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Measurement of K Vitamins in Human and Animal Plasma by High-Performance Liquid Chromatography with Fluorometric Detection

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A sensitive method for measuring endogenous vitamin K_1 (VK_1) and vitamin K_2 (MK-*n*) in human and animal plasma was developed based on high-performance liquid chromatography combined with coulometric reduction and fluorometric detection. The vitamins were extracted from plasma, purified with a silica Sep-Pak cartridge, and then measured by using the standard method. The detection limits of VK_1 and MK-4 were 5 pg/injection for each of the standard substances and 50 pg/ml for each of the two as components of human plasma.

Keywords——vitamin K_1; vitamin K_2; HPLC; electrochemical reduction; fluorometric determination; human plasma; animal plasma

For the measurement of K vitamins in biological materials, several high-performance liquid chromatographic (HPLC) methods based on electrochemical detection,[1-3] fluorometric detection[4,5] and ultraviolet detection[6,7] have been reported. However, these methods do not seem adequate to measure exactly the levels of K vitamins in blood plasma because of their low sensitivities. Recently, Langenberg and Tjaden[8] described a more sensitive HPLC method based on coulometric reduction combined with fluorometric detection to determine the vitamin K_1 level in human plasma.

In this study, we tried to improve this method and to use it to measure endogenous K vitamins in human and animal plasma.

Experimental

Apparatus——HPLC was performed with a system incorporating a Shimadzu LC-3A reciprocating pump, a Rheodyne 7125 injection valve, a stainless steel column (4.6 mm i.d. × 15 cm) packed with Nucleosil C_{18} (5 μ, Nagel), an Environmental Sciences Associates 5020 guard cell (which was placed between the pump and the injection valve to remove oxidizing substances from the mobile phase), an Environmental Sciences Associates 5100A coulochem equipped with an Environmental Sciences Associates 5010 analytical cell (Fig. 1), which was placed between the column and the fluorescence detector, a Hitachi 650-10S spectrofluorometer and a Shimadzu C-R1A recorder.

Materials——Vitamin K_1 was purchased from Wako Pure Chemical Ind., Ltd. Menaquinone (MK)-4, -6, -7, -9

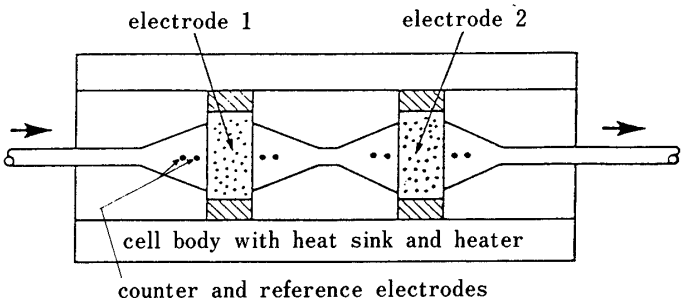

Fig. 1. Schematic Diagram of a Model 5010 Analytical Cell

and -10 were obtained from our laboratories. All other chemicals were of reagent grade. Blood plasma was obtained from Cynomolgus monkeys (8 years), beagle dogs (2—4 years), Crj-Hartley guinea pigs (7 weeks), Slc-SD rats (7 weeks), Jcl-ICR mice (7 weeks) and humans (22—44 years). A stock standard solution containing VK_1, MK-4, MK-6, MK-7, MK-9 and MK-10 was prepared by dissolving the K vitamins in ethanol at 100 μg/ml. This solution was stable for at least 1 month when stored in the dark. A working standard solution was obtained by diluting the stock standard solution with ethanol to the desired concentration before use.

High-Performance Liquid Chromatography——HPLC analysis of K vitamins was carried out by injecting 50 μl of the sample extract onto the column. The column was eluted isocratically with 92.5% ethanol containing 0.25% sodium perchlorate at a flow rate of 1 ml/min at room temperature. The effluent from the column was fed directly into a post-column reaction system to effect reduction of K vitamins, which were detected by fluorescence spectrophotometry at an excitation wavelength of 320 nm and an emission wavelength of 430 nm. The K vitamin concentration of the sample was measured by the peak area method and calculated from a calibration curve. The mobile phase was deaerated by bubbling argon gas through it during the experimental period.

Assay Procedure——A 1-ml plasma sample was placed in a brown glass centrifuge tube, then 1 ml of water, 4 ml of ethanol and 6 ml of *n*-hexane were added. The mixture was shaken for 5 min, followed by centrifugation for 5 min at 2000 rpm. A 5-ml portion of the upper layer was transferred into a brown glass centrifuge tube and evaporated to dryness under reduced pressure at room temperature. After dissolving the residue in 2 ml of *n*-hexane by shaking for 1 min, the solution was applied to a silica Sep-Pak cartridge (Waters) which had previously been cleaned by successive washing with 10 ml of *n*-hexane and 10 ml of an *n*-hexane and ether mixture (97 : 3, v/v). The Sep-Pak cartridge was washed with 10 ml of *n*-hexane, and K vitamins were eluted with 5 ml of an *n*-hexane and ether mixture (97 : 3, v/v). The latter was transferred into a brown conical centrifuge tube and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 200 μl of ethanol by shaking for 1 min, then the solution was filtered through a disposable 0.45 μm filter and 50 μl of the filtrate was injected into the HPLC system. At the same time, 50 μl of the working standard solution for the calibration curve was also injected into the system.

Results and Discussion

Reduction and HPLC Conditions

The fluorometric determination of K vitamins having a naphthoquinone structure requires their chemical or electrochemical reduction to the highly fluorescent naphthohydroquinone structure, since they do not possess native fluorescence. We chose to use electrochemical reduction and examined the reduction conditions together with the HPLC conditions in detail.

The relationship between the fluorescence intensity and the potential applied to the guard

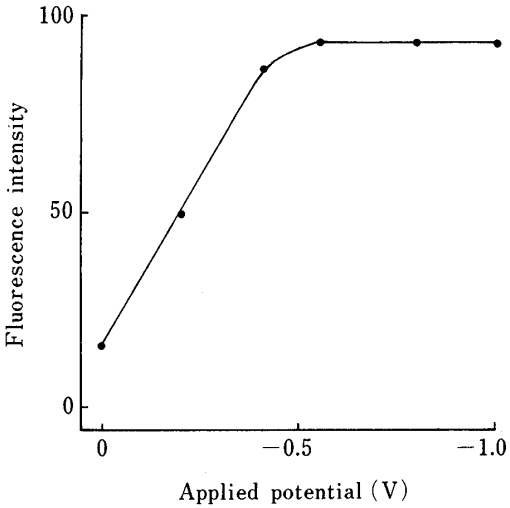

Fig. 2. Effect of Potential Applied to Electrode 2 on the Fluorescence Intensity

Working standard solutions of 50 μl containing 0.5 ng of vitamin K_1 were directly injected into the HPLC system with various applied potentials.

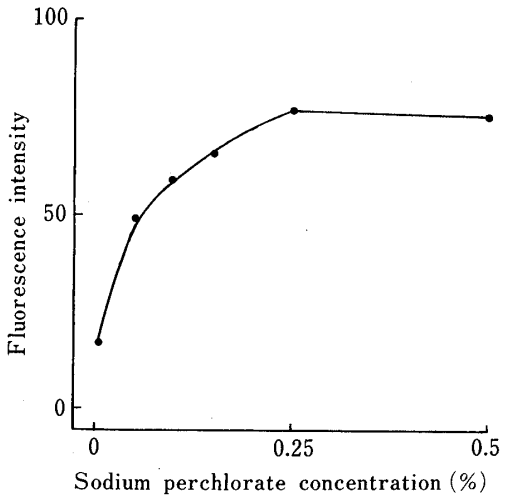

Fig. 3. Effect of Sodium Perchlorate Concentration on the Fluorescence Intensity

Working standard solutions of 50 μl containing 0.5 ng of vitamin K_1 were directly injected into the HPLC system with various concentrations of sodium perchlorate.

cell was examined with the electrode 1 and electrode 2 potentials being held constant at +0.25 and −0.55 V, respectively, and the guard cell potential being varied from 0 to −1.0 V. The fluorescence intensity was constant in the range of 0 to −1.0 V. An applied potential of −1.0 V was chosen for the guard cell since effective removal of oxidizing substances from the mobile phase was reguired. Next, the relationship between the fluorescence intensity and the potential applied to electrode 1 was also examined when the guard cell and the electrode 2 potentials were held constant at −1.0 and −0.55 V, respectively, and the electrode 1 potential was varied from 0 to +0.4 V. The maximum fluorescence intensity was obtained at +0.25 V, which was thus chosen as the applied potential for electrode 1 for effective removal of reducing substances from the mobile phase. Figure 2 gives the relationship between the fluorescence intensity and the potential applied to electrode 2, when the guard cell and the electrode 1 potentials were held at −1.0 and +0.25 V, respectively. When the electrode 2 potential was varied from 0 to −1.0 V, the maximum fluorescence intensity was in the range of −0.55 to 1.0 V. Thus, −0.55 V was chosen as the applied potential for electrode 2 to reduce the K vitamins.

The sodium perchlorate concentration in the mobile phase hardly influences the fluorescence intensity, as is clear from Fig. 3. The maximum intensity was obtained with 0.25% sodium perchlorate, which was thus chosen as the standard concentration for the procedure.

Oxygen must be absent from the mobile phase for the fluorometric detection of K vitamins. If it is present, it interferes with the reduction of K vitamins and the fluorescence development is depressed. Therefore, oxygen was removed by bubbling argon gas into the mobile phase during the experimental period. The mobile phase system was examined with

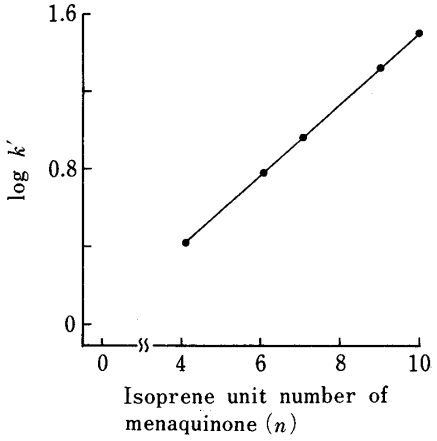

Fig. 4. Relationship between the Common Logarithm of the Capacity Ratio and Isoprene Unit Number for Menaquinone-*n*

A working standard solution of 50 μl containing 0.5 ng each of MK-4, MK-6, MK-7, MK-9 and MK-10 was directly injected into the HPLC system.

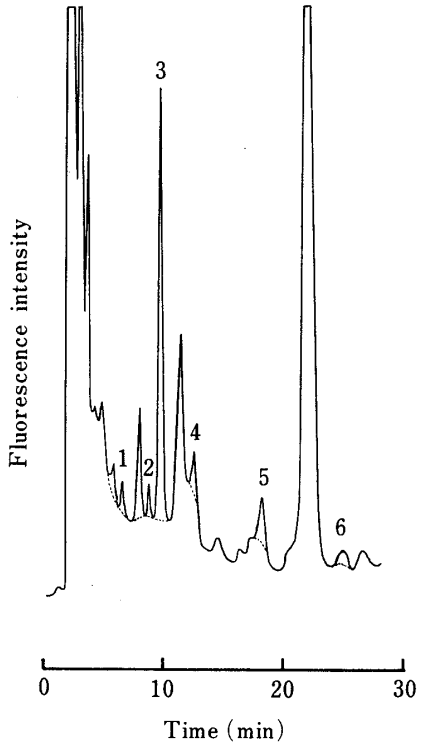

Fig. 5. Chromatogram of an Extract of Human Plasma

Human plasma, 1 ml, was treated according to the assay procedure. 1, MK-4; 2, MK-5; 3, VK_1; 4, MK-6; 5, MK-7; 6, MK-8. Solid line: reduction. Dotted line: no reduction.

TABLE I. Recoveries of Menaquinone-4 and Vitamin K_1 Added to Human Plasma

Sample No.	Added (ng/ml)		Found (ng/ml)	
	MK-4 (X_1)	VK_1 (X_2)	MK-4 (Y_1)	VK_1 (Y_2)
1	0.30	0.20	0.28	0.21
2	0.59	0.40	0.59	0.35
3	0.89	0.61	0.71	0.51
4	1.18	0.81	1.20	0.81
5	1.48	1.01	1.51	0.98
6	2.96	2.02	2.87	2.09

Regression equation: $Y_1 = 0.9846X_1 - 0.021$, $s = 0.09$, $r = 0.996$.
$Y_2 = 1.0559X_2 - 0.064$, $s = 0.05$, $r = 0.998$.

TABLE II. Endogenous Concentration of K Vitamins in Human and Animal Plasma Samples

Subject	VK (ng/ml)					
	VK_1	MK-4	MK-5	MK-6	MK-7	MK-8
Human	1.16	0.30	0.08	0.21	0.37	0.20
Monkey	0.21	0.65	ND	0.12	0.12	0.24
Dog	0.24	0.34	0.18	0.68	ND	ND
Guinea pig	1.84	2.38	0.07	0.26	ND	ND
Rat	2.02	0.30	0.12	0.22	ND	ND
Mouse	0.58	0.85	0.05	0.27	ND	ND

ND means the amount of vitamin K under 50 pg/ml. Each value is the average of determinations with four subjects.

respect to the selectivity between the K vitamins and the coextracted plasma components, and 92.5% ethanol gave the best results. Under these conditions, the detection limits of VK_1 and MK-4 are 5 pg/injection for each standard substance and 50 pg/ml for each of the two as components of human plasma.

Estimation and Determination of MK-5 and MK-8——When the common logarithms of the k' values of standard MK-*n* were plotted against their isoprene unit numbers (*n*), a linear relationship was obtained (Fig. 4). MK-*n* do not possess native fluorescence and only fluoresce after reduction. Moreover, the fluorescence intensities of MK-*n* were about the same in spite of the differences in their isoprene unit numbers.

These results show that MK-5 and MK-8 can be identified and quantified, even when standard samples are not available.

Application to Plasma Samples——The developed method was applied to the determination of VK_1 and MK-*n* in plasma samples from a human and several animals. The chromatogram of an extract of plasma taken from a healthy volunteer (Fig. 5) clearly shows that the endogenous VK_1 and MK-4 to MK-8 can be detected.

The recoveries of VK_1 and MK-4 from human plasma were determined by extracting plasma to which 0.20 to 2.02 ng/ml VK_1 and 0.30 to 2.96 ng/ml MK-4 had been added. As shown in Table I, the calculated relationship between the amounts added (X) and those found (Y) indicated about 100% recovery of both substances.

The endogenous concentrations of VK_1 and MK-*n* in human and several animals plasma were determined and the results are shown in Table II. Vitamin K_1, MK-4 and MK-6 were

found in all plasma samples, while MK-5 was found in all except monkey plasma. MK-7 and MK-8 were found in human and monkey plasma. These results indicate that the present method can be applied to the determination of endogenous K vitamins in plasma from various sources.

The present method is five times more sensitive than that of Langenberg and Tjaden[8] and can be applied to measure the endogenous levels of K vitamins in plasma samples from various sources.

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