Journal of Chromatography, 224 (1981) 389—397
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 883

QUANTITATION OF INDIVIDUAL TOCOPHEROLS IN PLASMA, PLATELETS, LIPIDS, AND LIVERS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received October 8th, 1980; revised manuscript received February 25th, 1981)

SUMMARY

A rapid, sensitive procedure is described for the analysis of the tocopherols (α-, β-, γ-, and δ-) in plasma, platelets, lipids, and liver using high-performance liquid chromatography and fluorometric detection. Excellent recoveries of these tocopherols in plasma were obtained — greater than 90%. Separation and quantitation of the four tocopherols required as little as 0.2 ml plasma.

INTRODUCTION

In order to ascertain vitamin E deficiency syndromes in laboratory animals and the nutritional status of man with respect to this vitamin, investigators should include an assessment of plasma vitamin E levels. In addition, in order to demonstrate or verify the absence of the nutrient in the diet, similar analyses for vitamin E of the dietary preparations should be performed. Investigators have employed serum creatine phosphokinase (ATP:creatine phosphotransferase, EC 2.7.3.2) activity [1—3] or urinary creatine [4] measurements as status parameters; creatinuria, however, may result from other conditions such as choline or potassium deficiencies [5]. In the past, however, tocopherol analyses required laborious, cumbersome techniques, including column or thin-layer chromatography, which often lead to poor recoveries. Most analytical procedures were based primarily on the Emmerie—Engel reaction [6]. Such determinations lack specificity because other substances are often present that will reduce the resultant chromogenic complex and the individual tocopherols

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0378-4347/81/0000—0000/$02.50

also react at varying rates to produce the reduced compound which is measured spectrophotometrically. The former limitation has been partially eliminated by elaborate sample preparation to remove reducing artifacts. Several investigators have also described a thin-layer chromatographic separation of these artifacts from the tocopherols and the use of gas—liquid chromatography for final separation and quantitation of the tocopherols [7, 8]; these procedures are rather lengthy.

Several investigators recently reported high-performance liquid chromatographic (HPLC) techniques that obviate such complicated methodologies for the quantitation of vitamin E. Hatam and Kayden [9] have employed a reversed-phase microparticulate column and fluorometric detection with excitation at 205 nm for the determination of tocopherol content of plasma and cellular components of blood. These investigators did not, however, report the separation of the positional isomers, β- and γ-tocopherols. Taylor et al. [10] measured total tocopherol content of homogenates and subcellular components from rat tissues using fluorometric analysis; these researchers employed 290 nm excitation instead of 215 nm because severe quenching problems reportedly limited the utility of the latter absorption maxima. Abe et al. [11] have also developed an HPLC technique for the determination of α-, β-, γ-, and δ-tocopherols using a spectrofluorometer with excitation at 295 nm; quantitative measurements for these components found in serum [12] and liver [13] were reported. Thompson and Hatina [14] have also reported the quantitation of the tocopherols as well as the tocotrienols in food and animal tissue homogenates employing fluorometric detection in conjunction with HPLC. Bieri et al. [15] have reported an HPLC procedure for simultaneous determination of α-tocopherol and retinol in plasma and the former in erythrocytes, using a reversed-phase microparticulate column and UV absorbance detection; the positional isomers were not separated.

The current investigators sought to verify the universality and reliability of an HPLC—fluorometric technique that was developed in this laboratory [16] for the quantitation of tocopherols found in plasma and casein. Because of the methodological inadequacies of earlier methods, the current investigators were prompted to develop a sensitive, reliable, versatile, and rapid technique. The coupling of fluorometric detection (with saponification) and HPLC provided a unique method for vitamin E analyses in a variety of biological materials.

EXPERIMENTAL

Analytical instrumentation

A Perkin-Elmer Model 1250 liquid chromatograph (Norwalk, CT, U.S.A.) in conjunction with a Fluorichrom filter fluorometer (Varian, Palo Alto, CA, U.S.A.), with a 25-μl cell volume and equipped with a deuterium lamp was employed in this study. Fluorescence was recorded on an Omniscribe B-500 1-mV strip chart recorder (Houston Instruments, Austin, TX, U.S.A.). The analytical column was a microparticulate 5-μm silica gel normal-phase column, Partisil PXS5 (Whatman, Clifton, NJ, U.S.A.), 25 cm × 4.6 mm I.D., preceded by a guard column, 10 cm × 4.6 mm I.D., packed with Corasil II (Waters Assoc., Milford, MA, U.S.A.). The mobile phase was 1.4% isopropanol in hexane

(MCB Manufacturing Chemists, Cincinnati, OH, U.S.A.). Flow-rates approximated 1.2 ml/min. An interference filter was used for excitation of column effluent at 200 nm and band pass filters for emission monitoring at 340 nm.

Four tocopherol standard (α-, β-, γ-, and δ-tocopherols) solutions containing 0.1 mg tocopherol per ml hexane were prepared; equal-volume aliquots of each were mixed together and serially diluted 1:10 with hexane. Standard calibration mixtures were injected every three or four sample injections in order to ensure the constancy of detector response. Sample concentrations were calculated using peak height and standard calibration curves. A typical calibration curve is depicted in Fig. 1. These calibration curves were calculated by linear regression. Excellent linearities were exemplified by correlation coefficients of 0.985, 0.994, 0.993, and 0.992 ($P < 0.001$) for α-, β-, γ-, and δ-tocopherols, respectively.

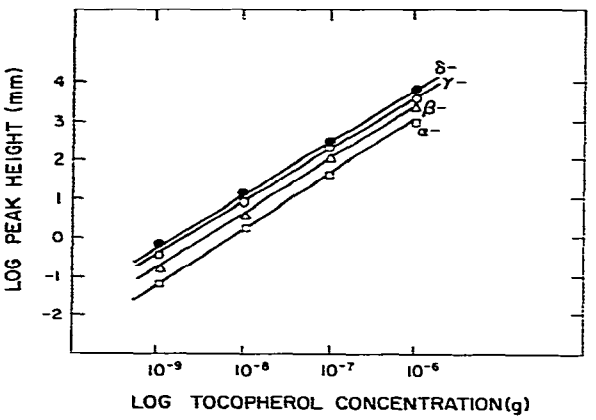

Fig. 1. Calibration curve for tocopherols by HPLC analysis.

Finally in order to ascertain the identity of the four tocopherols, standard solutions of each (approximately 1 mg tocopherol per ml hexane) were prepared for mass spectrometric (MS) analysis on a Hitachi Model RMU-7 electron impact mass spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). An equal-volume mixture of the tocopherol standards was also injected into the chromatograph and the eluate collected in fractions for MS analyses in order to verify the characteristic spectra of each tocopherol. A plasma extract was selected for MS analysis once the investigators observed both β- and γ-tocopherols present by chromatographic retention data. The plasma extract was then reinjected and the eluate collected in fractions which included all four tocopherol components; these fractions were then submitted to MS analysis. The β- and γ-tocopherols are known to be positional isomers which elute closely after one another [17]; the spectra for the β- and γ-tocopherols eluted from the column are shown in Fig. 2. Fragmentation patterns for the two tocopherols were quite similar except for differences in the relative intensity of the molecular ion peak at *m/e* 416 and the fragmentation peak at *m/e* 151. The spectra of all four tocopherols correspond closely to that obtained for each standard and to that reported by De Leenheer et al. [18].

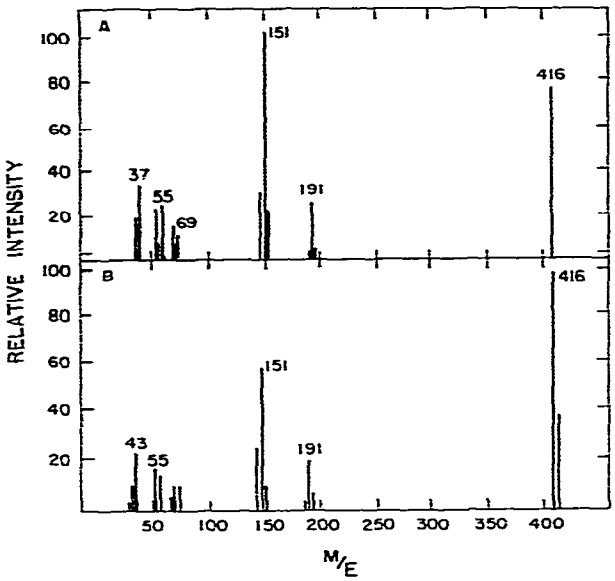

Fig. 2. Mass spectra of plasma HPLC eluates corresponding to (A) γ-tocopherol and (B) β-tocopherol.

Recoveries of the tocopherols were determined by standard additon; mixtures containing both 1 and 5 μg of each of the tocopherols were added to each type of sample matrix at the onset of sample preparation. Recoveries were calculated by comparing "spiked" values to duplicate analyses to which no tocopherols were added.

Plasma determinations

Blood samples (approximately 2 ml) from five male New Zealand white rabbits (Dutchland Laboratory Animals, Denver, PA, U.S.A.) 10 weeks of age were drawn from the central auricular artery using disposable syringes. Blood samples were also obtained from four males, 23—31 years of age. Blood was collected in polypropylene tubes containing 0.2 ml acid—citrate dextrose (ACD) as the anticoagulant. Tubes were stoppered and gently inverted to ensure adequate mixing of anticoagulant. Blood was stored in ice and subsequent analyses were performed in the dark. Blood was centrifuged at 3000 *g* for 10 min at 4°C. Plasma extraction of tocopherols was adapted from the procedures described by De Leenheer et al. [18] and by Abe and Katsui [19], except for the utilization of antioxidants in both aqueous and organic solvents — 3% pyrogallol in absolute ethanol and 0.025% butylated hydroxytoluene (BHT) in *n*-hexane. Following a two-fold hexane extraction, samples were subsequently evaporated under argon and redissolved in 0.2 ml *n*-hexane.

Platelet determinations

Blood, 5 ml, was obtained from rabbits by cardiac puncture or venipuncture from four young Caucasian males. Blood was collected in polypropylene tubes containing 0.6 ml ACD solution. The contents of these tubes were then centrifuged at 100 *g* for 15 min at 21°C to obtain platelet-rich plasma (PRP); following multiple washings of PRP the resultant platelet pellet was resuspended with 1 ml 0.154 *M* sodium chloride solution [20]. An aliquot

(6.6 μl) was then obtained for counting platelets on a Coulter Counter ZBI (Coulter Diagnostics, Hialeah, FL, U.S.A.) [21] and the platelet suspension was adjusted to contain roughly $1 \cdot 10^{10}$ cells.

The remaining resuspended platelet samples were mixed with 1 ml absolute ethanol containing 3% pyrogallol and tubes were allowed to sit for 5 min; 2 ml *n*-hexane containing 0.125% BHT were added to each sample tube. Tubes were then shaken for 10 min followed by centrifugation at 5000 *g* for 5 min at 21°C. The extraction was similar to that described above for plasma.

Liver determinations

Rabbit livers and commercially obtained calf liver, approximately 0.5 g, were homogenized in ice with 2 ml absolute ethanol and 2 ml 1% EDTA, disodium salt. The mixture was then preincubated at 70°C for 5 min. While samples were continuously flushed with argon, 1 ml of 60% aqueous potassium hydroxide solution was added; again contents were mixed vigorously and warmed for an additional 15 min in a 70°C water bath. Tubes were then placed in ice to cool and twice extracted with 6 ml *n*-hexane containing 0.125% BHT. The procedure was performed in a manner similar to those previously described.

Lipid determinations

Aliquots, about 0.5 g, of commercial oils or shortenings were transferred into test tubes; 2 ml of 3% pyrogallol in absolute ethanol were added to the contents of each tube. Contents were then mixed and flushed with argon. Samples were subsequently heated for 15 min in a 70°C water bath. Then 0.5 ml of 60% aqueous potassium hydroxide solution was added to each sample under a continuous stream of argon. Again the contents were heated for an additional 3 min, cooled in ice, and to each tube 2.5 ml deionized water and 3 ml *n*-hexane containing 0.125% BHT were added. Again, extraction was implemented as described earlier.

Dietary preparation determinations

Diets similar to that of Gaman et al. [22] were prepared according to specifications. Aliquots, approximately 1 g and 2.5 g, of pelleted rations were crushed and added to 4 ml absolute ethanol containing 3% pyrogallol in glass stoppered test tubes. Saponification and extraction were conducted in a similar fashion as described previously for liver.

RESULTS AND DISCUSSION

Typical chromatograms of extracts from plasma of a 16-week old rabbit and of an aliquot of control dietary preparation are depicted in Fig. 3. Very few plasma extracts were found to contain all four tocopherols; in fact, only one rabbit in each of two purchases of animals was observed to possess detectable quantities of β-tocopherol. α-Tocopherol is considered to be the most predominant form in plasma, followed by γ-, δ-, and β-tocopherols [23, 24]. In analyses of control diet aliquots, the present investigators observed significant quantities of both α- and γ-tocopherols. α-Tocopherol acetate does not fluoresce [14] but following saponification, quantitation of α-tocopherol was possible. Corn oil in the diet preparation was expected to contribute

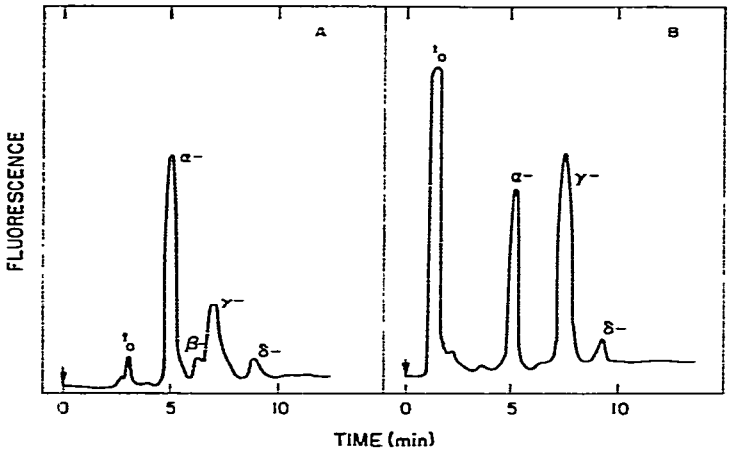

Fig. 3. HPLC separation of tocopherols from extracts of (A) plasma from control rabbit and (B) control diet.

considerable amounts of γ-tocopherol and lesser amounts of α- and δ-tocopherols; analyses of corn oil alone described later confirmed this expectation.

The tocopherol content of several animal tissues are given in Table I. Values for plasma tocopherols in rabbits are in agreement with those of Horn et al. [25] and Diehl and Kistler [26]. Horn et al. [25] utilized a microspectrophotometric technique based upon the Emmerie—Engel reaction for the determination of plasma total tocopherols in New Zealand white male rabbits fed a synthetic diet containing 225 mg DL-α-tocopherol per kg diet; plasma vitamin E levels of control rabbits were reported to be at least 1000 μg/dl. Diehl and Kistler [26] determined serum total tocopherol levels in New

TABLE I

VITAMIN E CONTENT OF SELECTED ANIMAL TISSUES

Values represent means ± S.D. Each analysis was performed in duplicate; the number of samples represents the number of animals used in independent analyses.

Sample	No. of samples	Tocopherol				
		α-	β-	γ-	δ-	Total
Rabbit plasma (mg/dl)	5	0.81 ±0.25	nd*	0.14 ±0.06	nd	0.95 ±0.23
Human plasma (mg/dl)	4	1.05 ±0.22		0.14 ±0.06	0.10 ±0.01	1.25 ±0.21
Human platelets ($\mu g/10^{10}$ cells)	3	4.04 ±0.82	nd	0.96 ±0.06	0.10 ±0.00	5.10 ±0.82
Rabbit liver (μg/g wet wt)	3	20.61 ±4.59	nd	0.74 ±0.06	0.50 ±0.00	21.85 ±4.27
Calf liver (μg/g wet wt)	3	14.23 ±2.67	nd	1.89 ±0.58	nd	16.12 ±3.73

*nd = not detected.

Zealand white rabbits fed a commercial rabbit chow reported to contain 8 mg/kg diet; a similar spectrophotometric procedure was employed and control rabbits exhibited a range of 0.5—1.0 mg tocopherol per dl sera. In contrast, Caravaggi [27] analyzed sera obtained from New Zealand white rabbits by a microspectrophotometric method and observed 0.34 ± 0.43 mg total tocopherol per dl ($\bar{X}$ ± S.D.); the dietary content of vitamin E was not reported. The plasma α-tocopherol level reported by Ishibashi et al. [12] for three rabbits of unknown strain (0.08 ± 0.01 mg/dl, $\bar{X}$ ± S.D.) was nearly 10 times smaller; α-tocopherol is the major component present in plasma. The latter investigators employed an HPLC technique similar to the current researchers with saponification and fluorometric detection; interestingly, human plasma α-tocopherol levels reported in the same study did approximate values reported in the current study.

With regard to platelet determinations, values obtained in the present study for total tocopherols are in agreement with those reported by other investigators. Hatam and Kayden [9], using an HPLC—fluorometric technique, observed 5.10 ± 0.63 μg α-tocopherol per 10^{10} platelets ($\bar{X}$ ± S.D.): in six healthy male subjects, these investigators reported that γ-tocopherol represented 11.4 ± 3.3% ($\bar{X}$ ± S.D.) of the total tocopherols in platelets. Nordφy and Strφm [20], using a spectrophotometric procedure, observed similar levels of total tocopherols in platelets from 12 male subjects.

Liver analyses for vitamin E content should be treated with caution; tocopherol measurements in wet aliquots are often different by 10-fold or greater. Additionally, data may be further subject to question because studies have demonstrated the diminutive effect of hepatic coccidiosis on the vitamin E status of rabbits [26, 28]. Values obtained for control rabbit livers in this study were slightly greater than those reported from non-infected rabbits in the study of Diehl [28]; the latter investigator used New Zealand rabbits of mixed age and sex which were given a commercial chow containing only 8 mg/kg diet. Draper and Csallany [29] observed a greater concentration of α-tocopherol (approximately 30 μg/g fresh tissue) in livers from rabbits; the strain was not reported and animals were of mixed age and sex; these animals were given a low tocopherol diet supplemented with α-tocopheryl acetate at 1 g/kg. Analyses included extraction and column chromatography, followed by a spectrophotometric determination. Factors that may have considerable influence on tissue tocopherol content are age, sex, dietary vitamin E content, and how long the diet was fed. Values for calf livers are similar to those reported in the literature [30, 31].

Since vegetable oils are known to contain significant quantities of the tocopherols, the present investigators analyzed representative oils obtained commercially (Table II). Note the large quantity of β-tocopherol present in wheat germ oil. Data obtained here are in agreement with those previously reported [31, 32]; such agreement exemplifies the degree of reliability and the advantages of such rapid methodology.

Also included in Table II are data obtained for tocopherol content of diets prepared commercially for the present study. Note only minute amounts of δ-tocopherol are present in the control ration. In the deficient preparation only β-tocopherol was detected.

TABLE II

VITAMIN E CONTENT OF SELECTED FOOD MATERIALS

Values represent means ± S.D.

	No. of replicates	Tocopherol (mg/100 g)				
		α-	β-	γ-	δ-	Total
Wheat germ oil, Rexall	5	120.18 ±10.96	71.4 ±4.6	9.42 ±0.17	nd*	201.00 ±10.03
Corn oil, Mazola	4	6.44 ±0.06	nd	68.97 ±3.89	0.92 ±0.03	76.33 ±3.65
Vegetable oil, Crisco	4	7.61 ±0.02	nd	40.23 ±1.72	9.46 ±0.13	57.30 ±1.52
Vegetable shortening, Crisco	4	9.12 ±0.31	nd	66.22 ±2.19	24.45 ±1.15	99.79 ±2.04
Diet, control	4	9.12 ±0.07	nd	3.66 ±0.04	0.31 ±0.01	13.09 ±0.05
Diet, deficient	4	nd	nd	0.00** ±0.00	nd	0.00 ±0.00

*nd = not detected.
**Actual mean was 0.052 μg/100 g.

Recoveries of the 1- and 5-μg spikes in all kinds of biological samples tested ranged from 94—99% for α-, 86—94% for β-, 93—99% for γ-, and 89—92% for δ-tocopherol. Typically the lower per cent recoveries were observed in liver samples.

This HPLC technique appears to be quick and reliable as well as sensitive. The wide applicability of such a method can be justified by the diversity of materials analyzed in this study. Such methodology undoubtedly merits greater consideration for researchers interested in the separation and quantitation of tocopherols.

ACKNOWLEDGEMENTS

The authors thank Hoffmann-La Roche, Nutley, NJ, U.S.A. and Eisai Research Labs., Tokyo, Japan for generously supplying tocopherol standards, Gary Anderson for assistance in rabbit phlebotomies, and Jorge Bedia for mass spectrometric analyses.

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