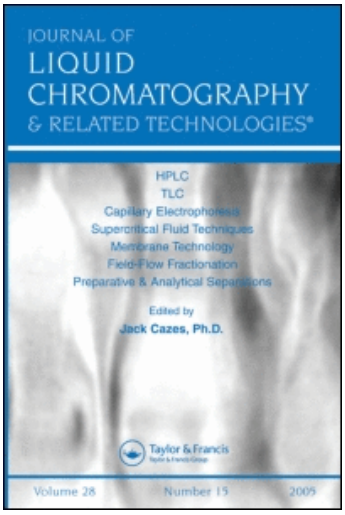

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Assay of Brain Tocopherols Using High Performance Liquid Chromatography

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ASSAY OF BRAIN TOCOPHEROLS USING HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The four natural tocopherols were separated using a μ-Bondapak-NH_2 column. For the analysis of brain tocopherols 5,7-dimethyltocol was used as an internal standard. α-Tocopherylquinone and other tocopherols than α-tocopherol were not detected. Rat cerebral cortex and cerebellum contained 19.3 μmol/g and 11.2 μmol/g of α-tocopherol, respectively. A chromatographic system with a reversed-phase column proved less suitable.

INTRODUCTION

Enhanced lipid peroxidation has been implicated as a mechanism in various types of tissue damage, *e.g.* during incomplete ischemia in brain (1). The peroxidative damage of brain membrane lipids during *in vitro* incubation has been characterized in this laboratory (2, 3). So far the role of tocopherol in these processes remains unclear. To some extent, this is due to the lack of rapid and sensitive analytical methods for studies of brain tocopherol levels and metabolism. Methods involving thin-layer chromatography and gas chromatography are often laborious, and tocopherols are very susceptible to oxidation if several steps of purification are involved. High performance liquid chromatography (HPLC) is

better suited to tocopherol analysis, especially since it employs specific detection methods and the exposure of tocopherols to air can be minimized.

Methods for the separation of α-tocopherol from serum or animal feed by reversed phase HPLC have previously been reported (4, 5, 6). Chromatography of other tocopherols have not been described except for the comigration of β- and γ-tocopherol (4). With Corasil columns instead, all four tocopherols have been resolved (7, 8, 9) and also the four tocotrienols (8). The objective of the present investigation was to compare different HPLC procedures for the separation of brain tocopherols and their metabolites such as α-tocopherylquinone.

EXPERIMENTAL

Apparatus

Waters Associates high performance liquid chromatography system (Model U6K injector, 6000A solvent delivery system, 440 UV absorbance detector) was used. The absorbance was measured at 254 nm and 280 nm.

Column Packing Materials

The packing material was Nucleosil 10 C18 (Macherey-Nagel & Co, Düren, West Germany, art. nr. 71215) with a mean particle diameter of 10 μm. A prepacked bonded amine phase column, with a mean particle size of 10 μm, μ-Bondapak-NH_2 (84040 Water associates, Milford, USA) was also used.

Column Tubings and Fittings

The Nucleosil column consisted of 6.4 mm O.D. x 200 mm length of 316 stainless steel tubing. The internal diameter was 5.0 mm. It was equipped with Parker-Hannifin compression fittings. Thin stainless steel mesh discs were placed at both ends of the column (part no. 206, hetp, Sutton, England). The μ-Bondapak-NH_2 column had the dimensions 300 x 4 mm I.D. in stainless steel. The loop

injector and detector were connected to the column via 1/16" O.D. (0.23 mm I.D.) stainless steel tubing.

Column Packing Technique

The column was packed according to the upward slurry packing technique (10). The Nucleosil 10 C18 material was slurried in chloroform and packed with acetone.

Chemicals

Solvents. n-Hexane, HPLC-grade, was obtained from Rathburn Chemicals (Walkersbrum, Peeblesshire, Scotland), methanol, p.a., from May & Baker (Dagenham, England) and ethanol, spectrographic grade, from Svensk Sprit AB (Sweden). Glass-distilled water was used.

Tocopherols. α-Tocopherol was obtained from Merck (Darmstadt, West Germany), 5,7-dimethyltocol from Koch-Light (Colnbrook, UK) and other tocopherols and α-tocopherolacetate from Hoffman-LaRoche (Basel, Switzerland). α-Tocopherylquinone was prepared from α-tocopherol according to Nair and Machiz (11).

Reagents. L(+)-Ascorbic acid, p.a., was from Merck (Darmstadt, West Germany) and tetra-n-butylammonium-hydroxide from BDH Chemicals Ltd (Poole, England).

Procedure

Animals. Male Wistar rats were fed a commercial pellet diet (Astra-Ewos, Sweden) containing 40 mg vitamin E/kg according to the manufacturer. Rats weighing 275-350 g were decapitated into liquid nitrogen. The brains were chiselled out during intermittent irrigation with liquid nitrogen and stored at -80°C until extraction.

Extraction procedures. A modification of a published procedure (12) was used. About 100 mg of the brain sample was put into a 10 ml homogenizer tube with a mixture of 1 ml 10 % aqueous solution of ascorbic acid, 1 ml ethanol and 2 ml hexane. 5,7-Dimethyltocol

was added as an internal standard (2.27 μg/sample). The mixture was homogenized for 3 min with a knife homogenizer (MSE 431) at the highest speed. The solution was transferred to a 15 ml centrifuge tube and was centrifuged at +4°C for 10 min at 10 000 rpm. The hexane phase was transferred to a test tube and was kept on ice until analyzed. Alternatively, the sample could be stored under nitrogen at -20°C for at least two weeks without loss of α-tocopherol. Fifty μl of the sample was injected into the chromatograph when the straight phase column was used. When the reversed phase column was used the extract was taken to dryness under nitrogen and the residue was dissolved in methanol.

Chromatographic conditions. All chromatographic experiments were performed at ambient temperature (approx. 20°C). The nonpolar C18-column was used with methanol/water, 98:2, as mobile phase. In some experiments tetra-n-butylammoniumhydroxide (0.25 mM, pH 7.8) was added to the mobile phase. The NH_2-column was used with n-hexane containing 0.8 % ethanol as mobile phase. The flow in the reversed phase system was 2.0 ml/min and in the straight phase system 1.0 ml/min.

RESULTS AND DISCUSSION

The Reversed Phase System (Nucleosil 10 C18)

In this system separation between α-tocopherol, β- + γ-tocopherol and δ-tocopherol was obtained (Fig. 1) in agreement with previous work (4, 6). The separation was improved if tetra-n-butylammoniumhydroxide was added to the methanol/water mobile phase, but changes in the methanol/water proportions or in the amount of quartenary amine did not give separation of β- and γ-tocopherol.

Another disadvantage with this system was that UV-absorbing substances in brain extracts migrated close to α-tocopherol. This probably explained the higher α-tocopherol content measured for rat brain using a nonpolar C18-column compared to the results obtained using a polar NH_2-column (Table 1). Furthermore, the

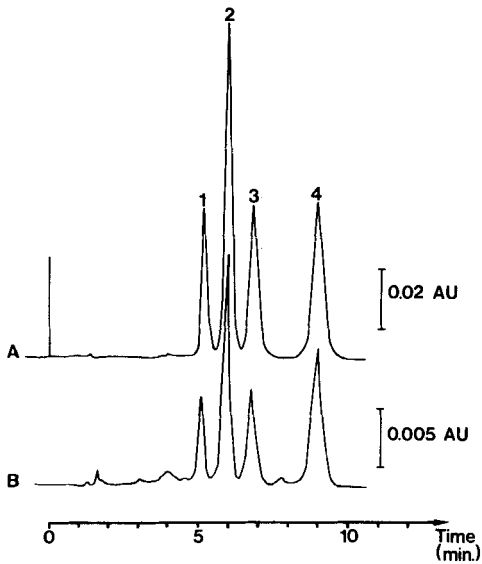

FIGURE 1. Separation of tocopherols using the reversed phase system. Nucleosil 10 C18 with methanol:water (98:2) as mobile phase. The flow rate was 2.0 ml/min. A is the absorption curve at 280 nm and B is the curve at 254 nm for the same sample. The peaks are: 1, δ-tocopherol; 2, β- + γ-tocopherol; 3, α-tocopherol; 4, α-tocopherolacetate. AU, absorbance units.

TABLE 1

Concentration of α-Tocopherol in Rat Brain Determined by HPLC. Data are expressed as μg/g wet tissue and are means ± S.E.

	Straight phase system	Reversed phase system
Cerebral cortex	19.3 ± 0.9 (n=9)	23.2 ± 0.6 (n=3)
Cerebellum	11.2 ± 0.3 (n=4)	-

redissolving of the brain lipids in the mobile phase prior to chromatography was incomplete. This chromatographic system is therefore not recommended.

The Straight Phase System (μ-Bondapak-NH_2)

Apart from reversed phase partition systems, HPLC of tocopherols has mainly been performed with silica columns *e.g.* Corasil. Chemically bonded polar phases have not been much used. Therefore, a column with a chemically bonded primary amine was tried (Fig. 2). All four tocopherols were resolved and in addition 5,7-dimethyltocol and α-tocopherylquinone could be separated from α-tocopherol. The degree of separation of different tocopherols obtained with μ-Bondapak-NH_2 (Fig. 2) was equal or superior to that previously reported for Corasil (7-9). α-Tocotrienol was, however, incomplete-

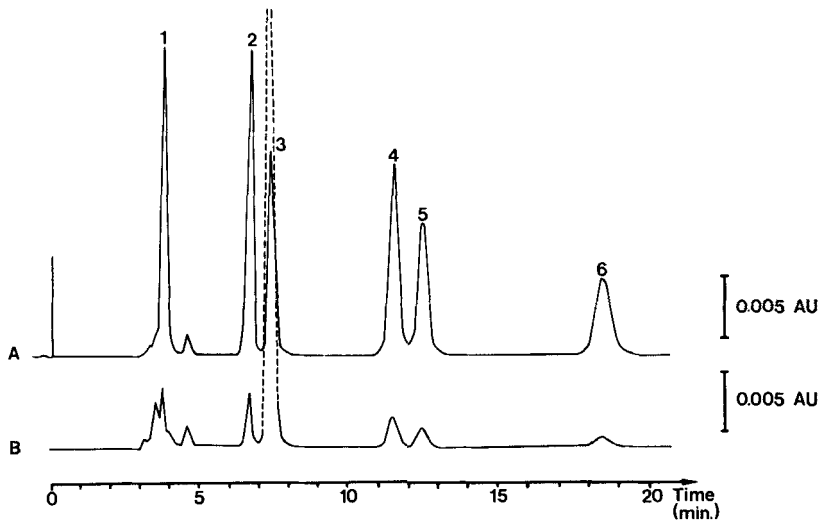

FIGURE 2. Separation of tocopherols using the straight phase system, μ-Bondapak-NH_2 with hexane : ethanol (99.2:0.8) was mobile phase. The flow rate was 1.0 ml/min. A is the absorption curve at 280 nm and B is the curve at 254 nm for the same sample. The peaks are: 1, α-tocopherolacetate; 2, α-tocopherol; 3, α-tocopherylquinone; 4, β-tocopherol; 5, γ-tocopherol; 6, δ-tocopherol.

ly resolved from α-tocopherol. The retention time of 5,7-dimethyltocol was close to that of α-tocopherol. This indicates that the number of methyl groups adjacent to the 6-hydroxyl group is important for the separation.

The effect of varying the proportion of ethanol in the mobile phase was studied (Fig. 3). At lower ethanol concentrations 5,7-dimethyltocol and α-tocopherylquinone were best resolved, but

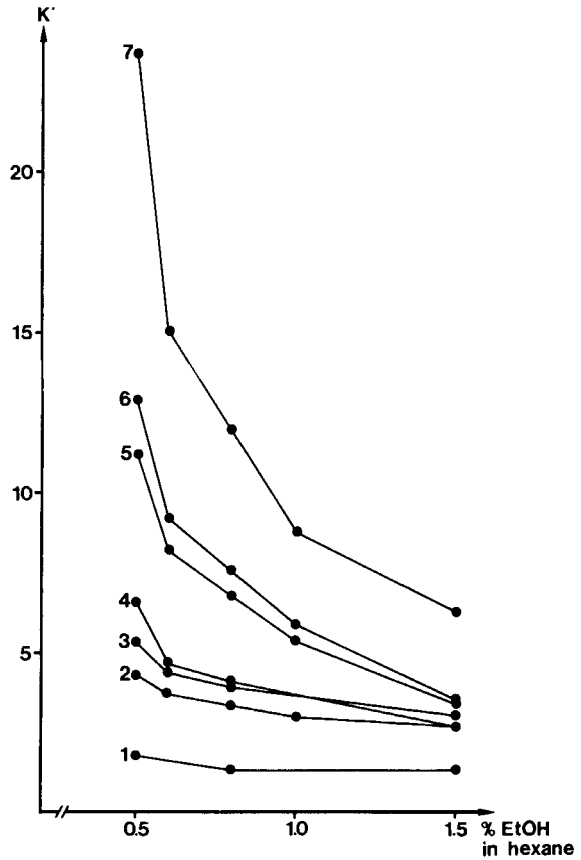

FIGURE 3. The capacity factor k' for tocopherols as a function of the amount of ethanol in the mobile phase in the straight phase system. 1, α-Tocopherolacetate; 2, α-tocopherol; 3, 5,7-dimethyltocol; 4, α-tocopherylquinone; 5, β-tocopherol; 6, γ-tocopherol; 7, δ-tocopherol.

under these conditions a disturbing peak broadening of α-tocopherol occurred, and therefore hexane:ethanol (99.2:0.8) was routinely used as mobile phase. A typical analysis of an extract from rat cerebral cortex in this system is shown in Fig. 4. Other tocopherols than α-tocopherol were not detected.

Quantitative Analysis

For the determination of α-tocopherol in brain, 5,7-dimethyltocol or α-tocopherolacetate were added as internal standards during the extraction. The linearity of the analysis was checked by chromatography of equal volumes of reference solutions containing identical amounts of 5,7-dimethyltocol and different amounts

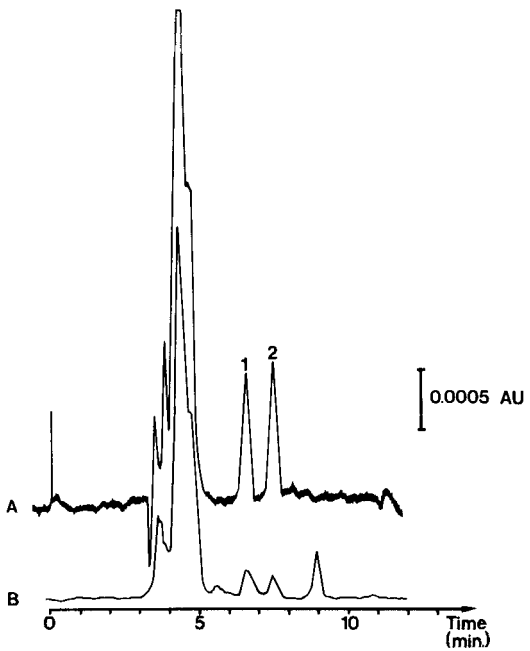

FIGURE 4. A chromatogram of an extract from rat cerebral cortex. For conditions see Fig. 2. The peaks are: 1, α-tocopherol; 2, 5,7-Dimethyltocol (internal standard).

of α-tocopherol (Fig. 5). Peaks were quantitated by peak height measurement and the standard curve was used for the quantitation of tocopherol in brain extracts. A similar curve was obtained using α-tocopherolacetate as internal standard. 5,7-Dimethyltocol was the most suitable internal standard. It is chemically similar to, and migrates close to α-tocopherol. However, it is difficult to acquire, and it is badly resolved from α-tocopherylquinone. Since UV-absorbing substances in brain extracts interfered with the quantitation of α-tocopherolacetate, this substance could not be used as internal standard. However, it may be a convenient internal standard if a fluorescence-detector is used.

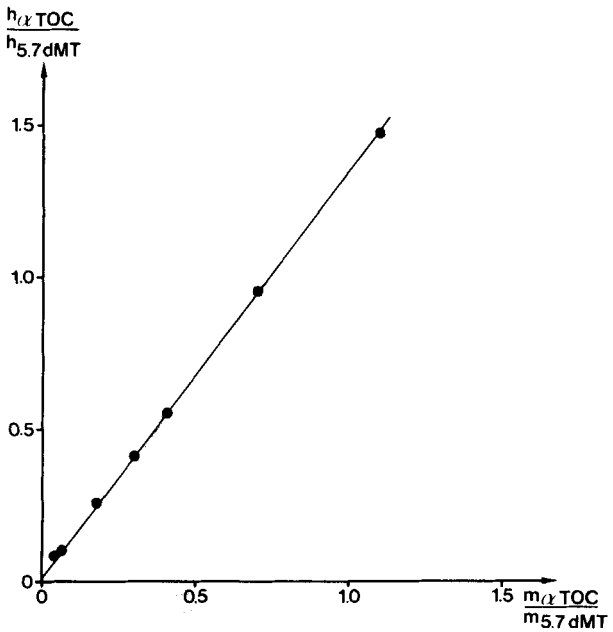

FIGURE 5. Standard curve used for quantitation of α-tocopherol with 5,7-dimethyltocol as internal standard. The reference solutions contained 2.27 μg 5,7-dimethyltocol and 0.13-2.50 μg α-tocopherol per ml of hexane. For chromatographic conditions see Fig. 2. Vertical axis, ratio of peak heights; horizontal axis, mass ratios.

Accuracy

The identity of α-tocopherol in brain extracts was established as follows. The k'-value of standard α-tocopherol was equal to that in brain extracts. α-Tocopherol added to brain extracts comigrated with the endogenous α-tocopherol and it was also quantitatively recovered (Table 2). The absorption of the eluent was routinely measured at both 280 and 254 nm. The ratio A_{280}/A_{254} was 5.11 ± 0.06 (mean ± S.E., n=8) for standard α-tocopherol and 5.12 ± 0.18 (n=14) for brain α-tocopherol.

Brain extracts were routinely analyzed twice and the coefficient of variance for duplicate analysis of α-tocopherol was 2.3 % (n=18). When duplicate samples of cortex from the same brain were analyzed, the coefficient of variance was 10.7 % (n=10). One sample, analyzed six times, had a ratio of α-tocopherol/5,7-dimethyltocol of 1.19 ± 0.006 (mean ± S.E.).

Brain Tocopherol Concentration

Rat cerebral cortex and cerebellum were analyzed (Table 1). As mentioned above analysis in the reverse phase system gave somewhat higher values. Cerebellum contained less α-tocopherol than cerebral cortex, which agrees with the recent report of Vatassery and Younoszai (13). Our values are higher, which proba-

TABLE 2

Recovery of α-Tocopherol Added to Extraction Mixtures in the Presence or Absence of Brain Tissue
Data are expressed as mean ± S.E.

Expt	Brain present	Amount of added α-tocopherol (μg)	Increment in α-tocopherol amount (μg)
I	+	1.17	1.18 ± 0.02 (n=4)
II	-	2.34	2.33 ± 0.05 (n=4)

bly is due to differences in rat diets. This point remains to be elucidated since data on dietary vitamin E was not given in the cited report (13).

In studies on α-tocopherol metabolism it is necessary to detect different metabolites. α-Tocopherylquinone could be separated from α-tocopherol in the straight phase system and some other oxidation products, probably dimers, could also be resolved.

α-Tocopherylquinone was badly resolved from 5,7-dimethyltocol but occurrence of α-tocopherylquinone in brain extracts would easily have been detected since the A_{280}/A_{254} ratio differed between the two compounds. 5,7-Dimethyltocol had a A_{280}/A_{254} ratio of 6.5 while the ratio for α-tocopherylquinone was 0.3. So far we have not detected any oxidation products in brain extracts.

It is possible that the NH_2-groups on the column could form stable Shiffs bases with the carbonyl group in the quinone, giving a false low value for α-tocopherylquinone in the brain extracts. In order to investigate that, a column with a bonded nitrile group (Nucleosil, 5 CN, Macherey-Nagel & Co., Düren, West Germany, art. nr. 71216) was used. There were no signs of a quinone peak in the brain extracts with this column either.

These data indicate that the straight phase system (μ-Bondapak-NH_2) was superior to the reversed phase system for the separation of tocopherol and related substances. Another advantage of the former system is that the extract can be injected onto the column directly without evaporation.

In summary, this report describes a rapid, sensitive technique for the analysis of brain tocopherols. The main advantages compared to previous studies are the rapid freezing of tissue in liquid nitrogen, minimizing postmortal changes, the use of different internal standards, and the high chromatographic resolution.

ACKNOWLEDGEMENTS

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