Determination of vitamin A in blood. Some practical considerations on the time of collection of the specimens and the stability of the vitamin[1,2]

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ABSTRACT The stability of vitamin A and retinol-binding protein (RBP) in serum separated from the clot at different times after blood extraction and the effect of ingesting a vitamin A-rich breakfast on postprandial serum levels of retinol, carotenoids, and RBP were investigated. In the stability trials, venous blood was drawn from 12 well-nourished young adult subjects and sample aliquots were maintained, respectively, at 4°C or at room temperature (26 to 28°C). The serum was separated from the clot either immediately after blood clotting or at 2, 4, 6, 12, and 24 h after blood extraction. Serum retinol and RBP were stable either at 4°C or at room temperature, even if the serum was separated from the clot 24 h after blood extraction. In the breakfast experiment, seven young adult subjects ingested a meal containing 291 μg of retinol equivalents. The serum levels of retinol, carotenoids, and RBP were measured pre- and postprandially up to a period of 4 h. The ingestion of the breakfast meal did not alter significantly the postprandial serum levels of retinol, carotenoids, or RBP. These results are of practical importance for the performance of vitamin A nutrition surveys involving field work, when, as is common, blood specimens cannot be collected under fasting and, in addition, they have to be transported from far away localities to the laboratory. *Am J Clin Nutr* 1983;37:147–151.

KEY WORDS Vitamin A, retinol, carotenoids, retinol-binding protein, vitamin A stability, retinol-binding protein stability, pre- and postprandial vitamin A levels, vitamin A field studies

Introduction

Since 1946, when Bessey et al. (1) published their analytical procedure for the uv light spectrophotometric measurement of blood serum retinol and carotenoids, their method has been used extensively by several laboratories in projects designed for assessing the nutritional status of population groups. Such application of the method usually involves the collection of blood specimens from population groups in small rural communities located far away from central laboratories where the biological materials are to be processed for analysis. These small rural communities ordinarily do not have facilities for separation of the serum from the blood cells. In many occasions, this implies that the clotted blood samples have to be transported to the central laboratory, and this phase of the procedure may take varied periods of time. In their original method, Bessey et al (1) have some brief notes regarding the stability of the retinol and of the carotenoids present in serum samples after the serum has been separated, and under different temperature conditions such as "room temperature," refrigeration at 4°C, or freezing at −20°C. However, nothing is mentioned in relation to the stability of the substances during various periods of time while the serum and the clot have not been separated. If the separation of these two components of the blood is done necessarily in a central laboratory, a period of time of several hours may elapse. It is

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important, therefore, to determine the stability of retinol and carotenoids under different conditions during this transportation step.

Another question that has been raised regarding the validity of the results obtained is the most appropriate time for collection. Ideally, it has been stated that the specimens should be collected during fasting, ie, before the subjects have had breakfast. Nevertheless, this condition is nearly impossible to fulfill in population field studies, particularly with children. A feasible recommendation, which in effect is the most common practice, has been to collect the blood samples throughout the morning, finishing the collection before lunch time, regardless of whether the subjects have had breakfast or not. In this regard, it was considered important to investigate whether the ingestion of a breakfast meal would affect the retinol values and to what extent. The objective of this report is to contribute information regarding the points previously mentioned. Furthermore, the determination of serum retinol-binding protein (RBP) has been suggested recently as a complementary parameter to be measured for the assessment of vitamin A nutritional status (2). Taking advantage of the present experiments, the stability of RBP was also determined in the specimens.

Methods

Stability trials

In a first experiment, a 12-ml fasting blood sample was obtained in the morning from the antecubital vein of each of six young adult subjects—males and females—using disposable syringes. Each sample was divided in six aliquots which were placed directly from the syringe into 13 × 100 mm test tubes. Each aliquot displaced about ¼ of the inner air-space of the tube. The tubes were immediately covered with vacutainer type rubber stoppers and, after labeling, they were stored for the study in the refrigerator at a temperature of approximately 4°C. The samples were protected from exposure to light at all times. Only dim indirect day light was allowed in the laboratory and, in addition, the specimens were covered with a dark cloth at all possible times. In a second experiment, similar size blood samples were obtained under the same conditions from another group of six young adults. The samples were handled as in the first experiment except that the aliquots of blood were maintained at all times throughout the period of study at room temperature. The temperature ranged in the laboratory from 26°C in early morning to 28°C in the afternoon. In both experiments, the serum was separated from the clot in each aliquot by centrifugation at about 1600 × *g* for 10 min in the following sequence: immediately after blood clotting (approximately 20 min after blood extraction), and, respectively, for each subject, at 2, 4, 6, 12, and 24 h after extraction of the blood. In the sera thus obtained, the concentrations of retinol and RBP were measured.

TABLE 1
Composition of the breakfast meal

Two scrambled eggs fried with margarine*
A portion of fresh cheese (34 g)
Two pieces of bread toast spread with margarine*
A cup of hot milk (175 ml) (2 teaspoons of sugar and one of instant coffee added)
A glass of papaya juice (200 ml)

* Commercial margarine fortified with vitamin A (17 μg β-carotene/g).

Effect of a breakfast meal

The purpose of this experiment was to evaluate the effect of ingesting a vitamin A-rich breakfast meal on postprandial serum levels of retinol, total carotenoids, and RBP. Seven young adult subjects of both sexes in apparent adequate health and nutritional status participated in the experiment. They came to the laboratory in early morning after an overnight fast, and a basal 2-ml venous blood sample was drawn from the antecubital vein of each subject using disposable syringes. The blood was placed in 13 × 100 mm test tubes, and centrifuged immediately after clotting for the separation of the serum as described in the previous section. Shortly after extraction of this basal blood sample, they ingested a complete breakfast meal designed to contain a relatively high amount of vitamin A. The composition of this meal is presented in **Table 1**. The food components were weighed and, according to food composition tables (3) and the results obtained from the laboratory analysis of the vitamin A content of the margarine (claimed by the manufacturer to be fortified with vitamin A in the form of β-carotene), the full meal was estimated to contain 291 μg of retinol equivalents (about 54% as retinol and 46% as carotenoids). This amount represents 29% for males and 36% for females of the adult dietary daily recommendations of the US National Academy of Sciences (4). After breakfast, the subjects were asked not to eat any other food while in the experimental period, and additional 2-ml blood samples were obtained from each subject at 1, 2, 3, and 4 h after ingestion of the meal. The blood samples were handled for the separation of the sera as described previously in this experiment. The serum concentration of retinol, total carotenoids, and RBP were determined in the samples.

Analysis of vitamin A and RBP

Retinol concentration was analyzed by uv inactivation as described by Bessey et al (1) using a serum sample size of 180 μl. By this method, since saponification is involved, both RBP-bound retinol and retinyl esters are measured together. Total carotenoids were determined in the same sample extracts by measuring their spectrophotometric absorbency at 460 nm. In the case of margarine, its carotene content was extracted with cyclohexane, separating the solids by centrifugation and filtration. The carotene concentration was then determined

by reading the sample extract at 460 nm. Serum RBP concentration was determined by the radial-immunodiffusion assay of Mancini et al (5).

Data analysis

All the data obtained from the different experiments were analyzed by a one-way analysis of variance.

Results

Stability trials

Table 2 shows the concentrations of retinol and RBP determined in serum separated from the clot at different times after blood extraction while the blood samples were maintained either at 4°C or at 26 to 28°C (room temperature). No significant changes in the levels of retinol or RBP were observed in the samples maintained at 4°C, whether the biochemical analyses were performed in serum separated immediately after clotting or at 2, 4, 6, 12, and even 24 h after extraction of the blood. Similar results were obtained when the blood samples were maintained at room temperature. Again, even at this temperature (26 to 28°C), which is usually considered improper for storage of blood, no significant changes in the serum concentration of retinol or RBP were observed up to 24 h of blood storage. These results indicate that retinol and RBP are stable under the described conditions, even if the serum is separated from the clot after 24 h of blood extraction.

TABLE 2
Stability of retinol and RBP at different times of separation of the serum

H after blood extraction	Retinol (μg/dl)		RBP (μg/ml)	
	4°C (n = 6)	26–28°C (n = 6)	4°C (n = 6)	26–28°C (n = 6)
Basal*	62.6 ± 9.8†	55.6 ± 10.3	57.8 ± 7.2	52.1 ± 7.3
2	62.9 ± 9.5	55.3 ± 10.8	57.6 ± 7.4	52.1 ± 7.8
4	63.0 ± 8.7	55.7 ± 10.5	57.8 ± 7.2	55.5 ± 7.6
6	62.2 ± 8.9	55.3 ± 10.0	57.6 ± 7.1	52.7 ± 7.5
12	62.8 ± 9.9	55.0 ± 11.5	57.5 ± 7.6	52.8 ± 7.6
24	62.8 ± 9.0	55.3 ± 10.5	57.4 ± 7.1	52.6 ± 7.5

* Immediately after clotting.
† $\bar{x}$ ± SD. In vertical columns, no significant changes were observed.

Effect of a breakfast meal

Table 3 shows the serum concentration of retinol, total carotenoids, and RBP before and after different times of ingestion of breakfast.

When comparing the levels of these parameters before and after the ingestion of the meal, no significant difference was found, even after 4 h postprandially. The results indicate that the ingestion of a breakfast relatively rich in vitamin A, under the conditions of the present experiment, does not influence the levels of these biochemical indicators of vitamin A nutritional status.

Discussion

The results obtained in the stability trials demonstrate that, in practice, the blood samples collected in the field can be maintained for temporary storage and transportation inside an icebox at ice temperature, even during a lapse of time extending for as long as 24 h, without detriment of the stability of retinol and RBP. Our study also indicates that if ice were not available, a room temperature as high as 26 to 28°C would still be harmless for as long as 24 h. Although the reason why the stability of the vitamin is maintained under these conditions cannot be established from the present experiment, it is possible that this phenomenon could be attributed to natural antioxidants present in blood.

Other studies have only addressed the question of stability of the vitamin during

TABLE 3
Effect of breakfast on postprandial serum levels of retinol, RBP, and total carotenoids

	Basal*	H after ingestion of breakfast			
		1	2	3	4
Retinol (μg/dl)	66.9 ± 12.2†	65.7 ± 12.0	65.9 ± 11.8	65.6 ± 11.0	67.3 ± 11.0 NS
RBP (μg/ml)	59.2 ± 9.8	59.0 ± 10.2	59.3 ± 9.7	59.0 ± 9.9	59.3 ± 10.5 NS
Total carotenoids (μg/dl)	138.2 ± 36.0	138.5 ± 36.3	136.8 ± 35.6	137.0 ± 35.9	139.7 ± 36.8 NS

* After overnight fast.
† $\bar{x}$ ± SD.

storage of the already separated serum or plasma (1, 6), and not during handling of the whole blood specimens as performed in the present experiment. Therefore, our stability results cannot be directly compared with previous experiments. At any rate, since under some climatic conditions the environmental temperature, especially inside transporting vehicles, may go higher than our reported experimental temperature, we believe that the requirement of having an icebox and sufficient ice to transport the samples at refrigeration temperature is an easy requirement to fulfill and ought to be respected wherever possible. It is important to mention that if methods other than the uv spectrophotometric of Bessey et al (1) are used to measure the retinol, the stability conditions presented and discussed may have to be revalidated. For instance, increases in apparent retinol values during storage of the serum have been reported when colorimetric methods are used (6).

Since it is known that exposure to light destroys vitamin A (6), another condition not particularly investigated in this experiment, but to be fully respected, is to conserve the samples in the dark at all times. This requirement is, again, so easily fulfilled that it hardly needs to be proved necessary. It should be accepted as an extra precaution with no difficulty for implementation, since only covering the samples with a dark cloth at all times will be sufficient. The need to prevent hard shaking or vibration of the samples during transportation is also obvious, since it has been shown that these undesirable factors would provoke hemolysis.

The results obtained also demonstrate that the ingestion of a breakfast meal with relatively liberal amounts of food sources of retinol and carotenoids does not raise the fasting levels of retinol, carotenoids, and RBP in the serum during the next 4 h after the meal. It may be possible that after ingesting this meal, the rate of gastric emptying was not fast enough to provide at one point in time an amount of vitamin A for intestinal absorption sufficient to cause detectable changes in the serum concentration of the vitamin. This, however, may not be the case when a single oral dose is given. When this latter approach has been used to measure vitamin A absorption, significant elevations in plasma retinol have been observed postprandially and the appearance of the maximal absorption peaks has varied in different studies from 2 to 6 h after the dose (7–9). One of these studies (9) has been performed in our own laboratory using the same analytical method as in the present investigation (1). These experiments, however, differ from the one reported here mainly in two aspects: *1*) the vitamin A was given as a single oral dose, and *2*) the amount of the vitamin dose was much larger. For example the dose used in adults by Ralli et al (7) has been of 30,000 μg. In children, McCoord et al (8) have used 2,100 μg of retinol per kg of body weight and Arroyave et al (9) a total dose of 75,000 μg. In our test meal there was a total of 291 μg of retinol equivalents and only 157 μg (54%) were derived from preformed retinol or retinyl esters, which in addition were not added, but were present as natural food's components. Under these conditions, gastrointestinal mechanics may differ. Gastric emptying may be expected to be slower, that is, extended through a longer period of time.

Our approach simulates, indeed, the form and order of magnitude in which vitamin A is commonly ingested, the reason being that our objective was to examine a situation as is ordinarily encountered in practice. Considering that the type of breakfast normally ingested by rural populations in underdeveloped settings, particularly by small children, is seldom as rich in retinol and carotenoids food sources as the meal used in this experiment, our results would indicate that specimens collected during the morning, before lunch, even if the subject has already eaten his breakfast, are representative of the basal preprandial blood levels.

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