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Determination of Vitamin K Analogues by High Performance Liquid Chromatography with Electrochemical Derivatization

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A simple, sensitive, and specific method for the determination of phylloquinone (PK) and menaquinone-4 (MK-4) in rat plasma was developed. PK and MK-4 extracted from biological materials were separated by high performance liquid chromatography using a Nucleosil® C_{18} column with acetonitrile–isopropyl alcohol as a mobile phase, monitored with a fluorescence detector (ex = 330 nm, em = 430 nm) after on-line electrochemical derivatization to fluorescent naphthohydroquinones in an electrochemical reactor. This method was successfully applied to the determination of very small quantities of PK and MK-4 in rat plasma.

Keywords——phylloquinone; menaquinone-4; HPLC; post-column electrochemical derivatization; fluorescence detection; electrochemical reduction; biological material

The analysis of vitamin K analogues in biological materials requires a highly sensitive and selective procedure since they are usually present at low concentrations. For the determination of vitamin K analogues, various high performance liquid chromatographic (HPLC) methods have been reported.[1,2] The previous HPLC methods with ultraviolet (UV) detection[1] lack both selectivity and sensitivity and those with fluorescence detection after post-column chemical reduction[2] require complicated analytical conditions. In a previous paper,[3] we reported a flow injection method for the fluorometric determination of thiamine based on electrochemical oxidation to fluorescent thiochrome in an electrochemical reactor (ECR).

In the present investigation, the ECR was applied to the post-column electrochemical derivatization of vitamin K analogues, which led to the development of an HPLC method for the fluorometric determination of phylloquinone and menaquinone-4 (MK-4) in rat plasma.

Experimental

Apparatus——The system consists of an HPLC pump (110A, Altex, Berkeley, CA, U.S.A.), an injector with a 100 μl sample loop (Rheodyne, Berkeley, CA, U.S.A.), a stainless steel column (4.6 mm i.d. × 15 cm) packed with Nucleosil® C_{18}, (5 μ, Macherey, Nagel & Co., Duren, G.F.R.), an electrochemical reactor made by us, a potentiostat (HA-101, Hokuto Denko, Tokyo, Japan), a fluorescence detector (FP-110, JASCO, Tokyo, Japan), and a recorder (U-228, Nippon Denshi Kagaku, Kyoto, Japan). The wavelengths for fluorescence detection were 330 nm for excitation and 430 nm for emission. Carbon cloth electrodes (1.5 × 5 cm, Type CH-n) and an ion-exchange membrane (2 × 6 cm, Celemion CMV) for the ECR were purchased from Hitachi Co., Ltd.

Materials——Phylloquinone (PK) and MK-4 were obtained from Eisai Co., Ltd. Menadione (MD) was purchased from Tokyo Kasei Co., Ltd. Eicosanyl naphthoate (EN), used as an internal standard, was synthesized according to the method of the previous paper,[4] and purified by column chromatography followed by recrystallization. All other chemicals were of reagent grade.

Assay Procedure——Male Sprague–Dawley rats weighing 220—300 g were used. After a single oral adminis-

tration (1.0 mg/kg) or an intravenous administration (0.2 mg/kg) of the mixture of PK and MK-4, rat blood was withdrawn with a heparinized syringe, transferred into a centrifuge tube and centrifuged at 3000 rpm for 15 min at room temperature. Extraction of PK and MK-4 from the plasma (0.5 ml) was performed with *n*-hexane (5.8 ml) in the same manner as described previously.[2]

After the addition of 1 ml of the internal standard (EN) solution (40 μg/ml *n*-hexane) to the *n*-hexane extract (4 ml), the mixture was evaporated to dryness under a nitrogen stream at 40 °C. The residue was dissolved in 100 μl of isopropyl alcohol and 20 μl of the solution was injected into the HPLC column. The mobile phase was prepared by dissolving 6.0 g of sodium perchlorate monohydrate in 1000 ml of acetonitrile–isopropyl alcohol (9 : 1) and adding 1.0 ml of 70% (w/v) perchloric acid solution; the mixture was deaerated by bubbling of argon gas.

Results and Discussion

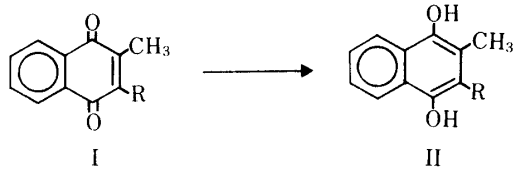
Vitamin K analogues have a naphthoquinone structure (I) which is known to be convertible to the highly fluorescent naphthohydroquinone structure (II) by chemical[2,5] or electrochemical[6] reduction (Fig. 1).

Figure 2 shows the flow diagram of our HPLC system for the post-column electrochemical derivatization of vitamin K analogues.

As shown in Fig. 3, carbon cloth was used as a working electrode and also as an auxiliary electrode in the ECR. Between the two electrodes, an ion exchange membrane was inserted to insulate the working and auxiliary electrodes. Two stainless steel screws were in contact with the surfaces of the electrodes. All the parts were fixed in place with teflon blocks (7 × 10 cm). The two screws were connected respectively to the positive and negative terminals of the potentiostat. Vitamin K analogues eluted from the column were electrochemically converted to the fluorescent naphthohydroquinones by passing the eluate through the carbon cloth working electrode and to the detector.

The relationship between the potential applied to the ECR and the intensity of the fluorescence signal is shown in Fig. 4. MD was more reducible than PK or MK-4. The difference in the reduction potentials is presumably due to the different side chains at position 3 of the naphthoquinones. We chose −1.4 V applied potential for the ECR.

Figure 5 shows the effect of perchloric acid concentration in the mobile phase on the



$R = -CH_2CH=CCH_2(CH_2CH_2CHCH_2)_3H$ (CH3 on the CCH2 carbon)

phylloquinone (PK)

$R = -(CH_2CH=CCH_2)_4H$ (CH3 on the CCH2 carbon)

menaquinone-4 (MK-4)

R = H menadione (MD)

Fig. 1. A Probable Scheme for the Reduction Reaction of Vitamin K Analogues

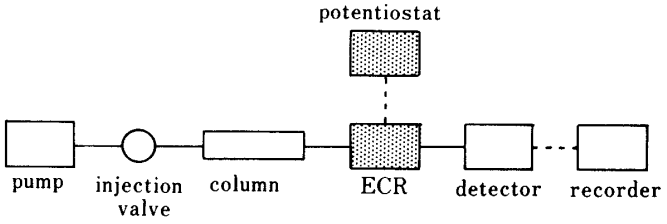


Fig. 2. Instrumental Set-up for the Post-column Electrochemical Derivatization HPLC System

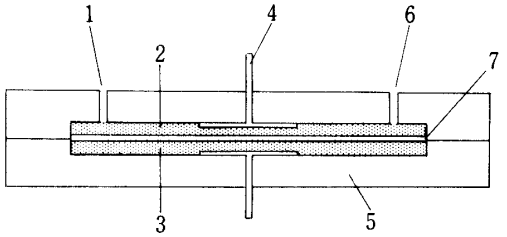


Fig. 3. Schematic Diagram of the Electrochemical Reactor (ECR)

1) inlet; 2) carbon cloth working electrode; 3) carbon cloth auxiliary electrode; 4) stainless steel screw; 5) teflon block; 6) outlet; 7) ion-exchange membrane.

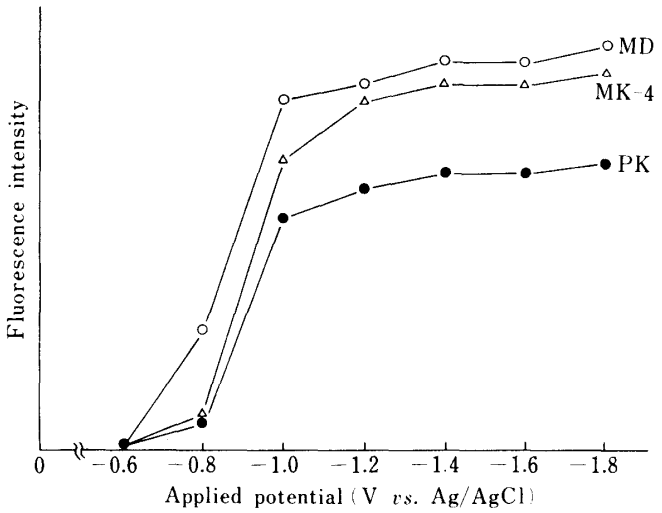


Fig. 4. Effect of Potential Applied to the ECR on the Fluorescence Intensity

Column, Nucleosil® C_{18} (5 μ), 4.6 mm i.d. × 15 cm; mobile phase, acetonitrile–isopropyl alcohol–perchloric acid (70% w/v) = 900 : 100 : 1, containing 0.05 M sodium perchlorate; applied potential, −1.4 V *vs.* Ag/AgCl; flow rate, 0.8 ml/min; detection, ex. 330 nm, em. 430 nm; injection amount, MD 3 ng, PK 10 ng, and MK-4 10 ng.

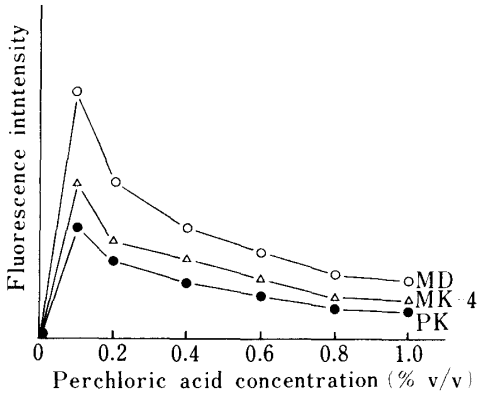


Fig. 5. Effect of Perchloric Acid Concentration in the Mobile Phase on the Fluorescence Intensity

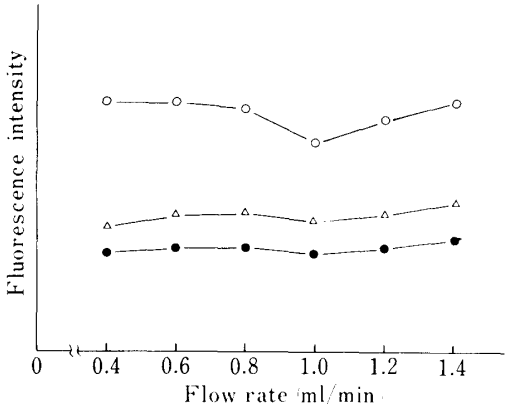


Fig. 6. Effect of Flow Rate

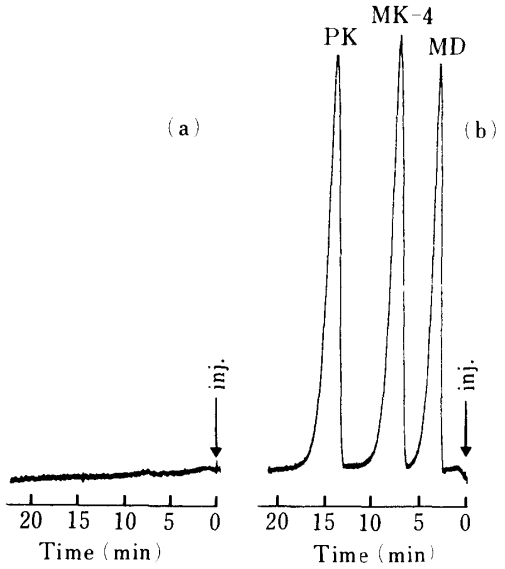


Fig. 7. Chromatograms of MD (3 ng), MK-4 (10 ng), and PK (10 ng)

(a) without applying potential to the ECR; (b) with −1.4 V potential applied to the ECR.

fluorescence intensity. MD, PK, and MK-4 all gave the strongest fluorescence intensity at 0.1% perchloric acid in the mobile phase.

The fluorescence intensity was almost unchanged when the flow rate was varied between 0.4 and 1.4 ml/min (Fig. 6). It was concluded that the electrochemical reduction of naphthoquinones to naphthohydroquinones in the ECR occurred rapidly.

The standard chromatograms of MD, PK, and MK-4 are shown in Fig. 7. When potential was not applied to the ECR, no peak signal was detected.

Acetonitrile was chosen as the main component of the mobile phase solvent, because it gave the strongest peak signal among the solvents (methanol, ethanol, isopropyl alcohol, dioxane, and acetonitrile) tested. Isopropyl alcohol was added to acetonitrile at a concentration of 10% (v/v) as a modifier to shorten the retention time of PK. The calibration curves of MD, PK, and MK-4 showed good linearity in the range of 1—100 ng. Under these conditions, about 90% of each was converted to the corresponding naphthohydroquinone of the vitamin K analogue, and the minimum detectable quantities of MD, PK, and MK-4 were 0.5, 1, and 1 ng, respectively.

The present method was applied to the determination of PK and MK-4 in plasma of rats after oral administration (1 mg/kg) or intravenous administration (0.2 mg/kg).

Figure 8 shows typical chromatograms of rat plasma taken after the administration of PK and MK-4 and of the plasma blank. The chromatogram of control plasma (no administration of PK and MK-4) showed only a front peak signal which probably represents biological materials, and no peak interfering with the determination of PK and MK-4 in rat plasma was detected. The PK and MK-4 concentrations were estimated by the peak height method. The recovery of PK and MK-4 throughout the whole procedure was better than 99.0%. The coefficients of variation ($n=8$) of the peak heights were 2.0% for 200 ng of PK and MK-4 in rat plasma.

Plasma concentration–time curves for PK and MK-4 are shown in Fig. 9. The results are in good agreement with those obtained by the HPLC–chemical reduction (HPLC–CR) method[2] (Table I).

The electrochemical reactor using carbon cloth electrodes is suitable for analytical use from the viewpoints of reaction efficiency and peak diffusion. Moreover, the adsorption of biological materials on the electrode surfaces is not a problem, because the surface area of the working electrode is very large. The intensity of the peak signal of the chromatogram did not

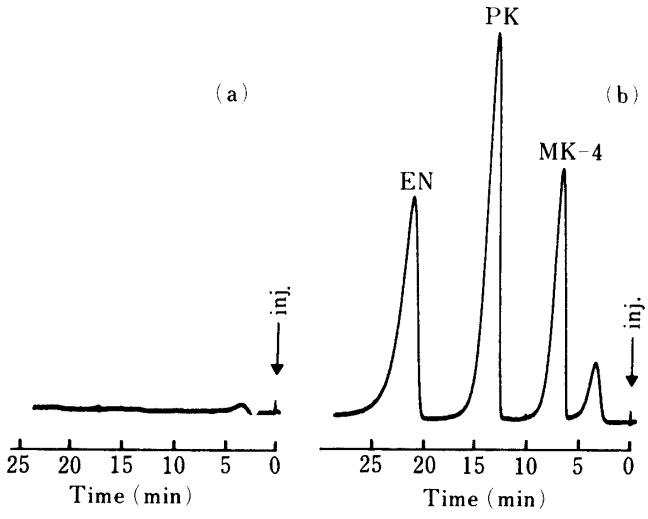


Fig. 8. Chromatograms of Rat Plasma

(a) plasma blank; (b) plasma taken after administration of PK and MK-4.

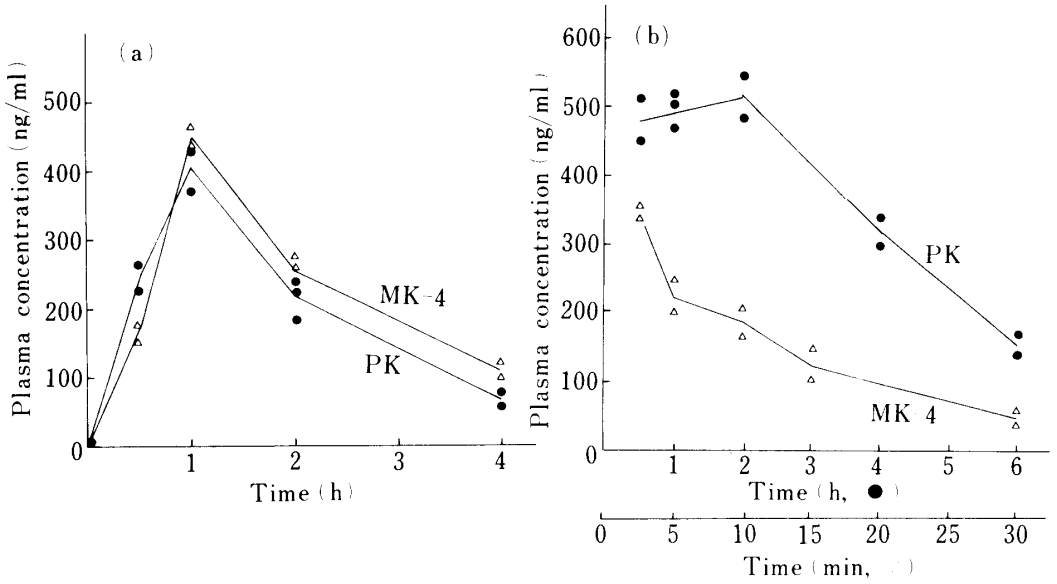


Fig. 9. Rat Plasma Concentrations of PK and MK-4 after Administration
(a) oral administration (1 mg/kg); (b) intravenous administration (0.2 mg/kg).

TABLE I. Comparison between the HPLC-ECR Method and the HPLC-CR Method for Determination of PK and MK-4 in Rat Plasma after Oral or Intravenous Administration

(a) Oral Administration (1 mg/kg)

Time (h)	PK (μg/ml) HPLC-ECR	PK (μg/ml) HPLC-CR	MK-4 (μg/ml) HPLC-ECR	MK-4 (μg/ml) HPLC-CR
0.5	0.24	0.28	0.17	0.18
1	0.40	0.43	0.46	0.41
2	0.22	0.21	0.27	0.27
4	0.07	0.10	0.12	0.15

(b) Intravenous Administration (0.2 mg/kg)

Time (h)	PK (μg/ml) HPLC-ECR	PK (μg/ml) HPLC-CR	MK-4 (μg/ml) HPLC-ECR	MK-4 (μg/ml) HPLC-CR
0.05	—	—	0.35	0.39
0.25	—	—	0.13	0.18
0.5	0.48	0.47	0.06	0.08
1	0.50	0.52	—	—
2	0.51	0.48	—	—
4	0.32	0.34	—	—
6	0.19	0.18	—	—

change after more than a hundred analyses of PK and MK-4 in rat plasma. The cleaning of the electrodes is very simple, *i.e.*, by passing 1% nitric acid over the electrodes for about an hour.

The HPLC method based on the electrochemical derivatization is more sensitive and selective than the previous UV detection method[1] and is much simpler than the method involving fluorescence detection after post-column chemical reduction.[2]

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