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Note

Quantitation of physiological α-tocopherol, metabolites, and related compounds by reversed-phase high-performance liquid chromatography

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Vitamin E, α-tocopherol, has been linked to a variety of seemingly unrelated pathological conditions in man. For example, in childhood, bronchopulmonary dysplasia, retrolental fibroplasia and hemolytic anemia have all been associated with a vitamin E deficient state [1]. High dose vitamin E has been used therapeutically in the treatment of non-congenital spherocytic hemolytic anemia secondary to glucose-6-phosphate deficiency [2].

At the present time, colorimetric, fluorometric, hemolytic estimate, paper chromatographic, gas chromatographic [3], and high-performance liquid chromatographic (HPLC) [4—8] methods have been used in the assay of α-tocopherol. In addition, some HPLC methods are capable of separating the naturally occurring vitamin E analogues α-, β-, γ- and δ-tocopherols.

Recently, we have investigated the potential protective effect of vitamin E in relation to anthracycline induced cardiomyopathy [9] and carcinogenicity [10]. In the study of these drug nutrient interactions the metabolism and distribution of α-tocopheryl acetate (major component of commercially available vitamin E) becomes an important topic. The HPLC system we describe was used to separate α-tocopherol, α-tocopheryl acetate, α-tocopheryl quinone, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and γ-tocopherol. The 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was used as a reference compound for retention time of water soluble metabolites.

EXPERIMENTAL

Reagents

Glass-distilled methanol and hexane were obtained from Burdick and Jackson

Labs. (Muskegon, MI, U.S.A.). α-Tocopheryl acetate was purchased from Sigma (St. Louis, MO, U.S.A.). Calbiochem-Behring (La Jolla, CA, U.S.A.) supplied the pure α-tocopherol. The α-tocopheryl quinone, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, and [^{14}C]α-tocopheryl acetate were the gifts of Hoffmann-La Roche (Nutley, NJ, U.S.A.).

Apparatus and samples

A Glenco HPLC system I (Houston, TX, U.S.A.) equipped with a Whatman Partisil 10-μm ODS 250 mm × 4.6 mm reversed-phase column was the base instrument used in isocratic elutions. A Laboratory Data Control (Riviera Beach, FL, U.S.A.) chromatographic system consisting of two Constametric III pumps and Gradient Master programmer were used in gradient elutions. The gradient system was equipped with a Custom LC Spherisorb 5 μm ODS 150 mm × 4.6 mm reversed-phase column. Ultraviolet detection at 275 or 285.5 nm was obtained on a variable-wavelength UV detector (Laboratory Data Control). Electronic integration of peak areas was obtained with a digital integrator CSI-38 (Columbia Scientific Industries, Austin, TX, U.S.A.). Fractions used in scintillation counting were collected with a fraction collector (LKB Instrument, Rockville, MD, U.S.A.) and counted with a Beckman LS 7500 liquid scintillation counter (Beckman Instruments, Fullerton, CA, U.S.A.). A Brinkman homogenizer was used in tissue processing.

Plasma and tissue samples assayed in this system were obtained from Sprague-Dawley (SD) rats. [^{14}C]α-Tocopheryl acetate was administered intraperitoneally (i.p.) daily to SD rats at a dose of 300 mg/kg (1.8 g/m^2) for one to four consecutive days according to experimental protocol. Plasma samples were also obtained from patients receiving high doses of α-tocopherol orally.

Procedures

For routine plasma analysis the isocratic system was used with a mobile phase of 87% methanol and 13% filtered distilled water. Eluent flow-rate was 1.30 ml/min with UV monitoring at 285.5 nm and 0.01 a.u.f.s. Analyses of tissue samples were usually performed using a convex gradient with an initial mobile phase of 88% methanol, 12% water increasing for 20 min to 100% methanol at a flow-rate of 2.0 ml/min. In tissue chromatograms, where quantitation of minimal amounts of α-tocopheryl quinone is a priority, UV monitoring was at 275 nm and 0.01 a.u.f.s. In elutions from which fractions were collected, chromatographic conditions were the same and fractions were collected every 40 sec at 0.8 ml per fraction.

Tissue and plasma samples were extracted in a similar manner in preparation for HPLC analysis. Plasma was extracted directly. Tissue samples were prepared for extraction by homogenizing 1 g of tissue in 4 ml of normal saline. One ml of tissue homogenate was extracted for routine analysis. Usually, 200 μl of plasma were added to a 12-ml conical centrifuge tube. Two hundred μl of double-distilled ethanol and 500 μl of hexane were then added to the tube. The contents were mixed and centrifuged at 9000 *g* for 10 min at 4°C. Four hundred μl of the hexane supernatant were removed and dried under nitrogen. At the time of HPLC analysis the sample was reconstituted in 100 μl of a mixture of 87% methanol and 13% propanol, centrifuged, and injected.

For the extraction of 1 ml of plasma or tissue the volume of ethanol and hexane added was proportionally increased. Mixing and centrifugation procedures were similar to those used for 200 μl of plasma.

Calibration and reproducibility

Calibration curves were constructed for both isocratic and gradient elutions. The isocratic elution calibration was performed for plasma analysis using a 200-μl sample size. Gradient elution was calibrated using a 1-ml sample size appropriate for tissue analysis. Saline, for the 200-μl sample size, and 4% bovine serum albumin in saline, for the 1-ml sample, were spiked with known amounts of standard.

Extraction methods were as outlined in the Procedures section. Table IA represents the results of the 200-μl calibration curve for the compounds listed using isocratic elution and monitoring at 285.5 nm. α-Tocopheryl acetate was not analysed because the compound is absent from plasma. Table IB represents the 1-ml calibration curves using gradient elution and monitoring at 275 nm. For both sections two samples were assayed at each concentration and four different concentrations were used. The lower limit of detectability and correlation coefficient were calculated from a least-squares linear regression. The lower limit of detectability was designated as 20,000 area units, where assay variability became excessive (n=6, C.V.=19%). Mean percentage recovery for both tables was determined by comparing the midpoint values of the extraction regression lines to midpoint values on regression lines from direct injection standard curves. The correlation coefficient for all direct injection curves was $>0.996, p<0.001$.

Assay reproducibility was determined using UV monitoring of α-tocopherol.

TABLE I

CALIBRATION CURVE DETECTION LIMITS, LINEARITY, AND RECOVERY

	Calibration range (mole/l)	Lowest detectable concentration (mole/l)	Correlation coefficient	Mean percentage recovery
A. 200-μl sample volume				
α-Tocopherol	$5\cdot10^{-6}$—$5\cdot10^{-5}$*	$3.7\cdot10^{-6}$	0.997	65
α-Tocopheryl quinone	$2.5\cdot10^{-5}$—$2.5\cdot10^{-4}$	$7.5\cdot10^{-6}$	0.999	58
γ-Tocopherol	$5\cdot10^{-6}$—$5\cdot10^{-5}$	$3.9\cdot10^{-6}$	0.997	63
B. 1-ml sample volume				
α-Tocopheryl acetate	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$1.3\cdot10^{-6}$	0.999	64
α-Tocopherol	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$1.2\cdot10^{-6}$	0.995	57
α-Tocopheryl quinone	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$6.7\cdot10^{-7}$	0.999	44
γ-Tocopherol	$2\cdot10^{-6}$—$5\cdot10^{-5}$	$2.1\cdot10^{-6}$	0.998	60

*Eight data points are used in each calibration curve.

Two hundred μl of a plasma sample were assayed repeatedly using the extraction and sample preparation procedure outlined previously: ten values were determined on five separate days. The following values were obtained shown in integrated unit area: mean 212,741; standard deviation, 21,526 (C.V.=10%).

A colorimetric procedure [9] was also used in analysis of plasma samples from patients and the results compared to our HPLC values. From a comparison of nineteen samples the mean value for the colorimetric assay was $7.1 \cdot 10^{-5}$ mole/l α-tocopherol and from the HPLC assay $8.9 \cdot 10^{-5}$ mol/l, a 20% higher value. By linear regression analysis of the two assays, r=0.96 with a slope of 1.09. One possible explanation for the higher HPLC values is overcompensation for carotene interference in the colorimetric assay.

RESULTS AND DISCUSSION

Fig. 1A represents a typical chromatogram of a patient plasma sample analysed using isocratic elution. Small quantities of α-tocopheryl quinone and γ-tocopherol can be detected in human plasma along with α-tocopherol. Of 24 patient plasma samples assayed by HPLC, γ-tocopherol was detected at a quantitatible level in five patients. The range of γ-tocopherol concentration was $3.7 \cdot 10^{-6}$ to $1.1 \cdot 10^{-5}$ mol/l representing from 3 to 24% of the total tocopherol detected. In the same group of samples a quantitatible level of α-tocopheryl quinone ($1.6 \cdot 10^{-5}$ mol/l) was found only in the patient with the highest level of α-tocopherol ($2.5 \cdot 10^{-4}$ mole/l). α-Tocopheryl acetate was not detectable in any plasma samples from experimental animals (or patients).

Fig. 1B is an analysis of liver from a rat treated with four i.p. doses of [^{14}C]α-tocopheryl acetate assayed using the gradient elution. Gradient elution dramatically increases the selectivity of our system without increasing analysis time substantially. Gradient elution also alleviates problems associated with late eluting materials present in some types of tissues. α-Tocopheryl acetate, γ-tocopherol, α-tocopherol, and α-tocopheryl quinone are detectable in the chromatogram by UV absorption, and radioactivity was present in areas corresponding to peaks 1, 3, and 4. A different liver sample was monitored simultaneously at 254 and 285.5 nm and the area ratio of 254:285.5 absorbance for peak 1 was compared to the ratio for known α-tocopheryl quinone. The ratios were equal within 5%, further verifying peak identity.

In general, α-tocopherol, α-tocopheryl quinone, α-tocopheryl acetate, and γ-tocopherol were detectable in liver, kidney, and heart samples from animals treated with α-tocopheryl acetate. In plasma and mammary fat only α-tocopherol and γ-tocopherol were detected by UV absorbance or radioactivity. Table II represents in part, data from our distribution study using the HPLC technique. Treated animals received four i.p. doses of [^{14}C]α-tocopheryl acetate at 300 mg/kg and were killed at the times indicated, after the last dose. Control animals were of comparable age and dietary regimen. Plasma samples were analyzed with an isocratic system and monitored at 285.5 nm. Heart samples were analyzed using a gradient system at 275 nm. Fractions were collected from the eluent and counted using liquid scintillation counting. By comparing the amount of compound detected by UV absorbance to the amount detected from radioactivity, a specific activity indicating radioactive

TABLE II

ANALYSIS OF RAT HEART AND PLASMA

Concentration (concn.) values are given in μg/ml ± S.D., for plasma and μg/g ± S.D. for heart. Specific activity (S.A.) is in mCi/mole ± S.D. *n* = number of samples analyzed.

No. of injections	Injected S.A.	Days after last injection	Plasma			Heart				
			α-Tocopherol			α-Tocopherol			α-Tocopheryl acetate concn.	α-Tocopheryl quinone concn.
			n	concn.	S.A.	*n*	concn.	S.A.		
0	—	—	6	23.1±5.2	—	2	26.0±5.9	—	B.Q.*	B.Q.
4	44.8	2	3	20.0±4.2	22.0±4.3	1	50.4	3.9	3.2	1.7
4	35.7	7	1	6.7	2.8	1	28.9	4.8	9.7	B.Q.
4	35.7	21	1	23.6	3.4	2	40.0±13	5.6±2.6	10.2±8.2	1.5
4	35.7	70	2	34.8±9.5	13.3±0.9	2	33.3±3.2	16.4±2.5	B.Q.	1.7±0.5

*B.Q. = Below reliable level of quantitation: <2.8 μg/g for α-tocopheryl acetate and <1.3 μg/g for α-tocopheryl quinone.

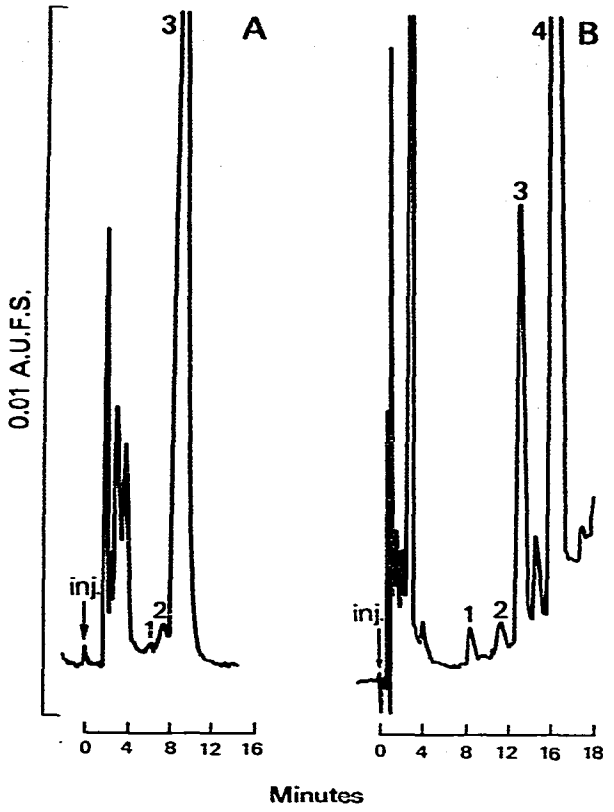

Fig. 1. (A) Injection (50 μl) of an extraction of 200 μl of plasma from a patient receiving high doses of α-tocopherol orally. Peaks: (1) α-tocopheryl quinone, 0.28 μg; (2) γ-tocopherol, 0.11 μg; (3) α-tocopherol, 3.53 μg. Conditions: Whatman Partisil ODS 10 μm 250 mm × 4.6 mm column; mobile phase, methanol—water (87:13); flow-rate, 1.30 ml/min; detection at 285.5 nm. (B) Injection (25 μl) of an extraction of 1 ml of liver homogenate containing 0.22 g of tissue from a rat receiving α-tocopheryl acetate. Peaks: (1) α-tocopheryl quinone, 0.04 μg; (2) γ-tocopherol, 0.2 μg; (3) α-tocopherol, 1.8 μg; (4) α-tocopheryl acetate, 24 μg. Conditions: Custom LC Spherisorb ODS 5 μm 150 mm × 4.6 mm; mobile phase, gradient elution from methanol—water (88:12) to 100% methanol in 20 min; flow-rate 2.0 ml/min; detection at 275 nm.

dilution was calculated. This dual detection method enables us to quantitate both the total amount of a compound and to determine what portion of the total pool has been replaced by radioactive material. From the data in Table II we see changes in the tocopherol pool that would not be readily apparent if quantitation were by UV absorption or radioactivity individually. This type of dynamic analysis may prove useful in determining rate of tissue uptake of administered compounds and may allow detection of otherwise non-detectable changes in distribution or metabolism.

The HPLC system outlined has been successfully used to quantitate α-tocopheryl acetate and its metabolic products in human plasma and in animal tissues and plasma. These types of separations and quantitations are invaluable in studying the metabolism and distribution of the compounds. With further refinement of techniques it is hoped that we can achieve the ability to monitor

tocopherol levels in other tissues along with testing the capabilities of our system in separating the remaining tocopherol analogues.

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