Simultaneous Determination of Retinol and \( \alpha \)-Tocopherol in Serum or Plasma by Liquid Chromatography

**Submitters:** George L. Catignani, Department of Food Science, North Carolina State University, Raleigh, NC 27650
John G. Bieri, Nutritional Biochemistry Section, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, MD 20014

**Evaluators:** William J. Driskell and Mark M. Bashor, Nutritional Biochemistry Branch, Clinical Chemistry Division, Center for Environmental Health, Centers for Disease Control, Atlanta, GA 30333
Charles P. Turley and Marge A. Brewster, Metabolic Lab., Arkansas Childrens Hospital, Little Rock, AR 72201

**Assigned Editor:** George E. Nicholads, Department of Obstetrics and Gynecology, University of Tennessee, Memphis, TN 38163

**Introduction**

Clinical interest in evaluating retinol (vitamin A) and \( \alpha \)-tocopherol (vitamin E) nutriture has increased appreciably in recent years. These vitamins are particularly important in premature infants, patients receiving long-term total parenteral nutrition, and patients with cancer or malabsorption such as is present in individuals with cystic fibrosis or who have undergone intestinal bypass surgery.

Currently, nutritional status with regard to retinol and \( \alpha \)-tocopherol is assessed by measurement of these vitamins in plasma (1). Fluorometric and colorimetric methods exist for both vitamins (2–8), and both a sequential (9) and a simultaneous fluorometric method (10) are available. These procedures, however, often exhibit one or more of the following difficulties:

- steps that result in variable sample losses, thus necessitating the use of individual recovery assays
- sample sizes as large as 2 mL
- interferences from endogenous compounds (e.g., \( \beta \)-carotene and phytofluene) and exogenous contaminants
- methods that are time consuming and require considerable analytical skill

These analytical problems are largely eliminated when the micronutrients are measured by "high-performance" liquid chromatography (HPLC). Several procedures for quantitating retinol and \( \alpha \)-tocopherol separately (11–15) and simultaneously (16–18) by this technique have been published. The HPLC method reported here is a rapid, easy assay for the concurrent determination of both vitamins in extracts prepared from microsamples of plasma or serum. Consequently, routine analyses either in clinical or survey settings are now more practicable.

**Principle**

Serum or plasma is deproteinized with ethanol that contains the internal standards (retinyl acetate and \( \alpha \)-tocopheryl acetate), and the lipid is extracted with hexane. After an aliquot of the solvent phase is evaporated, the residue is dissolved in diethyl ether and diluted with methanol. A portion of this solution is injected onto a C\(_18\) reversed-phase chromatographic column, and absorbances of the vitamins and internal standards are measured at 280 nm. Peak-height ratios are used to quantify each vitamin.

**Materials and Methods**

Sources of supply are given as a service; no endorsement is intended.

**Reagents**

**Solvents.** Hexane and methanol are liquid-chromatography grade. Anhydrous diethyl ether, absolute ethanol, and water are reagent grade; the ethanol should be redistilled before use.

**Standards.** Standard compounds were all-trans-retinol, all-trans-retinyl acetate, \( \alpha \)-tocopherol and \( \alpha \)-tocopheryl acetate. All standards are available from Eastman Organic Chemicals, Rochester, NY 14650. The vitamin \( E \) standards can be used without further purification.

**Purification of vitamin A standards.** Dissolve 100–150 \( \mu \)g of retinol or retinyl acetate in methanol and inject the solution into the chromatograph; collect the middle portion of the peak. Evaporate the eluate under a stream of nitrogen and dissolve the residue in ethanol.

*Note:* Evaluators C.P.T. and M.A.B. caution that they obtained one lot of retinyl acetate that did not purify easily because it unexplainably hydrolysed to retinol.

**Preparation of vitamin standards.** Prepare stock standards of retinol and retinyl acetate (100 mg/mL) and tocopherol and tocopheryl acetate (5 g/L) in ethanol. Dilute each stock standard 100-fold with ethanol to prepare working standards. Working standards of retinyl acetate and \( \alpha \)-tocopheryl acetate should be prepared weekly and biweekly, respectively. Retinol and \( \alpha \)-tocopherol standards are usually kept for only a few days while standard curves are being prepared. Confirm the concentration of each working standard spectrophotometrically by using their respective absorptivities \((a_{\lambda,\text{cm}})\) in ethanol: retinol 1780 at 325 nm, retinyl acetate 1510 at 328 nm, \( \alpha \)-tocopherol 75.8 at 292 nm, and \( \alpha \)-tocopheryl acetate 43.6 at 285 nm.

All vitamin standards should be stored at \(-20^\circ C\) and never exposed to natural illumination. Under these conditions the stock standard of retinyl acetate is stable for one

---

Paper no. 8054 of the Journal Series of the N.C. Agricultural Research Service, Raleigh, NC. The use of trade names in this publication does not imply endorsement of the products by the N.C. Agricultural Research Service or the American Association for Clinical Chemistry.
month; α-tocopheryl acetate stock standard is stable for several months.

Note: Evaluators W.J.D. and M.M.B. observe that retinol and α-tocopheryl standards are stable for at least 10 weeks at -20 °C. After about two weeks a slight shoulder on the retinyl acetate peak has sometimes been observed, together with a slight decrease in absorbance at 328 nm; these changes are avoided by preparing and storing the retinyl acetate solutions in acetic glassware.

Preparation of developing solution. Measure 950 mL of methanol with a glass graduated cylinder, dilute to 1000 mL with water, and mix. Filter through a 0.45-μm pore-size GA-6 membrane (Metrical Membrane Filter; Gelman Instrument Co., Ann Arbor, MI 48106) and de-gas by using reduced pressure from a water aspirator for 10 min with stirring.

Apparatus

Liquid chromatograph: Model 204 liquid chromatograph with a Model 6000A solvent delivery system, a U6K universal injector, and a Model 440, absorbance detector (all from Waters Associates, Inc., Milford, MA 01757).

Chromatography column: Reversed-phase μBondapak C18 (10-μm particle size) stainless steel column, 3.9 mm i.d. × 30 cm (Waters Associates, Inc.)

Note: Evaluators W.J.D. and M.M.B. have used the same column to analyze more than 2000 specimens, with a minimal decrease in resolution.

Guard column: Stainless steel 3.9 mm i.d. × 5 cm column packed with 10-μm μBondapak C18 (Waters Associates, Inc.) or Co Pell ODS (Whatman, Inc., Clifton, NJ 07014).

Recorder: 10-nV, 10-in. recorder (e.g., Model B-5217-2; Houston Instruments, Bausch and Lomb, Inc., Austin, TX 78753).

Collection and Stability of Specimens

Either plasma or serum can be used as sample. Specimens collected after an overnight fast are preferred. Direct exposure to natural illumination should be avoided. The samples can be stored at refrigerator temperature (4 °C) for as long as 10 days.

Note: Evaluators W.J.D. and M.M.B. report that retinol and α-tocopherol in serum are stable to repeated freezing (-20 °C) and thawing (17 cycles over a period of five weeks). They also note considerable variability in stability among different serum samples, but most remain stable for one day at 25 °C, four weeks at 4 °C, and one year at -20 °C or -70 °C. If stored for longer periods of time, it is extremely important to add the hexane immediately after the ethanol or to add ascorbic acid (1 g/L) to the ethanol. Otherwise, most of the retinol, tocopherol, and retinyl acetate are destroyed. These observations are currently being summarized for publication in Clinical Chemistry.

Procedures

Preparation of samples. All transfers involving solvents may be made with Lang-Levy pipets. Steps 2–4, involving hexane, should be carried out in an exhaust hood.

1. Pipet 50 μL each of retinyl acetate and α-tocopherol acetate working standards into a 6 × 50 mm disposable glass test tube. Add 100 μL of sample and vortex-mix vigorously for 10 s (sample sizes of 100–400 μL do not affect linearity of the assay, provided the proportion of ethanol to plasma in the initial precipitation of proteins is not changed).

2. Add 100 μL of hexane and vortex-mix intermittently and vigorously for 45 s.

3. Centrifuge at 800 × g for 5 min. Transfer 75 μL of the hexane layer to a 6 × 50 mm disposable glass test tube.

4. Evaporate the hexane under a stream of air or nitrogen. Tubes may be placed in a 60 °C water bath to speed evaporation (hexane boils at 69 °C).

5. Dissolve the lipid residue in 25 μL of diethyl ether. With gentle mixing, add 75 μL of methanol.

Note: Evaluators C.P.T. and M.A.B. find that these extracts are stable for at least two days at 4 °C. Evaluators W.J.D. and M.M.B. report that serum extracts stored in ethanol are stable for at least two weeks at -20 °C.

6. Using a 10-μL flush of methanol, inject 90 μL of the solution into the chromatograph (injection volumes of 30–90 μL do not affect linearity of the assay).

Chromatography conditions. Chromatography is performed at ambient temperature.

1. Set the detector wavelength to 280 nm and the sensitivity at 0.01 A full-scale. Should additional sensitivity be necessary due to limited sample size (less than 100 μL) or very low vitamin concentrations, the detector may be used at its higher attenuation (0.005 A full-scale).

Note: Evaluators C.P.T. and M.A.B. use a detector wavelength of 280 nm; W.J.D. and M.M.B. monitor the column effluent at 290 nm.

2. Set the flow rate of the solvent delivery pump at 2.5 mL/min.

Note: Evaluators C.P.T. and M.M.B. report that elution rates in excess of 4 mL/min result in deterioration of resolution.

3. Adjust the chart speed of the recorder to operate at 1 cm/min.

Standard curves and calculations. Retinol and α-tocopherol were quantified from standard curves of peak-height ratios vs weight ratios for each vitamin. To prepare the standard curves, a constant amount of the acetate form of each vitamin was combined with variable amounts of the corresponding alcohol form of each vitamin to give solutions with a threefold range of weight ratios. These solutions were chromatographed and the peak-height ratios recorded. Typical standard curves are shown in Figure 1.
Peak-height ratios of samples are converted to known quantities of retinol and α-tocopherol from the standard curves as follows:

\[
\frac{\text{vitamin peak height of sample}}{\text{vitamin acetate peak height (internal std.)}} = R_T
\]

\[
R_T = \frac{\text{slope of std. curve} \times \frac{\text{amount of added internal std.}}{\text{volume of sample size}}}{\text{vitamin concn}}
\]

Standard curves may also be constructed for peak-area ratio vs weight ratio. This method eliminates errors encountered when there are slight changes in retention times, such as from altered flow rates, day-to-day solvent inconsistencies, samples with high lipid content, and loss of column efficiency. Laboratories having an integrating recorder should find this method advantageous.

Results

An elution profile of a normal plasma sample with internal standards is shown in Figure 2. Each peak is well separated from the others. Negative peaks are not observed.

The intralaboratory within-day and day-to-day precisions (CVs) for the determinations of retinol and α-tocopherol are presented in Table 1. The Evaluators in each laboratory provided their own separate pooled plasma samples for these precision evaluations. Data concerning intralaboratory precision, based on a five-week study with three different common plasma pools, are given in Table 2. In all cases there was very good precision.

Analytical recovery of added retinol and α-tocopherol from plasma is summarized in Table 3; the average percent recovery was 101.6 ± 6.2 and 106.6 ± 10.8, respectively.

Note: Although individual data were not provided, Evaluators W.J.D. and M.M.B. found the percent recovery of retinol to be 97 ± 2 (n = 8) and the percent recovery of α-tocopherol to be 97.2 ± 2.5 (n = 8).

A comparison of results obtained by HPLC with those by accepted macro colorimetric methods for determining retinol (3) and α-tocopherol (4) is given in Table 4. For both vitamins the HPLC assay gave results that were not significantly different by linear regression analysis from the macro colorimetric procedures. The correlation coefficient (r) was slightly higher for tocopherol than for retinol.

Note: Evaluators W.J.D. and M.M.B. find that the two methods for vitamin A are highly correlated (r = 0.973, n = 300).

Discussion

Other forms of vitamins A and E exist in plasma but are not commonly measured as indices of nutriure. The procedure described herein quantifies the predominant forms of the two vitamins. More than 95% of the vitamin A in serum is all-trans-retinol (16). Esterified forms of vitamin A such as retinyl palmitate appear in the blood after ingestion of a vitamin A-containing meal and can be measured by this assay, but long retention times are involved and their normal concentrations are below detection in the postabsorptive state. There are several forms of vitamin E in serum, d-α-tocopherol representing greater than 95% of the vitamin E activity in the total pool (4), and β- and γ-tocopherol usually representing the remainder. Although not labeled in Figure 2, these latter two forms elute as a

<table>
<thead>
<tr>
<th>Table 1. Intralaboratory Precision of the Simultaneous Determination of Plasma or Serum Retinol (µg/L) and α-Tocopherol (mg/L) by HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Laboratory</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td><strong>Within-day</strong></td>
</tr>
<tr>
<td>G.L.C. and J.G.B.</td>
</tr>
<tr>
<td>W.J.D. and M.M.B.</td>
</tr>
<tr>
<td>C.P.T. and M.A.B.</td>
</tr>
<tr>
<td>Level 1</td>
</tr>
<tr>
<td>Level 2</td>
</tr>
<tr>
<td><strong>Day-to-day</strong></td>
</tr>
<tr>
<td>G.L.C. and J.G.B.</td>
</tr>
<tr>
<td>W.J.D. and M.M.B.</td>
</tr>
</tbody>
</table>

Fig. 2. Chromatogram of normal plasma with internal standards of retinyl acetate and α-tocopheryl acetate added: Peak 1, retinol; 2, retinyl acetate; 3, α-tocopherol; 4, α-tocopheryl acetate

<table>
<thead>
<tr>
<th>Table 2. Comparison of Simultaneous HPLC Analyses of Retinol (µg/L) and α-Tocopherol (mg/L) from Common Serum Pools by Different Laboratories</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Serum pool</strong></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Table 3. Analytical Recoveries of Added Retinol and α-Tocopherol from Plasma

<table>
<thead>
<tr>
<th>Concen in plasma (µg/L)</th>
<th>Concen added (µg/L)</th>
<th>Added concen (%)</th>
<th>Recovery, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol, µg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>630</td>
<td>500</td>
<td>540</td>
<td>108</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>520</td>
<td>104</td>
</tr>
<tr>
<td>460</td>
<td>500</td>
<td>470</td>
<td>94</td>
</tr>
<tr>
<td>500</td>
<td>500</td>
<td>480</td>
<td>96</td>
</tr>
<tr>
<td>400</td>
<td>500</td>
<td>530</td>
<td>106</td>
</tr>
<tr>
<td>α-Tocopherol, mg/L</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.10</td>
<td>7.00</td>
<td>8.09</td>
<td>116</td>
</tr>
<tr>
<td>6.84</td>
<td>7.00</td>
<td>6.73</td>
<td>96</td>
</tr>
<tr>
<td>8.10</td>
<td>7.00</td>
<td>8.12</td>
<td>116</td>
</tr>
<tr>
<td>8.36</td>
<td>7.00</td>
<td>6.57</td>
<td>94</td>
</tr>
<tr>
<td>5.06</td>
<td>7.00</td>
<td>7.74</td>
<td>111</td>
</tr>
</tbody>
</table>

Data from Submitters G.L.C. and J.G.B.

Table 4. Comparison of HPLC with Colorimetric Methods for Determination of Retinol and α-Tocopherol in Plasma

<table>
<thead>
<tr>
<th>Method</th>
<th>µg/L</th>
<th>SD</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>538</td>
<td>4.8</td>
<td>0.833</td>
</tr>
<tr>
<td>Colorimetric b</td>
<td>483</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HPLC</td>
<td>8.4</td>
<td>0.9</td>
<td></td>
</tr>
<tr>
<td>Colorimetric d</td>
<td>9.8</td>
<td>1.0</td>
<td>0.996</td>
</tr>
</tbody>
</table>

* Each mean is the average of 14 determinations, assayed in duplicate.
* a-Tocopherol acid method (3).
* Means are not significantly different (p > 0.3).
* Dipyridyl method after one-dimensional thin-layer chromatography (4).
* Means are not significantly different (p > 0.8).

single peak just before α-tocopherol, with a retention time of about 5 min.

When multiple analytes are measured spectrophotometrically in column chromatography, it is usual to monitor at the wavelength at which the absorption is maximum for the analyte having the least absorptivity. In this method that analyte is α-tocopherol, which absorbs significantly at 280 nm; this wavelength was chosen so that fixed-wavelength detectors could be used.

A known quantity of each standard should be run at the beginning of each day and the detector response measured. This control check requires only 10 to 12 min. Should the standard deteriorate prematurely or become concentrated through solvent evaporation, new standard solutions can be made without the loss of any samples. In addition, this procedure may detect equipment malfunction.

After a large number of samples have been assayed, the accumulation of neutral lipids within the guard column may cause a gradual loss of resolution. This is easily remedied by replacing the resin. Drugs and (or) contaminant interference may be suspected if there are distorted peaks, taller- than-usual peak heights, or vitamin concentrations inconsistent with a subject's history or therapy.

The use of retinyl acetate and α-tocopheryl acetate as internal standards compensates for possible losses caused by spillage or evaporation and for pipetting variability. These particular derivatives are stable, readily available from commercial sources, and have absorptivities similar to those of the analytes of interest. Furthermore, there are no interfering peaks in plasma with retention times close to those of the two acetate internal standards. A major difference between this procedure and other simultaneous HPLC assays for vitamins A and E with ultraviolet detection is the choice and number of internal standards. Single internal standards of both retinyl acetate (18) and dl-tocotrien (16) have been used successfully. The use of a single internal standard reduces the chromatography run time from 8 min to 6 min.

This assay is 20- to 25-fold more sensitive for retinol than α-tocopherol, owing to the much greater absorptivity of the former. Thus, 0.024 µg of retinol will give a peak height of 6 cm whereas 0.6 µg of α-tocopherol is needed to produce the same peak height. Because plasma ordinarily contains about 20-fold more α-tocopherol than retinol, the two peak heights are usually similar. Under the conditions described, a 100-µL sample generally gives peak heights ranging from 2 to 6 cm; these values represent retinol concentrations of 130 to 600 µg/L and α-tocopherol concentrations of 3.3 to 15.0 mg/L.

The detection limits of the assay are much lower than the concentrations of retinol and α-tocopherol in plasma that are associated with a state of deficiency.

Note: Evaluators W.J.D. and M.M.B. find detection limits of 100 µg/L for retinol and 0.8 mg/L for α-tocopherol. Plasma concentrations of < 200 µg/L of retinol and < 5 mg/L of α-tocopherol are considered to be inadequate (4).

The minor difference noted earlier in the correlation coefficients for retinol determinations (0.833 vs 0.973) done by HPLC and a macro colorimetric method is not surprising. The latter measures both retinol and retinyl esters. Also, the colorimetric assay requires making correction for the presence of carotenoids, and there is no reliable way of ascertaining the appropriate correction.

The simultaneous quantification of retinol and α-tocopherol by ultraviolet detection after separation by HPLC circumvents analytical difficulties inherent in previously published methods. The assay is reliable, fast, relatively inexpensive, and nondestructive. Other advantages of this simplified methodology are small sample volumes, freedom from interferences, and good sensitivity, specificity, and precision. Any laboratory with HPLC equipment and an ultraviolet detector can routinely determine retinol and α-tocopherol.

We thank Janice Butler for the manuscript preparation and express our appreciation to Richard N. Anderson and W. David Whybrey for their helpful comments. The assistance and cooperation of the Nutritional Biochemistry Branch, Centers for Disease Control, for providing the sample pools for evaluation of interlaboratory variation are most gratefully acknowledged.

References
8. Thompson JN, Erdy P, Maxwell WB. Chromatographic sepa-

Editor’s note: The reader is reminded that Selected Methods do not bear the official imprimatur of the Association. As detailed elsewhere (Clin. Chem. 19: 1207, 1973), these methods are offered here for criticism by the world community of users, and will be revised appropriately before being collected into a bound volume, Selected Methods of Clinical Chemistry. (Methods in the latest volume in this series, published by the Association in 1982, were not first published in Clinical Chemistry.) The Committee on Selected Methods attempts to select and evaluate methods that seem durable and generally useful, which have been checked by several Evaluators to determine both advantages and disadvantages. Thus sufficient information is provided to enable the user to know what to expect. Designation as a Selected Method does not imply superiority in all respects, nor is the procedure a selected proposed reference method unless it is so designated or has been developed as such by the AACC Standards Committee. The published procedure should be superior in terms of evaluation and thus of accurately describing to the user the characteristics of the method.