Notes

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High Performance Liquid Chromatography of Tocopherols and Their Model Compounds[1)]

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High performance liquid chromatography was employed to rapidly separate tocopherols and their model compounds, chroman-6-ols. Good separation of these compounds was obtained. By use of naphthalene as an internal standard, tocopherol was exactly quantitated in the range from 0.3 to 3.0 μg. Tocopherols and a model compound dispersed in a cell culture medium were analysed with ease.

Keywords——high performance liquid chromatography; tocopherol; vitamin E; chroman-6-ol; quantitative analysis; qualitative analysis; vitamin E-supplemented cell culture medium; naphthalene

In the sense of high resolution analysis at room temperature, high performance liquid chromatography (HPLC) may be most suitable for the determination of tocopherols (vitamin E), though various kinds of chromatography can be used to separate them.[3,4)] Niekerk has reported the separation of α-, β-, γ- and δ-tocopherols by HPLC on a Corasil II column.[5)] A β-tocopherol peak, however, seems to be close to a γ-tocopherol peak, and δ-tocopherol gives a broad peak under his conditions.

In this paper, HPLC data on a Micro Pak Si-10 column for the analysis of α-, β-, γ-, and δ-tocopherols and their model compounds, *i. e.* 2,2-dimethylchroman-6-ol, 2,2,5,7-, 2,2,5,8- and and 2,2,7,8-tetramethylchroman-6-ols, and 2,2,5,7,8-pentamethylchroman-6-ol are described. These compounds can be completely separated and quantitated by use of naphthalene as an internal standard. This method was employed for the determination of α- and γ-tocopherols and 2,2,5,7,8-pentamethylchroman-6-ol in a cell culture medium.

Experimental

Materials——dl-β-Tocopherol and chroman-6-ols were prepared in our laboratory according to the known method,[6)] and d-α-, d-γ- and d-δ-tocopherols were the gifts from Eisai Research Laboratories (Tokyo, Japan). Naphthalene, chloroform, diisopropyl ether, ethyl acetate, hexane and undecane were obtained from Wako Pure Chemical Industries (Osaka, Japan). Naphthalene was purified by recrystallization from ether. Diisopropyl ether was passed through a column of neutral aluminum oxide (E. Merck AG, Darmstadt, West Germany) to remove hydroquinone, a stabilizer of the ether. The other solvents were distilled before use.

Apparatus——Analyses were performed with a Varian 4200 liquid chromatograph (Varian Associates, Palo Alto, U.S.A.) equipped with a Varian Techtron 635 UV-visible spectrometer (Varian Techtron Pty. Ltd., Melbourne, Australia) and with a model 22 M recorder (Nippon Densi Kôgaku, Kyoto, Japan). The

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3) G. Katsui, "Seikagaku Zikken Kôza (Experiments in Biochemistry)," Vol. 13, eds. by Y. Nose, Y. Nishizuka, and Y. Kajiro, Tokyo Kagaku Dôjin, Tokyo, 1975, p. 627, 637.

4) R.H. Bunnell, *Lipids*, **6**, 245 (1971).

5) P.J.V. Niekerk, *Anal. Biochem.*, **52**, 533 (1973).

6) J.L.G. Nilsson, H. Sievertsson, and H. Selander, *Acta Chem. Scand.*, **22**, 3160 (1968).

column was a 50 cm × 2.2 mm i.d. stainless steel column packed with 10 μ particle size Micro Pak Si (Varian Associates). Specific HPLC conditions are given in the captions of Figures and Tables.

Quantitation of Tocopherols and Chromanols——The hexane solutions of tocopherols and chromanols containing naphthalene (an internal standard) were analysed, and the correlation of peak height to quantity of each compound was examined. The typical procedures are as follows: A series of the standard solutions of α-tocopherol were constituted at the following concentrations; 0.256, 0.639, 1.28, 1.92, 2.56 and 3.19 μg α-tocopherol in 5 μl hexane containing 2.84 μg naphthalene. The hexane solution (5 μl) was repeatedly injected on a Micro Pak Si column. Under the conditions (See Fig. 1), the peak height of 2.84 μg naphthalene was 3.05±0.34 graduations ($n=28$). The relation of peak height to quantity of α-tocopherol injected was linear. According to the usual internal standard method, a calibration curve was made (Fig. 1). The peak height ratio of α-tocopherol to naphthalene was plotted against the weight ratio of α-tocopherol to naphthalene. Each peak height ratio of α-tocopherol to naphthalene corresponding to the standard solutions was 0.20±0.02, 0.46±0.02, 0.88±0.03, 1.30±0.01, 1.72±0.04 and 2.13±0.03, respectively. Figure 1 represents the average values and the standard deviations for five injections. By using the calibration curve, a concentration of α-tocopherol (A: μg/μl) injected could be determined from the weight ratio of α-tocopherol to naphthalene (R) obtained graphically from the peak height ratio of α-tocopherol to naphthalene, that is, $A=R\times N$, where N is the concentration of naphthalene (μg/μl) added as an internal standard. For the quantitation of chromanols, the similar calibration curves were obtained by the same method as described above.

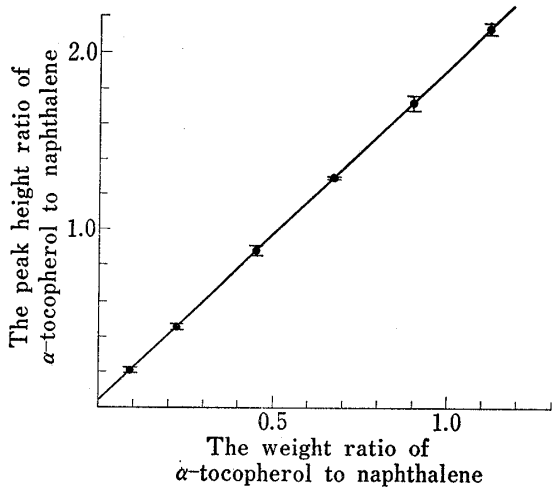

Fig. 1. The Calibrated Correlation of Peak Height to Quantity of α-Tocopherol

Each injection amount was estimated on the basis of the peak height of naphthalene which was added to α-tocopherol solution as an internal standard. Condition; Eluant: hexane–diisopropyl ether (8:2 v/v), Flow rate: 40 ml/hr at 600 psi., Temperature: ambient, Detector: full scale of OD 0.1 (damping) at 297 nm, Chart speed of a recorder: 0.5 cm/min.

Analytical Procedures for Cell Culture Media——Cell culture media were prepared in the following way: Tocopherol (1.14 mg) or 2,2,5,7,8-pentamethylchroman-6-ol in dimethyl sulfoxide (0.57 mg in 0.1 ml DMSO) was vigorously shaken with calf serum (4 ml, Microbiological Associates, Bethesda, U.S.A.). The mixture was added to Temin's modified Eagle medium (110 ml, Daigo Eiyô Kagaku, Osaka, Japan) in 0.3% Tryptose phosphate (Difco Laboratories, Detroit, U.S.A.) with penicillin G (500 u/ml, Meiji Seika Kaisha, Tokyo, Japan) and streptomycin (100 μg/ml, Kyôwa Hakkô Kôgyô, Tokyo, Japan). The medium was stored at 5° in the dark in an Erlenmeyer flask with a silicone rubber stopper. The upper part of the flask was covered with a piece of aluminum foil. An aliquot of the medium (4 ml each) was put in Lux plastic dishes (5.2 cm × 1.3 cm, Lux Scientific Corporation, Newbury Park, U.S.A.) and incubated at 37° under a 5% CO_2–95% air atmosphere. The medium (10 ml), ethanol (10 ml), and hexane (1 ml) were well shaken in a fractional funnel. The mixture was centrifuged at 0° at 3000 rpm for 3 min in a capped centrifuge tube. The water layer was discarded. An undecane solution (25 μl) of naphthalene (74.5 μg) was added to the hexane layer (0.5 ml). In the case of the chromanol-supplimented medium, the same procedure was employed except addition of ethanol. An aliquot (20 μl) of each hexane layer was analysed by HPLC under the same condition as described in Fig. 1.

Results and Discussion

Separation of Tocopherols and Their Model Compounds

Good separation of tocopherols and chromanols was obtained. The chromatograms illustrate the ability of a Micro Pak column to separate these compounds (Fig. 2 and 3). In terms of resolution and analysis time of tocopherols, the separation is a little more efficient than that reported by Niekerk[5] who employed a Corasil II column. Among the mobile phases of HPLC tested here, hexane–diisopropyl ether systems are the best for tocopherols and hexane–chloroform–ethyl acetate systems for chromanols. A Micro Pak CN or NH_2 column (Varian Associates) gave incomplete separation of β- and γ-tocopherol and of 2,2,5,8- and 2,2,7,8-tetramethylchroman-6-ols.

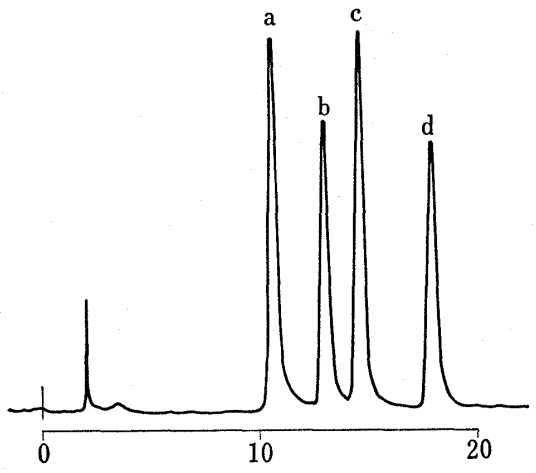

Fig. 2. Separation of Tocopherols

a: α-tocopherol, b: β-tocopherol, c: γ-tocopherol, d: δ-tocopherol. Condition: Eluant: solvent A=hexane, solvent B=diisopropyl ether, % of initial solvent B: 5.0, Slope of gradient (% of solvent B/min): 1.0, Flow rate: 40 ml/hr at 600 psi., Temperature: ambient, Detector: full scale of OD 1.0 at 297 nm, Chart speed of a recorder· 0.5 cm/min.

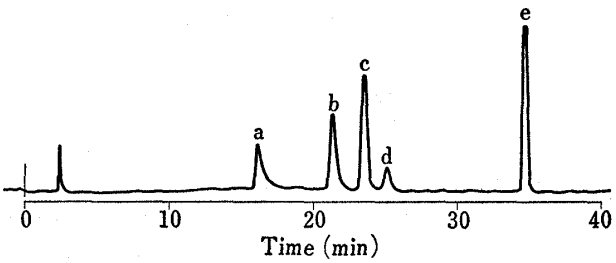

Fig. 3. Separation of Chromanols

a: 2,2,5,7,8-pentamethylchroman-6-ol, b: 2,2,5,7-tetramethylchroman-6-ol, c: 2,2,5,8-tetramethylchroman-6-ol, d: 2,2,7,8-tetramethylchroman-6-ol, e: 2,2-dimethylchroman-6-ol. Condition; Eluant: solvent A=hexane, solvent B=chloroform–ethyl acetate (8:2 v/v), % of initial solvent B: 5.0, Slope of gradient (% of solvent B/min): 0.5, Flow rate: 40 ml/hr at 600 psi., Temperature: ambient, Detector: full scale of OD 1.0 at 297 nm, Chart speeed of a recorder: 0.5 cm/min.

Quantitation of Tocopherols and Their Model Compounds

The relation of peak height to quantity of tocopherol injected has been found to be linear.[3,5] However, it is practically difficult to exactly know the injected amount of tocopherol on a column. The peak height of a constant injection volume of α-tocopherol solution (5 μl) was considerably fluctuated, *e.g.* 5.28±0.79 graduations for 2.56 μg α-tocopherol. Therefore, the injection amount was estimated on the basis of the peak height of naphthalene which was added to the tocopherol solution as an internal standard. On this procedure, the fluctuation of peak height of α-tocopherol was minimized and the linearity between the peak height and its quantity became much better (Fig. 1). Naphthalene was chosen as an internal standard from the following reasons; good separation from the tocopherols and the chromanols (*e.g.* the relative retention time under the conditions shown in Fig. 1: naphthalene 0.36, α-tocopherol 1.00, 2,2,5,7,8-pentamethylchroman-6-ol 1.43), a short retention time, a sharp peak without tailing, the sufficient optical density (log ε 2.57) at 297 nm[7] and easy availability for the pure and inexpensive preparation. Under the chromatographic conditions, tocopherols can be quantitated in the range from 0.3 to 3.0 μg and chromanols from 0.15 to 1.5 μg on a sample injection and as little as 30 ng of α-tocopherol can be detected. The most important thing for the precise quantitation is signal shape, *i.e.* sharpness and small tailing of peaks are of necessity. Gradient elution was not employed in the quantitative chromatography because of the aboidance of complex column condition. For the recovery of the initial column condition after an analysis with gradient elution, the initial solvent had to be flowed through the column for 20 min more. This procedure is very time-consuming. Thus, gradient elution is not suitable for continuous analysis of many samples although it offers increased resolution.

The Determination of α- and γ-Tocopherol and 2,2,5,7,8-Pentamethylchroman-6-ol in a Cell Culture Medium

It has been considered that tocopherols are important as antioxidants *in vivo*. Recently, the effect of tocopherol on cultured cells has been studied. For example, the extension of the

7) E. Clar, *Spectrochim. Acta*, **4**, 116 (1950).

Table I. The Stability of α- and γ-Tocopherols and 2,2,5,7,8-Pentamethylchroman-6-ol in a Cell Culture Medium

Exp. No.	Conditions for storage[a]			Recovery of the compounds from the medium (%)[b]		
	Time (days)	Temp. (°C)	Vessel	α-Tocopherol	γ-Tocopherol	2,2,5,7,8-Pentamethylchroman-6-ol
I	0	(r.t.)	Glass flasks	92.5	95.0	85.0
II	3	37	Plastic dishes	62.5	75.0	12.5
III	4	5	Glass flasks	86.5	91.0	85.0
IV	9	5	Glass flasks	70.0	85.0	50.0

a) Stored in the dark.
b) The compounds were analysed under the same conditions as described in Fig. 1.

lifespan of cultured human diploid cells with tocopherol was reported.[8] The stability of tocopherol in culture medium, however, has not been examined. Tocopherol is water-insoluble and it is difficult to obtain its stable and homogeneous suspension on a culture medium. We found that tocopherol (1 to 100 μM) could be homogeneously dispersed in a 3.5% calf serum medium when the tocopherol–serum mixture made by sharp shakes was added to the medium. For dispersion of 2,2,5,7,8-pentamethylchroman-6-ol, dimethyl sulfoxide was needed as a solubilizer. α- and γ-Tocopherols and the pentamethylchromanol dispersed in the medium were determined by HPLC. No peaks were observed on the chromatogram of the hexane extract of the control culture medium. As shown in Table I, more than 90% of α- and γ-tocopherols were retained in the medium in glass flasks at 5° for 4 days, while appreciable amounts of them were lost in plastic dishes at 37° for 3 days. Further, the chromanol decreased rapidly at 37°. Thus, on continuous administration of these compounds to cells, the medium should be changed at least every 3 days.

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8) L. Packer and J.R. Smith, *Proc. Natl. Acad. Sci. USA.*, **71**, 4763 (1974).