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DETERMINATION OF α-TOCOPHEROL IN RAT LIVER BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY UTILIZING ULTRAVIOLET DETECTION

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SUMMARY

A rapid, sensitive analytical procedure was developed to quantitate α-tocopherol in rat liver. Liver is homogenized in acetone, fractionated by reversed-phase high-performance liquid chromatography and α-tocopherol quantitated by monitoring the column effluent at 280 nm. A single analysis requires 18 min. The method is linear from 1.0 to greater than 30.0 μg α-tocopherol per g of liver (wet weight). The average relative standard deviation of the slope of the standard curve is 2.3% and the single-day coefficient of variation is 6.3%. The use of an acetone extraction, reversed-phase column chromatography and quantitation of α-tocopherol at 280 nm results in a sensitive and reproducible system for the determination of α-tocopherol concentrations in rat liver. Preliminary studies have shown the assay to be useful for investigation of the effects of age and diet on hepatic α-tocopherol concentration.

INTRODUCTION

The biological function of vitamin E (α-tocopherol) as a lipid antioxidant has been recognized for over 40 years. As a free radical scavenger within membrane phospholipids, vitamin E inhibits the destructive peroxidation of polyunsaturated lipids, which are vital components of cellular and organelle membranes [1]. Prior work from our laboratory has demonstrated evidence of hepatic lipid peroxidation in rats with chronic dietary iron overload [2, 3]. In order to determine the possible role of altered vitamin E metabolism in iron overload, it became necessary to develop a rapid, reliable method for measuring hepatic α-tocopherol concentrations. Previously described high-performance liquid chromatographic (HPLC) methods for measuring tissue α-tocopherol concentrations required the use of a fluorescence detector [4—8] or com-

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plicated extraction procedures [9]. Because of these disadvantages we sought to develop a simplified extraction procedure which would allow determination of hepatic α-tocopherol concentrations using an ultraviolet (UV) detector.

EXPERIMENTAL

Apparatus

Analyses were performed using a Varian Model 5020 high-performance liquid chromatograph interfaced with a UV-100 variable-wavelength detector (Varian, Palo Alto, CA, U.S.A.) and a 3390A recording integrator (Hewlett-Packard, Cleveland, OH, U.S.A.). Chromatography was performed using a 30 cm × 4 mm Varian Micropak MCH-10 octyldecylsilane C_{18} column preceded by a 4 cm × 4 mm guard column packed with 10-μm reversed-phase packing (Vydac SC®, Varian). A heater block maintained the column at 30°C. The flow-rate was 2.5 ml/min which developed a pressure of 100 bar. The detector wavelength was set at 280 nm with a 5-nm bandwidth.

Chemicals and reagents

The acetone, methylene chloride and methanol used as elution solvents were glass-distilled (Burdick & Jackson Labs., Muskegon, MI, U.S.A.). All other solvents and reagents were ACS-reagent grade. The *d*-α-tocopherol and the internal standard (I.S.), *d*-α-tocopheryl acetate (Fig. 1), were purchased from Eastman Organic Chemicals (Rochester, NY, U.S.A.).

Extraction procedure

A stock solution of the I.S., 50 μg/ml in ethanol, was prepared and kept in a brown bottle at 4°C to minimize oxidation and evaporation. Only glass labware was used. Liver tissue (250 mg) was homogenized in 3 ml of acetone using a PTFE-glass Potter-Elvehjem homogenizer. The homogenate was decanted into 100 × 16 mm borosilicate tubes. The homogenizing flask was washed once with 1 ml of acetone and the wash added to the homogenate. To each tube 250 μl of the I.S. stock solution were added. The samples were vortexed for 2 min and centrifuged at 1200 *g* for 15 min at 4°C. The acetone supernatant was removed from each tube with a pipette, placed in a 100 × 16 mm

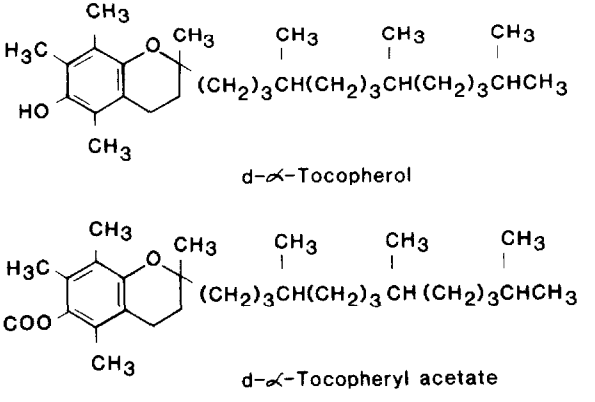

Fig. 1. Chemical structures of α-tocopherol and the internal standard, α-tocopheryl acetate.

borosilicate tube and evaporated under nitrogen in a 60°C water bath. The residue was redissolved in 150—500 μl methanol, filtered through a 0.45-μm alpha-metricel Gelman filter in a Swinney filter holder (Gelman Sciences, Ann Arbor, MI, U.S.A.) and 100 μl were recovered to load a 100-μl injector loop.

Mobile phase

The mobile phase in reservoir A was methanol—water (95:5). The mobile phase in reservoir B was methylene chloride.

Elution solvent gradient program

The isolation of α-tocopherol was accomplished isocratically with methanol—water (reservoir A). After analysis the column was briefly washed with methylene chloride (reservoir B) in the following fashion: for the first 12 min of the sample run the elution solvent was 100% reservoir A; during the 13th min the composition of the elution solvent was switched with a linear gradient to 100% reservoir B; after pumping 100% reservoir B during the 14th min the composition of the elution solvent was returned with a linear gradient to 100% reservoir A over a 1-min period.

Calculations

Peak areas of standards and unknowns were normalized by dividing the α-tocopherol peak area by the I.S. peak area for each sample. Standard curves were obtained by adding a constant amount of I.S. and varying amounts of *d*-α-tocopherol (ranging from 0.00 to 7.50 μg) to homogenized 250-mg liver samples from the same liver prior to analysis. To determine the α-tocopherol concentration of an unknown, the α-tocopherol-to-I.S. ratio was obtained and the quantity of α-tocopherol then calculated by interpolation from the standard curve.

Animals and diets

Male Sprague—Dawley rats were purchased from Zivic-Miller Labs. (Allison Park, PA, U.S.A.). Rats were housed in polyethylene cages with stainless-steel wire tops and were allowed diet and water ad libitum. All animals were sacrificed by decapitation after an overnight fast. Livers were quickly excised, rinsed, blotted dry, weighed and stored frozen at −70°C until analysis. The effect of age on hepatic α-tocopherol concentration was determined by sacrificing rats of three weeks (weanling), six weeks, six months and thirteen months of age that were fed a chow diet which contained 60 mg of α-tocopheryl acetate per kg diet (Purina Lab Chow, Ralston Purina, Chicago, IL, U.S.A.). To determine the effect of a vitamin-E-deficient diet on hepatic α-tocopherol concentration, groups of three- and six-week-old rats were fed either the chow diet or a vitamin-E-deficient diet which contained no measurable α-tocopheryl acetate per kg diet (Tocopherol-deficient diet, ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.) and sacrificed at weekly intervals for three weeks. The major biologically active form of vitamin E is α-tocopherol, which is derived from α-tocopheryl acetate in the diet.

Statistical methods

Data were analyzed using BMDP computer programs [10]. Analysis of variance was used to test for statistically significant differences among different-aged rats fed either a vitamin-E-deficient diet or a normal chow diet for one, two or three weeks. The hypothesis of equality of means was rejected at the 0.05-level of significance. To assure an overall significance level of 0.05, the Bonferoni multiple-comparisons procedure was applied to test for pairwise differences between the means of individual groups [11]. Data are presented as mean ± standard error of the mean (S.E.M.).

RESULTS

Extraction

The efficiency of extracting liver α-tocopherol in acetone versus a solution of ethanol, hexane and water containing 10% ascorbic acid (2:1:1), as described by Vatassery and Hagen [4], was determined. The liver α-tocopherol concentrations were 31% greater with the acetone extraction procedure (n=3). Repeated acetone extractions failed to increase the yield of α-tocopherol.

High-performance liquid chromatography

The use of an octyldecylsilane C_{18} column and an elution solvent consisting of methanol—water (95:5) permitted the separation of purified α-tocopherol and α-tocopheryl acetate (Fig. 2A). The absence of interference from co-eluting acetone-extractable hepatic compounds is demonstrated in Fig. 2B. Chromatograms used for quantitation of α-tocopherol in a normal rat liver and a liver from a rat fed a vitamin-E-deficient diet are shown in Fig. 2C and D, respectively. The capacity factors (k') of α-tocopherol and α-tocopheryl acetate were 6.8 and 9.4, respectively. When the column was not washed with methylene chloride after each sample run, three large unidentified peaks eluted at k' = 27, 34.2 and 41, respectively, and the total sample time was 42 min. With the methylene chloride wash all unwanted peaks eluted rapidly and the total sample time was reduced to 18 min.

The same column has been in use for twelve months without changes in pres-

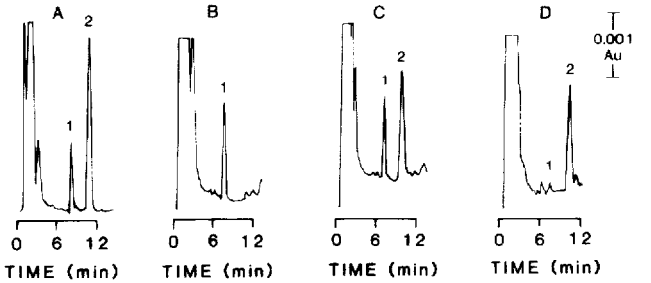

Fig. 2. Representative chromatograms of α-tocopherol and α-tocopheryl acetate. (A) Extracted α-tocopherol (2.5 μg) and α-tocopheryl acetate (12.5 μg); (B) extracted normal rat liver (α-tocopherol concentration 17.4 μg/g); (C) extracted normal liver (same liver as shown in B) with 12.5 μg of α-tocopheryl acetate added; (D) extracted liver from a vitamin-E-deficient rat with 12.5 μg of α-tocopheryl acetate added (hepatic α-tocopherol concentration 5.35 μg/g). Peaks: 1 = α-tocopherol; 2 = α-tocopheryl acetate.

sure requirements or resolution. The column was maintained at 30°C in order to avoid minor fluctuations in retention due to changes in ambient air temperature. In order to expand the applicability of the method to fixed-wavelength detectors, a wavelength of 280 nm was chosen for monitoring the column effluent rather than 292 nm, the absorption maximum for α-tocopherol.

Methodologic verification

In order to determine the stability and sensitivity of the assay, 0.50, 1.00, 2.50, 3.75, 5.00 and 7.50 μg of α-tocopherol were added to 250-mg liver samples for validation studies. These six standards plus a liver blank were run on each analysis day for a total of four days over one month. A standard curve was constructed each day as described in Experimental. The straight line obtained was described by the equation $y = 0.147x + 0.0087$, where the average regression coefficient had a value of 0.997 and the average relative standard deviation of the slope was 2.3%. Since the addition of 7.5 μg of α-tocopherol to a 250-mg liver sample was equivalent to increasing the α-tocopherol concentration of the tissue by 30 μg/g, linearity of the method was thus demonstrated to liver samples of 30 μg/g or more. Linearity of the method at low α-tocopherol concentrations was examined by constructing standard curves using livers from three rats fed a vitamin-E-deficient diet (hepatic α-tocopherol concentrations 0.9, 1.1 and 1.5 μg/g). Using a liver blank plus samples to which 0.50, 2.50 and 5.00 μg of α-tocopherol had been added, the straight lines obtained from these standard curves all had regression coefficients of 0.999. The within-day coefficient of variation (C.V.) for hepatic α-tocopherol concentrations from a rat fed a chow diet (α-tocopherol concentration 25.2 μg/g) was 6.3% (n=12). For the same liver assayed ten consecutive analysis days the C.V. was 7%. The C.V. between lobes of the liver was 4.6% (n=5). For liver from a rat fed a vitamin-E-deficient diet (hepatic α-tocopherol concentration 1.7 μg/g) the C.V. was 9.3% (n=10).

Animal studies

In order to determine the effect of age on hepatic α-tocopherol concentration, four groups of rats fed a standard chow diet were sacrificed at different ages and hepatic α-tocopherol concentrations determined. These results are shown in Table I. There is a progressive increase in the hepatic α-tocopherol concentration with increasing age. To study the effect of a vitamin-E-deficient

TABLE I

HEPATIC α-TOCOPHEROL CONCENTRATION IN RATS OF DIFFERENT AGES FED NORMAL CHOW DIETS (n=6)

Age (weeks)	Body weight range (g)	Hepatic α-tocopherol concentration (mean ± S.E.M.) (μg/g of liver)
3	60—90	11.1 ± 0.4
6	200—225	14.4 ± 0.8
26	650—1000	18.9 ± 1.4
56	800—1050	23.0 ± 0.6

diet on hepatic α-tocopherol concentration, three- and six-week-old rats were fed a tocopherol-deficient diet for three weeks and compared with controls. The results of these experiments are shown in Fig. 3. The control rats maintained normal α-tocopherol concentrations over the course of the three weeks. In both age groups there was a marked decrease in hepatic α-tocopherol concentrations after one week on the tocopherol-deficient diet ($P < 0.01$ compared to control) with a slight further decrease at two and three weeks ($P < 0.05$, ANOVA, grouping variable = time.)

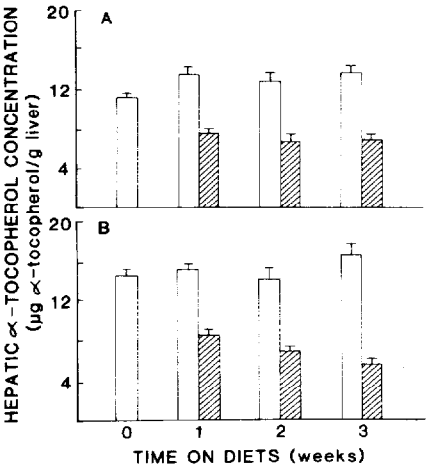

Fig. 3. Effect of tocopherol-deficient diet on hepatic α-tocopherol concentration. Male Sprague–Dawley rats of three (A) and six (B) weeks of age were fed a chow diet (open bars) or a tocopherol-deficient diet (hatched bars) for three weeks. Each bar represents the mean ± S.E.M. for that group. There were six rats in each dietary group at each time point. In both age groups (A and B) there was a marked decrease in hepatic α-tocopherol concentration after one week on the tocopherol-deficient diet ($P < 0.01$) with a slight further decrease at two and three weeks ($P < 0.05$).

DISCUSSION

Although other HPLC methods for the determination of α-tocopherol concentrations in tissues have been reported, they have certain disadvantages when compared to the method which we have described here. Several groups [4–8] have described methods requiring fluorescence detectors in order to avoid interference from unidentified peaks. In contrast, our method utilizes UV detection which provides two major advantages. First, an internal standard can be employed to improve reliability. Except for the method of Westerberg et al. [7], who describes the quantitation of brain tocopherols using 5,7-dimethyltocol as an internal standard, an internal standard was not used with the tissue methods utilizing fluorescence detection because of the absence of fluorescence by α-tocopheryl acetate. Secondly, high-performance liquid chromatographs are more commonly interfaced with UV detectors than with fluorescence detectors and hence our method should allow hepatic α-tocopherol determinations to be performed in a large number of laboratories not equipped

with fluorescence detectors. Hung et al. [9] have also described a method utilizing UV detection. However, their method requires a complicated sample preparation and does not utilize an internal standard. The method we describe has been used in our laboratory for over twelve months and is straightforward, rapid, accurate and stable. The use of gradient elution programming to purge the column allows for more rapid analyses but is not necessary if the liquid chromatograph is not programmable.

The hepatic α-tocopherol concentrations observed in our studies are similar to those reported by Bieri [12] who analyzed rat liver α-tocopherol concentrations by thin-layer chromatography. The gradual increase in hepatic α-tocopherol concentration over time and the prompt decrease while receiving a vitamin-E-deficient diet were also noted by Bieri [12]. Hepatic α-tocopherol concentrations analyzed by the HPLC method devised by Zaspel and Csallany [6] were somewhat higher than those in the studies reported here or by Bieri [12]. The differences between our results and those of Zaspel and Csallany may be related to differences in availability of dietary α-tocopheryl acetate (chow diet versus semi-purified diet).

In summary, this method is a rapid, accurate alternative to those previously described. It offers simple sample clean-up, UV detection and an internal standard for added reliability. This should facilitate investigations of vitamin E metabolism which require the determination of hepatic α-tocopherol concentrations.

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