[Chem. Pharm. Bull. 29(2) 451—455 (1981)]

Electrochemical Detector for High-Performance Liquid Chromatography. V.[1] Application to Adsorption Chromatography

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(Received August 7, 1980)

Electrochemical detection was applied to high-performance liquid chromatography (adsorption chromatography). The column eluate was mixed with methanol–ethanol mixture containing $NaClO_4$ as a supporting electrolyte and analyzed with an electrochemical detector. We applied this method to the determination of tocopherol isomers (TP) in wheat germ oil and phylloquinone isomers (PK) in rat plasma. The limits of detection were 1 ng and 10 ng for TP and PK, respectively. The present method can be applied to the determination of small amounts of geometricalisomers or stereoisomers.

Keywords——high-performance liquid chromatography; electrochemical detector; adsorption chromatography; isomer; tocopherol; estradiol; phylloquinone; menaquinone

It is generally known that geometricalisomers or stereoisomers of some drugs, such as tocopherols (TP), phylloquinone (PK), and estradiol, display dissimilar biochemical and pharmacological effects. For example, *trans*-PK has true vitamin K activity, whereas the *cis* isomer has little, if any, activity. Therefore, it is important to separate and determine these isomers in pharmacological or metabolic studies.

A small quantity of these isomers has been separated by high-performance liquid chromatography (HPLC) with an adsorption chromatographic column and detected with an ultraviolet or fluorometric detector.[3,4] However, ultraviolet detection shows poor specificity, and fluorometric detection can only be used for fluorescent substances. As a new, sensitive and specific method, we describe here the application of an electochemical detector (ECD) to HPLC; its use has previously been limited to reversed phase chromatography.[5]

In adsorption chromatography, hydrophobic solvents are generally used as the mobile phase, in which the supporting electrolyte is scarcely soluble. In this paper, we studied the applicability of ECD detection to adsorption chromatography by mixing the column eluate with alcoholic electrolyte solution at the outlet of the column. We also applied this method to the determination of TP and PK isomers in biological materials.

Experimental

Apparatus——Figure 1 illustrates the flow diagram. The HPLC system used was a Yanagimoto LC-2000 system with a Yanagimoto VMD-101 detector. For chromatographic separation, a 25 cm × 2.1 mm I.D. column packed with Zorbax SIL (Du Pont Instrument) was used. The column eluate was mixed with the electrolyte solution in a 30 cm × 0.5 mm I.D. Teflon coil, and the electrolyte solution was pumped with a Mini Micro Pump (Kyowa Seimitsu, KHD-W-52). HPLC measurements were performed at room temperature.

Materials——α-, β-, γ-, and δ-tocopherol (α-, β-, γ-, δ-TP), phylloquinone (PK) and menaquinone-4 (MK-4) were obtained from Tama Biochemical Co., Ltd. and Eisai Co., Ltd. *trans*-PK, *cis*-PK, *trans*-MK-4 and *cis*-MK-4 were prepared by column chromatography (LiChroprep Si 60 prepacked column) with isopropyl ether–*n*-hexane mixture (1 : 99) as a mobile phase. Estradiol-17α and estradiol-17β were purchased from Sigma Chemical Co., Ltd. Tocol(TC) was synthesized according to the published method.[6] $NaClO_4 \cdot H_2O$ used as a supporting electrolyte, was purchased from E. Merck Co., Ltd. Its purity was above 99%. All other chemicals were of reagent grade.

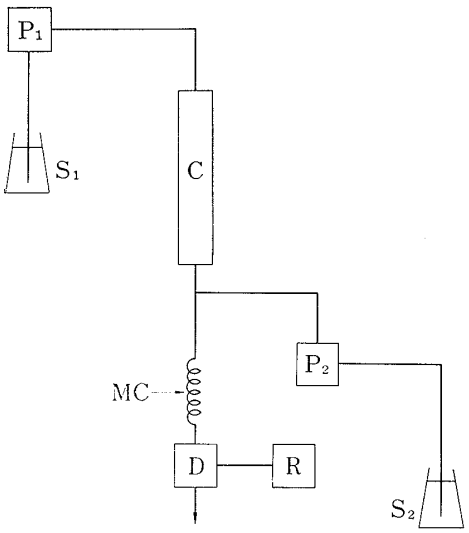

Fig. 1. Flow Diagram for High-Performance Liquid Chromatography

C: column, D: detector, R: recorder, P_1—P_2: pump, MC: mixing coil, S_1: mobile phase, S_2: electrolyte solution.

Assay Procedure——Tocopherols in Wheat Germ Oil: Wheat germ oil (150 mg) was dissolved in 20 ml of *n*-hexane containing 200 μg of TC as an internal standard. A 20 μl portion of the solution was injected into the HPLC column. An isopropyl ether–*n*-hexane mixture (12.5: 87.5) was used as the mobile phase, and the electrolyte solution was methanol–ethanol mixture (9: 1, v/v) containing 0.1 M $NaClO_4$. The flow rates of the mobile phase and the electrolyte solution were 0.5 ml/min and 1.0 ml/min, respectively. The applied potential was 0.7 V *vs.* Ag/AgCl. Phylloquinone in Rat Plasma: Adult male Sprague-Dawley rats, 220—300 g, were used. After a single oral administration of 10 mg/kg of *trans*-PK or *cis*-PK, blood was withdrawn with a heparinized syringe. Rat plasma (0.5 ml) was extracted with *n*-hexane according to the previous report.[7] The *n*-hexane extract (4 ml) was evaporated to dryness under a N_2 stream at 40°. The residue was dissolved in 100 μl of *n*-hexane and a 50 μl portion of the solution was injected into the HPLC column. As the mobile phase, *n*-hexane–isopropyl ether mixture (98.5: 1.5) was used. The electrolyte solution was methanol–ethanol–perchloric acid mixture (900: 100: 1, v/v) containing 0.1 M $NaClO_4$ and it was used after deaeration by N_2 gas bubbling. The flow rates of the mobile phase and the electrolyte solution were 0.5 ml/min and 1.5 ml/min, respectively. The applied potential was −0.3 V *vs.* Ag/AgCl.

Results and Discussion

Electrolyte Solution

The solvent for the electrolyte solution was selected on the basis of the conductivity and solubility of the supporting electrolyte, and the miscibility of the solution with the mobile phase. The best results were obtained with $NaClO_4$ as the electrolyte and methanol–ethanol

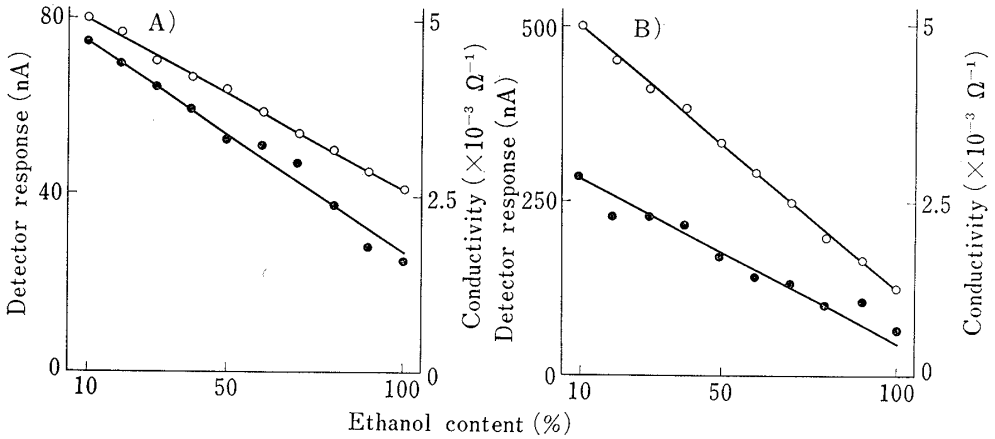

Fig. 2. Effect of Methanol-to-Ethanol Ratio of the Electrolyte Solution on the Detector Response and Conductivity

—○— detector response, —●— conductivity.

A) α-TP

Mobile phase: isopropyl ether–*n*-hexane (3: 7), 0.3 ml/min. Electrolyte solution: methanol–ethanol mixture containing 0.6% $NaClO_4 \cdot H_2O$, 1.25 ml/min. Injected amount: 370 ng. Applied potential: 0.7 V *vs.* Ag/AgCl.

B) *trans*-PK

Mobile phase: isopropul ether–*n*-hexane (1.35: 98.65), 0.5 ml/min. Electrolyte solution: methanol–ethanol mixture containing 0.6% $NaClO_4 \cdot H_2O$ and 0.1% $HClO_4$, 1.0 ml/min. Injected amount: 6 μg. Applied potential: −0.3 V *vs.* Ag/AgCl.

mixture as the solvent. Figure 2 illustrates the effect of the methanol to ethanol ratio of the electrolyte solution on the detector response and the conductivity after mixing the electrolyte solution with the mobile phase. Increasing ethanol ratio linearly decreases the detector response and the conductivity. These decreases are probably ascribable to the higher viscosity and lower dielectric constant of ethanol than of methanol. Although the theoretical basis remains unclear, measurement of the conductivity seemed to be useful for the selection of the solvent system. Figure 3 illustrates the plots of detector response against flow rate and $NaClO_4$ concentration of the electrolyte solution at a constant flow rate of the mobile phase. The detector response increased with increasing concentration of $NaClO_4$.

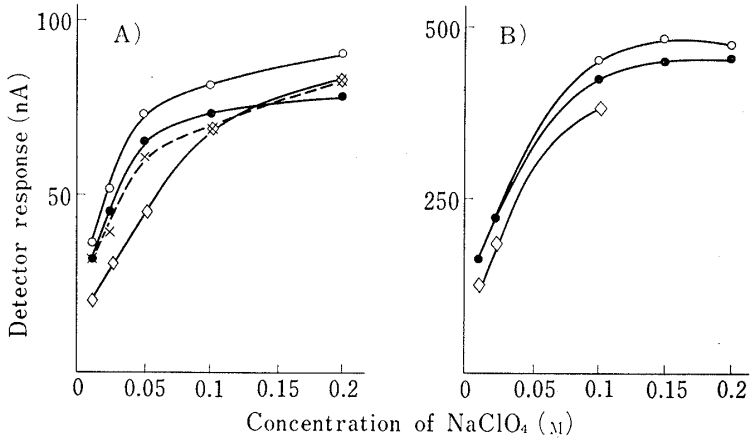

Fig. 3. Effect of $NaClO_4$ Concentration and Flow Rate of the Electrolyte Solution on the Detector Response

A) α-TP
Mobile phase: isopropyl ether–*n*-hexane (3: 7), 0.3 ml/min. Electrolyte: methanol–ethanol mixture (9: 1) containing $NaClO_4$. Flow rate of the electrolyte (ml/min): —◇— (0.25), --×-- (0.5), —○— (1.0), —●— (2.0). Injected amount: 370 ng. Applied potential: 0.7 V *vs.*Ag/AgCl.

B) *trans*-PK
Mobile phase: isopropyl ether–*n*-hexane (1.35: 98.65), 0.5 ml/min. Electrolyte solution: methanol–ethanol-$HClO_4$ mixture (900: 100: 1) containing $NaClO_4$. Flow rate of the electrolyte (ml/min): —◇— (1.0), —○— (1.5), —●— (2.0). Injected amount: 6 μg. Applied potential: —0.3 V *vs.*Ag/AgCl.

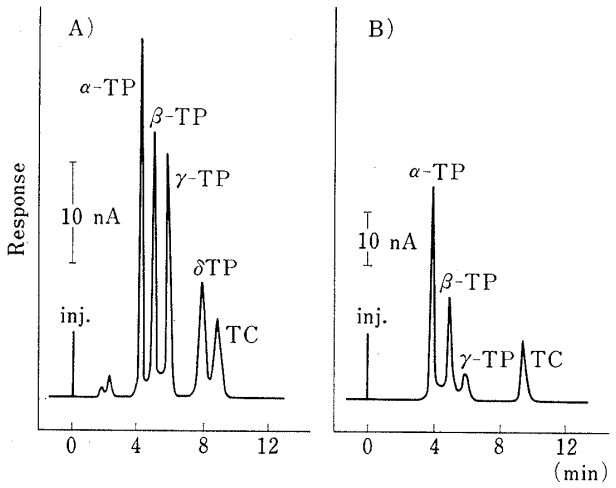

Fig. 4. Chromatogram of Tocopherols

Mobile phase: isopropyl ether–*n*-hexane (12.5: 87.5), 0.5 ml/min. Electrolyte: methanol–ethanol mixture (9: 1) containing 0.1 M $NaClO_4$, 1.0 ml/min. Applied potential: 0.7 V *vs.*Ag/AgCl.
A) Standard sample
Injected amount: 75 ng, in each case.
B) Wheat germ oil

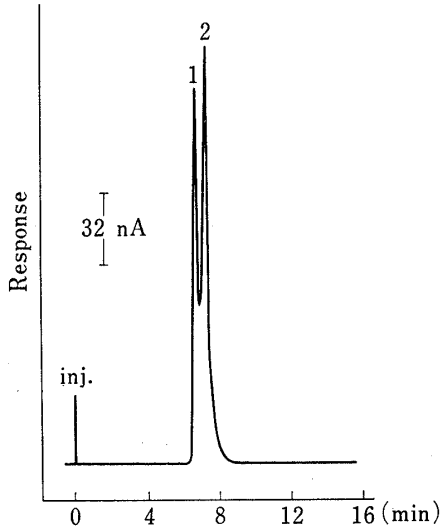

Fig. 5. Chromatogram of Estradiol

Mobile phase: CH_2Cl_2–methanol (98.5: 1.5), 0.5 ml/min. Electrolyte: methanol–ethanol (9: 1) containing 0.1 M $NaClO_4$, 1.5 ml/min. Injected amount (ng): 1. 17α-estradiol (250), 2. 17β-estradiol (350). Applied potential: 1.1 V *vs.* Ag/AgCl.

up to 0.1 M, and virtually reached a plateau above 0.1 M. The detector response was also enchanced with increasing flow rate of the electrolyte solution up to 1.0 ml/min but decreased at above 2.0 ml/min because of the decreased coulometric yield. A typical chromatogram of a mixture of TP isomers is shown in Figure 4-A. Figure 5 illustrates the applicability of this method to other solvent systems; in this case, estradiol-17α and estradiol-17β were separated by using CH_2Cl_2–methanol mixture (98.5: 1.5) as a moblie phase. A typical chromatogram for the mixture of PK and MK-4 is shown in Fig. 6-A.

Determination of TP in Wheat Germ Oil

TP isomers in wheat germ oil were determined by HPLC-ECD with TC as an internal standard. A chromatogram is shown in Fig. 4-B. The calibration curve of peak height ratio against ratio of α-TP to TC was linear from 1 ng to 100 ng injected. The limit of detection of α-TP was 1 ng injected, and the coefficient of variation of the peak height ratio was 3.0% for 2000 μg/g of α-TP. The contents of α-, β- and γ-TP in wheat germ oil were 1586, 769 and 100 μg/g, respectively, which are in good agreement with the values reported by others.[8]

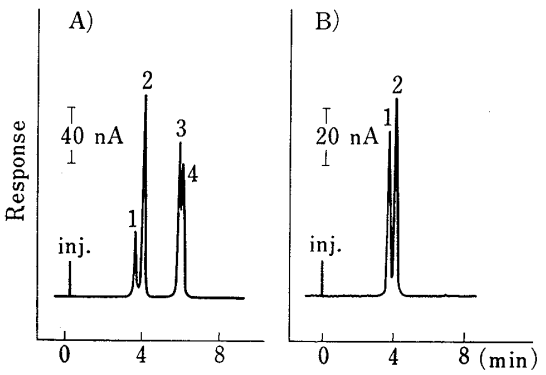

Fig. 6. Chromatogram of PK and MK-4 Isomers

Mobile phase: isopropyl ether–*n*-hexane (1.35: 98.65), 0.5 ml/min. Electrolyte: methanol–ethanol–$HClO_4$ (900: 100: 1) containing 0.1 M $NaClO_4$, 1.5 ml/min. Applied potential: −0.3 V *vs.* Ag/AgCl.
A) Standard sample
Injected amount (μg): 1. *cis*-PK (0.5), 2. *trans*-PK(1.5), 3. *cis*-MK-4 (1.5), 4. *trans*-MK-4 (1.2).
B) Rat plasma mixture after single oral administration of *cis*-PK and *trans*-PK
1. *cis*-PK, 2. *trans*-PK.

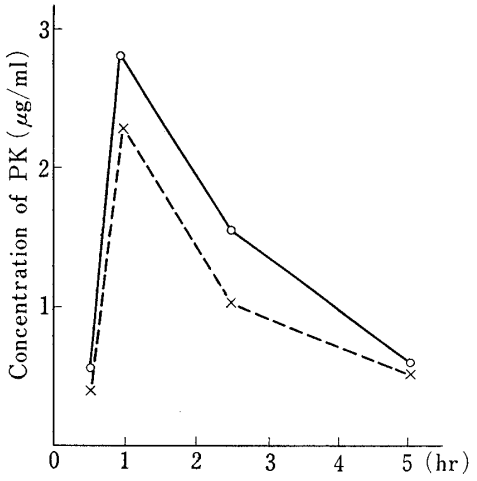

Fig. 7. Concentration of Phylloquinone in Plasma after Oral Administration of 10 mg/kg to Rats

—○—: *trans*-PK, —×—: *cis*-PK.

Determination of PK in Rat Plasma

PK in rat plasma was extracted according to our previous report,[7] and its concentration was estimated from a calibration curve of peak height against concentration of PK. The calibration curve was linear from 10 ng to 1000 ng injected. The limit of detection was 10 ng of PK injected. The recoveries through the whole procedure were 99.3% and 99.5% for *cis*-PK and *trans*-PK, respectively. The coefficients of variation of the peak height were 5.2% and 5.4% for 3 μg/ml plasma of *trans*-PK and *cis*-PK, respectively. Figure 6-B shows a chromatogram of rat plasma mixture after single oral administration of *cis*-PK and *trans*-PK. Figure 7 showed the time courses of *cis*-PK and *trans*-PK concentrations in rat plasma from 0.5 to 5 hr. No conversion of *cis* to *trans* or *trans* to *cis* was observed.

As mentioned above, it was shown that ECD could be applied to HPLC (adsorption chromatography) by adding alcoholic electrolyte solution to the mobile phase at the outlet of the column. The limit of detection was about 10 times poorer than in the case of reversed phase chromatography. The decrease of the sensitivity was probably due to the decrease of the

coulometric yield. This ECD detection method should be widely applicable to the determination of geometricalisomers or stereoisomers.

Acknowledgement The authors thank Mr. M. Takada of Eisai Co., Ltd. for his valuable assistance.

References and Notes

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