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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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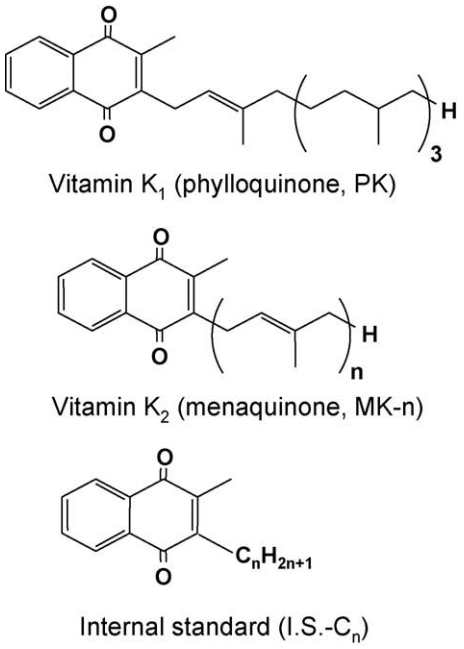

Fig. 1. Structures of natural Vitamin K_1, K_2 and internal standards.

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 ^{1}H 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)

^{1}H 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

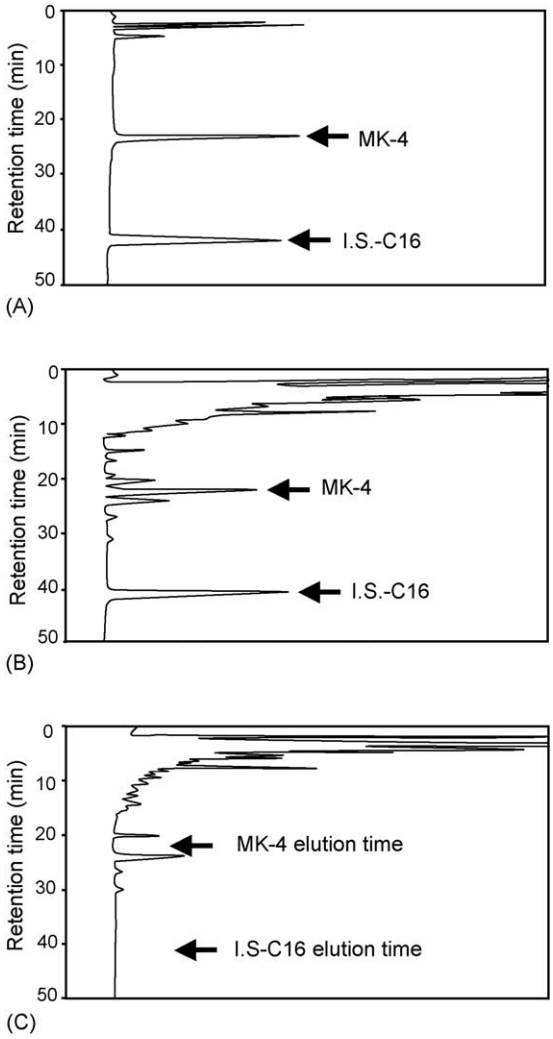

Fig. 2. Chromatograms of authentic standards and a plasma sample in System 1. (A) authentic standards of MK-4 and I.S.-C16; (B) a plasma sample obtained from a healthy subject with reduction by the platinum-reduction column; and (C) a plasma sample obtained from a healthy subject without reduction by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

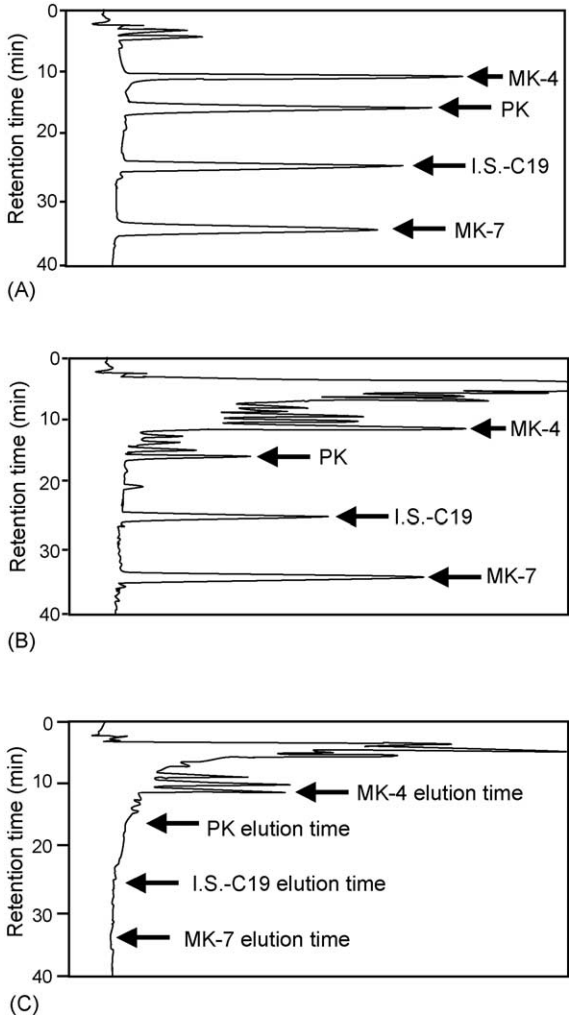

Fig. 3. Chromatograms of authentic standards and a plasma sample in System 2. (A) authentic standards of MK-4, PK, I.S.-C19 and MK-7; (B) a plasma sample obtained from the same subject as shown in Fig. 2 with reduction by the platinum-reduction column; and (C) a plasma sample obtained from the same subject 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

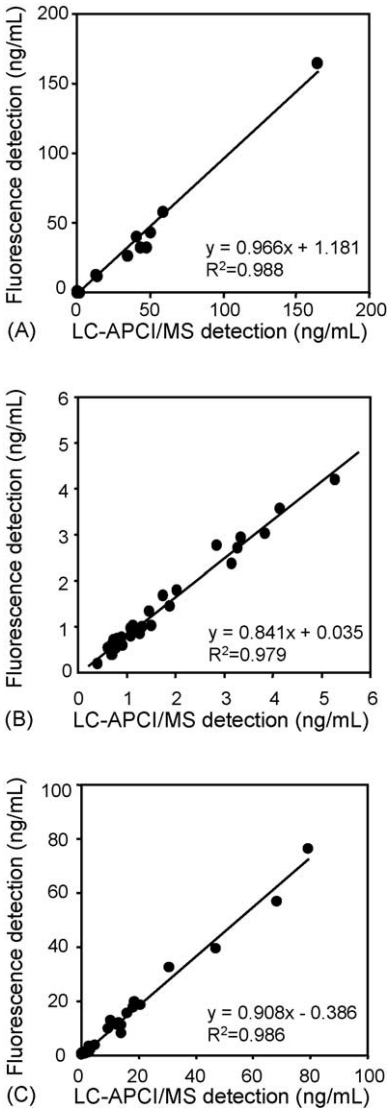

Fig. 4. Correlation between the values obtained by HPLC with fluorescence detection using internal standards and those obtained by LC-APCI/MS. (A) MK-4; (B); PK; and (C) MK-7.

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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References

[1] P.A. Friedman, N. Engl. J. Med. 310 (1984) 1458.
[2] P.M. Gallop, J.B. Lian, P.V. Hauschka, N. Engl. J. Med. 302 (1980) 1460.
[3] Y. Sato, Y. Honda, H. Kuno, K. Oizumi, Bone 23 (1998) 291.
[4] I. Iwamoto, S. Kosha, S. Noguchi, M. Murakami, T. Fujino, T. Douchi, Y. Nagata, Maturitas 31 (1999) 161.
[5] A.M. Craciun, J. Wolf, M.H. Knapen, F. Brouns, C. Vermeer, Int. J. Sports Med. 19 (1998) 479.
[6] Y. Somekawa, M. Chigughi, M. Harada, T. Ishibashi, J. Clin. Endocrinol. Metab. 84 (1999) 2700.
[7] Y. Akiyama, K. Hara, I. Ohkawa, T. Tajima, Jpn. J. Pharmacol. 62 (1993) 145.
[8] K. Hara, Y. Akiyama, I. Ohkawa, T. Tajima, Bone 14 (1993) 813.
[9] T. Tomiuga, M. Kobayashi, Y. Nakajima, M. Bessho, Y. Katoh, K. Hara, Y. Akiyama, T. Nakamura, T. Tajima, Jpn. J. Pharmacol. 65 (1994) 35.
[10] Y. Ehara, H. Takahashi, Y. Hanahisa, M. Yamaguchi, Res. Exp. Med. (Berl.) 196 (1996) 171.
[11] Y. Akiyama, K. Hara, M. Kobayashi, T. Tomiuga, T. Nakamura, Jpn. J. Pharmacol. 80 (1999) 67.
[12] S. Matsunaga, H. Ito, T. Sakou, Calcif. Tissue Int. 65 (1999) 285.

[13] M. Yamaguchi, H. Taguchi, Y.H. Gao, A. Igarashi, Y. Tsukamoto, J. Bone Miner. Metab. 17 (1999) 23.
[14] M.J. Shearer, Blood Rev. 6 (1992) 92.
[15] J.W. Suttie, J. Am. Diet Assoc. 92 (1992) 585.
[16] S.J. Hodges, M.J. Pilkington, M.J. Shearer, L. Bitensky, J. Chayen, Clin. Sci. 78 (1990) 63.
[17] P.L. Donnahey, V.T. Burt, H.H. Rees, J. Chromatogr. 170 (1979) 272.
[18] G.H. Dialameh, R.E. Olson, Anal. Biochem. 32 (1969) 263.
[19] G.G. Dolnikowski, Z. Sun, M.A. Grusak, J.W. Peterson, S.L. Booth, J. Nutr. Biochem. 13 (2002) 168.
[20] E.M. Kirk, A.F. Fell, Clin. Chem. 35 (1989) 1288.
[21] T. Sakano, T. Nagaoka, A. Morimoto, K. Hirauchi, Chem. Pharm. Bull. 34 (1986) 4322.
[22] Y. Haroon, D.S. Bacon, J.A. Sadowski, J. Chromatogr. 384 (1987) 383.
[23] M. Shino, Analyst 113 (1988) 393.
[24] Y. Usui, N. Nishimura, N. Kobayashi, T. Okanoue, M. Kimoto, K. Ozawa, J. Chromatogr. 489 (1989) 291.
[25] K. Hirauchi, T. Sakano, S. Notsumoto, T. Nagaoka, A. Morimoto, K. Fujimoto, S. Masuda, Y. Suzuki, J. Chromatogr. 497 (1989) 131.
[26] J.P. Hart, M.J. Shearer, P.T. McCarthy, S. Rahim, Analyst 109 (1984) 477.
[27] Y. Haroon, C.A. Schubert, P.V. Hauschka, J. Chromatogr. Sci. 22 (1984) 89.
[28] J.P. Hart, M.J. Shearer, P.T. McCarthy, Analyst 110 (1985) 1181.
[29] H. Wakabayashi, K. Onodera, S. Yamato, K. Shimada, Nutrition 19 (2000) 661.
[30] Y. Sano, K. Kikuchi, K. Tadano, K. Hoshi, Y. Koshihara, Anal. Sci. 13 (1997) 67.
[31] Y. Suhara, M. Kamao, N. Tsugawa, T. Okano, Anal. Chem., in press.
[32] K.W. Devidson, J.A. Sadowski, Methods Enzymol. 282 (1997) 408.
[33] L. Benli, G. Lianzuan, Z. Jingling, Recl. Trav. Chim. Pays-Bas 110 (1991) 99.