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MEASUREMENT OF VITAMIN K IN HUMAN LIVER BY GRADIENT ELUTION HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING PLATINUM-BLACK CATALYST REDUCTION AND FLUORIMETRIC DETECTION

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SUMMARY

A sensitive and precise method for measuring endogenous phylloquinone (K_1) and menaquinone (MK-n) in human liver was developed, based on gradient elution high-performance liquid chromatography using platinum-black catalyst reduction and fluorimetric detection. Subnanogram levels of vitamin K compounds in 1 g of liver specimen were detectable. We measured vitamin K concentrations in 38 human resected livers. K_1 and MK-4 to MK-13 were detected. The concentrations of MK-10 to MK-12 in livers with chronic hepatitis ($n=10$) and cirrhosis ($n=22$) were significantly lower than in normal livers ($n=6$). It is suggested that the decreased concentrations indicate functional damage of the hepatocytes.

INTRODUCTION

Vitamin K is a cofactor for the synthesis of blood-clotting factors in the liver. Phylloquinone (vitamin K_1, K_1) and menaquinone (vitamin K_2, MK-n according to the number of isoprenoid units in the side-chain at the 3 position) have been found in the human liver using UV detection and mass spectrometry [1,2]. Although the concentration of K_1 has been estimated from the radioisotope recovery (14%) [2], that of MK-n has yet to be determined. A highly sensitive method is required for the measurement of their very low concentrations in tissues.

High-performance liquid chromatography (HPLC) with fluorimetric detection [3–7] or electrochemical detection (ED) [8] offers high sensitivity and selectivity. Since the quinone forms of vitamin K compounds lack native fluorescence, they have to be reduced to their hydronaphthoquinone forms for fluorescence detection. There are several methods for the reduction of vitamin K

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[3–7]. We have measured plasma vitamin K levels fluorimetrically after electrochemical reduction or catalytic reduction with a platinum-black column [9]. Platinum-black catalyst reduction is simple and requires no modifiers in the mobile phase. Therefore, we employed a platinum-black catalyst column for the reduction in this study.

EXPERIMENTAL

Chemicals

K_1 was purchased from Wako (Osaka, Japan) and MK-*n* (MK-4 to MK-13) were kindly provided from Eizai (Tokyo, Japan). HPLC-grade solvents were obtained from Kanto (Tokyo, Japan).

High-performance liquid chromatography

The high-performance liquid chromatograph consisted of two reciprocating pumps (LC-6A, Shimadzu, Kyoto, Japan) for gradient elution controlled by a system controller (SCL-6A, Shimadzu), a fluorimeter (RF-535, Shimadzu), a column oven (CTO-6A, Shimadzu) and an automatic sample injector (SIL-6A, Shimadzu). A 50-μl sample of the extract was injected onto a reversed-phase column (Nucleosil $5C_{18}$, 250 mm × 4.6 mm I.D., Macherey-Nagel, Düren, F.R.G.) and eluted at a flow-rate of 1.0 ml/min. A stainless-steel column packed with platinum-black powder (RP-10, 10 mm × 4.0 mm I.D., IRICA, Kyoto, Japan) was used for post-column reduction. Detection was at an excitation wavelength of 320 nm and an emission wavelength of 430 nm. Fig. 1 shows a schematic diagram of the system.

Assay procedures

We prepared ca. 1 g of liver sample by the method of Hirauchi et al. [10]. This method incorporates the purification on a Sep-Pak cartridge developed by Hirauchi et al. [5] and that using thin-layer chromatography (TLC) reported by Sakano et al. [11], with slight modifications. Briefly, 1 g of liver tissue was homogenized with 5 ml of 66% 2-propanol, and then 6 ml of hexane were added. The mixture was shaken and a 5-ml portion of the upper layer was transferred to another brown centrifuge tube and dried under reduced pressure. After the resi-

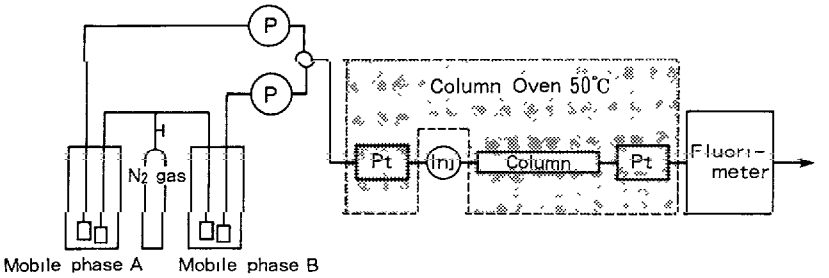

Fig. 1. Schematic diagram of the gradient HPLC system. P = pump; Inj = automatic sample injector; Pt = platinum-black catalyst column for reduction.

due had been dissolved in 2 ml of hexane, the solution was applied to a silica gel Sep-Pak cartridge (Waters Assoc., Milford, MA, U.S.A.), which had previously been successively washed with 10 ml of 4% of diethyl ether in hexane and 10 ml of hexane. The Sep-Pak cartridge was washed with 10 ml of hexane before the vitamin K compounds were eluted with 5 ml of 4% of diethyl ether in hexane. The eluate was dried under reduced pressure and dissolved in 150 μl of hexane. The solution was applied to a silica gel 60 F_{254} plate (20 cm$\times$20 cm, E. Merck, Darmstadt, F.R.G.) as a 13 cm long band. At the same time, 10 μl of hexane containing 1 μg of K_1 were spotted as a marker at 2 cm from the band on both sides. The plate was developed with a mixture of petroleum ether and diethyl ether (85:15, v/v) for 20 min over 12 cm in a developing chamber. After the markers were detected by UV illumination (254 nm), with reference to the R_F values of vitamin K compounds, portions of silica gel ca. 3 cm wide were scraped into a brown centrifuge tube. Vitamin K compounds were extracted with 7 ml of chloroform by shaking. A 5-ml portion of the chloroform layer was transferred into another brown centrifuge tube and dried under reduced pressure. The residue was dissolved in 200 μl of ethanol, and 50 μl of the solution were injected into an HPLC system.

RESULTS

Reduction activity of the platinum-black column

We examined the reduction activity with several solvents after the removal of oxygen. The reduction activity was stable with methanol, ethanol, 2-propanol and small amounts of water as the mobile phase. However, the reduction activity gradually decreased when acetonitrile or tetrahydrofuran was used as the mobile phase, especially when reagent-grade or not freshly prepared HPLC-grade solvents were used. The decrease in activity was probably due to the production of

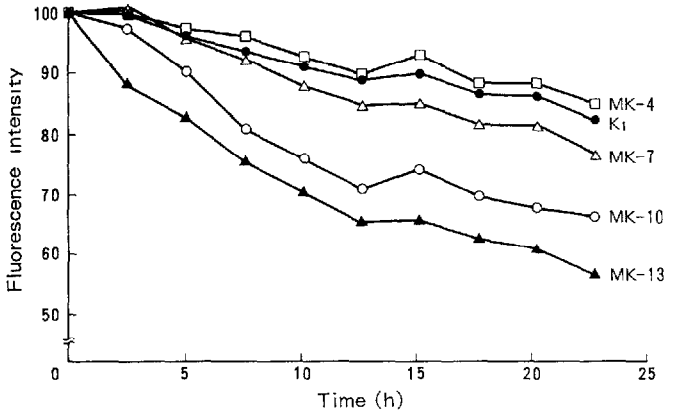

Fig. 2. Decrease in fluorescence intensity without the removal of oxygen from the mobile phase. The behaviour of MK-5, MK-6, MK-8, MK-9, MK-11 and MK-12 was the same as vitamin K compounds in this figure. Gradient elution was carried out using methanol as mobile phase A and 2-propanol–ethanol (4·1, v/v) as mobile phase B.

peroxides in the solvents. We thus chose methanol, ethanol and 2-propanol as the mobile phase. With these solvents, the reduction power of the platinum-black catalyst column was equal to that of the electrochemical method described by Hirauchi et al. [5].

Fig. 2 shows the decrease in the fluorescence intensity when oxygen was present. However, when oxygen was removed by nitrogen bubbling, the reduction activity was stable during continuous analysis. We also found that the reduction activity became even more stable when another platinum-black column was placed between the pumps and the injector, as described by Tejada et al. [12] for a platinum–rhodium catalyst column. We used an automatic sample injector.

Gradient conditions and column heating

In the isocratic elution method for vitamin K compound in human liver, the elution times of MK-*n* with long isoprenoid units in the side-chain were long. Fig. 3 shows a chromatogram of vitamin K in human liver obtained by isocratic elution with methanol–ethanol (7:3, v/v) as the mobile phase. The capacity ratios (k') of vitamin K compounds ranged from 1.6 to 60. [A linear relationship between log k' for MK-*n* and their isoprenoid unit numbers (n) was observed.] Contaminating peaks interfered with the analysis of MK-5 and MK-6. The unknown peak (*) near that of MK-11 was considered to be MK-9 (H_{10}) based on the retention times of MK-*n* reported by Tamaoka et al. [13]. Neither the peak of MK-11 nor of this unknown substance appeared in the analysis without reduction. If the concentration of ethanol was increased, a shorter analysis time was obtained. However, the resolution of vitamin K was decreased and the unknown peak was still not separated from the MK-11 peak.

At room temperature, it was difficult to separate efficiently this peak from that

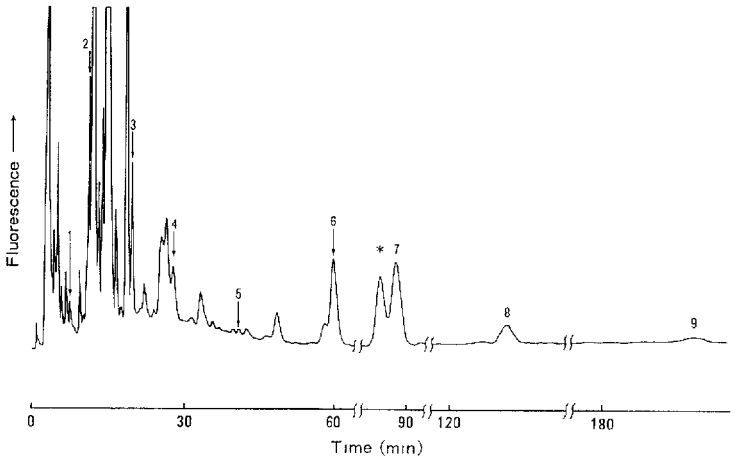

Fig. 3. Isocratic chromatogram of the human liver sample. Analytical column, Nucleosil $5C_{18}$ (250 mm×4.6 mm I.D.); mobile phase, methanol–ethanol (7 3, v/v); flow-rate, 1.0 ml/min; room temperature. Peaks: 1=MK-4 (0.7 ng/g); 2=K_1 (7.0 ng/g); 3=MK-7 (25.0 ng/g); 4=MK-8 (7.0 ng/g), 5=MK-9 (0.7 ng/g); 6=MK-10 (51 ng/g); 7=MK-11 (59 ng/g); 8, 9=MK-12, MK-13 (could not be quantified because of broadness).

of MK-11 by gradient elution. However, the two peaks could be separated easily when the analytical column was heated to 50°C. A large baseline drift was observed during the gradient analysis at elevated temperatures. However, when 100% methanol was used as mobile phase A and 2-propanol–ethanol (4:1, v/v) as mobile phase B, the baseline drift was smaller. The most efficient gradient conditions for separating vitamin K compounds in the human liver were obtained when the concentration of mobile phase B was increased linearly from 0% (15 min) to 80% (90 min). Fig. 4 shows the gradient profile (mobile phase B) and a chromatogram of the standards. Under these conditions, the relative standard deviations of the peak areas and retention times were within ±2% and ±1%, respectively, during continuous analysis for at least 48 h ($n=5$); the day-to-day precision of peak areas and retention times was within ±3% ($n=5$).

Fig. 5 shows chromatograms of a liver sample analysed by gradient elution with and without reduction. The peaks were identified as vitamin K compounds by the coincident retention times with those of the standards and by the disappearance of the peaks during the analysis without reduction (by removal of platinum-black columns from the system). The MK-11 peak is readily separated from the unknown peak (*) by column heating to 50°C. MK-5 and MK-6 could not be measured because of interferences from other peaks. However, their amounts were small (subnanogram levels) when we employed isocratic analysis by electrochemical reduction using acetonitrile–methanol (4:1, v/v) as the mobile phase. The patient studied received Kaytwo (MK-4, Eizai) at the preoperative stage. The peaks, denoted by dotted line, have retention times very close to that of MK-

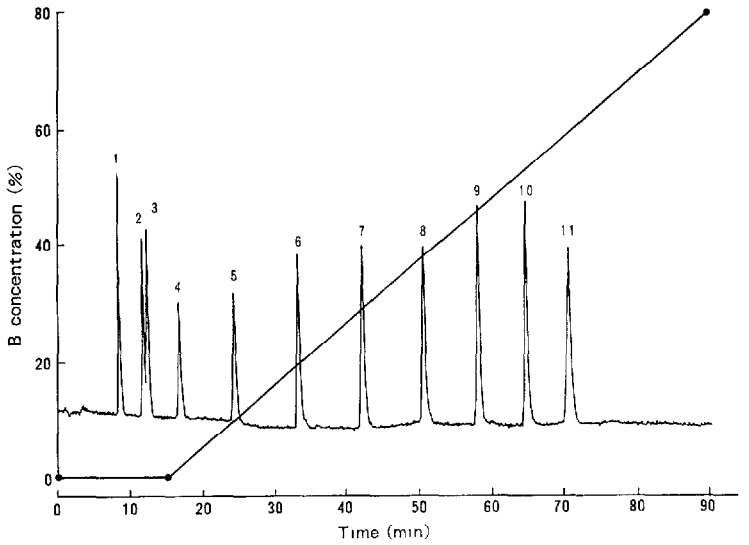

Fig. 4. Chromatogram of vitamin K standards analysed by gradient elution, and the gradient profile in terms of mobile phase B. Peaks: 1 = MK-4; 2 = MK-5; 3 = K_1; 4–11 = MK-6–MK-13. Mobile phase A, 100% methanol; mobile phase B, 2-propanol–ethanol (4 1, v/v); temperature, 50°C. The concentration of mobile phase B was increased linearly from 0% (15 min) to 80% (90 min). Injected amounts: K_1 and MK-4–MK-6, 200 pg each; MK-7, 350 pg; MK-8 and MK-9, 500 pg each; MK-10, 600 pg; MK-11, 700 pg; MK-12 and MK-13, 800 pg each.

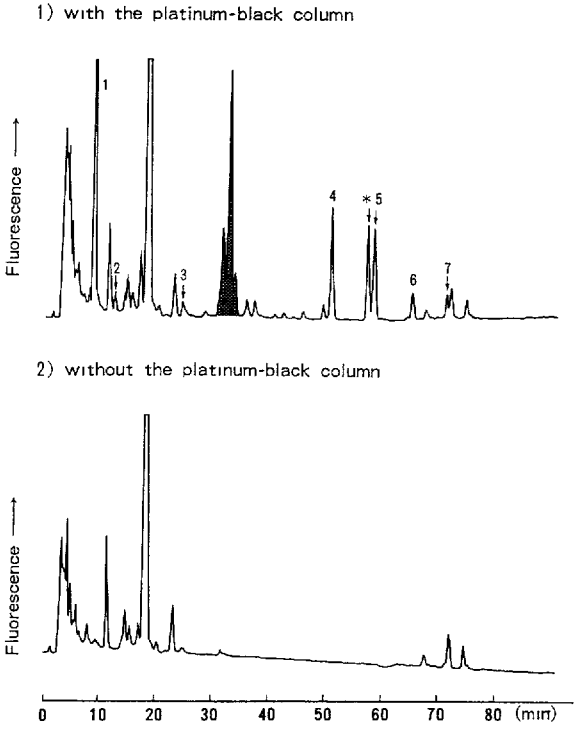

Fig. 5. Gradient elution chromatograms of a human liver sample analysed with and without reduction. Peaks: 1 = MK-4 (34.4 ng/g); 2 = K_1 (2.0 ng/g); 3 = MK-7 (1.7 ng/g); 4 = MK-10 (16.1 ng/g); 5 = MK-11 (12.1 ng/g); 6 = MK-12 (3.1 ng/g); 7 = MK-13 (3.4 ng/g). See Figs 1 and 4 for HPLC conditions.

8 and disappeared during the analysis without reduction. They are not MK-8, but could be degradation products of MK-4 because they were also found in small amounts of Kaytwo itself. In our measurements of many human liver samples from subjects administered Kaytwo, MK-4 was not converted into MK-8. In addition, the reduction potential of the platinum column and the resolution of the analytical column were stable after more than 200 analyses, and after two years of use.

Detection limits and quantification

The detection limit of the standard compounds was 5 pg for MK-4, 10 pg for K_1, MK-5 and MK-6, 20 pg for MK-7 to MK-9 and 40 pg for MK-10 to MK-13 (at a signal-to-noise ratio of 3); they were ten times higher when measured in the liver sample (1 g): 50, 100, 200 and 400 pg, respectively. The gradient elution system described offers a considerable enhancement in sensitivity over isocratic elution using methanol–ethanol (7:3, v/v) for MK-n with $n \geqslant 9$. The concentrations of vitamin K in human liver were calculated by peak-area measurements using calibration curves, each of which showed good linearity.

The calibration curves of vitamin K compounds were linear over the range 0.02–100 ng per injection (corresponding 0.2–1000 ng/g in the liver sample). The regression equations for K_1, MK-4, MK-7, MK-10 and MK-13 were as follows.

For K_1, $y=171\,000x-2200$, S.D.$=2660$, $r=0.99999$; for MK-4, $y=182\,000x-1690$, S.D.$=5530$, $r=0.99999$; for MK-7, $y=111\,000x-4860$, S.D.$=5850$, $r=0.99999$; for MK-10, $y=81\,100x-871$, S.D.$=9500$, $r=0.99998$; for MK-13; $y=67\,100x-7485$, S.D.$=9290$, $r=0.99998$ ($n=15$; $x=$injected amount; y, peak-area counts; $r=$correlation coefficient). The correlation coefficients for other MKs were also more than 0.99995.

Recoveries of vitamin K compounds from liver homogenates

Table I shows the recoveries of K_1 and MK-4 to MK-13 supplemented to liver homogenates ($n=5$). Those of MK-5 and MK-6 are not shown because of interferences by other peaks. The coefficient of variation (C.V.) of MK-7 and MK-11 were relatively high, but those of the others were satisfactory. A large amount of

TABLE I

RECOVERIES OF VITAMIN K_1 AND MK-4 TO MK-13 SUPPLEMENTED TO HUMAN LIVER HOMOGENATE

Human liver sample (1 g) was prepared by the method of Hirauchi et al. [10]. See Figs. 1 and 4 for HPLC conditions.

Vitamin	Added (ng)	Found (ng)						Recovery (%)	C.V. (%)
		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Mean ± S.D.		
K_1	26.1	28.3	28.6	26.6	28.8	28.0	28.0 ± 0.9	107.5	3.0
MK-4	25.4	24.8	24.2	22.5	23.9	22.6	23.6 ± 1.0	93.1	4.4
MK-7	26.8	28.5	26.1	23.4	26.5	24.4	25.8 ± 2.0	96.3	7.7
MK-8	26.5	27.6	26.1	24.5	27.4	25.3	26.2 ± 1.3	98.8	5.1
MK-9	22.0	24.2	24.1	22.3	24.5	22.7	23.6 ± 1.0	107.4	4.2
MK-10	34.9	34.7	35.0	31.3	35.0	32.7	33.7 ± 1.7	96.6	5.0
MK-11	41.4	41.5	40.3	35.6	39.2	36.6	38.6 ± 2.5	93.5	6.5
MK-12	23.7	21.9	21.6	20.0	22.2	19.7	21.1 ± 1.2	88.9	5.5
MK-13	30.6	29.1	27.5	26.3	27.0	25.4	27.0 ± 1.4	88.5	5.1

TABLE II

VITAMIN K CONCENTRATIONS IN HUMAN LIVER

See Figs. 1 and 4 for HPLC conditions.

Specimen	n	Concentration (mean ± S.E.M.) (ng/g wet mass)							
		K_1	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13
Normal	6	8.3 ± 1.8	79.9 ± 40.0	8.2 ± 1.5	3.3 ± 1.2	82.1 ± 14.2	87.0 ± 24.3	21.4 ± 5.8	8.4 ± 3.4
Chronic hepatitis	10	7.6 ± 2.0	14.7 ± 3.6	9.7 ± 6.9	2.1 ± 1.7	12.3 ± 2.2[a]	9.9 ± 1.9[a]	2.4 ± 0.8[b]	1.6 ± 1.0
Liver cirrhosis	22	11.0 ± 2.2	40.6 ± 17.2	5.5 ± 1.1	1.5 ± 0.4	11.3 ± 2.6[a]	9.8 ± 2.6[a]	2.4 ± 0.7[b]	1.2 ± 0.4

[a] $p<0.05$ compared with normal group.
[b] $p<0.01$ compared with normal group.

biological contaminants in the liver sample was removed by the method of Hirauchi et al. [10] and recoveries of above 88% were obtained.

Vitamin K concentrations in human liver

We obtained 38 human resected liver specimens from patients with primary or metastatic liver cancer. Vitamin K compounds were also measured in non-cancerous portions of the specimens. They were histologically classified into normal livers ($n=6$), livers with chronic hepatitis ($n=10$) and liver cirrhosis ($n=22$) groups. No patients had portal vein obstructions. All samples were stored at $-20\,^{\circ}C$ in the dark until analysis. Table II shows the results for each group [mean $\pm$ standard error of the mean (S.E.M.)]. MK-4 was excluded from the table because many patients received MK-4 preoperatively. In the normal liver group, there were small amounts of K_1 and large amounts of MK-*n*. The MK-7 concentration (128.4 and 256.7 ng/g) was very high for two specimens in the normal group, and also for two specimens in the liver cirrhosis group (102.9 and 384.4 ng/g). If these specimens are excluded, the values for MK-7 are 23.6 ± 7.4 and 20.3 ± 4.1 ng/g, respectively. MK-7 is found in high concentrations in natto (fermented soybean) [7], which is an integral part of the Japanese diet. The concentrations of MK-10, MK-11 and MK-12 of the chronic hepatitis and liver cirrhosis groups were significantly different from those of the normal group, acccording to the Student's *t*-test.

We also analysed the subcellular distribution of vitamin K using 10 g of the non-cancerous part of a human liver specimen, which had been resected because of metastatic liver cancer. The specimen was immediately cut into small pieces and homogenized with ice-cold medium containing 0.3 mol/l mannitol and 0.1 mmol/l EDTA. The subcellular fractions were isolated by the conventional method of differential centrifugation. Nuclei and cellular debris were obtained by centrifugation at 400 *g* for 7 min. Human liver mitochondria were prepared by the method of Ozawa et al. [14]. We confirmed by electron microscopy that the mitochondrial fraction thus obtained was virtually free from contamination by any microsomal components. For the preparation of the microsome, after the mitochondrial fraction was obtained, the supernatant was centrifuged at 105 000 *g* for 80 min. Vitamin K was measured in each fraction. Fig. 6 shows the chromatograms of vitamin K in the mitochondrial and microsomal fractions. The values for vitamin K in both fractions are expressed in nanograms per injection. The sensitivity of the fluorimeter was twice as high as that in Fig. 5.

From our preliminary experiments, MK-10 and MK-11 concentrations in the mitochondrial fraction (between 400 *g* and 6500 *g* for 15 min) were 1.32 and 1.14 pmol/mg of protein, respectively. (Protein was measured by the method of Lowry et al. [15].) They were four times higher than the concentrations in the microsomal (pellets, 105 000 *g* for 80 min) and the nuclear (pellets, 400 *g* for 10 min) fractions. However, the three fractions had the same K_1 concentration (ca. 0.1 pmol/mg of protein). All vitamin K compounds in the cytosol fraction (supernatant, 105 000 *g* for 80 min) were much lower than in other fractions.

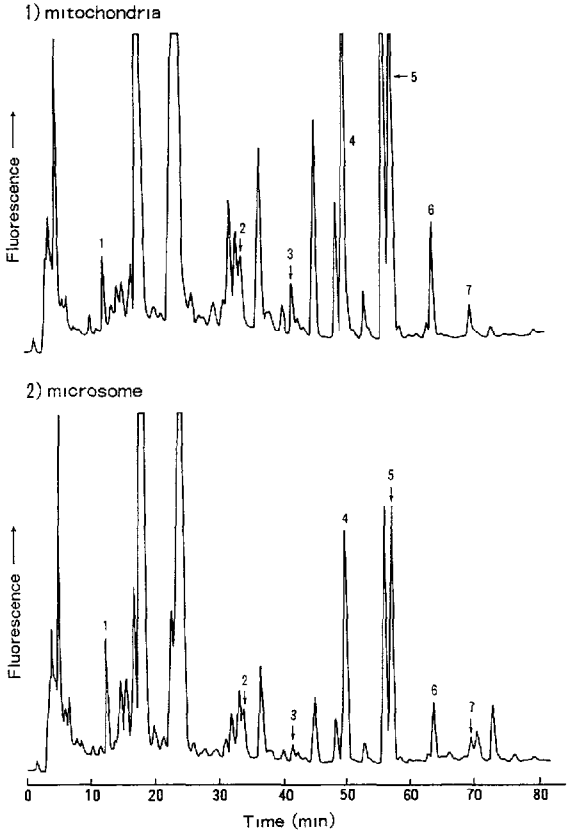

Fig. 6. Vitamin K in mitochondrial and microsomal fractions. Peaks: 1 = K_1 (0.2 for the mitochondria, 0.3 ng per injection for the microsome); 2 = MK-8 (0.4, 0.2 ng); 3 = MK-9 (0.3, 0.1 ng); 4 = MK-10 (4.4, 1.9 ng); 5 = MK-11 (4.1, 1.8 ng); 6 = MK-12 (0.8, 0.4 ng); 7 = MK-13 (0.2, 0.1 ng). See Figs. 1 and 4 for HPLC conditions.

DISCUSSION

Haroon and Hauschka [16] reported a method for the preparation of rat liver, which consisted of the chromatographic collection of K_1. However, they did not measure MKs. Many vitamin K analogues have been detected in human liver [1,2], which contains many more biological substances than the rat liver. Although there is no adequate method for preparation of human liver, vitamin K compounds in the liver of several animals have recently been measured by Hirauchi et al. [10] after purification on a Sep-Pak cartridge and TLC. We have applied this method to the measurement of vitamin K in human liver, with satisfactory recoveries and C.V. values for human liver homogenate, as shown in Table I.

It is difficult to separate vitamin K compounds by isocratic elution because of the wide range of their capacity ratios. Gradient elution systems with fluorimetric detection after post-column reduction with zinc metal [6] or an electrochemical cell [17] have been reported. However, the former method has not been used to

measure vitamin K compounds in the biological samples. With regard to the electrochemical reduction method, there is a risk of decreased reduction power, because of the coating of electrodes caused by continuous analysis of many liver samples.

The platinum-black column is not consumed and reduces the vitamin K catalytically, like a platinum oxide catalyst column [7] or a platinum–rhodium catalyst column [12] without any modifier. We developed a new gradient HPLC method for the measurement of vitamin K compounds in the liver using fluorimetric detection and platinum-black catalyst reduction. This method has the advantages of high sensitivity (subnanogram for each vitamin K in the liver), stable reduction power and simplicity (no modifier required).

Vitamin K is known to be a cofactor of γ-carboxylase for the synthesis of blood-clotting factors in the liver. Some investigators [18] doubt whether MK-*n* in the human intestine is utilized, but the present study revealed large amounts of menaquinones in the normal liver, especially MK-10 and MK-11, which were probably derived from intestinal flora via the portal vein and were trapped. They have also been found in high concentrations in the human faeces [11]. MK-10, MK-11 and MK-12 concentrations in livers with chronic hepatitis and cirrhosis were significantly lower than those in normal livers. Fick et al. [19] reported that both starvation and reduction of the intestinal bacteria were necessary to induce vitamin K deficiency in humans but fasting alone for four to five weeks did not result in vitamin K depletion. It is suggested that MK-*n*, with its long isoprenoid chain, is utilized by humans and the decrease in vitamin K in the liver may be one cause of haemorrhagic tendency in liver diseases.

The subcellular distribution of K_1 and MK-4 has been investigated [20–23]. Suttie [24] suggested that vitamin K is localized in various cellular membranes and that significant amounts of vitamin K are probably found in non-target membranous fractions. However, MK-*n* was not investigated. From our analysis, MK-10 and MK-11 concentrations in the mitochondrial fraction were higher than those in other fractions.

Menadione, which is an analogue of vitamin K with no isoprenoid units in the 3 position, was found to mediate electron transfer in the mitochondrial respiratory chain in rat liver [25]. In the present study, the concentrations of MK-10 and MK-11 were high compared with other vitamin K compounds in normal human livers; they were lower in livers with chronic hepatitis and cirrhosis. It is suggested that MK-10 and MK-11 act in the electron-transport system and that their decrease in chronic hepatitis and cirrhotic livers may reflect the functional damage of the hepatocytes. Further investigation is required to determine the relationship between the hepatocyte function and MK-*n* in the liver.

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