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MEASUREMENT OF K VITAMINS IN ANIMAL TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

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SUMMARY

A highly sensitive method for measuring endogenous phylloquinone and menaquinones in animal tissues was developed, based on high-performance liquid chromatography with coulometric reduction and fluorimetric detection, following extraction from tissue homogenate and purification on a Sep-Pak silica cartridge followed by thin-layer chromatography. The detection limits of phylloquinone, menaquinone-4, -6, -10 and -13 were 40, 40, 50, 70 and 80 pg/g in rat liver, respectively.

INTRODUCTION

A highly sensitive method for measuring phylloquinone (vitamin K_1, K_1) and vitamin K_2 (menaquinone-*n*, MK-*n*) in animal tissue samples was developed, based on high-performance liquid chromatography (HPLC) with coulometric reduction and fluorimetric detection, following extraction from tissue homogenate and purification on a Sep-Pak silica cartridge followed by thin-layer chromatography (TLC). The method, a combination of previous methods [1–3], was utilized to determine K_1 and K_2 levels in animal tissues. The detection limits of K_1, MK-4, MK-6, MK-10 and MK-13 were 40, 40, 50, 70 and 80 pg/g in rat liver, respectively.

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EXPERIMENTAL

Apparatus

The HPLC system consisted of a Shimadzu LC-3A reciprocating pump, a Rheodyne 7125 injection valve, a stainless-steel column (15 cm × 4.6 mm I.D.) packed with Nucleosil C_{18} (5 μm, Macherey-Nagel, Düren, F.R.G.), an Environmental Sciences (ESA, Bedford, MA, U.S.A.) 5020 guard cell, an ESA 5100A Coulochem equipped with an ESA 5010 analytical cell, which consisted of two porous graphite electrodes in series and was used as a reduction reactor, a Hitachi 650-10S spectrofluorimeter and a Shimadzu C-R3A recorder. Each component was tightly connected with stainless-steel tubing through which the mobile phase was passed.

Chemicals and materials

K_1 was purchased from Wako (Osaka, Japan). MK-4, -6, -7, -9, -10 and -13 were obtained from our laboratories. All other chemicals were of analytical-reagent grade. Tissue samples were obtained from Holstein bovines (5–6 years), thoroughbred horses (7–8 years), cross-breed pigs (2–3 years), Crj-Hartley guinea pigs (8–9 weeks), white leghorn chickens (27–28 weeks), Slc-SD rats (7–8 weeks) and Jcl-ICR mice (8 weeks). A stock standard solution containing K_1, MK-4, MK-6, MK-7, MK-9, MK-10 and MK-13 was prepared by dissolving them in ethanol at 10 μg/ml. This solution was stable for at least 2 months when stored in the dark. A working standard solution was obtained by diluting the stock standard solution with ethanol to the desired concentration.

Assay procedure

A 1.0-g tissue sample was homogenized with 5 ml of 66% 2-propanol in a brown glass centrifuge tube using an Ultra-Turrax (Janke & Kunkel, Staufen, F.R.G.). The homogenate was mixed with 6 ml of *n*-hexane. The mixture was shaken for 5 min, followed by centrifugation at 800 *g* for 5 min. A 5-ml portion of the upper layer was transferred to 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 Sep-Pak silica cartridge (Waters Assoc., Milford, MA, U.S.A.), which had previously been cleaned by successive washing with 10 ml of *n*-hexane–diethyl ether (96:4, v/v) and 10 ml of *n*-hexane. The Sep-Pak cartridge was washed with 10 ml of *n*-hexane, and K vitamins were eluted with 5 ml of *n*-hexane–diethyl ether (96:4, v/v). The eluate was transferred to 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 as a 13-cm band to a Silica Gel 60 F_{254} plate (20 cm × 20 cm × 0.25 mm, Merck, Darmstadt, F.R.G.), which had previously been cleaned by developing with chloroform. At the same time, 10 μl of

n-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 light petroleum–diethyl ether (85:15, v/v) for 20 min (ca. 12 cm) in a developing chamber saturated with solvent vapour, and then was dried for 5 min in air at room temperature. After detection of the marker by UV illumination (254 nm), the silica gel layer of the 13-cm rectangular portion, 1.5 cm wide, from the line passing through both centres of the marker was scraped into a brown glass centrifuge tube, and the materials were extracted with 7 ml of chloroform by shaking for 5 min, followed by centrifugation at 800 *g* for 5 min. A 5-ml portion of the chloroform layer was transferred to 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 were injected into the HPLC system. At the same time, 50 μl of the working standard solution for the calibration graph were also injected into the system.

Chromatography

A sample extract was injected onto the column and eluted with 92.5 or 97.5% ethanol containing 0.25% sodium perchlorate at a flow-rate of 1.0 ml/min. The effluent from the column was fed into a post-column reaction system to reduce the vitamins. Detection was performed by spectrofluorimetry at an excitation wavelength of 320 nm and an emission wavelength of 430 nm. The applied potentials for the upstream electrode and the downstream electrode of the analytical cell were +0.25 and −0.55 V, respectively, and that for the guard cell was −1.0 V. The K vitamin concentrations in the sample extract were measured by the peak-height method and calculated from their calibration graphs. The mobile phase was deaerated by bubbling argon gas through it from 2 h before and throughout the measurements at a flow-rate of 300 ml/min.

RESULTS AND DISCUSSION

The HPLC and assay conditions used in the present method have been discussed elsewhere [1–3]. The chromatogram of an extract of bovine liver obtained by the method is shown in Fig. 1. The identification of the peaks as K vitamins in the chromatogram was performed as follows. When standards were available, the peaks were identified by comparison of retention times with those of the standards and the disappearance of peaks without reduction, and when standards were not available, the peaks were identified by the previously described method [1]. The existence of MK-5 is difficult to determine because its peak overlaps with those of the biological materials, but the peaks of K_1, MK-4 and MK-6 to MK-14 are clearly visible, demonstrating that the method can be applied to their determinations in bovine liver. Similar chromatographic patterns were obtained for other tissues regardless of the kind of animal.

The recoveries of K_1 and MK-4 from rat tissues were determined by extract-

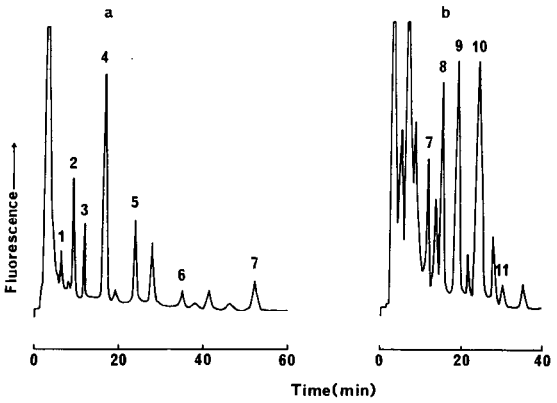

Fig. 1. Reversed-phase high-performance liquid chromatograms of an extract of bovine liver with different mobile phases: (a) 92.5% ethanol containing 0.25% sodium perchlorate; (b) 97.5% ethanol containing 0.25% sodium perchlorate. Peaks: 1 = MK-4; 2 = K_1; 3 = MK-6; 4 = MK-7; 5 = MK-8; 6 = MK-9; 7 = MK-10; 8 = MK-11; 9 = MK-12; 10 = MK-13; 11 = MK-14.

TABLE I

RECOVERIES OF VITAMIN K_1 AND MENAQUINONE-4 FROM RAT TISSUES

RE = regression equation; S.D. = standard deviation; r = correlation coefficient; x = amount added; y = amount found.

Tissue	K_1			MK-4		
	RE	S.D.	r	RE	S.D.	r
Liver	$y=0.743x+0.81$	0.68	0.999	$y=0.788x-1.69$	2.03	0.989
Kidney	$y=0.826x-0.89$	0.79	0.999	$y=0.735x+0.88$	0.94	0.998
Heart	$y=0.838x+0.93$	1.63	0.995	$y=0.918x+0.30$	2.07	0.994
Spleen	$y=0.851x-0.28$	1.97	0.991	$y=0.804x+0.30$	0.89	0.998
Muscle	$y=0.795x-0.42$	1.56	0.995	$y=0.809x-1.22$	0.90	0.998

ing tissues to which 4.89–48.9 ng/g K_1 ($n=7$) and 5.08–50.8 ng/g MK-4 ($n=7$) had been added. The values obtained for K_1 and MK-4 were between 74.3% (liver) and 85.1% (spleen) and 73.5% (kidney) and 91.8% (heart), respectively (Table I).

The determination of MK-n without standard sample was performed according to a previously described method [1]. The detection limits of K_1, MK-4, MK-6, MK-10 and MK-13 were 40, 40, 50, 70 and 80 pg/g in rat liver, respectively. The concentrations of K_1 and MK-n in the tissues of several animals were determined, and the results are shown in Tables II–VI. MK-4, which was not detected in the classical studies of Matschiner and co-workers [4,6–8], was detected in the hepatic and non-hepatic tissues in this study, and this

TABLE II

CONCENTRATIONS OF K VITAMINS IN ANIMAL LIVER

Each value is the average of determinations with four subjects. N.D. = not detectable, less than the detection limits of the K vitamins described in this paper. UK = unknown whether or not MK-5 was present.

Animal	Concentration (ng/g)											
	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13	MK-14
Bovine	18.4	8.2	UK	24.5	181.8	48.4	15.3	66.3	136.4	215.6	436.9	22.6
Horse	76.7	2.1	UK	1.0	2.3	1.2	1.4	0.5	N.D.	N.D.	N.D.	N.D.
Pig	0.5	5.9	UK	0.4	6.1	5.6	2.6	5.1	7.2	4.4	4.9	0.3
Guinea pig	39.3	17.0	UK	7.8	5.1	1.5	3.0	13.3	2.4	0.2	N.D.	N.D.
Chicken	25.2	39.6	UK	0.3	N.D.	0.9	0.4	0.3	N.D.	N.D.	N.D.	N.D.
Rat	4.7	7.7	UK	26.8	N.D.	1.5	1.0	4.8	N.D.	N.D.	N.D.	N.D.
Mouse	1.2	2.6	UK	5.4	N.D.	N.D.	N.D.	1.2	0.5	N.D.	N.D.	N.D.

TABLE III

CONCENTRATIONS OF K VITAMINS IN ANIMAL SPLEEN

Each value is the average of determinations with four subjects. N.D. = not detectable, less than the detection limits of the K vitamins described in this paper. UK = unknown whether or not MK-5 was present.

Animal	Concentration (ng/g)											
	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13	MK-14
Bovine	2.8	7.9	UK	2.0	1.4	0.4	0.6	7.8	10.3	16.8	42.9	1.3
Horse	23.7	0.7	UK	1.1	0.9	0.8	0.4	N.D.	N.D.	N.D.	N.D.	N.D.
Pig	0.5	6.3	UK	0.4	3.9	3.5	2.4	3.0	3.3	2.0	1.7	0.1
Guinea pig	20.9	32.0	UK	2.4	0.5	N.D.	N.D.	5.6	1.7	N.D.	N.D.	N.D.
Chicken	7.7	133.1	UK	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Rat	2.5	6.1	UK	5.5	N.D.	N.D.	N.D.	2.1	N.D.	N.D.	N.D.	N.D.
Mouse	0.6	1.3	UK	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

TABLE IV

CONCENTRATIONS OF K VITAMINS IN ANIMAL KIDNEY

Each value is the average of determinations with four subjects. N.D. = not detectable, less than the detection limits if the K vitamins described in this paper. UK = unknown whether or not MK-5 was present.

Animal	Concentration (ng/g)										
	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13
Bovine	1.9	21.2	UK	0.8	2.1	0.1	N.D.	1.2	1.9	5.8	18.8
Horse	5.8	1.5	UK	N.D.	N.D.	N.D.	N.D.	1.6	N.D.	N.D.	N.D.
Pig	0.2	13.0	UK	0.2	0.7	0.5	2.2	2.4	0.1	0.1	0.2
Guinea pig	12.4	56.4	UK	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Chicken	3.3	50.2	UK	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Rat	1.7	28.2	UK	1.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Mouse	0.4	5.3	UK	0.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

TABLE V

CONCENTRATIONS OF K VITAMINS IN ANIMAL HEART

Each value is the average of determinations with four subjects. N.D. = not detectable, less than the detection limits of the K vitamins described in this paper. UK = unknown whether or not MK-5 was present.

Animal	Concentration (ng/g)										
	K_1	MK-4	MK-5	MK-6	MK-7	MK-8	MK-9	MK-10	MK-11	MK-12	MK-13
Bovine	2.4	21.7	UK	2.8	0.9	N.D.	N.D.	0.9	0.9	1.3	4.1
Horse	14.5	0.4	UK	0.2	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Pig	0.3	1.2	UK	0.2	1.1	N.D.	0.2	1.5	N.D.	N.D.	N.D.
Guinea pig	47.8	263.6	UK	1.4	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Chicken	5.5	142.6	UK	0.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Rat	5.4	13.4	UK	1.1	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Mouse	1.0	4.4	UK	0.6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

TABLE VI

CONCENTRATIONS OF K VITAMINS IN ANIMAL MUSCLE

Each value is the average of determinations with four subjects. N.D. = not detectable, less than the detection limits of the K vitamins described in this paper. UK = unknown whether or not MK-5 was present.

Animal	Concentration (ng/g)				
	K_1	MK-4	MK-5	MK-6	MK-7
Bovine	3.1	34.3	UK	0.3	0.3
Horse	14.9	2.0	UK	0.2	2.3
Pig	1.4	9.4	UK	0.3	0.3
Guinea pig	14.2	158.5	UK	0.2	N.D.
Chicken	2.3	89.9	UK	N.D.	N.D.
Rat	5.2	9.8	UK	N.D.	N.D.
Mouse	1.0	4.3	UK	0.2	N.D.

vitamin was perhaps derived from menadione (vitamin K_3, K_3) in feed given to domestic and laboratory animals [11,12]. By our measurements, the content of K_3 in premixes for domestic animals and in feed for laboratory animals was ca. 100–840 and 0.02–9.3 μg/g, respectively [13]. The contents of K vitamins in tissues (Tables II–VI) varied on the basis of the type of animal, the relative standard deviation being of the order of 20–45%.

These results indicate that the present method can be applied to the determination of K vitamins in tissues from various sources. These values of K vitamins in the tissues, with the exception of those in the liver [4–10], are, to the best of our knowledge, the first to have been measured.

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