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

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A high-performance liquid chromatographic (HPLC) method was developed for measuring endogenous K vitamins in human and animal feces, and these vitamins in human feces were also identified by mass spectrometry (MS). K vitamins extracted from human and animal feces were purified by thin-layer chromatography (TLC) and then measured by reversed-phase HPLC with fluorometric detection. Vitamin K_1 (VK_1) and menaquinone (MK)-4 to MK-15 were found and their amounts were determined in human, monkey and rabbit feces. K vitamins extracted from human feces were purified successively by column chromatography, TLC, reversed-phase HPLC and normal-phase HPLC, and then identified as VK_1 and MK-4 to MK-15 by MS, confirming the validity of the HPLC method.

Keywords——vitamin K_1; vitamin K_2; high-performance liquid chromatography; chemical reduction; fluorometric determination; human feces; animal feces; identification; menaquinone

In a previous study,[1] we established a highly sensitive method for the assay of K vitamins by high-performance liquid chromatography (HPLC) combined with coulometric reduction and fluorometric detection, and applied it to measure endogenous K vitamins in human and animal plasma. In this paper, we describe a method for assaying K vitamins in human and animal feces using an HPLC system which is a slight modification of that of Abe *et al.*[2] and we also present the results of identification of endogenous K vitamins in human feces by mass spectrometry (MS), which was done to confirm the HPLC findings.

Experimental

Apparatus——HPLC was performed with a system incorporating a Shimadzu LC-3A reciprocating pump, an Eldex A-30S reciprocating pump which was used to supply the reducing agent, a Rheodyne 7125 injection valve, a stainless steel column (4.6 mm i.d. × 15 cm) packed with Nucleosil C_{18} (5 μ, Nagel), a stainless steel coil (0.8 mm i.d. × 2 m) which was used as a reactor, a Hitachi 650-10S spectrofluorometer and a Shimadzu C-RlA recorder. Mass spectra (MS) were taken on a Hitachi M-68 spectrometer.

Materials——Vitamin K_1 (VK_1) and sodium borohydride were purchased from Wako Pure Chemical Ind., Ltd. Menaquinone (MK)-4, -6, -7, -9, -10 and -13 were obtained from our laboratories. All other chemicals were of reagent grade. Feces samples were obtained from Cynomolgus monkeys (8—11 years), beagle dogs (2—4 years), Crj-Hartley guinea pigs (8—9 weeks), Slc-SD rats (7—8 weeks), Jcl-ICR mice (7 weeks), JW-NIBS rabbits (15—16 weeks), white leghorn chickens (28 weeks) and humans (24—41 years). The feces was used as a powder after drying under reduced pressure for 40 h in a desiccator containing phosphorus pentoxide. A stock standard solution containing VK_1, MK-4, MK-6, MK-7, MK-9, MK-10 and MK-13 was prepared by dissolving the K vitamins in ethanol at a concentration of 100 μg/ml. The 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. The reducing agent was prepared by dissolving sodium borohydride in ethanol at 0.025% (w/v) before use.

HPLC——HPLC analysis of the K vitamins was carried out by injecting 50 μl of the sample extract onto the

column. The column was eluted isocratically with 92.5 or 97.5% ethanol at a flow rate of 1 ml/min at room temperature. The effluent from the column was mixed with the reducing agent and 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 flow rate of the reducing agent was 0.4 ml/min. The K vitamin concentration of the sample was measured by the peak area method and calculated from a calibration curve.

Assay Procedure——A 0.3-g sample of dried feces powder corresponding to about 1 g of normal feces was placed in a brown glass centrifuge tube, then 5 ml of 75% isopropanol 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 conical centrifuge tube and evaporated to dryness under reduced pressure at room temperature. The residue was dissolved in 150 μl of *n*-hexane by shaking for 1 min, and the solution was applied to a Silica gel 60 F_{254} plate (20 × 20 cm, Merck) as a 13-cm band. At the same time, 10 μl of *n*-hexane containing 1 μg of VK_1 was spotted as a marker at 2 cm from the band on both sides. The plate was developed with a petroleum ether and ether mixture (85 : 15, v/v) for 20 min (*ca.* 12 cm) in a developing chamber saturated with solvent vapor, and then the plate was dried for 5 min in air at room temperature. After detection of the marker by ultraviolet (UV) illumination (254 nm), the silica gel layer of the 13-cm rectangular portion, 1.5 cm wide, from the line passing through both centers of the marker was scraped into a brown glass centrifuge tube, and the material was extracted with 7 ml of chloroform by shaking for 5 min, followed by centrifugation for 5 min at 2000 rpm. A 5-ml portion of the chloroform layer 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 and 50 μl of the solution 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.

Purification and Fractionation of K Vitamins in Human Feces for the Measurement of MS——Extraction: The dried feces powder (100 g) from a healthy human was shaken (5 min) with an *n*-hexane and 75% isopropanol mixture (6 : 5, v/v, 1.65 l) and centrifuged (2000 rpm, 5 min). The upper layer was filtered and the filtrate was evaporated to dryness under reduced pressure.

Purification: The residue from the above step was dissolved in *n*-hexane (6 ml) and subjected to column chromatography (180 g, Kieselgel 60, Merck, 5 cm i.d.) with an *n*-hexane and ether mixture (94 : 6, v/v) as an eluent. A yellow eluate (200 ml) containing K vitamins was collected and evaporated to dryness under reduced pressure. The residue was dissolved in *n*-hexane (6 ml) and subjected to thin-layer chromatography (TLC, 30 plates, Silica gel 60 F_{254}) according to the assay procedure. The scraped silica gel (2.1 g) was extracted with chloroform (210 ml), the extract was filtered and the filtrate was evaporated to dryness under reduced pressure.

Fractionation: The residue from the above step was dissolved in ethanol (6 ml) and subjected to HPLC (60 injections; column, Nucleosil$_{5\mu}$C18, 4.6 mm i.d. × 15 cm; solvent system, 97.5% ethanol; flow rate, 1 ml/min; detection, UV absorption at 249 nm), giving six fractions, fr. 1 (VK_1 and MK-4 to MK-10 mixture, t_R = 5—13 min, 540 ml), fr. 2 (MK-11 rich, t_R = 16 min, 60 ml), fr. 3 (MK-12 rich, t_R = 21 min, 60 ml), fr. 4 (MK-13 rich, t_R = 26 min, 60 ml), fr. 5 (MK-14 rich, t_R = 34 min, 60 ml) and fr. 6 (MK-15 rich, t_R = 43 min, 60 ml), based on their retention times. Fraction 1 was evaporated to dryness under reduced pressure, and the residue was dissolved in ethanol (4 ml) and subjected to HPLC (40 injections; column, Nucleosil$_{5\mu}$C18, 4.6 mm i.d. × 15 cm; solvent system, 92.5% ethanol; flow rate, 1 ml/min; detection, UV absorption at 249 nm), giving seven fractions, fr. 1′ (MK-4 rich, t_R = 7 min, 40 ml), fr. 2′ (VK_1 and MK-5 mixture, t_R = 9—10 min, 80 ml), fr. 3′ (MK-6 rich, t_R = 13 min, 40 ml), fr. 4′ (MK-7 rich, t_R = 19 min, 40 ml), fr. 5′ (MK-8 rich, t_R = 27 min, 40 ml), fr. 6′ (MK-9 rich, t_R = 41 min, 40 ml) and fr. 7′ (MK-10 rich, t_R = 61 min, 40 ml), based on their retention times. Fraction 2′ was evaporated to dryness under reduced pressure, and the residue was dissolved in *n*-hexane (1 ml) and subjected to HPLC (10 injections; column, LiChrosorb$_{5\mu}$Si 60, 4 mm i.d. × 25 cm; solvent system, cyclohexane : chloroform = 4 : 6, v/v; flow rate, 1 ml/min; detection, UV absorption at 270 nm), giving two fractions, fr. 1″ (VK_1 rich, t_R = 7 min, 10 ml) and fr. 2″ (MK-5 rich, t_R = 10.5 min, 10 ml), based on their retention times. Finally, the 13 fractions (frs. 2, 3, 4, 5, 6, 1′, 3′, 4′, 5′, 6′, 7′, 1″ and 2″) were individually evaporated to dryness under reduced pressure, and the residues were individually dissolved in *n*-hexane (1 ml) and subjected to HPLC (10 injections; column, LiChrosorb$_{5\mu}$Si60, 4 mm i.d. × 25 cm; solvent system, *n*-hexane : chloroform : ethyl acetate = 65 : 35 : 0.2, v/v; flow rate, 1 ml/min; detection, UV absorption at 270 nm). In each case, the fractions (5 ml each) comprising the main peak were combined and evaporated to dryness under reduced pressure. The MS of the residue was determined.

Results and Discussion

Purification

K vitamins were purified from the coextracted feces components by using a Silica gel 60 F_{254} plate and four kinds of petroleum ether and ether mixtures. K vitamins could be effectively separated from other feces components by using the petroleum ether and ether mixture of 85 : 15 (v/v) as a developing solvent. In this system, the *Rf* values of standard K

vitamins were in the range of 0.53 (MK-4) to 0.61 (MK-13).

Reduction and HPLC Conditions

K vitamins had to be reduced for their fluorometric determination because they do not possess native fluorescence. The optimum concentration of sodium borohydride in ethanol was examined in the range of 0.025 to 0.10% (w/v). The fluorescence intensity was almost the same over the whole range, but the stability of the fluorescence decreased with increasing concentration of sodium borohydride. Therefore, 0.025% was chosen as the standard concentration for the procedure.

A mobile phase of 92.5% ethanol was suitable for measuring VK_1 and MK-4 to MK-10,[1] but was unsuitable for MK-11 to MK-15 because the retention times were too long. Thus, MK-11 to MK-15 were measured using 97.5% ethanol. Under these conditions, the detection limits of VK_1 and MK-4 were 50 pg/injection for each standard substance and 500 pg/0.3 g for each of the two as components of dried human feces.

Estimation and Determination of MK-*n* without Standard Sample

This was performed according to a reported method.[1]

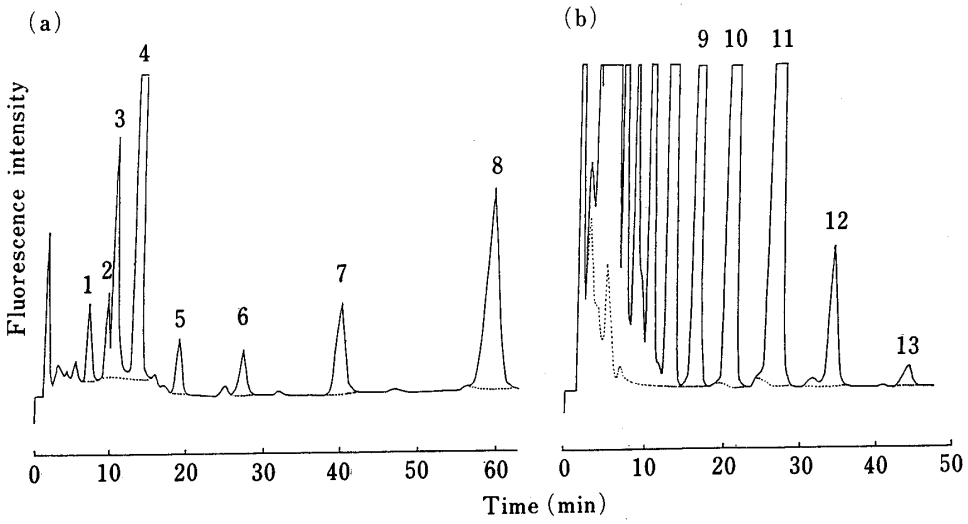

Fig. 1. HPLC Chromatogram of an Extract of Monkey Feces

Monkey dried feces, 0.3 g, were treated according to the assay procedure.
1, MK-4; 2, MK-5; 3, VK_1; 4, MK-6; 5, MK-7; 6, MK-8; 7, MK-9; 8, MK-10; 9, MK-11; 10, MK-12; 11, MK-13; 12, MK-14; 13, MK-15.
Solid line, reduction; dotted line, no reduction.
a, 92.5% ethanol was used; b, 97.5% ethanol was used.

TABLE I. Recoveries of VK_1 and MK-4 Added to Human Feces

Sample No.	Added (ng/0.3 g)		Found (ng/0.3 g)	
	VK_1 (X_1)	MK-4 (X_2)	VK_1 (Y_1)	MK-4 (Y_2)
1	50.5	20.72	40.8	16.40
2	101.0	31.08	73.8	25.87
3	202.1	51.80	155.0	42.27
4	303.1	62.16	225.2	50.66
5	404.2	82.88	315.5	60.71
6	505.2	93.24	349.5	74.58

Regression equation: $Y_1=0.7113X_1+7.632$, $s=13.94$, $r=0.995$. $Y_2=0.7591X_2+1.826$, $s=2.53$, $r=0.995$.

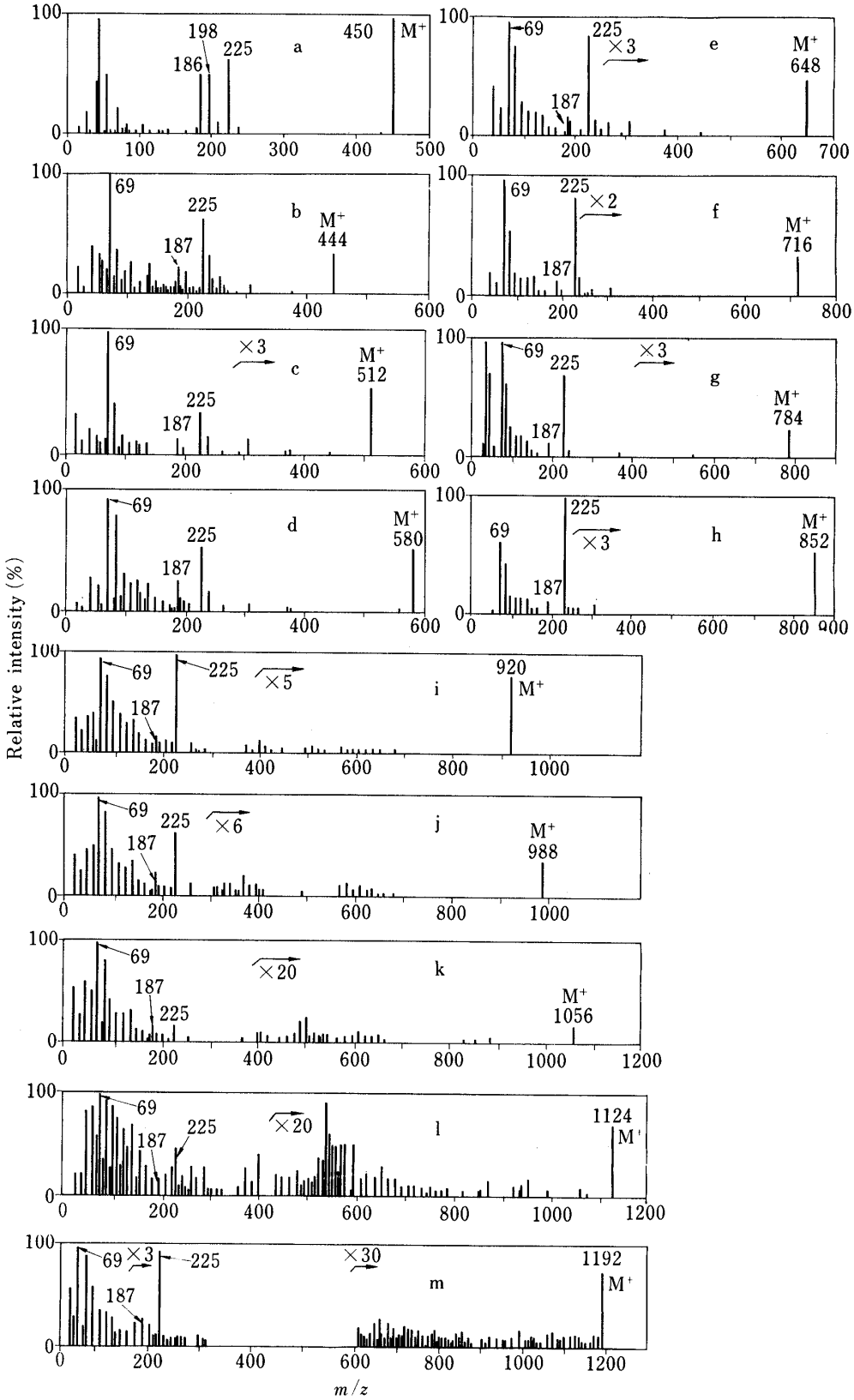

Fig. 2. MS of the 13 Purified Fractions Obtained from a Healthy Human

a, fr. 1″; b, fr. 1′; c, fr. 2″; d, fr. 3′; e, fr. 4′; f, fr. 5′; g, fr. 6′; h, fr. 7′; i, fr. 2; j, fr. 3; k, fr. 4; l, fr. 5; m, fr. 6.

TABLE II. Endogenous Concentrations of K Vitamins in Human and Animal Dried Feces

Subject	VK (ng/0.3 g)												
	VK_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13	MK-14	MK-15
Human	557	49	67	249	236	60	236	2266	1084	1011	1087	171	18
Monkey	346	112	136	2208	108	202	476	1813	3795	7818	13377	1259	77
Dog	26	27	21	430	34	76	120	704	920	1252	754	22	ND
Guinea pig	3677	144	108	165	88	90	152	2022	1150	59	25	9	ND
Rat	321	16	16	22	37	173	260	1414	530	29	85	9	ND
Mouse	20	UK	14	111	2	4	8	104	46	3	6	ND	ND
Rabbit	4373	2	110	310	60	30	102	544	200	50	8	5	8
Chicken	74	UK	4	5	131	42	10	22	6	8	2	ND	ND

UK means that the peak of MK-4 in the chromatogram was not sufficiently resolved to be determined. ND means that the amount of vitamin K was under 0.5 ng/0.3 g. Each value is the average of determinations with four subjects.

Application to Feces Samples

The method developed was applied to the determination of VK_1 and MK-*n* in feces samples from a human and several animals. The chromatogram of an extract of feces taken from a healthy monkey (Fig. 1) clearly shows that endogenous VK_1 and MK-4 to MK-15 can be detected.

Recoveries of VK_1 and MK-4 from human feces were determined by extraction from feces to which about 50 to 500 ng/0.3 g VK_1 and about 21 to 93 ng/0.3 g MK-4 had been added. As shown in Table I, the calculated relationship between the amounts added (X) and those found (Y) indicated recoveries of about 71% for VK_1 and 76% for MK-4.

The endogenous concentrations of VK_1 and MK-*n* in the feces of humans and several animals were determined (Table II). VK_1 and MK-4 to MK-15 were found in human, monkey and rabbit feces, VK_1 and MK-4 to MK-14 in dog, guinea pig and rat feces, and VK_1 and MK-5 to MK-13 in mouse and chicken feces. These results indicate that the present method can be applied to the determination of endogenous K vitamins in feces from various sources.

Identification of K Vitamins in Human Feces

K vitamins in human feces were identified on the basis of the relationship between the common logarithm of the capacity ratio and the isoprene unit number of MK-*n*,[1] and the MS. The MS of the 13 purified fractions obtained from the feces of a healthy human are shown in Fig. 2. Vitamin K_1 in fr. 1″ was identified from its molecular ion at *m/z* 450 and other characteristic ions at *m/z* 225, 198 and 186[3] (Fig. 2, a). MK-4 to MK-15 in frs. 1′, 2″, 3′, 4′, 5′, 6′, 7′, 2, 3, 4, 5 and 6 were also identified from their molecular ions at *m/z* 444, 512, 580, 648, 716, 784, 852, 920, 988, 1056, 1124 and 1192 and other characteristic ions at *m/z* 225, 187 and 69[3] (Fig. 2, b—m). These results showed that VK_1 and MK-4 to MK-15 were present in the human feces examined, supporting the validity of our HPLC method.

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