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Articles

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 detec-

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tion 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 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.

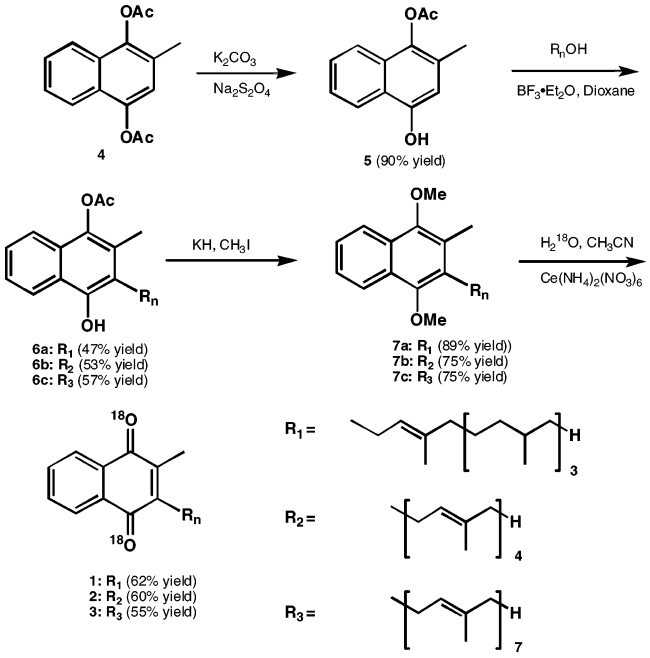
We used HPLC-fluorescence detection to measure the concentrations of vitamin K homologues (PK, MK-4, menaquinone-7 (MK-7)) in human plasma and found that PK and MK-7 could be clearly detected as a single peak. However, the peak of MK-4 overlapped with impurities in the plasma and especially appeared 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

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



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.

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.

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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.

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 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_4 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 $BF_3{\cdot}Et_2O$ (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

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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, 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 $C_{33}H_{52}{}^{16}O_2$ (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

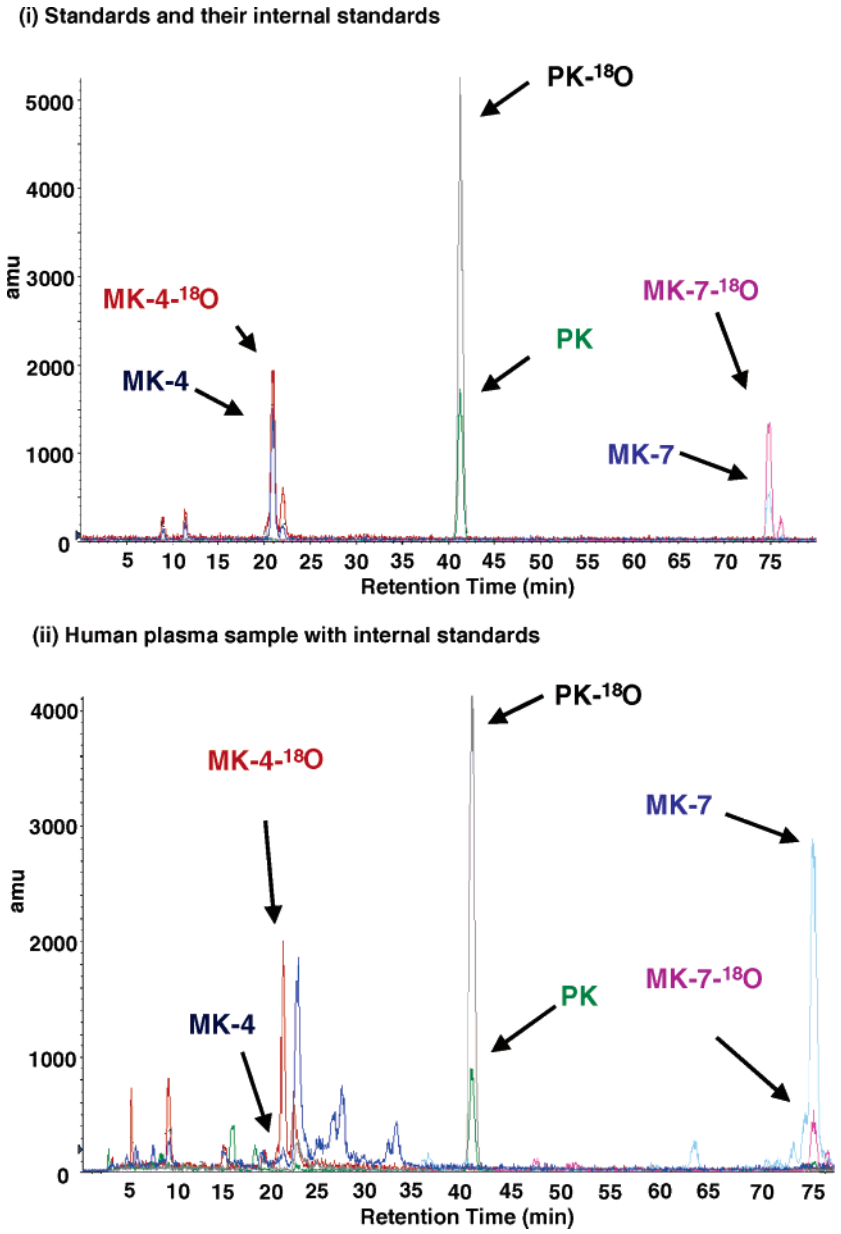

Figure 1. LC-APCI-MS/MS chromatograms of the analytes of vitamin K Homologues for the injection of (i) standard mixture and (ii) human plasma sample with internal standards. PK, phylloquinone; MK-4, menaquinone-4; MK-7, menaquinone-7; PK-^{18}O, ^{18}O-labeled phylloquinone; MK-4-^{18}O, ^{18}O-labeled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

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 [$^{2}H_{7}$]MK-4 (deuterated MK-4) and [$^{2}H_{7}$]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 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

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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: $n = 5$.

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.

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 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

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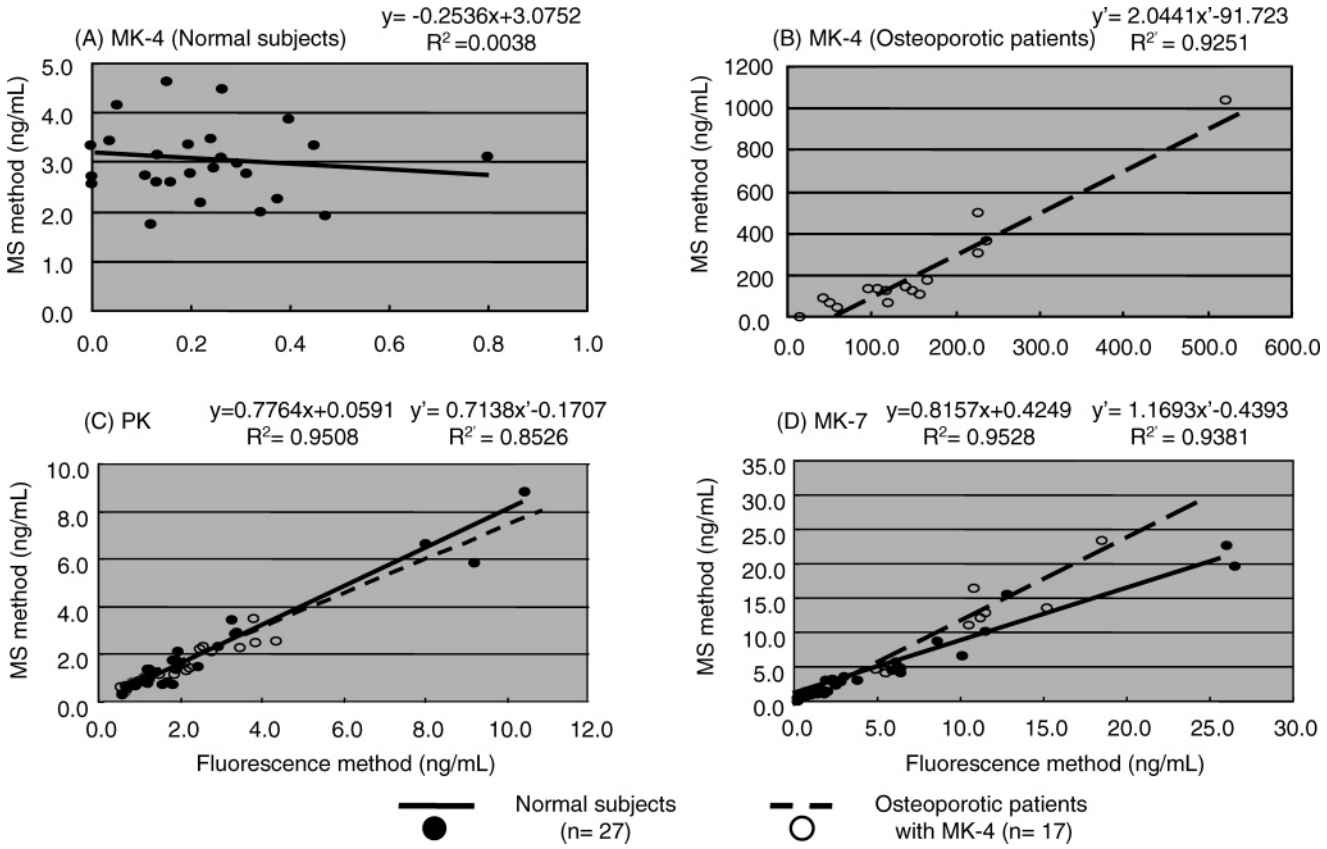

Figure 2. Comparison of assayed values (ng/mL) of human plasma vitamin K homologues between the LC-APCI-MS/MS method (MS method) and HPLC with fluorescence detection (fluorescence method). (A) MK-4 (normal subjects); (B) MK-4 (osteoporotic patients); (C) PK (normal subjects and osteoporotic patients);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

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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