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Note

Simultaneous determination by high-performance liquid chromatography of tocopherol isomers, α-tocopheryl quinone, and cholesterol in red blood cells and plasma

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In recent years numerous authors [1—9] have reported methods for the analysis of α-tocopherol in both plasma and red blood cells (RBC). Several of these methods have involved high-performance liquid chromatography [1—5], but other techniques such as ultraviolet (UV) spectrophotometry [6, 7] and gas—liquid chromatography [8, 9] have also been employed. Among these methods the reported plasma levels are relatively consistent, but those of the red cells vary significantly, yielding values for the RBC to plasma ratio which range from 0.13 [5] to 0.30 [9]. This discrepancy appears to arise primarily from the oxidation of α-tocopherol during its extraction from red cells when insufficient precautions have been taken to prevent this reaction. This problem does not occur in plasma during normal extraction procedures. To prevent oxidation, pyrogallol or ascorbic acid is usually added either to the red cell suspension or to the alcohol which is added to facilitate the extraction. The amounts added vary widely among the published procedures.

One purpose of this study was to develop a method which would allow us to detect any significant oxidative loss during the extraction of α-tocopherol from red cells. The improved sensitivity afforded by the measurement of α-tocopheryl quinone at 265 nm enables the detection of as little as 0.5% formation of the quinone during red cell extraction. This capability has enabled us to detect conversion of α-tocopherol to the quinone which might occur during previously reported extraction procedures. We report here a method which reduces this oxidation to an undetectable amount, achieving greater accuracy and reproducibility. This method is suitable for rapid analysis of large

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numbers of samples and also for the determination of α-tocopherol in samples of limited size, such as RBC from mice.

EXPERIMENTAL

Materials

α-Tocopherol, γ-tocopherol and α-tocopheryl acetate were the generous gift of Hoffmann-La Roche (Nutley, NJ, U.S.A.). α-Tocopheryl quinone was from ICN Chemicals (Cleveland, OH, U.S.A.). Cholesterol was from Fisher Scientific (Philadelphia, PA, U.S.A.).

Red cells and plasma were obtained from subjects receiving medical care at Mount Sinai Medical Center with informed consent and institutional approval of the research protocol. Blood samples from Swiss-Webster mice were obtained from animals maintained on a standard laboratory diet.

High-performance liquid chromatography

The chromatograph was a Hewlett-Packard Model 1084B, equipped with a programmable, variable-wavelength detector with stopped-flow scan capability, and with an autosampler and integrator. The column was a 25 cm × 4.6 mm I.D. ODS reversible (5-μm particle size) from Regis Chemical (Morton Grove, IL, U.S.A.). A 5 cm × 4.6 mm I.D. guard column packed with Pelliguard LC-18 from Supelco (Bellefonte, PA, U.S.A.) was attached before the analytical column. Elution was performed with methanol—water (96:4) at a flow-rate of 1.05 ml/min, at 30°C. The eluent was monitored at 265 nm for α-tocopheryl quinone, at 292 nm for β- + γ-tocopherol, α-tocopherol, and α-tocopheryl acetate, and at 215 nm for cholesterol.

Sample preparation

Whole blood samples were drawn in heparinized, evacuated tubes (Becton-Dickinson, Rutherford, NJ, U.S.A.).

Red blood cells. The separated red cells were washed with 0.9% saline and brought to a measured hematocrit with 0.9% saline—0.5% pyrogallol. Two milliliters of a methanol solution containing internal standard (α-tocopheryl acetate) and 1.0% pyrogallol were added to 1 ml of the RBC suspension and this mixture was extracted with pesticide grade petroleum ether. The petroleum ether layer was evaporated to dryness with nitrogen; the residue was dissolved in methanol—ethanol (80:20) and an aliquot injected onto the HPLC.

Plasma. This procedure is derived from that of De Leenheer et al. [10]. A methanol solution containing internal standard (α-tocopheryl acetate) was added to an equal volume of plasma and the mixture extracted with petroleum ether. The petroleum ether layer was then treated as described above.

Quantification

Cholesterol, α-tocopherol, and the sum of β- + γ-tocopherol were quantified from a standard curve of their peak height ratios versus α-tocopheryl acetate. A linear relationship was found between the peak height ratios (standard/internal standard) and the concentration ratios (standard/internal standard) for each of the compounds. No detectable α-tocopherol was formed from the internal standard during the extraction.

RESULTS

A typical chromatogram of a red cell extract with no added internal standard or standards is shown in Fig. 1. The same sample with added α-tocopheryl quinone, γ-tocopherol, α-tocopherol, α-tocopheryl acetate, and cholesterol is shown in Fig. 2. Baseline separation is achieved for all peaks of interest.

Ten compounds of interest in the tocopherol system which are well separated under the described analytical conditions are listed in Table I. The characteristics of the method are listed in Table II. The mean values, standard deviations, and ranges for the red cell cholesterol, β- + γ-tocopherol and α-tocopherol concentrations in sixteen normal subjects are shown in Table III. With the exception of the concentraton of α-tocopherol in red cells and its ratio to α-tocopherol in plasma, these levels are in good agreement with those reported in the literature [1—5]. These apparent anomalies are discussed below. The red cell α-tocopherol is plotted against the plasma α-tocopherol for these subjects in Fig. 3. The regression equation for these values is: [α-tocopherol (RBC)] = (1.46 ± 0.31)* μg/ml + (0.11 ± 0.03) [α-tocopherol (plasma)] μg/ml, with significance at the 0.002 level.

The ability of this method to determine tocopherols in small samples was studied by the analysis of red cells and plasma from mice. The concentration of α-tocopherol in 12-week-old Swiss-Webster mice was found to be 1.72 ± 0.28

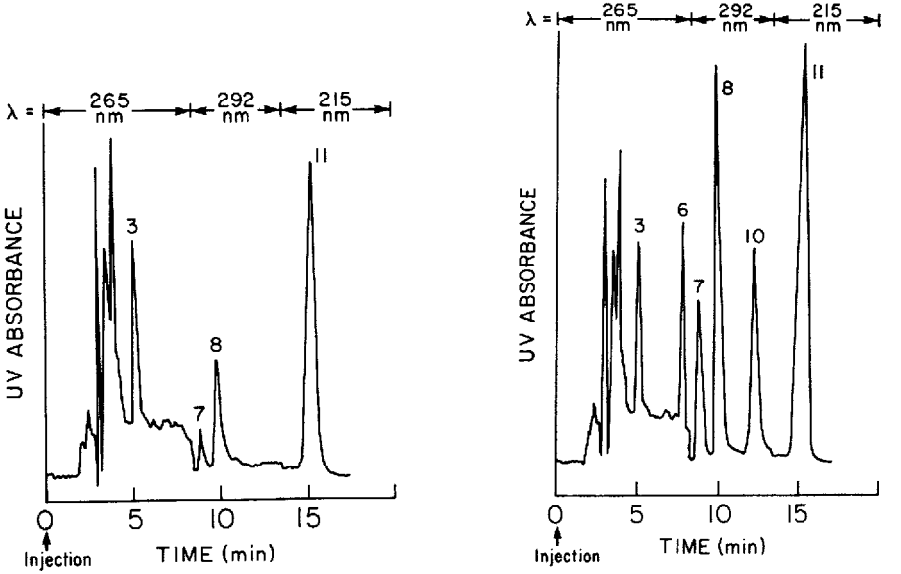

Fig. 1. Chromatogram of a red cell extract with no added standards or internal standard. Peaks: 7 = β- + γ-tocopherol; 8 = α-tocopherol; and 11 = cholesterol. The attenuation is changed from 2^4 to 2^5 at 14 min.

Fig. 2. Chromatogram of the same concentration of red cells as in Fig. 1 but with 0.25 μg of α-tocopheryl quinone (peak 6), 0.9 μg of γ-tocopherol (peak 7), 3.1 μg of α-tocopherol (peak 8), 20.0 μg of α-tocopheryl acetate (peak 10), and 280 μg of cholesterol (peak 11) added. The attenuation is changed from 2^4 to 2^5 at 11.7 min, and then to 2^9 at 14 min.

*Mean ± S.D.

TABLE I

SIGNIFICANT CHROMATOGRAPHIC PEAKS

Peak No.	Retention time (min)	Relative retention time	UV absorbance maximum (nm)	Identification
1	3.1	0.24	256 (SH)	Quinone decomposition product
2	4.9	0.39	292	α-Tocopherol hydroquinone
3	5.3	0.42	—	Unknown
4	5.7	0.45	250 (SH)	Quinone decomposition product
5	6.7	0.53	260	γ-Tocopherol oxidation product
6	7.7	0.61	265	α-Tocopheryl quinone
7	9.2	0.72	295	β- + γ-Tocopherol
8	10.2	0.81	292	α-Tocopherol
9	11.5	0.91	301	α-Tocopherol oxidation product
10	12.6	1.00	285	α-Tocopheryl acetate
11	15.9	1.25	<210	Cholesterol

TABLE II

CHARACTERISTICS OF THE HPLC PROCEDURE FOR TOCOPHEROL ISOMERS AND CHOLESTEROL IN RBC AND PLASMA

	β- + γ-Tocopherol	α-Tocopherol	Cholesterol
Coefficient of variation (8 replicate plasma determinations)	4.4%	2.9%	4.4%
Coefficient of variation (8 replicate RBC determinations)	6.4%	4.1%	2.6%
Correlation coefficient (typical plasma standard curve)	0.999	0.998	0.990
Correlation coefficient (typical RBC standard curve)	0.9995	0.9998	0.9995
Rate of recovery from red cells	N.D.*	92.3% ± 0.4%**	95.0% ± 3.4%***
Limit of detection (μg/ml packed RBC)§	0.1	0.1	N.D.*

*Not determined.
**Average of 2 samples.
***Average of 3 samples.
§Limit for α-tocopheryl quinone is 0.02 μg/ml.

μg/ml in plasma (n = 12) and 0.84 ± 0.13 μg/ml in RBC (n = 7). No β- + γ-tocopherol was detected in either the plasma or red cells. The detection limits were 0.02 μg/ml and 0.05 μg/ml, respectively.

Addition of pyrogallol to the red cell suspension but not to the added alcohol has been used by others [2] to prevent the oxidation of α-tocopherol during extraction. Between 2% and 8% of the α-tocopherol is converted to the quinone under these conditions. The quinone is not the only product formed, corresponding to only 15—25% of the total tocopherol lost. Thus, if the α-tocopheryl quinone is measured at a less than optimal wavelength such as 280 nm [2], an undetectable amount of quinone can correspond to a 10% loss of the

TABLE III

CONCENTRATIONS OF TOCOPHEROL ISOMERS AND CHOLESTEROL FOR 16 NORMAL SUBJECTS

	Mean	Standard deviation	Range
RBC β- + γ-tocopherol (μg/ml)	0.52	0.27	0.15 —1.02
RBC α-tocopherol (μg/ml)	2.61	0.41	1.89 —3.42
RBC cholesterol (mg/ml)	1.21	0.13	1.03 —1.51
Plasma β- + γ-tocopherol (μg/ml)	2.0	1.2	0.40 —3.84
Plasma α-tocopherol (μg/ml)	10.2	2.6	4.93 —14.40
α-Tocopherol RBC/plasma ratio	0.267	0.054	0.181—0.381
β- + γ-Tocopherol RBC/plasma ratio	0.299	0.143	0.180—0.717

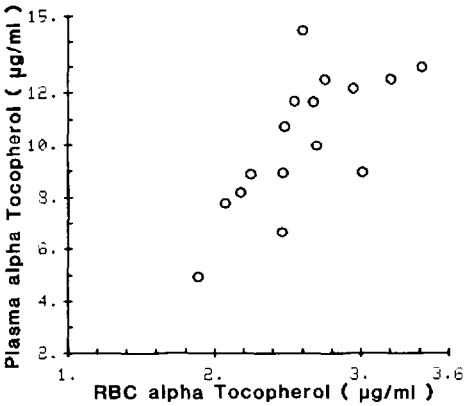

Fig. 3. α-Tocopherol concentrations (μg/ml) in the red cells and plasma of sixteen normal subjects. The R value of the regression fit is +0.71.

tocopherol originally present. Addition of pyrogallol to the alcohol added during extraction allows less than 0.2% (none detected) conversion of the original tocopherol to the quinone. γ-Tocopherol and α-tocopheryl acetate exhibited no detectable oxidation when pyrogallol was added to the red cell suspension and not to the methanol.

DISCUSSION

The prevention of oxidative loss of α-tocopherol during red cell extraction is dependent not only on the anti-oxidant added, but also on the order of the addition of the alcohol and the antioxidant. The oxidative reaction proceeds rapidly upon addition of alcohol to the cell suspension (data not shown). The ten-fold greater sensitivity for α-tocopheryl quinone at 265 nm versus 280 nm allows the detection of minute losses of α-tocopherol. Monitoring the chromatographic effluent at 265 nm also allows sensitive detection of the in vivo quinone which has been postulated to exist [11]. The present method yields values for the α-tocopherol RBC/plasma ratio which are significantly greater than many of the previously described procedures [2, 4—6]. Insufficient

protection against oxidative loss during RBC extraction could lead to 10—50% loss of the membrane α-tocopherol, accounting for these differences.

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