

Craft Technologies is developing a method that quantifies retinol by measuring the fluorescence of holo-RBP (27). Efforts are now focused on enhancing the sensitivity of this portable fluorometry test. Approximate cost of the method using anti-RBP antibodies will be USD 1 per sample. PATH and collaborators are working on a so-called "RBP dipstick" method that could semiquantitatively assess the RBP concentration in whole blood, using the previously mentioned EIA method (24). The costs of this method will be approximately USD 0.25 per sample. Both methods will be field tested this year.

When ≥15% of preschool age children (6–71 mo) in a population have a serum retinol concentration <0.70 μmol/L, VAD is a public health problem in the population surveyed. Serum retinol concentration has to be analyzed by HPLC.

Serum RBP concentration would also be a very good biochemical indicator for determining whether VAD is a public health problem, because it is much easier to analyze and it correlates well with serum retinol concentration. However, a cut-off cannot yet be proposed, and RBP can therefore only be used for those populations for which the relationship with serum retinol concentration has been determined in a subsample.

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