A Re-Examination of the Stability of Retinol in Blood and Serum, and Effects of a Standardized Meal

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We examined the stability of retinol in blood and serum samples, kept in the dark, under different handling procedures. Samples not protected from contact with air oxygen were highly unstable, even when kept at ice temperature. Samples collected under anaerobic conditions, with Vacutainer Tubes, or treated with nitrogen after collection to displace the air from the tubes were stable during the usual interval between collection and freezing or analysis in biochemical surveys. Ingestion of a moderate amount of vitamin A significantly increased serum retinol concentrations in normal volunteers, showing the importance that survey samples be preprandial.

Additional Keyphrase: sample handling

Data on retinol in serum are currently used to assess vitamin A nutriture, especially at the prepathological level (1-5). However, the classification into "deficient" (<100 μg/L), "low" (100-200 μg/L), and "adequate" (200-400 μg/L) (3) is highly controversial, mainly owing to the very wide range of "normal" values obtained by different laboratories (3, 6-16). On the other hand, this indicator and the presence or absence of clinical signs of vitamin A deficiency rarely coincide except in advanced pathological stages. This seriously diminishes the value of measuring retinol in serum (5, 6).

One factor contributing to the poor reliability of this biochemical indicator is the lack of standardization of procedures for handling and storage of blood and serum samples. Recommendations may be quite liberal, for example, "samples should be stored at ice temperature and taken to the laboratory as soon as possible" (1, 3), or very strict, demanding analysis or freezing of the sera immediately after collection and centrifugation (5).

Another potential confounder, especially under field conditions, is the possibility of including postprandial samples for determination of total retinol. This would mean including, in the results, significant amounts of retinyl esters, which readily enter the blood stream after ingestion.

We collected data over several years that show that handling and storage procedures should receive special attention. Also, we repeatedly observed that information on the fasting state of the subjects is most important when the data are assessed. This study was designed to document these observations in a more systematic manner.

Materials and Methods

Samples

Blood was sampled from apparently normal adult volunteers, all members of the staff of our department. The samples were collected into disposable syringes or Vacutainer Tubes (Becton-Dickinson, Rutherford, NJ 07070) as indicated.

Procedures

After collection, the blood samples were incubated for 7-10 min at room temperature, to allow clotting. The clotted samples were then centrifuged (5 min, 3000 rpm). The sera were immediately analyzed to obtain "time 0" values. Appropriate aliquots of blood or serum, in the presence of air or nitrogen, were kept in the dark under refrigeration or at room temperature, for analysis at different intervals after collection. When nitrogen was used to displace the air in the tubes, it was bubbled into the sera, or gently blown above the blood samples, to avoid hemolysis. All the tubes were kept tightly capped until analysis.

Samples collected into Vacutainer Tubes were not treated with nitrogen, but were kept under reduced pressure, with special precautions taken to prevent air from getting in when aliquots were withdrawn for analysis.

Effect of a Standardized Breakfast on Serum Retinol Concentrations

A preprandial sample of blood was collected from all volunteers, who then had a breakfast as similar as possible to the one offered in a previous, similar study (17) (two eggs fried in butter, 37 g cheese, 200 g papaia, a glass of milk, and bread and butter), which supplied 304 retinol equivalents (RE) as pre-formed retinol and 60 RE in the form of carotenoids (18).

Analytical Methods

Total retinol was determined by a modification (19) of the spectrophotometric method of Bessey et al. (20). Assessment by liquid chromatography (5) was performed to verify that the substance in the extracts absorbing at 326 nm was, in fact, retinol.

Data Analysis

The stability of retinol in the samples was evaluated through the coefficient (k) of an exponential model of destruction:

\[ \ln C_0 - \ln C_t = kt \]

where \( C_0 \) and \( C_t \) are the retinol concentrations at times 0 and \( t \), respectively.

The destruction curve is a multiexponential, but calculating the different coefficients was not deemed necessary because the first component of the curve is several times higher than the others and because our objective was to determine whether or not the samples are stable under different conditions of handling and storage, rather than to assess the degree of instability.

Our criterion for stability was a reasonably narrow 95% confidence interval for \( k \), including 0.

Appropriate software were used for statistical analysis of the data.

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Results

The best procedure we found to ensure the quality of the results was to immediately centrifuge the blood after clotting, storing at -20 °C any sera that cannot be promptly analyzed. Samples thus treated, and analyzed at daily intervals during 30 days, and at 6 and 12 months after collection, yielded very consistent results, with a coefficient of variation of 3.5% for values in the 250 μg/L range and of 2.5% for values in the 400 to 600 μg/L range.

Samples of serum sent to other laboratories were first treated with nitrogen to displace the air from the tubes, and were then shipped in sealed ampoules and placed with solid CO₂ for shipping. Samples thus treated yielded identical values when analyzed in our laboratory, and six to nine months later in other laboratories (one of them at the Massachusetts Institute of Technology, Cambridge, MA).

Table 1 shows that samples of blood or serum, kept either at room temperature or refrigerated, may assay 23 to 48% lower than initially after as short an interval as 1 h. In all cases, when no special precautions were taken (such as the use of Vacutainer Tubes or displacement of the air with nitrogen or immediate freezing of the sera), the exponential coefficient of destruction ranged between 0.16 and 0.64. Treatment with nitrogen, or the use of Vacutainer Tubes, leaves little doubt as to the cause of destruction: even in blood samples, in which bubbling with nitrogen causes hemolysis, displacement of the air significantly decreased retinol loss from 48.3 to 1.1% per hour. In the case of sera, either treatment (use of Vacutainers or displacement of the air with nitrogen) decreased the exponential constant to the desired value: zero.

As regards the effects of a breakfast containing an amount of vitamin A that cannot be considered overly large (360 RE), Table 2 shows that values for serum retinol were significantly altered. The method most used in surveys determines total retinol, so it would be irrelevant that most of the increase observed was probably ascribable to retinyl esters (21-24). Analysis of variance and the t-test for nonpaired values showed this increase to be significant (P ≤ .05).

<table>
<thead>
<tr>
<th>Sample/treatment</th>
<th>r</th>
<th>k* (95% confidence interval)</th>
<th>Loss, %/h*</th>
<th>k# (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>26</td>
<td>0.66 (0.64-0.68)</td>
<td>48.3</td>
<td>1.0</td>
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<tr>
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<td>2.1</td>
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<tr>
<td>Vacutainer</td>
<td>0</td>
<td>0.01 (-0.08-0.10)</td>
<td>1.1</td>
<td>64.9</td>
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<tr>
<td><strong>Serum</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>23.8</td>
<td>2.6</td>
</tr>
<tr>
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<td>1.8</td>
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<td>0.02 (0.00-0.03)</td>
<td>1.57</td>
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</tr>
</tbody>
</table>

Discussion

One of the methods most widely used to determine retinol in serum, and in other biological samples, is the colorimetric one (1-3), based on the formation of a blue complex between retinol and a Lewis acid (trifluoroacetic or trichloroacetic acid). This classic method (25, 26) has several drawbacks, including the use of highly hygroscopic and corrosive reagents. It requires great skill from the operator, because the blue complex is very unstable. A single reading would not suffice to determine the retinol concentration because the absorbance values must be extrapolated to time 0 (21). Moreover, the slope of the curve representing the destruction of the complex is not the same for samples and standards, being affected by the handling and storage conditions of both (5). Finally, stored samples, even those stored at -20 °C, produce spuriously high values (5, 27).

These drawbacks would be enough to explain differences between laboratories and the lack of correlation with clinical signs. Some of these problems also affect the spectrophotometric method (5, 20, 21, 28, 29), thus it is not surprising that the reliability of this indicator is considered rather poor (5).

Although vitamin A is highly sensitive to heat, light, and oxygen, blood samples placed in crushed ice have been described as being highly stable (17, 30), which completely disagrees with our experience.

There are few reported data on the stability of retinol in biological samples. The authors of the spectrophotometric method of analysis ran only one stability test (20) in which one sample was, and another was not, stable under refrigeration. Later, it was also shown that the presence of air in the sample tubes affected the results of colorimetry (27).

From an analyst's viewpoint, our results have not disclosed any new fact. This work was deemed necessary for practical rather than scientific reasons. Despite the well-known lability of vitamin A to heat, photodestruction, and oxidation, statements still appear in the literature to the effect that samples for retinol analysis "were kept at ice temperature for 24 to 48 h." Thus, it is not surprising that retinol concentrations in serum, especially when considered alone, are not much credited as an indicator of vitamin A status. The data in Table 1 show that great precautions must be taken to protect serum and blood samples from oxidation by air oxygen as well as from heat and light. Prompt analysis, immediate freezing, immediate displacement of the air from the tubes with sera, or collection of the samples under anaerobic conditions, constitute the only
alternatives to ensure the quality of retinol analysis.

The experiments reported here were run under a variety of different conditions and setups, and with samples from very different individuals. Thus we cannot ascribe the discrepancy between published results (17, 30) and ours to peculiarities of the volunteers, such as the presence, in blood, of substances that could “protect” retinol against oxidation.

A study from the Johns Hopkins Hospital (31) compared samples “properly” and “improperly” treated, after several hours of storage. Results were very similar, and it should be noted that the samples were collected and kept under anaerobic conditions (31). Those results and ours seem to be in agreement; they both suggest that the effect of air on the stability of retinol in samples is more important than that of temperature. Also in agreement with our observations are those of Driskell et al. (28, 29), who showed that precautions must be taken to prevent oxidation when thawing samples (collected under vacuum) that have been stored for several years.

With regard to the effect of a breakfast on total serum retinol levels, we can offer no explanation for the difference between our results and those previously reported (17, 30). Vitamin A enters rapidly the thoracic duct and the kinetics of its absorption (32) are in keeping with our results.

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References


