Measurement of α-, β-, and γ-Tocopherol in Serum by Liquid Chromatography

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A liquid-chromatographic assay for α-, β-, and γ-isomeric tocopherols in human serum is reported. The tocopherols and the internal standard (tocol) are adsorbed onto a silica gel column and are eluted in less than 10 min with n-hexane/isopropanol (99.4/0.6, by vol) at a flow rate of 1 mL/min. The complete analysis requires no longer than 30 min. Within-day precision (CV) was 1.4% (x̄ = 13.18 mg/L, n = 24), 7.4% (x̄ = 0.214 mg/L, n = 14), and 1.3% (x̄ = 1.01 mg/L, n = 14) for α-, β-, and γ-tocopherol, respectively. Day-to-day precision (CV) was 4.4% (x̄ = 9.85 mg/L, n = 10) for α-tocopherol, 9.1% (x̄ = 0.222 mg/L, n = 20) for β-tocopherol, and 3.8% (x̄ = 1.00 mg/L, n = 20) for γ-tocopherol. Extraction recoveries for α-, β-, and γ-tocopherol averaged 92.4 ± 2.9% (n = 5), 91.4 ± 7.6% (n = 5), and 92.0 ± 4.1% (n = 4), respectively. The smallest injected amount detectable is estimated to be 0.03 μg for α-, and 0.04 μg for β- and γ-tocopherol.

Additional Keyphrases: vitamin E • "high-performance" liquid chromatography • tocol as internal standard

Of all the tocopherols, α-tocopherol is considered the biologically most active, and of the various isomers in serum it is present in the highest concentration. We have reported a method for its determination by reversed-phase column chromatography (1). Concentrations in serum vary between 6.6 and 15.0 mg/L, whereas β-tocopherol concentrations reportedly range from 0.0 to 0.2 mg/L, and γ-tocopherol from 0.7 to 2.7 mg/L (2).

Separating the two positional isomers, β- and γ-tocopherol, poses a challenging analytical problem. For gas–liquid chromatography the tocopherols must be oxidized to their corresponding β-quinones (3) or must be subjected to preliminary thin-layer chromatography (4). Standard solutions of α-, β-, and γ-tocopherol have also been separated as their trimethylsilyl ethers on a PZ-176 open tubular capillary column in about 55 min, but tocopherols other than α-tocopherol were not observed in plasma samples (5). For separation by liquid chromatography an adsorption column (6, 7) has been used and applied to the analysis of drugs and cell-culture media. Abe and colleagues have used a normal-phase partition column for the assay of tocopherols in sera (8), liver (9), and erythrocytes (10).

We propose a faster and improved procedure for determining the isomeric tocopherols in serum, based on adsorption chromatography with ultraviolet (294 nm) detection.

Materials and Methods

Apparatus and Instrumental Conditions

We used a Hewlett-Packard model 1084A liquid chromatograph (Hewlett-Packard, Böblingen, Germany) and a Varichrom Multiple Wavelength Detector (Varian Associates, Palo Alto, CA). The chromatographic support (5-μm microparticulate silica) and the column tubing (150 × 3.2 mm, Lichroma SS) were obtained from RSL, St. Martens-Latem, Belgium. The column was packed by a slurry technique under the following conditions: slurry liquid, tetrachloromethane/methanol (9/1, by vol); slurry concentration, 40 g/L; pump, Varian model 8500; packing pressure, 35 MPa; pressurizing liquid, n-hexane. At the optimum flow rate (0.2 mL/min) an initial efficiency of 6000 theoretical plates (reduced plate height, h = 5) was obtained for β-tocopherol.

Serum extracts were chromatographed in n-hexane/isopropanol (99.4/0.6, by vol) at a flow rate of 1 mL/min and an operating pressure of 4.4 MPa. The column effluent was monitored at 294 nm at a sensitivity of 32 × 10⁻⁴ A/cm for α-tocopherol and 16 × 10⁻⁴ A/cm for β- and γ-tocopherol. Oven temperature was set at 30 °C.

Reagents

Ethanol, n-hexane, and isopropanol, all analytical-grade reagents from Merck, Darmstadt, Germany, were used without further purification. d-α- and d-β-tocopherol were purchased from Eastman-Kodak (Rochester, NY 14650), and d-γ-tocopherol was obtained from Supelco (Bellefonte, PA 96823). The internal standard, dl-tocopherol, was from two sources: purchased from Koch-Light Laboratories (Colnbrook-Bucks, Great Britain) and a gift from Eisai Research Laboratories, Tokyo, Japan.

Procedure

To determine α-tocopherol, transfer 100 μL of serum (or plasma), 10 μL of a tocol internal standard solution (0.2 g/L of ethanol), and 100 μL of pure ethanol to a teflon-capped centrifuge tube (100 × 10 mm). Mix the contents of the tube with a vortex-type mixer. Add 500 μL of n-hexane as extraction solvent and cap the tube. Extract by interrupted mixing on the vortex-type mixer for 4 min. After centrifugation (10 min, 1500 g), transfer the supernate to a conical 10 × 100 mm glass tube and evaporate at 40 °C under a gentle stream of nitrogen. Dissolve the residue in 20 μL of the mobile phase and inject on top of the silica column.

The same technique is used for the β- and γ-tocopherol assay except the volumes are changed to 500 μL of serum, 10


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μL of a different tocol internal standard solution (0.1 g/L of ethanol), 500 μL of ethanol, and 1.5 mL of n-hexane.

Results

Figures 1 and 2 show typical chromatograms obtained for serum α-, β-, and γ-tocopherol. Retention characteristics are given in terms of capacity ratios ($k'$) in Table 1. Total elution of the tocopherols and the internal standard takes less than 10 min.

We standardized the procedure by adding known amounts of α-tocopherol (range: 5–25 mg/L), β-tocopherol (0.05–0.4 mg/L), and γ-tocopherol (0.5–6 mg/L) to aliquots of a serum pool. The relationship between peak-area ratios (peak area of the tocopherol/peak area of the internal standard, I.S.) and tocopherol concentrations was linear (Figures 3–5). We used the calibration curves after subtracting the intercept, which represents the endogenous level of the corresponding tocopherol in the serum pool, and then easily determined concentrations in unknown serum samples by calculating the peak-area ratios.

The mean extraction recoveries (Table 2) were determined by adding known amounts of the tocopherols to samples of a serum pool; the internal standard was added only after extraction with 2.00 mL of n-hexane but before evaporation of the transferred organic layer (1.00 mL).

We estimate the detection limit at 0.03 μg for α-tocopherol, and 0.04 μg for β- and γ-tocopherol (total injected amount). Analytical precision, illustrated by the within-day and day-to-day precisions, was determined on samples of a normal serum pool stored at −18°C, under nitrogen; results are given in Table 3. Serum tocopherol peaks were identified on the

<table>
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<th>Table 1. Capacity Ratios ($k'$) of Chromatographic Peaks</th>
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<td>α-Tocopherol</td>
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<td>β-Tocopherol</td>
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<td>γ-Tocopherol</td>
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<td>Tocol (internal standard)</td>
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$k'$ is the ratio of the weight of the solute (sample) in the stationary phase to that in the mobile phase of the column. It is a retention parameter.
basis of their retention characteristics. Retention times of the serum peaks matched those from a standard mixture containing the different tocopherols.

Discussion

Our liquid chromatographic assay of all serum tocopherols offers several major advantages over the assays currently in use.

The separation of $\alpha$- and $\gamma$-tocopherol by gas−liquid chromatography of their corresponding $p$-quinone derivatives on packed columns (3) was performed only on standard solutions; its application to serum samples requires preliminary removal of sterols, triglycerides, and long-chain alcohols. Moreover, high temperatures are needed if the chromatography is to be done within reasonable time (about 30 min). In another gas−liquid-chromatographic method, which requires a preliminary thin-layer chromatography of 3-mL plasma samples as a purification step (4), the derivatized tocopherols elute within 20 min at 240 °C. Both of these approaches are rather cumbersome—they provoke losses of material (multiple consecutive manipulations) and increase the analysis time.

In liquid chromatography, separation of the $\beta$- and $\gamma$-tocopherol isomers on a reversed-phase column appears impossible (1). Yet reversed phase is recommended for routine analysis because it can be easily operated, the mobile phase can be rapidly changed, and column retentions are highly reproducible (11). For separation of all serum tocopherols ($\alpha$, $\beta$, and $\gamma$-tocopherol), adsorption chromatography on silica gel is essential, even though this technique is known to be more sensitive to retention variation (11). The latter inconvenience can be partly overcome by very precise preparation of eluting solvent mixtures and by calibration with peak-area ratios.

Because our method could be partly overcome by very precise preparation of eluting solvent mixtures and by calibration with peak-area ratios. As is well known, peak-area measurements are less sensitive to changes in mobile-phase composition than are peak heights (12).

When Eriksson and Sörensen (6) used an adsorption column to determine vitamin E in pharmaceutical raw materials and finished products, the three tocopherols eluted in about 12 min in diisopropyl ether/n-hexane (5/95, by vol); our method elutes them in less than 5 min. Matsuo and Thara (7) chromatographed $\alpha$-, $\beta$-, and $\gamma$-tocopherol standards on a silica gel column by a gradient elution in about 20 min. Abe and colleagues separated all tocopherols on a normal-phase partition column by diisopropyl ether/n-hexane (2/98, by vol) in human sera (8), liver (9), and erythrocytes (10).

This, at present, is in fact the only liquid chromatographic method applied to human serum. In their method, elution of the three tocopherols takes more than 10 min, whereas we elute them in half the time. They also used fluorometry for online detection. This increases the method’s sensitivity, but fluorescence detection is more subject to problems than is the usual absorption detection technique. We cannot judge their day-to-day CV’s either, as they were not mentioned. Nevertheless, generally the reproducibility is better with a UV detector. If a fluorometer is used as a detection device, it should be calibrated daily because the fluorescence yield varies widely from day to day.

Our reported determination of the isomeric tocopherols in serum is based on "high-performance" liquid chromatography plus an adsorption technique. The addition of a known amount of tocot to an internal standard before the extraction compensates for possible losses caused by spilling and improves the precision of the method. The on-line uv monitoring of the eluate at 294 nm affords sensitivity. One could consider using our method for the simultaneous determination of the three isomeric tocopherols; however, because $\alpha$-tocopherol appears for about 88% of the total vitamin E in serum, whereas only 2% occurs as the $\beta$-form and about 10% as $\gamma$-tocopherol, it is not possible to use a single adequate amount of internal standard that will allow simultaneous measurement of all three components. Therefore, the overall procedure is rather

| Table 2. Extraction Recoveries of $\alpha$-, $\beta$-, and $\gamma$-Tocopherol |
|----------------|-------|-------|-----|------|
|                | $\bar{X}$, % | SD, % | CV, % | n | Range, mg/L |
| $\alpha$-Tocopherol | 92.4 | 2.9 | 3.1 | 5 | 5–25 |
| $\beta$-Tocopherol | 91.4 | 7.6 | 8.4 | 5 | 0.05–0.4 |
| $\gamma$-Tocopherol | 92.0 | 4.1 | 4.4 | 4 | 0.5–6 |

| Table 3. Analytical Precision of the Procedure |
|----------------|-------|-------|-----|
|                | $\bar{X}$ | SD | CV, % |
| **Within-day precision** |
| $\alpha$-Tocopherol | 13.18 | 0.19 | 1.4 |
| $\beta$-Tocopherol | 0.214 | 0.016 | 7.4 |
| $\gamma$-Tocopherol | 1.01 | 0.01 | 1.3 |
| **Day-to-day precision** |
| $\alpha$-Tocopherol | 9.85 | 0.44 | 4.4 |
| $\beta$-Tocopherol | 0.222 | 0.020 | 9.1 |
| $\gamma$-Tocopherol | 1.00 | 0.04 | 3.8 |
simple and short, we prefer to split the analysis into separate \( \alpha \)-tocopherol and \( \beta + \gamma \)-tocopherol determinations.

So far no interference in the assay has been found, although, of course, interference from exogenous compounds is always possible. Selectivity is promoted by the combination of extraction, chromatography, and high wavelength detection, as illustrated by the successful separation of four structurally related compounds.

References