Table 1 Characteristics of biotin assays.

名前		感度	利点	欠点
微生物学的定量法	比濁法	0.2 ng/ml	感度が高い。また，特異性が高いので，試料を完全に精製しなくて良い。	大部分が結合型であるので，総ビオチン量を定量する時には，前処理として加水分解や酸素処理を行い，遊離型にする必要がある。また再現性があまりよくない。微生物の増殖を利用するため，培養に時間を要し，迅速性にかける。（8～15時間）
	ろ紙ディスクプレート法	10 ng/ml	多くの試料を一度に簡単に測定できる。	
	ATP法	0.05 ng/ml	感度が高い。また，特異性が高いので，試料を完全に精製しなくて良い。	
理学的定量法	DACA法	1～10 μg/ml	操作が簡単で迅速に定量できる。	感度が低い。
	アビジン－HABA法	0.4 μg/ml		
	同位体希釈法	1 ng/ml	感度が高く，操作が簡単で迅速に定量できる。	放射性物質を使用して測定を行うので，測定できる場所が限定される。
	高速液体クロマトグフィ法	3～400 ng/ml	構造的に類似したビオチン関連物質がよく分離されるため同時に分別定量できる。	精製の必要があり，前処理の複雑さが問題である。
酸素定量法		0.05 ng/ml	特異的で感度が高い。また，迅速に測定できる。	試料に酸素反応の妨害物質が存在すると不正確になるので，注意が必要である。また，放射性物質を使用して測定を行うので，測定できる場所が限定される。

結　語

今回開発した微生物学的定量法は，乳酸菌が産生するATPを利用した方法である。本ATP法は，検出限界が50pg/mlと，従来の濁度法と比べ，高感度である。測定範囲は50pg/ml～800pg/ml，添加回収率は94.1%，同時再現性は8%であった。

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Measurement of 3-Hydroxyisovaleric Acid in Urine of Biotin-Deficient Infants and Mice by HPLC[1]

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ABSTRACT We developed an assay for measuring urinary 3-hydroxyisovaleric acid (3-HIA) using HPLC after derivatization with 2-nitrophenylhydrazine hydrochloride (2-NPH · HCl). The derivatized 3-HIA was extracted into *n*-hexane and separated isocratically on a C8 reversed-phase column for fatty acids (YMC-Pack FA). We used this method to measure 3-HIA in urine extracts from mice fed a biotin-deficient diet for >4 wk and in an infant who was fed a special Japanese formula and was suspected of being biotin deficient. Urinary 3-HIA could be assayed within the range of 0.42–8.5 mmol/L with high accuracy by this method, as an indicator of biotin deficiency. Therefore, the HPLC method for 3-HIA described here may be a useful tool clinically as well as in the research laboratory. J. Nutr. 135: 615–618, 2005.

KEY WORDS: • *3-hydroxyisovaleric acid* • *HPLC* • *biotin deficiency* • *methylcrotonyl CoA carboxylase* • *urine*

Biotin is a cofactor for several carboxylases used in fatty acid synthesis, gluconeogenesis, and BCAA metabolism. The major biotin-containing enzymes are β-methylcrotonyl-CoA carboxylase (MCC),[3] propionyl-CoA carboxylase, pyruvate carboxylase, and acetyl CoA carboxylase. MCC catalyzes an essential step in the degradation of leucine, which converts β-methylcrotonyl-CoA to 3-methylglutaconyl-CoA. The reduced activity of MCC leads to an elevated excretion of 3-methylcrotonic acid, the product of its hydration (3-hydroxyisovaleric acid: 3-HIA), and 3-methylcrotonylglycine, formed by conjugation with glycine. The increased urinary excretion of these abnormal metabolites reflects reduced activity of MCC or is due to dietary biotin depletion in genetically normal individuals.

Conditions that cause biotin deficiency include the following: long-term consumption of undenatured egg white; infant diets that do not include biotin; deficiency of one or more of the biotin-dependent carboxylases in inherited metabolic disorders; biotinidase deficiency and holocarboxylase synthetase deficiency; and biotin transporter deficiency (1,2). One of the most important criteria for the diagnosis of biotin deficiency is the detection of organic acids in urine, such as 3-HIA and 3-hydroxypropionic acid, which are elevated in biotin deficiency (3–5).

In general, 3-HIA in the urine and/or serum of biotin-deficient patients and experimental animals has been assayed by GC/MS, which is specific for assaying 3-HIA in screening tests for biotin deficiency. However, the procedure is complex and difficult. Therefore, we devised an assay for urinary 3-HIA by HPLC, using equipment that is available in most laboratories. We also discuss the usefulness of this method for assaying 3-HIA in the urine of biotin-deficient and biotin-supplemented mice and human infants.

MATERIALS AND METHODS

Animal care and urine collection. Male mice (ICR/Jcl) were obtained from CLEA Japan at 8 wk of age. The biotin-deficient diet was purchased from Oriental Yeast in pelleted form. The components of the biotin-deficient diet (g/kg) were as follows: egg white, 245; cornstarch, 465; sucrose, 100; nonnutritive cellulose, 50; corn oil, 60; mineral mix, 70; and vitamin mix, 10. The control diet was made by supplementing the biotin-deficient diet with biotin (5.0 mg of biotin/kg diet). Mice were kept in an animal room maintained at constant temperature (23 ± 2°C) with a 12-h light:dark cycle (0700–1900 h).

The mice consumed a biotin-deficient or biotin-supplemented diet ad libitum and had free access to distilled water for 6 wk. The body weights of the 14-wk-old mice were 27.9 ± 2.5 and 28.2 ± 2.0 g in the biotin-deficient and biotin-supplemented groups, respectively. Consumption of the diet was also confirmed to be approximately the same in both diet groups, as shown in our previous study (6). Urine was collected from 3 mice fed the biotin-deficient or biotin-supplemented diet every week for 6 wk. The urine of the mice in the individual metabolic cages was collected for 24 h and stored at −40°C until use. All procedures were performed in accordance with the standards related to the care and management of experimental animals of the Japanese Prime Minister's Office (7).

Urine collection in an infant. Because biotin is not yet registered in Japan as a food additive, except in some foods such as dietary supplements, it cannot be added to infant formulas. We demonstrated previously that the biotin concentration of special formulas for medical treatment and prevention of disease in Japan was less than a fifth of the level in American products (8). Therefore, it has often been

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[3] Abbreviations used: 3-HIA, 3-hydroxyisovaleric acid; MCC, methylcrotonyl CoA carboxylase; 2-NPH, 2-nitrophenylhydrazide; RT, retention time.

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reported that biotin deficiency develops in infants with food allergies or inborn errors of metabolism who have been fed special Japanese formulas (9).

In the present case, the infant studied was a 5-mo-old Japanese male. At 4 wks of age, he was diagnosed as having dyspepsia and started to receive maternal milk and/or special formula called Elemental Formula (Meiji Milk Products). Afterwards, prominent erythematous skin lesions developed on his eyelids, perioral region, and neck. Dietary biotin deficiency was strongly suspected and oral treatment with 1 mg/d biotin was started. After 2 wk, the skin lesions disappeared rapidly, and the infant recovered and remained well. Urine was collected 1 wk before and after the biotin treatment. This study was performed in accordance with the ethical principles for medical research involving human subjects (Declaration of Helsinki, World Medical Association). Informed consent was obtained from the parents at enrollment.

Reagents. For the HPLC analysis of standards, 3-HIA was obtained from Tokyo Kasei Kogyo. A kit (X8RFAR 01) for the analysis of short- and long-chain FFA by HPLC (YMC) was used for the pretreatment of the 3-HIA standard solution and urine samples. This kit contains a derivatized reagent that converts the carboxyl moiety of FFA into 2-nitrophenylhydrazide (2-NPH). Sensitive detection in the UV-visible range thus becomes possible after a simple derivatization procedure. Analytical reagent-grade acetonitrile was obtained from Wako Chemical Industries. The control urine "AUTION CHECK II," which is prepared from human urine and is used to monitor the precision of urinalysis, was obtained from Arkray.

Derivatization procedures. The derivatization procedure for fatty acids, including 3-HIA, was a modification of a previously reported technique (10,11) and was as follows:

1. Sample and standard solutions of 3-HIA (100 μL), 200 μL of 2-NPH · HCl (20 mmol/L) solution, and 200 μL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (250 mmol/L) solution were added in turn to a 15-mL tube. The mixture was heated at 60°C for 20 min.
2. After the addition of 200 μL of 15% KOH solution (in 20% methanol), the mixture was heated at 60°C for 15 min and then cooled to room temperature.
3. To the resulting mixture, 4 mL of 0.033 mmol/L phosphate buffer (pH 6.4)-0.5 mol/L HCl (3.8:0.4 v:v) and 5 mL of *n*-hexane were added and mixed on a vortex mixer.
4. The *n*-hexane layer, separated after centrifugation at 2800 × *g* for 5 min, was taken to remove the long-chain fatty acids. This step was repeated.
5. Diethyl ether (5 mL) was added and mixed on a vortex mixer. After centrifugation at 2800 × *g* for 5 min, the diethyl ether layer was collected in a new 15-mL tube.
6. Milli-Q water (3 mL) was added to the diethyl ether layer, mixed on a vortex mixer, and centrifuged at 2800 × *g* for 5 min. The diethyl ether layer was collected in a fresh tube.
7. The diethyl ether layer was collected and dried under decompression at room temperature. The residue was dissolved in 1 mL of methyl alcohol, and an aliquot (10 μL) was used as a sample for measurement.

Chromatographic analyses and conditions. Chromatographic separations were performed using a HITACHI HPLC system (Hitachi). The HPLC column was a C8 reversed-phase column for fatty acids (YMC-Pack FA), which was packed with Si 60 (particle size 5 μm, 250 × 4.6 mm) only for carboxylic acid.

The solvent system for elution from the YMC-Pack FA column consisted of acetonitrile:methyl alcohol:Milli-Q water (30:16:54, by vol). Solvents were adjusted to pH 4.5 with 0.01 mol/L HCl; they were filtered through a 0.45-μm membrane filter and degassed under decompression with ULVAC (Sinku Kiko). Separations were made at a flow rate of 1.0 mL/min. The column temperature was kept constant at 50°C. The elution patterns were monitored at 230 nm to detect the derivatized carbonyl moiety of 3-HIA. The retention time (RT, in min) on a YMC-Pack FA column ranged from 5 to 11 min.

Biochemical analyses. The biotin concentrations in the urine were quantified using a microtiter plate adaptation of the microbiological assay with *Lactobacillus plantarum* ATCC 8014. The microbioassay lacks specificity compared with biotin analysis using an HPLC/streptavidin assay. Urinary specimens were filtered through 0.45-μm membranes and assayed without hydrolysis. The creatinine concentrations were measured in all urine samples with the picric acid method using a Creatinine Test Wako kit (Wako Chemical Industries). The concentrations of urinary biotin and 3-HIA were expressed as μmol/mol creatinine and mmol/mol creatinine, respectively.

Statistical analyses. For statistical evaluation of the data in mice, repeated-measures ANOVA and Student's *t* test were used. Differences of $P < 0.05$ were considered significant. StatView 5.01 (SAS Institute) software was used for all statistical analyses. Values in the text are means ± SD.

RESULTS

In the chromatograms of the 3-HIA standard solution and of the urine analyzed by HPLC, the peak of authentic 3-HIA was identified as the position with an RT of 8.32 min (**Fig. 1***a*). In the chromatogram of the extract of the urine of mice fed a biotin-deficient diet for 6 wks, a high peak was detected at 8.33 min and low unknown peaks appeared (Fig. 1*b*). 3-HIA was

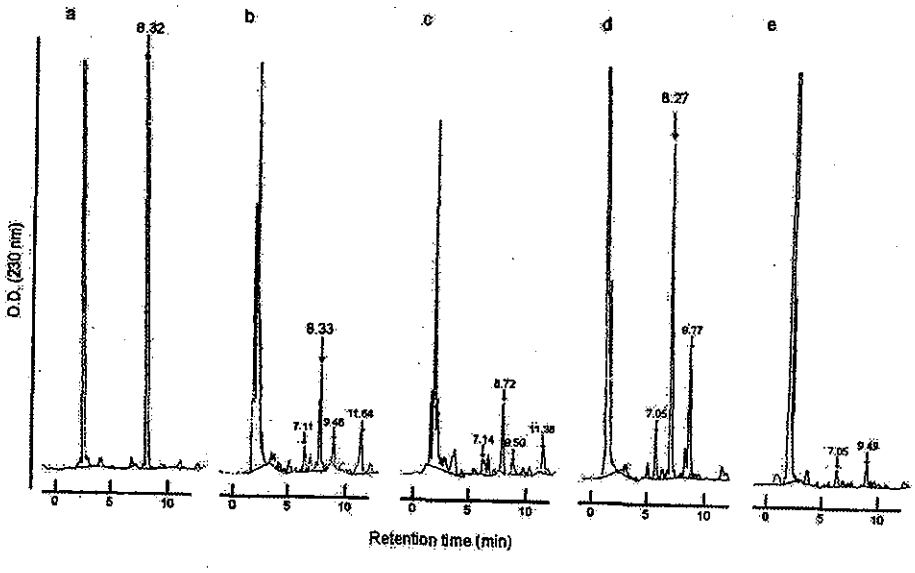

FIGURE 1 Chromatograms (HPLC) of derivatized 3-HIA in biotin-deficient and -supplemented mice and in an infant suspected of being biotin deficient. (*a*) Standard 3-HIA; (*b*) Urinary extract of biotin-deficient mice; (*c*) Urinary extract of biotin-supplemented mice. A new peak appeared at 8.72 min at a position markedly different from that of the 3-HIA peak; (*d*) Extract of the infant's urine collected before biotin treatment; (*e*) Extract of the infant's urine collected after biotin treatment. Large arrows and figures indicate the peak of 3-HIA.

separated completely from other fatty acids in each sample. A peak was detected at 8.72 min in the urine of the biotin-supplemented mice at a position markedly different from the peak of 3-HIA (Fig. 1c). No peak was present at 8.33 min.

Standard 3-HIA concentrations ranging from 0.42 to 8.5 mmol/L and the peak area were correlated ($r = 0.999$, $P < 0.01$) (**Fig. 2a**). In mice fed the biotin-deficient diet for 6 wk, the concentration of urinary 3-HIA was 114.2 ± 69.6 mmol/mol creatinine, which was higher than the concentration of 35.2 ± 23.8 mmol/mol creatinine in mice fed the biotin-deficient diet for 4 wk ($P < 0.046$; Table 1). However, no profound clinical signs of biotin deficiency, such as loss of hair or dermatitis, were observed in these biotin-deficient mice during the experimental period.

3-HIA was detected by HPLC in the urine of biotin-deficient mice (3-HIA–positive mice) (Table 1). The incidence of 3-HIA–positive mice increased with the length of time the biotin-deficient diet was fed. There were no 3-HIA–positive mice within 3 wk of feeding, but all biotin-deficient mice excreted 3-HIA in their urine after 4 wk of feeding. Urinary biotin and 3-HIA concentrations in biotin-deficient mice were inversely correlated ($r = -0.58$, $P = 0.021$; Fig. 2b). When the biotin concentration in the urine was <15 μmol/mol creatinine, 3-HIA was detected in the urine of 6 of 7 mice. On the other hand, no 3-HIA was detected in the urine of 8 biotin-deficient mice that had a urinary biotin level > 15 μmol/mol creatinine. Moreover, no 3-HIA was detected in the urine of the biotin-supplemented mice (Table 1).

The chromatogram of urine obtained from the infant fed a special formula manufactured in Japan and suspected of being biotin deficient had its highest peak at 8.27 min(Fig. 1d). Another peak was detected at 8.77 min. However, these peaks were not detected in the urine after biotin treatment (Fig. 1e).

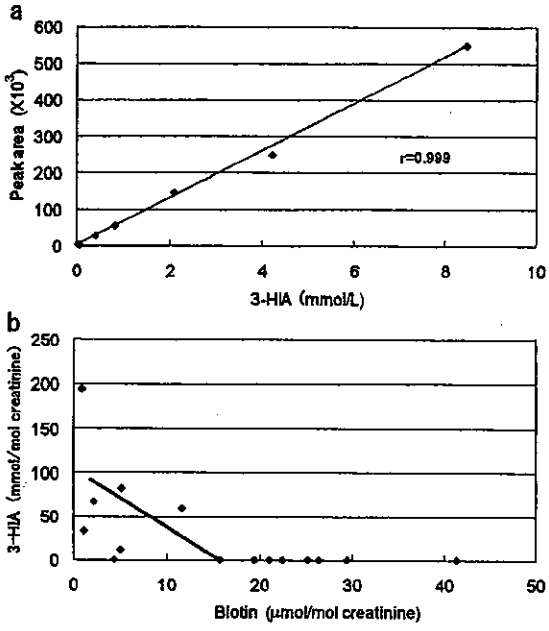

FIGURE 2 Development of the standard curve (*panel a*) and assessment of the relation between urinary biotin and 3-HIA concentrations in biotin-deficient mice (*panel b*, $r = -0.581$, $P = 0.021$).

TABLE 1

Effects of biotin deficiency on the excretion of 3-HIA in urine of mice[1]

Duration of feeding	Mice excreting 3-HIA/mice examined	3-HIA	Biotin
wk	*n/n*	*mmol/mol creatinine*	*μmol/mol creatinine*
Biotin deficient			
1	0/3	ND[2]	22.3 ± 3.6
2	0/3[3]	ND	29.7 ± 10.3
3	0/3	ND	16.5 ± 12.6
4	3/3	35.2 ± 23.8	5.9 ± 5.3
6	3/3	114.2 ± 69.6	2.8 ± 2.2
Biotin supplemented			
1	0/3	ND	263 ± 175
2	0/3	ND	772 ± 476
3	0/3	ND	1020 ± 680
4	0/3	ND	591 ± 387
6	0/3	ND	493 ± 319

1 Values are *n* or means ± SD.
2 ND, not detected.
3 3-HIA was positive in only 1 mouse with a urinary level of 12.0 mmol/mol creatinine, a value below the limit of detection.

DISCUSSION

GLC was used previously to detect 3-HIA and β-methylcrotonylglycine after hydrogenation of urinary extracts from humans with MCC deficiency (12). Subsequently, the first application of GC/MS for the quantitative detection of 3-HIA in urine was reported by Mock et al. (13). Derivatized 3-HIA was detected in urine extracts of patients with biotin deficiency. However, in addition to the high cost of the necessary equipment, after repeated extraction and the separation of the organic acids in the urine, a derivative for separation by GC must also be made (14). In addition, measurement of urinary 3-HIA is a time-consuming process.

Several HPLC methods were developed for the analysis of fatty acids in serum and urine; these employ precolumn derivatization techniques to increase the sensitivity and specificity of detection (10,11,15,16). In the present study, the preprocessing, including labeling, was simplified by the use of the derivatization technique, and a method for 3-HIA measurement was established using a standard HPLC system. The lower limit of detection of urinary 3-HIA was 0.042 mmol/L. This sensitivity was sufficient to measure 3-HIA in urine samples from an infant suspected of being biotin deficient. A relatively higher peak of 3-HIA was obtained before biotin treatment compared with other fatty acids present in the urine. These findings suggest that this HPLC method is sufficiently sensitive for assaying urinary 3-HIA in screening tests for biotin deficiency in humans.

The recovery of 3-HIA was in the range of 90.1–108.8%. This indicates that the present method does not have completely satisfactory precision for analyzing the 3-HIA concentration in urine because the method used here has several extraction and separation steps, which may affect the recovery of 3-HIA. The normal range of urinary 3-HIA in humans was reported to be from 5.1 to 10.7 mmol/mol creatinine as assessed by GC/MS, and it is increased several-fold by biotin deficiency (17). In the present study, the urinary 3-HIA concentration was 78.6 mmol/mol creatinine in an infant fed a special Japanese formula. However, no 3-HIA was detected after biotin treatment of this infant.

The concentrations of biotin and organic acids, and the activity of carboxylase in the serum and urine are generally used as indicators of biotin status (18,19). Mock et al. (3–5) demonstrated that decreased urinary biotin and increased urinary 3-HIA are sensitive indicators of early biotin deficiency, but methylcrotonylglycine and isovalerylglycine, which are also produced due to the decreased activity of MCC, are not. 3-HIA is detected in urine before the appearance of clinical signs of biotin deficiency; thus, it is expected to be a useful indicator of early biotin deficiency. The measurement of 3-HIA in urine may thus be useful for the diagnosis of biotin deficiency.

3-HIA is also a sensitive indicator of biotin deficiency in mice. 3-HIA was detected within a short time after the beginning of the feeding of a biotin-deficient diet, along with a decrease of biotin in the urine. Thus, the HPLC method for 3-HIA described here can be a useful tool clinically as well as in the research laboratory.

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Method for the Determination of Vitamin K Homologues in Human Plasma Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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We report here the development of a precise and sensitive method for the determination of vitamin K homologues including phylloquinone (PK), menaquinone-4 (MK-4), and menaquinone-7 (MK-7) in human plasma using HPLC-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS). The method involves the use of stable isotope ^{18}O-labeled internal standard compounds, which were synthesized in our laboratory, and the selection of a precursor and product ion with a MS/MS multiple reaction monitoring method. The average intraassay and interassay variation values for PK, MK-4, and MK-7 were <10%. Average spiked recoveries from authentic compounds added to normal human plasma samples for PK, MK-4, and MK-7 were 98−102%. Mean plasma concentrations of PK, MK-4, and MK-7 from healthy subjects ($n = 20$) were 1.22 ± 0.57, 0.39 ± 0.46, and 6.37 ± 7.45 ng/mL, respectively. We conclude that this novel LC-APCI-MS/MS method should be useful for the evaluation of vitamin K status in postmenopausal women and elderly subjects and provides useful information for the treatment and prevention of osteoporosis with vitamin K.

There is growing interest in the role, biochemical function, and metabolism of vitamin K in vivo. Vitamin K is a blood clotting agent.[1] It serves as an essential cofactor of the carboxylase involved in the activation of the blood coagulation cascade proteins.[2,3] Recent investigations indicate that vitamin K is required for the synthesis of another calcium-binding protein, osteocalcin, which is important for mineralization in bone. To activate calcium-binding proteins, vitamin K participates in the carboxylation of glutamyl residues of osteocalcin to form γ-carboxyglutamyl residues.[4,5] Thus, several of the biological activities of vitamin K homologues such as phylloquinone (PK, vitamin K_1) isolated from green plants, menaquinones (MK-*n*, vitamin K_2) synthesized by microorganisms, and menadione (vitamin K_3) have been reported. Recently, it was reported that menaquinone-4 (MK-4) was the most potent analogue, and all vitamin K derivatives were converted to MK-4 in vivo.[6,7] In Japan, MK-4 has been given to osteoporotic patients, and phylloquinone has been used as a therapeutic agent for vitamin K-deficient syndromes such as hypoprothronmbinemia in newborn babies and in antibiotic-treated patients. However, information on the physiological and pharmacological roles of vitamin K in vivo is still limited.[8] One reason for this is that the detection and monitoring of vitamin K homologues in plasma and organs have been difficult on account of quite small concentrations and many kinds of impurities even though measurement of the vitamin K plasma concentration is essential to optimize therapy. For pharmacokinetic and epidemiological purposes, specific, accurate, and sensitive analytical methods are required that allow assays at low (ng/mL) plasma levels.

Several assay techniques have been described for the measurement of vitamin K concentrations in human plasma. Initially, high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was the first choice for measuring the individual forms of vitamin K.[9] This method offered more selectivity than the traditional chick bioassay commonly used to measure vitamin K status; however, its sensitivity was still insufficient. More recent methods include electrochemical techniques, fluorescence detection after postcolumn reduction,[10−13] and gas chromatography-mass spectrometry with HPLC.[14] These techniques provide greater selectivity and sensitivity than UV detection. The most common

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and conventional method is HPLC-fluorescence detection. For the measurement, naphthoquinone in vitamin K is converted to a hydroquinone analogue by a platinum oxide catalyst or electrochemical reduction after separation by HPLC. Then the hydroquinone, which fluoresces when exposed to ultraviolet light of a wavelength of 320 nm, can be detected with a fluorescence detector with high sensitivity. Most studies about the quantitation of vitamin K in human or rat plasma have been carried out with this method.

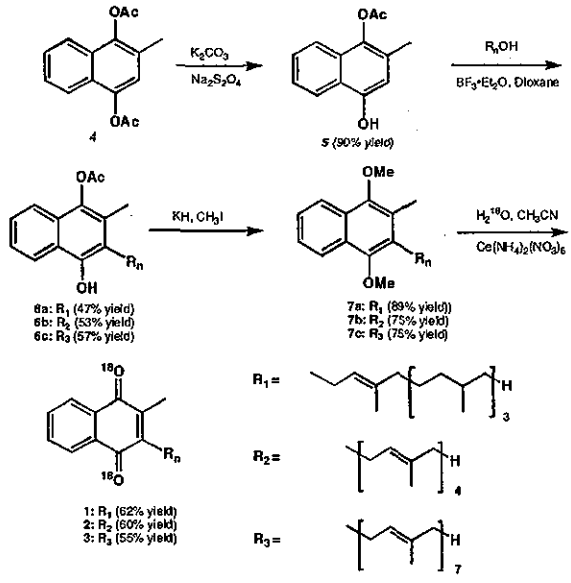
We used HPLC-fluorescence detection to measure the concentrations of vitamin K homologues (PK, MK-4, menaquinone-7 (MK-7)) in human plasma and found that PK and MK-7 could be clearly detected as a single peak. However, the peak of MK-4 overlapped with impurities in the plasma and especially appeared in the shoulder of a large peak of impurity. These results indicate that detection of the plasma MK-4 level with this method depends on the threshold, and the accuracy is uncertain. However, it is essential to accurately determine plasma concentrations of vitamin K homologues. If human plasma vitamin K levels could be clearly determined, the data would provide significant information related to applications to clinical trials. For example, investigating the relationship between plasma vitamin K and undercarboxylated osteocalcin levels might elucidate a therapeutic effect for osteoporosis. Furthermore, analyzing the distribution of vitamin K homologues in vivo and their metabolic pathway should provide valuable information for the development of new drugs. Against this background, we evaluated a new method of quantitating plasma vitamin K levels using HPLC-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) system. One of the advantages of this method is the much greater sensitivity and selectivity in comparison with other LC–MS techniques. In this report, we validated the accuracy and sensitivity of the LC-APCI-MS/MS method using a multiple reaction monitoring mode (MRM) for the determination of vitamin K homologues in human plasma.

EXPERIMENTAL SECTION

Reagents and Chemicals. HPLC-grade solvents and reagents for chemical synthesis were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries, Ltd. (Osaka, Japan). The ^{18}O-labeled vitamin K derivatives used as internal standards were synthesized in our laboratory, and the chemical identity of the products was confirmed by nuclear magnetic resonance (NMR) spectrometry and high-resolution MS spectra (HREIMS) (Scheme 1). Control human serum was purchased from Wako Pure Chemical Industries, Ltd.. PK, MK-4, and MK-7, as standard samples, were kindly donated by Eisai Co., Ltd. (Tokyo, Japan).

***Preparation of* Standard Solutions.** A standard mixture (PK, MK-4, MK-7) stock solution of 100 μg/mL for the reference compounds was prepared in ethanol according to the solubility of the solute and stored in the dark at −30 °C prior to use. For the analytical curves, working solutions of the standard mixture, ranging from 25 to 400 ng/mL, were prepared by dilution of the stock solution with ethanol. Stock solutions of 10 μg/mL for ^{18}O-labeled internal standards (PK-^{18}O, MK-4-^{18}O, MK-7-^{18}O) as shown in Scheme 1 were prepared by dilution in ethanol and stored in the dark at −30 °C prior to use. Dilution of the solution with ethanol gave working internal standard solutions of 3.6 and 100 ng/mL, respectively. The 3.6 ng/mL mixture was used for determination of vitamin K homologues in human plasma samples. An equal amount of the standard solution of 25–400 ng/mL and ^{18}O-labeled internal standard solution of 100 ng/mL gave the solution used for the standard curve. The final concentration ranged from 12.5 to 200 ng/mL in the case of the standard and contained 50 ng/mL internal standards.

Scheme 1. Synthetic Route to ^{18}O-Labeled Vitamin K Homologues 1–3 and Their Chemical Yield at Each Step: 1, ^{18}O-Labeled PK; 2, ^{18}O-Labeled MK-4; 3, ^{18}O-Labeled MK-7



Sample Preparation. A liquid control serum (Wako Pure Industries, Ltd., Lot. No. DG118) was used for validation of the LC-APCI-MS/MS method. The serum or donated human plasma (0.5 mL) in a brown screw-capped Pyrex tube was supplemented with 3.6 ng (in 1 mL of ethanol) of PK-^{18}O, MK-4-^{18}O, and MK-7-^{18}O, respectively, as an internal standard. Extra ethanol (1 mL) was then added to denature the protein and 3 mL of hexane was added, followed by shaking for 5 min. The solution was centrifuged at 3000 rpm for 5 min, and the upper layer was separated. The supernatant was applied to Sep-Pak silica (Waters, USA), which was washed with 10 mL of hexane, and then eluted with 5 mL of hexane/diethyl ether (97:3). The eluate was evaporated under reduced pressure. The dried sample was reconstituted in 60 μL of ethanol and vortexed for 10 s. The solutions were transferred to microvials, capped, and placed in a SIL-10AD vp autosampler rack. Aliquots (30 μL) were automatically injected into the HPLC system.

Apparatus and HPLC Conditions. The HPLC analyses were conducted with a Shimadzu HPLC system (Simadzu, Kyoto, Japan) consisting of a binary pump (LC-10AD liquid chromatography), automatic solvent degasser (DGU-14A degasser), and autosampler (SIL-10AD autoinjector). Separations were carried out using a reversed-phase C_{18} analytical column (Capcell PAK C_{18} UG120, 5 μm; 4.6 mm i.d. × 250 mm) (Shiseido, Tokyo, Japan) with a solvent system consisting of an isocratic solvent A (25 min) and then a linear gradient from 0 to 50% ethanol (50 min). Solvent A

Table 1. LC-APCI-MS/MS Parameters

compound	precursor ion (m/z)	collision energy (V)	product ion(s)[a] (m/z)	Rt (min)
MK-4-^{18}O	449.3	29	191.2	20.8
MK-4	445.4	31	187.2	20.8
PK-^{18}O	455.4	35	191.3	41.3
PK	451.3	33	187.1	41.3
MK-7-^{18}O	653.5	39	191.3	74.9
MK-7	649.5	45	187.2	74.9

[a] Ion sequence according to descending abundance.

contained methanol/0.1% acetic acid aqueous (95:5, v/v) and was delivered at 1.0 mL/min. This mobile phase was passed through the column at 1.0 mL/min. The column was maintained at 40 °C with a column oven (CTO-10AC column oven). The HPLC system was controlled by a SCL-10A System Controller (Shimadzu). Acetic acid both functioned as an ion pair reagent during reversed-phase HPLC and facilitated formation of protonated vitamin K, $[M + H]^+$, in the positive ion mode with an APCI. The autosampler was maintained at 25 °C.

Apparatus and Mass Spectrometry. Mass spectrometry was performed with an API3000 LC–MS/MS System (Applied Biosystems, Foster City, CA), equipped with an APCI electrospray interface. All MS data were collected in the positive ion mode, The following settings were used: corona discharge needle voltage, 5.5 kV; vaporizer temperature, 400 °C; sheath gas (high-purity nitrogen) pressure, 50 psi; auxiliary gas, none; and transfer capillary temperature, 220 °C. The electron multiplier voltage was set at 850 eV. Identification and quantitation were based on MS/MS-MRM. The range for the parent scan was 400–500 atomic mass units (amu) in the case of PK, MK-4, and their ^{18}O-labeled compounds and 600–700 amu for MK-7 and MK-7-^{18}O. A complete overview of the MRM transitions, collision energy, retention time, and corresponding segment used for each analyte is given in Table 1.

Quantitation. A quantitative analysis was carried out using MS/MS-MRM of the precursor ion of vitamin K homologues (m/z 445 (MK-4), 449 (MK-4-^{18}O), 451 (PK), 455 (PK-^{18}O), 649 (MK-7), 651 (MK-7-^{18}O)), and their product ion (m/z 187 (natural vitamin K analogues), m/z 191 (^{18}O-labeled vitamin K analogues)) with a dwell time of 500 ms. Calibration, using internal standardization, was done by linear regression analysis using five different concentrations, 12.5, 25, 50, 100, and 200 ng/mL. The points were given by the calculated peak area ratio of standard and internal standard.

Precision and Accuracy. Interassay and intraassay precision and accuracy were evaluated using human control serum samples.

Sample Collection. Blood samples (10 mL) were collected into heparinized tubes from a convenient forearm vein. The samples were centrifuged at 2000*g* for 5 min at room temperature, and the plasma was separated and stored at −30 °C with shading until assay of vitamin K content.

Chemical Synthesis and Data Analysis. The synthetic method and the data on ^{18}O-labeled vitamin K homologues as internal standards are shown in Scheme 1 and described as follows. The data for compounds **5**, **6a**, **6b**, and **6c** were previously reported.[15,16] The 500-MHz ^{1}H NMR spectra of the synthetic compounds were measured on a Varian VXR-500. All compounds were dissolved in deuterized chloroform ($CDCl_3$) (Merk). Chemical shifts are given in ppm (δ) using tetramethylsilane as the internal standard. Mass spectra were registered on a JMS SX-102A instrument. Column chromatography was carried out on silica gel 60 F_{254} (Merk). Unless otherwise noted, all reagents were purchased from commercial suppliers and used as received.

2-Methyl-1,4-naphthalenediol, 1-Acetate (5). Vitamin K_1 monoacetyl derivative **5** was prepared by deacetylation of diacetyl **4** as the reported method.[16, 17] In short, a suspension of diacetate **4** (25.0 g, 97 mmol), K_2CO_3 (4.7 g, 34 mmol), and $Na_2S_2O_4$ (5.0 g, 29 mmol) in 15% aqueous MeOH (300 mL) was kept for 1 h at 30–40 °C with stirring and then poured into 1 L of cold water. The solution was extracted with ethyl acetate (3 × 300 mL), and the organic layers were combined, dried, and concentrated. The residue was purified by silica gel column chromatography using 5:1 hexane/ethyl acetate as eluent and then recrystallized from hexane and ethyl acetate to give **5** (18.8 g) in 90% yield.

4-Acetoxy-3-methyl-2-phytyl-1-naphthaleneol (6a). Phytol (500 mg, 1.7 mmol) was added dropwise to a solution of **5** (250 mg, 1.2 mmol) and BF_3·Et_2O (30 μL) in dry dioxane (300 μL) and ethyl acetate (300 μL). Then the mixture was heated to 50 °C for 3 h. The brown reaction mixture was poured into ice-cold water (30 mL) and extracted with ether (3 × 50 mL). The ether extracts were combined and washed with water (25 mL), dried, and concentrated. Purification by flash chromatography using 10:1 hexanes/ethyl acetate as eluent afforded **6a** (266 mg, 47% yield) as a pale yellow oil.

4-Acetoxy-2-geranylgeranyl-3-methyl-1-naphthaleneol (6b). Alcohol **6b** (360 mg, 53% yield) was obtained according to the procedure described above for **6a**.

4-Acetoxy-2-geranylgeranylfarnesyl-3-methyl-1-naphthaleneol (6c). Alcohol **6c** (800 mg, 57% yield) was obtained as described above for **6a**.

2-Methyl-3-phytyl-1,4-dimethoxynaphthalene (7a). A solution of **6a** (400 mg, 809 μmol) in THF (10 mL) was added, using a cannula, to a suspension of 30% potassium hydride, dispersion in mineral oil (128 mg, 3.20 mmol) in THF (20 mL), under argon at 0 °C. An additional 3 mL of THF was used to ensure a complete transfer. The dark green reaction mixture was warmed to room temperature. After 20 min, methyl iodide (230 μL, 3.71 mmol) was added and the mixture was stirred overnight. During this time a white precipitate appeared. The reaction mixture was cooled to 0 °C and quenched by careful addition of saturated aqueous ammonium chloride (10 mL), diluted with water (40 mL), and extracted with ether (4 × 25 mL). The ether layers were combined, dried, filtered, and concentrated to yield 412 mg of **7a** as pale yellow oil. Purification by flash chromatography using 5% ethyl acetate in hexane yielded 346 mg (89% yield) of **7a** as a pale yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81 (s, 3H), 0.82 (s, 3H), 0.85 (s, 3H), 0.87 (s, 3H), 1.00–1.38 (m, 16H), 1.81 (s, 3H), 1.94–1.98 (m, 2H), 2.38 (s, 3H), 3.57 (dd, J = 1.0, 6.5 Hz, 2H), 3.86 (s, 3H), 3.88 (s, 3H), 5.08–5.11 (m, 1H), 7.44–7.47 (m, 2H), 8.04–8.06 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.4, 16.3, 19.7, 22.6, 22.7, 24.5, 24.8, 25.4, 26.3, 28.0, 32.7, 32.8, 36.7, 37.3,

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37.4, 39.4, 40.0, 61.3, 62.2, 122.1, 122.3, 122.6, 125.2, 125.4, 126.9, 127.3, 127.5, 131.0, 136.1, 149.7, 150.1; HREIMS calcd for $C_{38}H_{52}{}^{16}O_2$ (M^+), 480.3967, found 480.3967.

2-Methyl-3-geranylgeranyl-1,4-di-methoxynaphthalene (7b). The compound **6b** (70 mg, 143 μmol) was converted to dimethoxynaphthalene (**7b**) (51 mg) in 75% yield as a colorless oil according to the procedure described above for obtaining **7a** from **6a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (s, 3H), 1.57 (s, 3H), 1.59 (s, 3H), 1.67 (s, 3H), 1.82 (s, 3H), 1.92–2.10 (m, 12H), 2.37 (s, 3H), 3.57 (dd, J = 0.5, 6.0 Hz, 2H), 3.86 (s, 3H), 3.88 (s, 3H), 5.06–5.13 (m, 4H), 7.43–7.46 (m, 2H), 8.03–8.06 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.4, 15.9, 16.0, 16.4, 17.6, 25.7, 26.3, 26.5, 26.6, 26.7, 39.7, 61.3, 62.1, 122.1, 122.2, 122.8, 124.0, 124.2, 124.4, 125.2, 125.4, 126.9, 127.2, 127.5, 130.9, 131.2, 134.9, 135.1, 135.7, 149.7, 150.1; HREIMS calcd for $C_{33}H_{48}{}^{16}O_2$ (M^+), 474.3498, found 474.3479.

2-Methyl-3-geranylgeranylfarnesyl-1,4-dimethoxynaphthalene (7c). The compound **6c** (80 mg, 101 μmol) was converted to dimethoxynaphthalene (**7c**) (58 mg) in 75% yield as a colorless oil according to the procedure described above for obtaining **7a** from **6a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.57 (s, 6H), 1.59 (s, 12H), 1.67 (s, 3H), 1.82 (s, 3H), 1.92–2.07 (m, 24H), 2.38 (s, 3H), 3.57 (d, J = 7.0 Hz, 2H), 3.87 (s, 3H), 3.88 (s, 3H), 5.06–5.13 (m, 7H), 7.44–7.46 (m, 2H), 8.03–8.06 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.4, 16.0, 16.4, 17.7, 25.7, 26.3, 26.6, 26.7, 26.8, 39.7, 61.3, 62.2, 122.1, 122.2, 122.8, 124.0, 124.2, 124.4, 125.2, 125.4, 126.9, 127.2, 127.5, 130.9, 131.3, 134.9, 135.1, 135.7, 149.7, 150.1; HREIMS calcd for $C_{48}H_{70}{}^{16}O_2$ (M^+), 678.5376, found 678.5374.

Phylloquinone-^{18}O (1). A solution of **7a** (60 mg, 124 μmol) in degassed acetonitrile (0.5 mL) and ether (0.1 mL) was added using a cannula to a solution of ceric ammonium nitrate (205 mg, 0.374 mmol) in degassed $H_2{}^{18}O$ (0.1 mL, >95% ^{18}O). An extra 0.3 mL of acetonitrile was used to ensure a complete transfer. After 20 min at room temperature, the reaction mixture was treated with water (10 mL) and ether (10 mL). The aqueous layer was extracted with ether (25 mL), and the ether layers were combined, washed with water (4 × 15 mL), dried, filtered, and concentrated to yield 35 mg (62% yield) of yellow oil. The crude material of **1** was purified by flash chromatography through silica gel using 5% ethyl acetate in hexane as the eluent. Analysis of LC-APCI-MS/MS showed a 95.5% incorporation of ^{18}O; ^{13}C NMR showed 95.3% labeling: ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.81 (s, 3H), 0.82 (s, 3H), 0.83 (s, 3H), 0.87 (s, 6H), 0.98–1.41 (m, 16H), 1.78 (s, 3H), 1.92–1.96 (m, 2H), 2.20 (s, 3H), 3.37 (d, J = 7.0 Hz, 2H), 5.01 (dt, J = 1.0, 7.0 Hz, 1H), 7.67–7.71 (m, 2H), 8.06–8.10 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.7, 16.3, 19.7, 22.6, 22.7, 24.4, 24.5, 24.8, 25.3, 26.0, 28.0, 32.6, 32.8, 36.6, 37.3, 37.4, 39.4, 40.0, 118.8, 126.2, 126.3, 132.16, 132.20, 133.26, 133.32, 138.0, 143.3, 146.2, 184.5, 185.4; HREIMS calcd for $C_{31}H_{46}{}^{18}O_2$ (M^+), 454.3583, found 454.3588.

Menaquinone-4-^{18}O (2). The compound **7b** (70 mg, 147 μmol) was converted to menaquinone-4 (**2**) (40 mg) in 60% yield as a pale yellow oil according to the procedure described above for obtaining **1** from **7a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.55 (s, 3H), 1.56 (s, 3H), 1.59 (s, 3H), 1.67 (s, 3H), 1.79 (s, 3H), 1.91–2.09 (m, 12H), 2.19 (s, 3H), 3.37 (d, J = 7.5 Hz, 2H), 5.00–5.10 (m, 4H), 7.67–7.70 (m, 2H), 8.06–8.10 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.7, 15.95, 16.01, 16.4, 17.7, 25.7, 26.0, 26.5, 26.6, 26.7, 39.7, 119.1, 123.9, 124.2, 124.4, 126.2, 126.3, 131.2, 132.2, 133.26, 133.32, 134.9, 135.2, 137.6, 143.3, 146.2, 184.5, 185.4; HREIMS calcd for $C_{31}H_{40}{}^{18}O_2$ (M^+), 448.3113, found 448.3111.

Menaquinone-7-^{18}O (3). The compound **7c** (80 mg, 118 μmol) was converted to menaquinone-4 (**3**) (42 mg) in 55% yield as a pale yellow oil according to the procedure described above for obtaining **1** from **7a**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.56 (s, 6H), 1.59 (s, 12H), 1.67 (s, 3H), 1.79 (s, 3H), 1.91–2.09 (m, 24H), 2.19 (s, 3H), 3.37 (d, J = 7.0 Hz, 2H), 5.00–5.13 (m, 7H), 7.67–7.70 (m, 2H), 8.07–8.09 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.7, 15.95, 16.01, 16.4, 17.7, 25.7, 26.0, 26.5, 26.6, 26.7, 26.8, 39.7, 119.1, 123.9, 124.2, 124.4, 126.2, 126.3, 131.2, 132.2, 133.27, 133.28, 134.9, 135.2, 137.6, 143.4, 146.2, 184.5, 185.5; HREIMS calcd for $C_{46}H_{64}{}^{18}O_2$ (M^+), 652.4991, found 652.4984

RESULTS AND DISCUSSION

Method Development. The separation of vitamin K derivatives and the respective internal standards in human plasma was achieved in 80 min. After a wash of the column and reequilibration period of 40 min, the next sample was injected. The reliability of the LC method was evaluated based on the variation in retention times. The relative standard deviation (RSD), calculated from retention times obtained from over 30 injections, proved to be less than 1.0% for all compounds, indicating good chromatographic stability.

The precursor and product ion(s) for each analyte of interest was determined by the direct infusion of single-analyte solutions (1 μg/mL in ethanol). After optimization of the separation process and selection of a unique precursor–product ion combination for each compound, a quantitative LC-APCI-MS/MS method was developed based on MRM.

To ensure maximum sensitivity in the MS analysis, the chromatographic run was divided into seven segments. Each segment was optimized for the compounds of interest eluted within a given time period. The following mass spectrometric parameters were specified within each segment: transfer capillary voltage, tube lens voltage, ion optic voltage, collision energy, and MRM scan events. Table 1 shows an overview of the MS parameters including MRM transitions, collision energy, and retention time. The MRM chromatograms for the target analytes were obtained from the injection of a standard mixture (10 ng). The retention times of the standard and internal standard peaks completely matched; namely, this result proved that the compounds had the same chemical properties.

^{18}O-Labeled Internal Standards. As a preliminary experiment, we tried to detect PK and MK-n (n= 1–10) in several human plasma samples using LC-APCI-MS/MS. PK, MK-4, and MK-7 were found as major peaks. Therefore, we chose stable isotope-labeled vitamins as internal standards to measure these three kinds of vitamin K. The stable isotopes are nonradioactive forms of elements that occur naturally within the environment and have applications for human research. There has been much recent study using stable isotope-labeled vitamins and provitamins, such as [$^2H_7$]MK-4 (deuterated MK-4) and [$^2H_7$]PK (deuterated PK), to examine metabolic pathways in vitro and in vivo using mass spectrometry.[14,18] We used isotope ^{18}O-labeled vitamin K

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analogues as internal standards, namely, MK-4-^{18}O, PK-^{18}O, and MK-7-^{18}O.[19,20] The efficiency of extraction can be adjusted accurately since the chemical properties of the labeled analogues are almost the same as those of the original substrates. The synthesis of the requisite ^{18}O-labeled vitamin K homologues was carried out as shown in Scheme 1. These analogues are much easier and more convenient to synthesize than, for example, deuterated vitamin K. First, we chose monoacetate **4** to afford a coupling intermediate with an alkyl side chain, though there are many different schemes for the synthesis of vitamin K derivatives.[15,21] The compound **5** was prepared from 1,4-hydroquinone diacetate **4** by selective hydrolysis of 4-*O*-acetate with sodium hydrosulfite.[16,17] Treatment of the monoacetate **5** with an alkyl side chain alcohol (phytol, geranylgeraniol, geranygeranylfarnesol) in the presence of boron trifluoride etherate yielded **6a**–**6c** as reported.[16] To afford the dimethyl ether **7a**–**7c**, the monocetate **5** was treated with an excess amount of potassium hydride followed by methyl iodide to give **7a**–**7c** in good yield. Finally, oxidation of the naphthohydroquinone methyl ether **5** with Ce-$(NH_4)_2(NO_3)_6$ in $H_2{}^{18}O$ gave the desired ^{18}O-labeled vitamin K homologues **1**–**3** in good yield. Thus, three kinds of stable isotope-labeled compounds were prepared for LC-APCI-MS/MS detection.

Calibration and Validation. Calibration using internal standardization with standard samples and ^{18}O-labeled analogues was performed. Under the stated conditions, stock solutions proved to be stable for at least 3 months. Analyte recoveries in the stability experiments were within the variability range obtained for precision and accuracy. No significant loss or deterioration of any of the compounds of interest was observed. Analytes were stable during sample pretreatment at room temperature.

The method fulfilled our analytical standard criteria. MRM provided high specificity for all of the compounds, and no cross-talk interference with the ^{18}O-labeled internal standards was observed. The positive precursor ion APCI-MS of PK, MK-4, MK-7, and their ^{18}O-labeled forms showed base peaks at m/z 451, 445, 649, 455, 449, and 653 corresponding to protonated molecules, respectively. While the product ion of PK, MK-4, and MK-7 was m/z 187, that of their labeled analogues was m/z 191.[22] Each of the calibration curves could be drawn as linear through zero. All values were calculated as ratios (intensity of analyte area)/(that of internal standard area). Over the range of vitamin K concentrations, 12.5–200 ng/mL, positive ion APCI produced a linear response and the each correlation coefficient of the calibration curves in PK, MK-4, and MK-7 were 0.9993, 0.9997, and 0.9999. LC-APCI-MS/MS was used throughout this investigation because of its wide dynamic range and linearity of detector response. Thus, vitamin K could be directly detected without conversion to other derivatives.

Figure 1 shows the MRM chromatograms for the target analytes obtained from the injection of human plasma samples. We examined recovery and accuracy using commercially available pooled plasma. The injection samples were obtained according to the procedure described above. All measurements were well performed, and each peak of the vitamin K analogues was clearly afforded as a single peak.

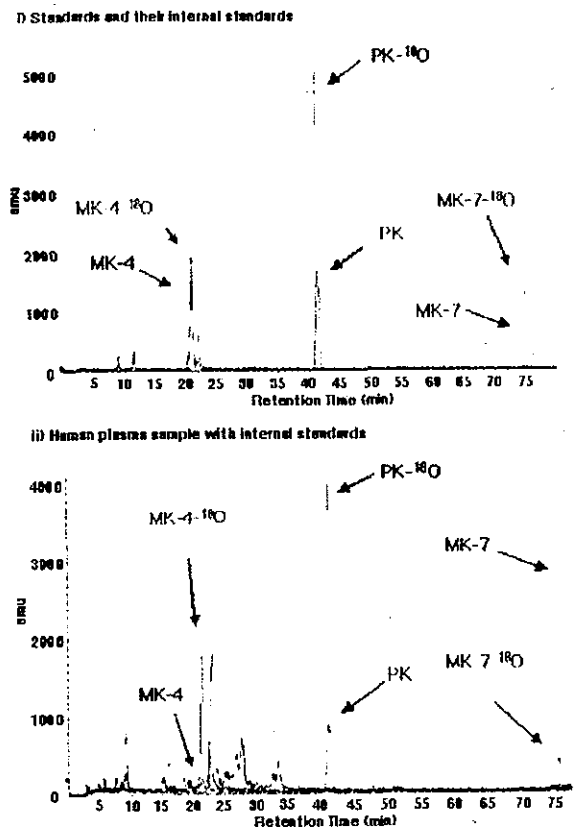

Figure 1. LC-APCI-MS/MS chromatograms of the analytes of vitamin K Homologues for the injection of (i) standard mixture and (ii) human plasma sample with internal standards. PK, phylloquinone; MK-4, menaquinone-4; MK-7, menaquinone-7; PK-^{18}O, ^{18}O-labeled phylloquinone; MK-4-^{18}O, ^{18}O-labeled menaquinone-4; MK-7-^{18}O, ^{18}O-labeled menaquinone-7.

After the analytes were extracted from 0.5 mL of plasma sample including 3.6 ng/mL internal standard, they were concentrated to 60 μL in an ethanol solution. The concentrations of both internal standards in the samples and calibration curves were 50 ng/mL. The data were converted to the concentration in 1 mL of plasma according to the following equation: vitamin K in plasma concentration (ng/mL) = measured data × (6/5) × (60/500).

As shown in Table 2, the average intraassay and interassay variation (RSD) for PK, MK-4, and MK-7 was less than 10%. Average spiked recoveries from authentic compounds (PK, 0.95 ± 0.04 ng/mL; MK-4, 0.27 ± 0.01 ng/mL; MK-7, 1.44 ± 0.08 ng/mL) added to normal human pool serum samples for PK, MK-4, and MK-7 were 98–102%. With this method, the lower quantitation limits were less than 0.1 ng/mL (PK, 40 pg/mL; MK-4, 50 pg/mL; MK-7, 80 pg/mL). The quantitation limits of plasma vitamin K concentrations are different from species. Indeed, the signal-to-noise ratio depends on impurities in plasma samples. As far as we know, the rat plasma or serum vitamin K concentrations were easy to determine in comparison with human plasma because

(19) Naganathan, S.; Hershline, R.; Ham, S. W.; Dowd, P. *J. Am. Chem. Soc.* **1994**, *116*, 9831–9839.
(20) Dowd, P.; Ham, S.-W.; Hershline, R. *J. Am. Chem. Soc.* **1992**, *114*, 7613–7617.
(21) Naruta, Y. *J. Org. Chem.* **1980**, *45*, 4097–4104.
(22) Di Mari, S. J.; Supple, J. H.; Rapoport, H. *J. Am. Chem. Soc.* **1966**, *88*, 1226–1232.

Table 2. Summary of Assay Method for Vitamin K[a]

		PK	MK-4	MK-7
quantitation limit (pg/mL)		40	50	80
recovery	mean ± SD (ng/mL)	1.89 ± 0.05	0.43 ± 0.02	3.19 ± 0.20
	RSD (%)	2.65	4.65	6.27
	recovery (%)	98 ± 3	102 ± 5	102 ± 6
intraassay control serum	mean ± SD (ng/mL)	0.95 ± 0.04	0.27 ± 0.01	1.44 ± 0.08
	RSD (%)	6.21	4.79	5.85
interassay control serum	mean ± SD (ng/mL)	1.03 ± 0.06	0.21 ± 0.02	1.51 ± 0.04
	RSD (%)	6.21	9.10	2.97

[a] Recovery, intraassay, and interassay: $n = 5$.

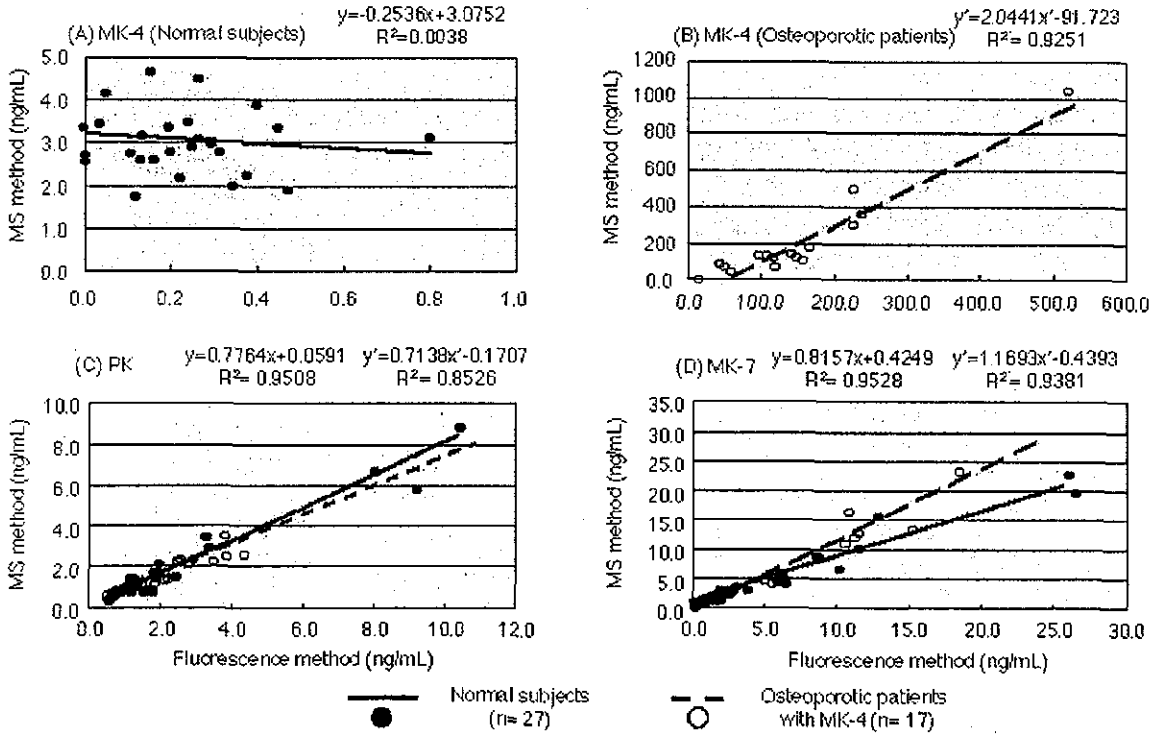

Figure 2. Comparison of assayed values (ng/mL) of human plasma vitamin K homologues between the LC-APCI-MS/MS method (MS method) and HPLC with fluorescence detection (fluorescence method). (A) MK-4 (normal subjects); (B) MK-4 (osteoporotic patients); (C) PK (normal subjects and osteoporotic patients); (D) MK-7 (normal subjects and osteoporotic patients).

impurities were not observed (data are not shown). These results proved our system was reliable and reproducible for the measurement of plasma vitamin K.

Plasma Concentration Profile of Vitamin K Analogues. Next we examined healthy subjects ($n = 20$) using this method. The accuracy of determination was improved according to the internal standards. The mean plasma concentrations of PK, MK-4, and MK-7 from the subjects were 1.22 ± 0.57, 0.39 ± 0.46, and 6.37 ± 7.45 ng/mL, respectively. While plasma levels of vitamin K analogues in osteoporotic patients on a MK-4 supplement were 1.90 ± 0.78, 172.90 ± 138.56, and 7.03 ± 5.79 ng/mL.

To compare the conventional fluorescence method and LC-APCI-MS/MS detection, we examined another 27 plasma samples using both methods. The concentrations of PK and MK-7 correlated although the fluorescence method gave slightly higher concentrations than the LC-APCI-MS/MS method as shown in Figure 2C and D. However, the plasma levels of MK-4 were not related at all. We also examined 17 plasma samples from osteoporotic patients given an MK-4 supplement. The concentrations of all vitamin K derivatives were correlated to some extent (Figure 2B–D). This result suggested that the detection of MK-4 using these methods was not related to the low concentration range (less than 1.0 ng/mL) (Figure 2A) but correlated with the higher concentration range (Figure 2B). Presumably, impurities in the plasma sample are at least partly responsible for the result.

Our LC-APCI-MS/MS system has much greater sensitivity and selectivity in comparison with the conventional method. As we described above, the conventional method has various disadvantages such as interference from impurities in human plasma and correction of extractive efficiency. Most conventional methods do not use any internal standards or often use another kind of vitamin K homologue. Extractive efficiency is important for accurate

determination of plasma concentration. In our method, extractive efficiency from plasma can be completely adjusted using ^{18}O-labeled vitamin K analogues as internal standards, which have the same chemical properties as the original vitamin K homologues. Therefore, the accuracy of determination was remarkably improved. Though the conventional method is certainly a common system, our method is useful to make gold standards of plasma vitamin K concentrations.

CONCLUSION

This study shows that LC-APCI-MS/MS provides a rapid and relatively easy-to-use approach to the quantitation of vitamin K analogues in human plasma without compromising assay sensitivity. This approach overcame major disadvantages of previous methods. For the analysis of both the analyte and internal standard, the method has been thoroughly validated. The sensitivity has been shown to be excellent, with no interference from impurities. The interday precision for the analyte was less than 10% RSD. Furthermore, throughout our study, the HPLC column used remained stable.

We conclude that this novel LC-APCI-MS/MS method using ^{18}O-labeled internal standards should be convenient for the evaluation of vitamin K status in human plasma; therefore, the data will provide useful information for the treatment and prevention of osteoporosis with vitamin K.

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SUPPORTING INFORMATION AVAILABLE

Calibration curves for vitamin D homologues. This material is available free of charge via the Internet at http://pubs.acs.org.

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Determination of plasma Vitamin K by high-performance liquid chromatography with fluorescence detection using Vitamin K analogs as internal standards

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Abstract

A HPLC fluorescence determination method for Vitamin K derivatives (Vitamin K_1, phylloquinone, PK and K_2, menaquinones, MK-4 and MK-7) using post-column reduction and internal standards was developed. Selectivity and reproducibility were increased by optimized chromatography conditions and satisfactory precision and accuracy were attained by using synthetic internal standards. After addition of internal standards to plasma samples, lipids were extracted with ethanol and hexane. Chromatography was performed by isocratic reverse phase separation on a C18 column. Vitamin K derivatives were detected at 430 nm with excitation at 320 nm for MK-4 and 240 nm for PK and MK-7. The detection limits for MK-4, PK and MK-7 were 4, 2 and 4 pg, respectively. The recoveries of MK-4, PK and MK-7 were greater than 92% and the inter- and intra-assay R.S.D. values were 5.7–9.2% for MK-4, 4.9–9.6% for PK and 6.3–19.3% for MK-7. The data showed good correlation between proposed method and LC-APCI/MS method for MK-4 ($R^2 = 0.988$), PK ($R^2 = 0.979$) and MK-7 ($R^2 = 0.986$). The method allows the determination of Vitamin K for evaluating their clinical and nutritional status.

Keywords: Vitamin K; Internal standards; Fluorescence detection

1. Introduction

Vitamin K is a cofactor for an enzyme that converts specific glutamyl residues in several proteins such as plasma clotting factors II (prothrombin), VII, IX and X, protein C, S and Z, osteocalcin (bone Gla protein), matrix Gla protein to γ-calboxylglutamyl (Gla) residues. These Vitamin K-dependent proteins play crucial roles in homeostasis and calcification [1,2]. It is well known that neonatal and infantile Vitamin K deficiency causes melena neonatorum and intracranial hemorrhagic disorders. In addition, several reports indicate an important role for Vitamin K in bone health. Administration of Vitamin K results in an increase in bone-mineral density and a reduction in bone resorption in humans [3–6] and rodents [7–13].

There are two major forms of Vitamin K in nature. Vitamin K_1 (phylloquinone, PK) is produced by plants and algae, and Vitamin K_2 series (menaquinones, MKs) is synthesized by bacteria (Fig. 1). The length of the isoprenoid side-chain in menaquinones is defined by its carbon number, or the number of isoprenoid units. The major dietary form of Vitamin K has been considered to be PK, which is contained in green and leafy vegetables [14,15]. In contrast, MKs are found in fermented food and in the colon, where they are synthesized by the intestinal microflora [16]. However, little is known about Vitamin K status in humans. It is difficult to determine plasma Vitamin K because basal concentrations of Vitamin K in plasma are very low. In addition, MK-4 has received governmental approval for use as an agent for the treatment of osteoporosis in Japan. Thus, measurement of MK-4

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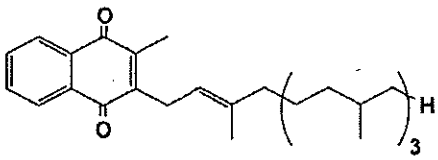
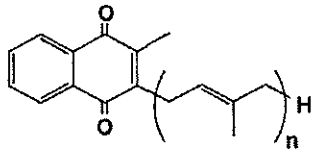
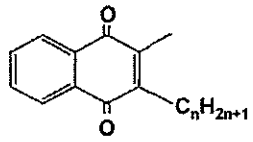


Vitamin K_1 (phylloquinone, PK)



Vitamin K_2 (menaquinone, MK-n)



Internal standard (I.S.-C_n)

Fig. 1. Structures of natural Vitamin K_1, K_2 and internal standards.

concentration in plasma of osteoporotic patients is important in therapeutic drug monitoring.

Previously, several methods for separation and determination of Vitamin K by thin layer chromatography (TLC) [17], gas liquid chromatography (GLC) [18,19] and high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection [20], fluorescence detection [21–25], electrochemical detection [26–29] and mass spectrometric detection [30] were reported. Recently, we have developed a method for the determination of Vitamin K in human plasma by liquid chromatograpy-atmospheric pressure chemical ionization/mass spectrometry (LC-APCI/MS) [31]. Although this method has great advantage in high sensitivity and accuracy, it is very expensive for routine assay. In contrast, the separation and detection by HPLC with fluorescence detection using post-column chemical reduction is relatively high sensitive, convenient and stable. Vitamin Ks are reduced by the platinum-reduction column and converted into Vitamin K hydroquinones, which are highly fluorescent. However, there is still the problem in separation of Vitamin K from interfering compounds in plasma. Especially, it is difficult to determine MK-4 accurately in a routine assay, because basal plasma concentration of MK-4 is markedly lower than PK and elute at the same retention time of a number of interfering compounds in plasma. Also, it is assumed that use of internal standards is necessary due to the loss of Vitamin K during extraction. One of natural Vitamin K derivatives, MK-6, has been used widely as internal standard [32], because human circulating levels are undetectable. However, synthetic compounds are more suitable for internal standards in terms of application to various samples such as animal tissue or foods. In this paper, we describe improved highly sensitive assay method for Vitamin K in human plasma using two kinds of HPLC systems, optimized for determination of MK-4 and less polar derivatives, PK and MK-7. We also synthesized Vitamin K analogs with different length of the alkyl side-chain (Fig. 1) as internal standards and selected suitable synthetic Vitamin K analogs for determination of MK-4, PK and MK-7.

2. Experimental

2.1. Chemicals and reagents

PK, MK-4, MK-5, MK-6, MK-7, MK-8, MK-9 and MK-10 were kindly provided by Eisai Pharmaceuticals (Tokyo, Japan). The Vitamin K analogs with different length of the alkyl side-chain as internal standards were synthesized in our laboratory as described below. ^{18}O-labeled MK-4, PK and MK-7 which are replaced both oxygen atoms at quinone structure to ^{18}O were also synthesized in our laboratory as described previously [31]. The isotopic purity of ^{18}O-labeled MK-4, PK and MK-7 are 95%, respectively. HPLC-grade solvents and reagents for chemical synthesis were purchased from Nacalai Tesque, Inc. (Kyoto, Japan) and Wako Pure Chemical Industries Ltd. (Osaka, Japan). Control human serum was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

2.2. Synthesis of internal standards

The synthetic method of Vitamin K analogs with different length of the alkyl side-chain was previously reported [33]. A solution of 1 g $(NH_4)_2S_2O_8$ in 10 mL water was added dropwise over 90–120 min to a stirred suspension of 10 mL water, 20 mL acetonitrile, 0.25 g $AgNO_3$, 0.2 g Vitamin K_3 (2-methyl-1,4-naphthoquinone) and 1.5 mmol fatty acid ($n = 13$, 14, 15, 16, 18, 19, 20, 23) at 65–75 °C. After stirring for another 30 min, the resulting mixture was cooled, extracted with ether, washed with water, dried, filtered, and concentrated. Purified products with yield of 50–65% as yellow needles were obtained after flash chromatography through silica gel 60 (Merck, Darmstadt, Germany) using hexane-ethyl acetate (20:1, v/v) and recrystallization. The 500 MHz ^1H NMR spectra of the synthetic compounds were measured on a Varian VXR-500. All compounds were dissolved in 0.3 mL of deuterated chloroform ($CDCl_3$, Merk). Chemical shifts are given in ppm (δ) using tetramethylsilane (TMS) as the internal standard. Mass spectra were obtained using M-4100 (Hitachi, Tokyo, Japan).

2.2.1. 2-Methyl-3-trydecyl-1,4-naphthoquinone (Code: I.S.-C13)

^1H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, $J = 6.8$, 3H), 1.15–1.38 (m, 16H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, $J = 7.8$, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.11, 22.68, 27.10, 28.76, 29.34, 29.43, 29.53, 29.62, 29.63, 29.66, 29.99, 31.91,

126.15, 126.25, 132.16, 132.21, 133.26, 133.29, 143.07, 147.59, 184.72, 185.40; HREIMS calcd for $C_{24}H_{34}O_2$ (M^+), 354.2559, found 354.2561.

2.2.2. 2-Methyl-3-tetradecyl-1,4-naphthoquinone (Code: I.S.-C14)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=7.0, 3H), 1.15–1.38 (m, 18H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ12.63, 14.11, 22.68, 27.10, 28.76, 29.34, 29.43, 29.54, 29.62, 29.64, 29.65, 29.68, 30.00, 31.91, 126.15, 126.25, 132.16, 132.21, 133.26, 133.29, 143.07, 147.59, 184.72, 185.40; HREIMS calcd for $C_{25}H_{36}O_2$ (M^+), 368.2715, found 368.2717.

2.2.3. 2-Methyl-3-pentadecyl-1,4-naphthoquinone (Code: I.S.-C15)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=6.8, 3H), 1.15–1.38 (m, 20H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.11, 22.68, 27.10, 28.76, 29.35, 29.43, 29.54, 29.62, 29.64, 29.66, 29.67, 29.69, 30.00, 31.91, 126.15, 126.25, 132.16, 132.21, 133.26, 133.29, 143.07, 147.59, 184.72, 185.40; HREIMS calcd for $C_{26}H_{38}O_2$ (M^+), 382.2872, found 382.2866.

2.2.4. 2-Methyl-3-hexadecyl-1,4-naphthoquinone (Code: I.S.-C16)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=6.8, 3H), 1.15–1.38 (m, 22H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.12, 22.69, 27.11, 28.76, 29.35, 29.43, 29.54, 29.63, 29.65, 29.66, 29.67, 29.68, 29.69, 30.00, 31.91, 126.16, 126.26, 132.16, 132.21, 133.26, 133.30, 143.07, 147.60, 184.73, 185.41; HREIMS calcd for $C_{27}H_{40}O_2$ (M^+), 396.3028, found 396.3025.

2.2.5. 2-Methyl-3-octadecyl-1,4-naphthoquinone (Code: I.S.-C18)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=6.8, 3H), 1.15–1.38 (m, 26H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.11, 22.68, 27.11, 28.76, 29.35, 29.43, 29.54, 29.62, 29.64, 29.65, 29.66, 29.69, 30.00, 31.92, 126.16, 126.26, 132.16, 132.21, 133.26, 133.29, 143.07, 147.59, 184.72, 185.40; HREIMS calcd for $C_{29}H_{44}O_2$ (M^+), 424.3341, found 424.3344.

2.2.6. 2-Methyl-3-nonadecyl-1,4-naphthoquinone (Code: I.S.-C19)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=6.8, 3H), 1.15–1.38 (m, 28H), 1.40 (m, 2H), 1.47 (m, 2H), 1.56 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.12, 22.69, 27.11, 28.77, 29.36, 29.43, 29.55, 29.63, 29.65, 29.67, 29.68, 29.69, 30.00, 31.92, 126.16, 126.26, 132.16, 132.21, 133.26, 133.30, 143.07, 147.60, 184.73, 185.41; HREIMS calcd for $C_{30}H_{46}O_2$ (M^+), 438.3498, found 438.3497.

2.2.7. 2-Methyl-3-icosyl-1,4-naphthoquinone (Code: I.S.-C20)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=7.0, 3H), 1.15–1.38 (m, 30H), 1.40 (m, 2H), 1.47 (m, 2H), 1.57 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.11, 22.69, 27.11, 28.76, 29.35, 29.43, 29.54, 29.63, 29.65, 29.66, 29.68, 29.69, 30.00, 31.92, 126.16, 126.26, 132.16, 132.21, 133.26, 133.30, 143.07, 147.60, 184.73, 185.41; HREIMS calcd for $C_{31}H_{48}O_2$ (M^+), 452.3654, found 452.3658.

2.2.8. 2-Methyl-3-tricosyl-1,4-naphthoquinone (Code: I.S.-C23)

^{1}H NMR (500 MHz, $CDCl_3$) δ 0.88 (t, J=6.8, 3H), 1.15–1.38 (m, 36H), 1.40 (m, 2H), 1.47 (m, 2H), 1.55 (m, 2H), 2.19 (s, 3H), 2.63 (t, J=7.8, 2H), 7.69 (m, 2H), 8.08 (m, 2H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 12.63, 14.11, 22.65, 27.11, 28.76, 29.35, 29.43, 29.54, 29.63, 29.65, 29.67, 29.68, 29.69, 30.00, 31.92, 126.16, 126.27, 132.18, 132.23, 133.26, 133.30, 143.08, 147.61, 184.73, 185.41; HREIMS calcd for $C_{34}H_{54}O_2$ (M^+), 494.4124, found 494.4126.

2.3. Sample preparation

For the developmental work on the assay, control serum (Wako Pure Chemical Industries Ltd.) and plasma of healthy subjects were used. The plasma samples from healthy subjects and osteoporotic patients treated with MK-4 were obtained through the kind help of Dr. M. Shiraki, the Research Institute and Practice for Involutional Disease. Exactly 0.5 mL of serum or plasma sample was placed in a brown tube and diluted with distilled water to 1 mL. After addition of internal standards solution (I.S.-C16 and I.S.-C19, 1 ng/50 μL each), the diluted sample was extracted with 1.9 mL of ethanol and 3 mL of hexane. The mixture was shaken for 5 min before centrifuging at 3000 rpm for 5 min, 2.5 mL of hexane layer was passed through a Sep-Pak silica cartridge (Waters, Milford, MA, USA) that was washed with 10 mL of hexane. Vitamin K was eluted with 5 mL of hexane-diethyl ether (97:3). The eluate was evaporated under reduced pressure, and the residue was dissolved with 200 μL of ethanol. Forty microlitre aliquots were subjected to two kinds of HPLC, System 1 for determination of MK-4 and System 2 for determination of PK and MK-7. The recoveries of MK-4, PK and MK-7 were evaluated using control serum spiked MK-4 (0.1, 0.2 or 0.4 ng/0.5 mL serum), PK (0.4, 0.8 or 1.6 ng/0.5 mL serum) and MK-7 (0.75, 1.5 or 3.0 ng/0.5 mL serum) (n=5). To evaluate the precision (intraassay and interassay), control serum and plasma of healthy subject were used. To examine the correlation with LC-APCI/MS method, plasma samples from healthy subjects (n=20, age 59–82) and osteoporotic patients treated with MK-4 (n=10; age, 47–85; daily dose of MK-4, 45 mg; attending period, 4.0–12.2 years)

were used. For LC-APCI/MS analyses, ^{18}O-labeled MK-4, PK and MK-7 were used as internal standards.

2.4. HPLC apparatus and conditions for fluorescence detection

The HPLC system consisted of a LC-10AD$_{VP}$ pump (Shimadzu, Kyoto, Japan), a SIL-10AD$_{VP}$ auto injector (Shimadzu), a CTO-10AD$_{VP}$ column oven (Shimadzu) set to 35 °C, and a RF-10A$_{XL}$ fluorescence detector set to an excitation wavelength of 320 nm (System 1: for determination of MK-4) or 240 nm (System 2: for determination of PK and MK-7) and an emission wavelength of 430 nm. The data acquired by C-R8A chromatopac (Shimadzu) were processed by CLASS-PR10 software (Shimadzu). Separations were performed on a CAPCELL PAK C18 UG120 (4.6 mm × 250 mm, 5 μm, Shiseido Co. Ltd., Tokyo, Japan). A RC-10 platinum-reduction column (4.0 mm × 15 mm, Irica, Kyoto, Japan) was located between HPLC column and the fluorescence detector for postcolumn reduction.

Analysis was performed using an isocratic eluent system. For determination of MK-4, the mobile phase was a 95:5 (v/v) mixture of methanol and water (System 1). For determination of PK and MK-7, the mobile phase was a 95:5 (v/v) mixture of methanol and ethanol (System 2). The flow-rate was 1.0 mL/min in both systems.

For determination of MK-4, standard solutions containing MK-4 (1, 5 and 10 ng/mL), and I.S.-C16 (5 ng/mL) were used. For determination of PK and MK-7, standard solutions containing PK and MK-7 (1, 5 and 10 ng/mL each), and I.S.-C19 (5 ng/mL) were used. The calibration curve was constructed by plotting the peak-area ratio of Vitamin K to internal standard versus molar ratio of Vitamin K to internal standard. The concentration of MK-4, PK and MK-7 in plasma (ng/mL) was calculated using the following formula:

$$\text{Concentration of MK-4, PK or MK-7} = \frac{RS}{V}$$

where R is quantitative ratio of Vitamin K to internal standard obtained from calibration curve, S is added amount of internal standards (1 ng), V is volume of a sample (mL) taken for assay (0.5 mL).

2.5. LC-APCI/MS apparatus and conditions

The HPLC system consisted of a SCL-10ADvp system controller (Shimadzu), a LC-10AD$_{VP}$ pump (Shimadzu), DGU-14A automatic solvent degasser (Shimadzu), a SIL-10AD$_{VP}$ auto injector (Shimadzu), and a CTO-10AD$_{VP}$ column oven (Shimadzu) set to 40 °C. Separations were carried out using a CAPCELL PAK C18 UG120 (4.6 mm × 250 mm) with a solvent system consisting of an isocratic solvent A (methanol–0.1% acetic acid, 95:5, v/v) in 25 min and then a linear gradient from 0 to 50% ethanol in 50 min. Mass spectrometry was performed with an API3000 LC/MS/MS System (Applied Biosystems, Foster City, CA, USA), equipped with an APCI electrospray interface. All MS data were collected in positive ion mode. The following APCI/MS parameter settings were applied: corona discharge needle voltage, 4.5 kV; vaporizer temperature, 400 °C; sheath gas (high-purity nitrogen) pressure, 70 psi; no auxiliary gas; and transfer capillary temperature, 220 °C. The electron multiplier voltage was set at 850 eV. The scan range for the parent scan was 400–500 atomic mass units (amu) in case of PK, MK-4, and their ^{18}O-labeled compounds, 600–700 amu in MK-7 and ^{18}O-MK-7. Quantitative analysis was carried out using MS/MS-multiple reaction monitoring (MRM) of precursor ion of Vitamin K homologues (*m/z* 445, MK-4; 449, ^{18}O-MK-4; 451, PK; 455, ^{18}O-PK; 649, MK-7; 651, ^{18}O-MK-7) and their product ion (*m/z* 187, natural Vitamin Ks; 191, ^{18}O-labeled Vitamin Ks) with a dwell time of 500 ms. Calibration, using internal standardization, was done by linear regression analysis using four different concentration ranges from 12.5 to 200 ng/mL. The concentration of MK-4, PK and MK-7 in plasma was calculated as fluorescence detection.

3. Results and discussion

3.1. Optimal HPLC systems and internal standards

PK, MK-4 and MK-7 were detected in almost all plasma samples from healthy subjects tested here. In contrast, MK-5, MK-6, MK-8, MK-9 and MK-10 were not detected in all samples. Thus, it was decided to measure PK, MK-4 and MK-7 in this study using two kinds of HPLC systems. In case of determination of MK-4 in control serum and plasma of healthy subjects, MK-4 was not separated completely from interfering compounds in serum or plasma by a mixture of methanol and ethanol, which was used as mobile phase generally (data not shown). In addition, the peak which has retention times very close to that of MK-4 was observed during the analysis without reduction. To separate MK-4 from interfering compounds, HPLC System 1 (retention time of MK-4, 23.74 min) was used. In System 1, unknown peaks, which eluted at the same retention time as MK-4 was not observed with or without reduction column. For quantitative analysis of MK-4, I.S.-C16, which was found as a single peak, was chosen as an internal standard. The retention times of Vitamin K analogs in System 1 were as follows: I.S.-C13, 21.88 min; I.S.-C14, 27.41 min; I.S.-C15, 32.43 min; I.S.-C16, 41.98 min. Fig. 2 shows the chromatographic profiles of authentic MK-4 and I.S.-C16 (Fig. 2A), a representative plasma sample from healthy subject with post-column reduction (Fig. 2B) and the same plasma sample without post-column reduction (Fig. 2C) in System 1.

Utilizing a 95:5 (v/v) mixture of methanol and ethanol as mobile phase and the detection at 430 nm with excitation at 320 nm were effective for the separation of PK from other substances in serum or plasma, however, MK-7 was not

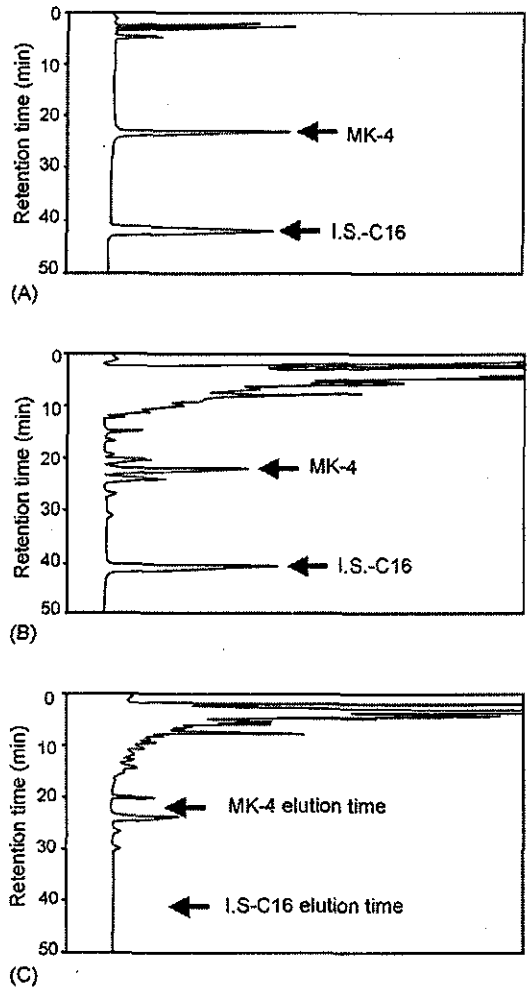


Fig. 2. Chromatograms of authentic standards and a plasma sample in System 1. (A) authentic standards of MK-4 and I.S.-C16; (B) a plasma sample obtained from a healthy subject with reduction by the platinum-reduction column; and (C) a plasma sample obtained from a healthy subject without reduction by the platinum-reduction column.

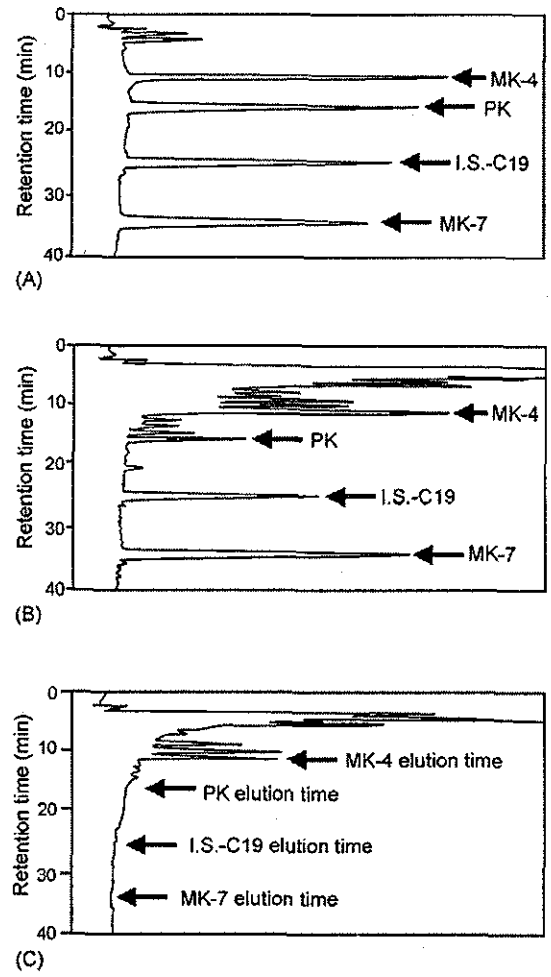


Fig. 3. Chromatograms of authentic standards and a plasma sample in System 2. (A) authentic standards of MK-4, PK, I.S.-C19 and MK-7; (B) a plasma sample obtained from the same subject as shown in Fig. 2 with reduction by the platinum-reduction column; and (C) a plasma sample obtained from the same subject as shown in Fig. 2 without reduction by the platinum-reduction column.

successfully isolated from deterrent. HPLC chromatograms were obtained by injecting plasma samples and verifying the excitation wavelength from 230 to 320 nm and the optimum excitation wavelength for the detection of PK and MK-7 was set at 240 nm (data not shown). For quantitative analysis of PK and MK-7 in System 2, I.S.-C19, which was found as a single peak between PK and MK-7, was chosen as an internal standard. The retention times of PK, MK-7 and Vitamin K analogs in System 2 were as follows: PK, 16.99 min; MK-7, 34.48 min; I.S.-C13, 11.47 min; I.S.-C14, 13.04 min; I.S.-C15, 15.42 min; I.S.-C16, 17.81 min; I.S.-C18, 24.41 min; I.S.-C19, 25.54 min; I.S.-C20, 31.01 min; I.S.-C23, 36.35 min. Fig. 3 shows the chromatographic profiles of authentic MK-4, PK, IS-C19 and MK-7 (Fig. 3A), a representative plasma sample from healthy subject with post-column reduction (Fig. 3B) and the same plasma sample without post-column reduction (Fig. 3C) in System 2. MK-4 was not separated from interfering compounds in plasma.

For multiple assay of MK-4, PK and MK-7, a gradient eluent system after elution of MK-4 is available. It has been

Table 1
The recoveries of MK-4, PK and MK-7

	MK-4	PK	MK-7
Low ($n=5$)			
Mean ± S.D. (%)	93 ± 8.6	103 ± 5.2	97 ± 6.1
R.S.D. (%)	9.2	5.0	6.3
Middle ($n=5$)			
Mean ± S.D. (%)	101 ± 2.5	99 ± 2.3	101 ± 4.6
R.S.D. (%)	2.5	2.3	4.6
High ($n=5$)			
Mean ± S.D. (%)	99 ± 5.2	99 ± 2.0	104 ± 3.2
R.S.D. (%)	5.3	2.0	3.1

Table 2
Intra- and inter-assay/R.S.D. values of assay method for Vitamin K

	MK-4	PK	MK-7
Intra-assay ($n = 10$)			
Control serum			
Mean ± S.D. (ng/mL)	0.179 ± 0.014	0.145 ± 0.014	0.067 ± 0.013
R.S.D. (%)	7.5	9.6	19.3
Plasma of normal subject			
Mean ± S.D. (ng/mL)	0.353 ± 0.021	1.270 ± 0.084	0.693 ± 0.061
R.S.D. (%)	6.0	6.6	8.8
Inter-assay ($n = 10$)			
Control serum			
Mean ± S.D. (ng/mL)	0.191 ± 0.011	0.152 ± 0.008	0.076 ± 0.011
R.S.D. (%)	5.7	5.3	13.9
Plasma of normal subject			
Mean ± S.D. (ng/mL)	0.332 ± 0.031	1.193 ± 0.058	0.598 ± 0.038
R.S.D. (%)	9.2	4.9	6.3

confirmed that MK-4, PK and MK-7 were successfully determined without interruption of co-eluting compounds by using following HPLC system; column, CAPCELL PAK C18 UG120 (4.6 mm × 250 mm); reduction column, RC-10 (4.0 mm × 15 mm); mobile phase, an isocratic elution of 95:5 (v/v) mixture of methanol and water for 30 min, and a linear gradient from 0 to 100% ethanol following 50 min; flow-rate, 1.0 mL/min; excitation wavelength, 240 nm; emission wavelength, 430 nm; retention time of MK-4, 23.74 min; I.S.-C16, 38.49 min; PK, 44.73 min; I.S.-C19, 55.12 min; MK-7, 65.79 min.

3.2. Sensitivity, precision and accuracy

A calibration curve of MK-4, PK and MK-7 gave a linearity between 2 and 500 pg in Systems 1 and 2 described previous section. The detection limits of MK-4 and MK-7, based on a signal-to-noise ratio of 3: 1, were 4 pg per injection, and that of PK was 2 pg per injection, respectively. The recoveries of MK-4, PK and MK-7 calculated by measurements of control serum spiked Vitamin K at low (MK-4, 0.1 ng; PK, 0.4 ng; MK-7, 0.75 ng/0.5 mL serum), middle (MK-4, 0.2 ng; PK, 0.8 ng; MK-7, 1.5 ng/0.5 mL serum) and high (MK-4, 0.4 ng; PK, 1.6 ng; MK-7, 3.0 ng/0.5 mL serum) concentration were about 92–105% (Table 1). Intra- and inter-assay R.S.D. values calculated by measurements of control serum and plasma of healthy subject were 5.73–9.21% for MK-4, 4.86–9.64% for PK and 6.32–19.31% for MK-7 (Table 2). We speculate that intra- and inter-assay R.S.D. values of control serum for MK-7 were high because concentration of MK-7 was low and close to the limit of quantitation. The sensitivity and an overall recovery combined with reproducibility allowed the measurement of three kinds of Vitamin K, MK-4, PK and MK-7 with only 0.2 mL of plasma, which is smaller than in previous.

3.3. Concentration of Vitamin K in healthy subjects and osteoporotic patients treated with MK-4

This method was applied to the plasma samples obtained from 20 healthy subjects and 10 osteoporotic patients. Plasma levels of MK-4, PK and MK-7 in healthy subjects were 0.15 ± 0.17 ng/mL (mean ± S.D.), 1.81 ± 1.10 ng/mL and 16.27 ± 20.58 ng/mL, respectively (Table 3). Plasma levels of MK-4, PK and MK-7 in osteoporotic patients treated with MK-4 were 46.83 ± 46.41 ng/mL, 0.62 ± 0.25 ng/mL and 4.18 ± 6.28 ng/mL, respectively. The plasma levels of MK-4 in patients treated with MK-4 were significantly higher than that of healthy subjects ($p < 0.05$). The plasma levels of PK and MK-7 in patients treated with MK-4 were significantly lower than that of healthy subjects ($p < 0.001$ and $p < 0.05$, respectively). However, it is conceivable that the individual difference derived from diet exists in plasma levels of PK and MK-7. We confirmed that the plasma concentration of MK-7 was markedly increased after intake of fermented soybean (data not shown).

Table 3
Concentration of Vitamin K in healthy subjects and osteoporotic patients treated with MK-4

	MK-4	PK	MK-7
Healthy subjects ($n = 20$) (mean ± S.D. (ng/mL))			
Fluorescence detection	0.149 ± 0.172	1.814 ± 1.107	16.27 ± 20.58
LC-APCI/MS detection	0.392 ± 0.457	2.163 ± 1.340	17.53 ± 22.55
Osteoporotic patients ($n = 10$) (mean ± S.D. (ng/mL))			
Fluorescence detection	46.83 ± 46.41	0.621 ± 0.245	4.179 ± 6.281
LC-APCI/MS detection	51.89 ± 44.88	0.850 ± 0.272	4.128 ± 6.373

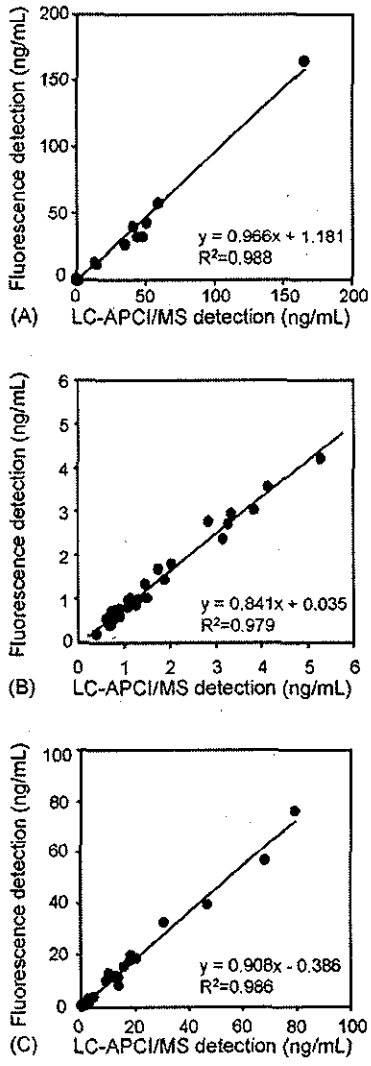


Fig. 4. Correlation between the values obtained by HPLC with fluorescence detection using internal standards and those obtained by LC-APCI/MS. (A) MK-4; (B); PK; and (C) MK-7.

3.4. Correlation with LC-APCI/MS method

To compare the proposed method with LC-APCI/MS method, the same plasma samples of healthy subjects and osteoporotic patients treated with MK-4 were measured by both methods (Table 3). Fig. 4 shows correlation between the values obtained by HPLC with fluorescence detection using internal standards and those obtained by LC-APCI/MS. The data showed good correlation between two methods for MK-4 (Fig. 4A, $y=0.966x+1.181$, $R^2=0.988$), PK (Fig. 4B, $y=0.841x+0.035$, $R^2=0.979$) and MK-7 (Fig. 4C, $y=0.908x-0.386$, $R^2=0.986$), respectively. These results suggest that the accuracy of quantitative determination of Vitamin K by this method was sufficient for nutritional and clinical applications.

4. Conclusions

Here we show an improved HPLC method for determination of Vitamin K with fluorescence detection using post column reduction. Synthetic internal standards were synthesized and used and validated to quantify MK-4, PK and MK-7. The proposed method has several advantages in comparison with previously reported method: high selectivity and reproducibility attained by using two kinds of HPLC system optimized for determination of MK-4 and less polar derivatives, PK and MK-7; satisfactory precision and accuracy attained by using synthetic Vitamin K analogs as internal standards; small sample required for determination of three major Vitamin K derivatives, MK-4, PK and MK-7. Therefore, the proposed method is suitable for both clinical and nutritional studies and the routine assay for MK-4, PK and MK-7 in plasma. Due to its high sensitivity, the assay may provide a useful tool for elucidation of importance of Vitamin K in bone metabolism, for example, analysis of Vitamin K in bone tissue extracts obtained from animals.

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