83.7 μg/d. However, there are great differences between individuals in natto consumption. In this study, 42.4% of participants consumed natto and mean total vitamin K intake for them was about twofold higher than that of non-natto eaters. It was also demonstrated that the largest contributor to the total vitamin K intake of natto eaters was pulses including fermented soybean products unlike for non-natto eaters. Thus, natto consumption can influence total vitamin K intake. Kaneki et al. (*30*) reported that serum MK-7 levels were significantly higher in frequent natto eaters and a statistically significant inverse correlation was observed between the incidence of hip fractures in women and natto consumption in Japan. Ikeda et al. (*31*) also showed significant positive associations between natto intake and the rates of changes in BMD in postmenopausal women. Moreover, we demonstrated that plasma concentration of MK-7 correlated inversely with under carboxylated osteocalcin concentration as well as PK in Japanese (*32*). Thus, PK derived from vegetables and oils and MK-7 derived from natto may have a nutritionally important role in maintaining the bone health in Japanese. Further epidemiological study on vitamin K intake, including MKs and bone, and reconsideration of the dietary habit-based vitamin K requirement are thought to be required.

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Quantification of fat-soluble vitamins in human breast milk by liquid chromatography–tandem mass spectrometry

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Abstract

Sensitive quantification method for fat-soluble vitamins in human breast milk by liquid chromatography–tandem mass spectrometry was developed. Vitamins A, D and E were extracted from 10.0 mL of breast milk after saponifying by basic condition. Vitamin K derivatives were extracted from 3.0 mL of breast milk after lipase treatment. The corresponding stable isotope-labeled compounds were used as internal standards. For the determination of vitamin D compounds, derivatization with a Cookson-type reagent was performed. All fat-soluble vitamins were determined by liquid chromatography–tandem mass spectrometry in the positive ion mode. The detection limits of all analytes were 1–250 pg per 50 μL. The recoveries of fat-soluble vitamins were 91–105%. Inter-assay CV values of each vitamin were 1.9–11.9%. The mean concentrations of retinol, vitamin D_3, 25-hydroxyvitamin D_3, α-tocopherol, phylloquinone and menaquinone-4 were 0.455 μg/mL, 0.088 ng/mL, 0.081 ng/mL, 5.087 μg/mL, 3.771 ng/mL, and 1.795 ng/mL, respectively ($n = 82$). This method makes possible to determine fat-soluble vitamins with a wide range of polarities in human breast milk. The assay may be useful for large-scale studies.

Keywords: Vitamin A; Vitamin D; Vitamin E; Vitamin K; Fat-soluble vitamins; Liquid chromatography–tandem mass spectrometry; Breast milk

1. Introduction

International agencies and health organizations of various countries recommend breast-feeding as the preferred method of infant feeding for the first several months of life and thereafter as long as is beneficial to the relationship between mother and infant [1]. Term infants nursed by nutritionally adequate mothers are provided with sufficient energy and the proper profile of nutrients to support normal growth and development during the first 6 months except for vitamins D and K in the immediate newborn period [2,3].

There have been reports of clinical rickets in breast-fed infants, especially nursed by mothers who restrict their intake of vitamin D-rich foods (i.e. strict vegetarians) [4]. Also, in countries where climate or custom lead to low levels of exposure of the child or the mother to sunlight, infant serum concentrations of 25-hydroxyvitamin D [25(OH)D] may be sub-optimal [5,6]. In previous reports, the concentrations of vitamin D_3 (cholecalciferol, D_3), 25-hydroxyvitamin D_3 [25(OH)D_3], 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] and 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] in human breast milk were 0.03–0.12, 0.28–0.34, 0.04–0.28 and

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0.005–0.02 ng/mL, respectively [7–9]. These results indicate that most antirachitic activity is attributable to D_3 and 25(OH)D_3, and the transfer of vitamin D and its metabolites from plasma to milk is limited.

Vitamin K is also a possible problem for breast-fed infants. Transplacental transfer of vitamin K is minor and infants are born with low tissue stores. Human breast milk contains only a low concentration of vitamin K and there is strong evidence of increased incidence of late haemorrhagic disease in breast-fed infants [10]. It was reported that the vitamin K_1 (phylloquinone, PK) concentration of human breast milk ranges from 1 to 9 ng/mL [11,12]; however, there are few reports associated with other important vitamin K derivatives, vitamin K_2 (menaquinone, MK), contents [13].

Regional or individual nutritional problems with other fat-soluble vitamins in breast-fed infants are also reported. In the developing world, subclinical vitamin A deficiency has been observed in infants fed with breast milk. Several reports have indicated an association between the vitamin A content of the diet in pregnancy and lactation, and vitamin A concentration in breast milk. The vitamin A content of milk in poorer populations in developing countries such as India, Ceylon and Jordan, where intake is marginal, is lower than in North America and Europe [14]. Meanwhile, there has been no report of problems with vitamin E for breast-fed infants.

In this manner, surveys of the concentrations of fat-soluble vitamins in human breast milk have important implications for the promotion of breast-feeding. However, there are several problems in sensitivity, specificity and accuracy with determination of fat-soluble vitamins in breast milk. Especially, it is difficult to determine vitamin D compounds using standard assay methods such as HPLC with ultraviolet detection [15], competitive protein binding assay (CPBA) [16], radio immunoassay (RIA) [17] and enzyme immunoassay (EIA) [18], because concentrations of them are markedly low. In this study, we have developed a high-sensitive quantification method of fat-soluble vitamins in human breast milk using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Also, we applied this method to a nutrition survey for lactating mothers.

2. Experimental

2.1. Chemicals and reagents

Retinyl palmitate and β-carotene were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA). 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). D_3, vitamin D_2 (ergocalciferol, D_2), 25(OH)D_3 and 25-hydroxyvitamin D_2 [25(OH)D_2] were obtained from Duphar B.V. (Amsterdam, The Netherlands). α-Tocopherol (α-Toc), PK, MK-4 and MK-7 were kindly provided by Eisai Pharmaceuticals (Tokyo, Japan). Four internal standards, d_6-retinyl acetate, d_6-β-carotene, d_7-D_3, and d_6-α-Toc were synthesized as described below. d_6-25(OH)D_3, [$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7

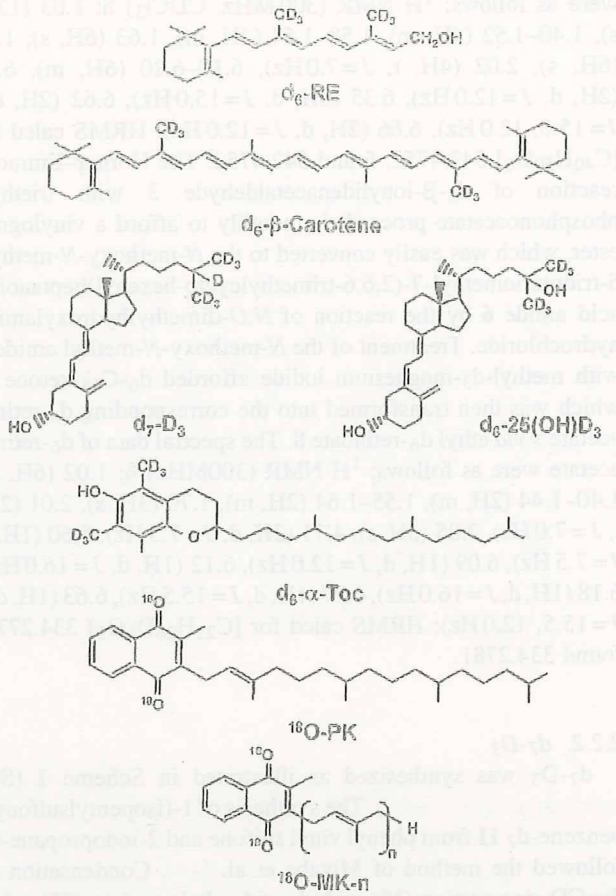

Fig. 1. Chemical structures of internal standards.

were synthesized as described in our previous reports [19,20]. The chemical structures of the internal standards are shown in Fig. 1.

2.2. Synthesis of internal standards

The structure of the target compounds were confirmed by ^{1}H NMR and MS. The 500 MHz and 300 MHz ^{1}H NMR spectra were measured on a Varian VXR-500 and VXR-300, respectively. All compounds were dissolved in 0.3 mL of deuterated chloroform ($CDCl_3$, Merck, Darmstadt, Germany). 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. d_6-Retinyl acetate and d_6-β-carotene

d_3-Labeled β-ionone 2 was prepared from *N*-methoxy-*N*-methyl-3-(2,6,6-tri-methylcyclohexenyl)propanoic acid amide 1 [21] by treatment with methyl-d_3-magnesium iodide and was then converted to the corresponding d_3-β-ionylidenacetaldehyde 3 by the previously reported method [22]. Polyene chain elongation of d_3-aldehyde 3 by a standard method [17] gave d_3-retinal 4. Reductive coupling of d_3-retinal 4 with lithium aluminum hydride and titanium tetrachloride [23] afforded d_6-β-carotene 5. The spectral data of d_6-β-carotene

were as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ; 1.03 (12H, s), 1.40–1.52 (4H, m), 1.58–1.64 (4H, m), 1.63 (6H, s), 1.93 (6H, s), 2.02 (4H, t, J = 7.0 Hz), 6.10–6.20 (6H, m), 6.25 (2H, d, J = 12.0 Hz), 6.35 (2H, d, J = 15.0 Hz), 6.62 (2H, dd, J = 15.0, 12.0 Hz), 6.66 (2H, d, J = 12.0 Hz); HRMS calcd for [$C_{40}H_{50}D_6$] 542.4752, found 542.4752. The Horner–Emmons reaction of d_3-β-ionylidenacetaldehyde **3** with triethylphosphonoacetate proceeded smoothly to afford a vinylogous ester, which was easily converted to the *N*-methoxy-*N*-methyl-5-triduteriomethyl-7-(2,6,6-trimethylcyclo-hexenyl)heptanoic acid amide **6** by the reaction of *N*,*O*-dimethylhydroxylamine hydrochloride. Treatment of the *N*-methoxy-*N*-methyl amide **6** with methyl-d_3-magnesium iodide afforded d_6-C_{18}-ketone **7**, which was then transformed into the corresponding d_6-retinyl acetate **9** *via* ethyl d_6-retinoate **8**. The spectral data of d_6-retinyl acetate were as follows: ^{1}H NMR (300MHz) δ; 1.02 (6H, s), 1.40–1.44 (2H, m), 1.55–1.64 (2H, m), 1.70 (3H, s), 2.01 (2H, t, J = 7.0 Hz), 2.05 (3H, s), 4.71 (2H, d, J = 7.5 Hz), 5,60 (1H, t, J = 7.5 Hz), 6.09 (1H, d, J = 12.0 Hz), 6.12 (1H, d, J = 16.0 Hz), 6.18 (1H, d, J = 16.0 Hz), 6.27 (1H, d, J = 15.5 Hz), 6.63 (1H, dd, J = 15.5, 12.0 Hz); HRMS calcd for [$C_{22}H_{26}D_6O_2$] 334.2773, found 334.2781.

2.2.2. d_7-D_3

d_7-D_3 was synthesized as illustrated in Scheme 1 (See supplementary material). The synthesis of 1-(isopentylsulfonyl) benzene-d_7 **11** from phenyl vinyl sulfone and 2-iodopropane-d_7 followed the method of Miyabe et al. [24]. Condensation of the CD-ring portion (**10**) with the side-chain moiety (**11**) using *n*-butyllithium as a base in the presence of hexamethylphosphoramide furnished a mixture of C-23 epimeric sulfones, *tert*-butyldimethyl(octahydro-7α-methyl-1-((*R*)-6-methyl-4-(phenylsulfonyl)heptan-2-yl)-1*H*-inden-4-yloxy)silane-d_7 **12** as a previous report [25]. Desulfonylation with sodium amalgam in a buffered mixture of methanol and tetrahydrofuran (THF) produced *tert*-butyldimethyl(octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-inden-4-yloxy)silane-d_7 **13**. Removal of the protecting group in **13** with toluenesulfonic acid afforded octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-inden-4-ol-d_7 **14**. The resulting secondary alcohol was oxidized with tetrapropylammonium perruthenate and 4-methylmorpholine *N*-oxide to give octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)inden-4-one-d_7 **15**. Bromomethylenation of **15** furnished the requisite CD-ring synthon, (*E*)-4-(bromomethylene)-octahydro-7α-methyl-1-((*R*)-6-methylheptan-2-yl)-1*H*-indene-d_7 **16**. The coupling reaction of the A-ring enyne (**17**) which was obtained according to the reported method [25] with the CD-ring portion (**16**) catalyzed by tetrakis(triphenylphosphine)palladium and triethylamine in toluene under reflux for 2 h, followed by deprotection with *tetra*-butylammonium fluoride in THF gave a diastereomer mixture (3α and 3β) of deuterated D_3 **18a–18b**. 3β-Deuterated D_3 (**18b**, d_7-D_3) was used as an internal standard. The spectral data of 3α and 3β deuterated D_3 were as follows: (18a) ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.55 (3H, s), 0.92 (3H, d, J = 6.5 Hz), 1.11–1.68 (16H, m), 1.82–1.92 (2H, m), 1.96–2.02 (2H, m), 2.15 (1H, m), 2.27 (1H, dd, J = 8.5, 13.0 Hz), 2.40 (1H, m), 2.58 (1H, dd, J = 4.0, 13.0 Hz), 2.82 (1H, dd, J = 4.0, 12.5 Hz), 3.88 (1H, m), 4.84 (1H, m), 5.06 (1H, m), 6.04 (1H, d, J = 11.5 Hz), 6.24 (1H, d, J = 11.0 Hz); HRMS calcd for [$C_{27}H_{37}D_7O$] 391.3824, found 391.3832 (18b) ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.54 (3H, s), 0.92 (3H, d, J = 6.5 Hz), 1.04–1.68 (16H, m), 1.82–1.92 (2H, m), 1.96–2.02 (2H, m), 2.18 (1H, m), 2.29 (1H, dd, J = 7.5, 13.5 Hz), 2.40 (1H, m), 2.58 (1H, dd, J = 3.0, 13.0 Hz), 2.82 (1H, dd, J = 4.0, 12.5 Hz), 3.93 (1H, m), 4.82 (1H, m), 5.05 (1H, m), 6.03 (1H, d, J = 11.5 Hz), 6.24 (1H, d, J = 11.5 Hz); HRMS calcd for [$C_{27}H_{37}D_7O$] 391.3824, found 391.3822.

2.2.3. d_6-α-Toc

d_6-α-Toc was synthesized as illustrated in Scheme 2 (See supplementary material). Minor modifications gave the desired d_6-α-Toc **20** from γ-tocopherol as previously reported in the method by Hughes et al. [26]. d_6-α-Toc **20** was prepared from γ-Tocopherol **19** by treatment with $SnCl_2$, DCl (35% in D_2O) and $(CD_2O)_n$ in isopropyl ether under reflux for 2.5 h. The spectral data of d_6-α-Toc were as follows: ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.83 (3H, s), 0.84 (3H, s), 0.85 (3H, s), 0.88 (3H, s), 1.01–1.28 (16H, m), 1.48–1.61 (4H, m), 1.57 (3H, s), 1.78-1.82 (3H, m), 2.10 (3H, s), 2.60 (2H, t, J = 6.0 Hz), 4.17 (s, 1H). HRMS Calcd for [$C_{29}H_{44}D_6O_2$] 436.4181, found 436.4174.

2.3. Standards and sample preparation

2.3.1. Preparation of retinol and d_6-retinol

Retinol (RE) and d_6-RE solutions were prepared by saponifying retinyl palmitate and d_6-retinyl acetate before use. Forty microgram of retinyl palmitate and d_6-retinyl acetate were dissolved in 1.5 mL of pyrogallol–ethanol (7%, w/v). After the addition of 0.5 mL of NaCl solution (1%, w/v) and 0.8 mL of KOH solution (60%, w/v), the mixture was incubated at 70 °C for 30 min. RE and d_6-RE were extracted with hexane-diethyl ether (90:10, v/v), evaporated under reduced pressure, and the residue was dissolved with 2-propanol. Concentrations of RE and d_6-RE were determined spectrophotometrically using a molar extinction coefficient, ε = 52,480. The purity was checked by high-performance liquid chromatography (HPLC) consisting of an LC-10AD$_{VP}$ pump, a SIL-10AD$_{VP}$ auto injector, a CTO-10AD$_{VP}$ column oven set to 35 °C, and an RF-10A$_{XL}$ fluorescence detector set to an excitation wavelength of 340 nm and an emission wavelength of 460 nm (Shimadzu, Kyoto, Japan). Separation was performed on a Capcellpak C18 UG120 (4.6 mm × 250 mm, Shiseido Co. Ltd., Tokyo, Japan) eluted with ethanol:H_2O (95:5) at a flow rate of 0.4 mL/min.

2.3.2. Sample collections

Human breast milk samples were collected from March 2005 to October 2006 from 82 lactating mothers aged 18–39 years (30.8 ± 4.5 years) at 3–265 d (49.1 ± 57.6 d) post-partum living in Japan. Written informed consent was obtained from each subject prior to enrollment in this study according to the conditions of the Helsinki Declaration and approved by the ethics committee of our university. Approximately 50 mL of human breast milk was collected by manual expression at an interme-

diate time during suckling and immediately frozen at −20 °C. Before extraction of fat-soluble vitamins, frozen breast milk was thawed and sonicated in ice water twice for 15 min. For the developmental work on the assay, pooled human breast milk prepared by mixing breast milk from seven healthy subjects was used.

2.3.3. Extraction of fat-soluble vitamins except for vitamin K derivatives

Ten milliliters of breast milk samples was placed in a 50-mL screw-top vial. After the addition of 50 μL of internal standard solution (d_6-RE, d_6-β-carotene, d_7-D_3, d_6-25(OH)D_3 and d_6-α-Toc, 50 ng/50 μL ethanol each), 20 mL of pyrogallol–ethanol (7%, w/v), 6 mL of NaCl solution (1%, w/v) and 10 mL of KOH solution (60%, w/v), the mixture was incubated at 70 °C for 60 min. Then, the mixture was transferred to a 200-mL of separating funnel containing 38 mL of NaCl solution (1%, w/v) and fat-soluble vitamins were extracted twice with 30 mL of hexane-ethyl acetate (9:1, v/v), washed with water, and dehydrated with Na_2SO_4. The eluate was evaporated under reduced pressure, and the residue was dissolved with 2.5 mL of hexane-ethyl acetate (9:1, v/v). For the determination of RE, β-carotene and α-Toc, 1.0 mL of 2.5 mL was evaporated, and the residue was dissolved with 100 μL of ethanol, 50 μL of which was subjected to liquid chromatography–tandem mass spectrometry (LC–MS/MS). For determination of vitamin D (D) and 25(OH)D, another 1.5 mL was concentrated and purified by normal phase HPLC. HPLC was carried out using a model 600 pump and a model 996 photodiode array detector (Waters Associates, Milford, MA). Elution was performed on a Zorbax SIL column (4.6 mm × 250 mm, Agilent, Santa Clara, CA), using hexane-2-propanol-methanol (88:10:2, v/v/v), at a flow rate of 1.0 mL/min. The eluates corresponding to D_3 and D_2 (D fraction, 3.5–5.0 min) and 25(OH)D_3 and 25(OH)D_2 (25D fraction, 5.0–8.0 min) were collected.

2.3.4. DMEQ-TAD derivatization of D and 25(OH)D

DMEQ-TAD derivatization was performed according to the method of Higashi et al. [27]. D and 25(OH)D fractions were dried and then dissolved in 150 μL of ethyl acetate containing DMEQ-TAD (60 μg). The mixture was kept at room temperature for 30 min, then an additional reagent (60 μg/150 μL of ethyl acetate) was added and the entire mixture was further kept at room temperature for 1 h. After the addition of 1.5 mL of EtOH to decompose excess reagent, the solvent was evaporated and the residue was dissolved in 100 μL of acetonitrile, 30 μL of which was subjected to LC–MS/MS.

2.3.5. Extraction of vitamin K derivatives

Three milliliters of breast milk samples was placed in a 50 mL screw-top vial. After the addition of internal standard solution ([$^{18}O_2$]-PK, [$^{18}O_2$]-MK-4 and [$^{18}O_2$]-MK-7, 25 ng/25 μL ethanol each), 12 mL of phosphate buffer (pH 7.7) and 0.3 g of lipase, the mixture was incubated at 37 °C for 90 min with stirring. Then, 12 mL of ethanol was added and vitamin K derivatives were extracted twice with 12 mL of hexane. The mixture was shaken for 5 min before centrifuging at 3000 rpm for 5 min. The extracts were combined and evaporated under reduced pressure, and the residue was dissolved with 3 mL of hexane. The resultant extract was passed through a Sep-Pak Vac silica cartridge (Waters, Milford, MA, USA) that was washed with 10 mL of hexane. Vitamin K derivatives were 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, 50 μL of which was subjected to LC–MS/MS.

2.4. LC–MS/MS

2.4.1. Apparatus

The HPLC system consisted of a SCL-10ADvp system controller, two LC-10ADvp pumps, a DGC-14A automatic solvent degasser, a SIL-10ADvp auto injector, and a CTO-10ADvp column oven set to 35 °C (Shimadzu). The HPLC system was coupled to an API 3000 triple-quadrupole tandem mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) source. Analyst (Ver. 1. 3. 2; Applied Biosystems/MDS SCIEX) was used for data acquisition and analysis.

2.4.2. Chromatographic conditions

Separations were performed on a Capcellpak C18 UG120. For the determination of RE, β-carotene, α-Toc and vitamin K derivatives, a solvent system consisting of an isocratic solvent A (methanol–H_2O, 90:10, v/v) in 10 min and then a linear gradient from 0 to 90% acetonitrile in 30 min was used (Condition 1). The injection volumes of standard and sample solutions were 50 μL. For the determination of DMEQ-TAD derivatives of D and 25(OH)D, a solvent system consisting of a mixture of acetonitrile and H_2O (30:70, v/v) in 5 min and then a linear gradient from 30 to 95 % acetonitrile in 30 min was used (Condition 2). The injection volumes of standard and sample solutions were 30 μL. Acquisition settings were optimized by the infusion of a 1 μg/mL solution of each compound at a rate of 20 μL/min as shown in Table 1. Curtain gas (8 psi), nebulizer gas (8 psi), collision gas (4 psi), nebulizer current (2 μA) and ion source temperature (400 °C) were identical for all analytes. The mass spectrometer was operated in the positive ion mode. All analytes were detected in the MS/MS-multiple reaction monitoring (MRM) with unit resolution at both Q1 and Q3.

2.5. Statistical analysis

All statistical analyses were performed using JMP statistical software (version 5.0.1 J: SAS Institute Inc, Cary, NC, USA). For cross-sectional analyses, simple regression analysis was performed.

3. Results and discussion

3.1. Acquisition settings and chromatography

The mass spectrometer was used in MRM mode to optimize selectivity and sensitivity. The selected molecular transitions are listed in Table 1. MRM chromatograms of human breast milk sample for the determination of RE, β-carotene and α-Toc in Condition 1 are shown in Fig. 2. Under these conditions, MRM

Table 1
Retention times, molecular weights and optimized instrument settings

Analyte	Retention time (min)	MW	Transitions, m/z		DP (V)	FP (V)	CE (V)	CXP (V)
			Parent ion	Product ion				
Condition 1								
RE	9.73	286.5	269.1	213.4	21	80	19	14
d_6-RE	9.48	292.5	275.2	192.4	16	70	19	14
β-Carotene	87.01	536.9	537.6	177.2	31	100	27	12
d_6-β-Carotene	86.24	542.9	543.6	180.2	31	110	25	12
α-Toc	38.72	430.7	430.4	165.2	51	180	43	10
d_6-α-Toc	38.44	436.7	436.5	171.2	56	180	41	10
PK	49.15	450.7	451.5	187.1	41	140	33	12
[$^{18}O_2$]-PK	49.13	454.7	455.4	191.2	41	140	33	12
MK-4	32.72	444.7	445.5	187.3	21	80	31	12
[$^{18}O_2$]-MK-4	32.66	448.7	449.4	191.2	26	100	31	12
MK-7	86.74	649.0	649.7	187.2	41	150	47	12
[$^{18}O_2$]-MK-7	86.67	653.0	653.7	191.1	36	130	43	12
Condition 2								
DMEQ-TAD-D_3[b]	36.46	729.9	730.5	468.3	61	200	35	32
DMEQ-TAD-D_2[b]	36.43	742.0	742.6	468.3	56	170	35	34
DMEQ-TAD-d_7-D_3[b]	36.30	737.0	737.6	468.2	56	210	33	8
DMEQ-TAD-25(OH)D_3[b]	22.21	745.9	746.5	468.1	61	210	37	16
DMEQ-TAD-25(OH)D_2[b]	21.92	758.0	758.5	468.2	56	180	37	16
DMEQ-TAD-d_6-25(OH)D_3[b]	22.08	752.0	752.5	468.1	56	190	39	16

[a] DP, declustering potential; FP, focusing potential; CE, collision energy; CXP, collision cell exit potential.
[b] The retention times of the derivatives are those of the 6*S*-isomer.

provided high specificity for all compounds, and no crosstalk interference with d_6-labeled internal standards was observed. The linearity of calibration curves of RE (up to 62500 ng/mL, $r^2 = 0.9993$), α-tocopherol (up to 62500 ng/mL, $r^2 = 0.9998$) and β-carotene (up to 2500 ng/mL, $r^2 = 0.9989$) was confirmed (data not shown). LC–MS/MS MRM chromatograms of human breast milk sample for the determination of PK, MK-4 and MK-7 in Condition 1 are shown in Fig. 3. Vitamin K derivatives were successfully detected without interruption of co-eluting compounds in breast milk and interference of their ^{18}O-labeled internal standards. The linearity of calibration curves of PK (up to 2500 ng/mL, $r^2 = 1.0000$), MK-4 (up to 2500 ng/mL, $r^2 = 0.9998$) and MK-7 (up to 2500 ng/mL, $r^2 = 1.0000$) was confirmed (data not shown). LC–MS/MS MRM chromatograms of human breast milk samples after DMEQ-TAD derivatization for the determination of D_3, D2, 25(OH)D_3 and 25(OH)D_2 in Condition 2 are shown in Fig. 4. In the reaction with DMEQ-TAD, vitamin D compounds produce two C6-epimeric derivatives. In the case of D_3, D_2, 25(OH)D_3 and 25(OH)D_2, the 6*S* isomer was the main product (6*S*:6*R* = 3:1). Thus, 6*S* isomer was used for the determination of each vitamin D compound. The linearity of calibration curves of the DMEQ-TAD derivatives of D_3 (up to 50 ng/mL, $r^2 = 0.9999$), D_2 (up to 50 ng/mL, $r^2 = 1.0000$), 25(OH)D_3 (up to 50 ng/mL, $r^2 = 0.9999$) and 25(OH)D_2 (up to 50 ng/mL, $r^2 = 0.9991$) was confirmed (data not shown).

At present, standard assay methods are HPLC with fluorescence detection for RE [28], tocopherol [29] and vitamin K derivatives [30] and HPLC with visible detection for β-carotene [31]. For the determination of vitamin D compounds, HPLC with ultraviolet detection [15], competitive protein binding assay (CPBA) [16], radio immunoassay (RIA) [17] and enzyme immunoassay (EIA) [18] are used widely; however, in the comprehensive evaluation of the nutritional status of fat-soluble vitamins, each vitamin needs to be measured separately. Our proposed method, including two extraction methods and sensitive LC–MS/MS detection using stable isotope-labeled internal standards, makes it possible to determine the principal fat-soluble vitamins in breast milk which contain more interfering compounds compared to plasma or serum samples. Vitamin K cannot be extracted along with other fat-soluble vitamins because of its lability under basic condition. In addition, D and 25(OH)D could be measured by LC–MS/MS after DMEQ-TAD derivatization with the equivalent of only 6 mL of breast milk. DMEQ-TAD, a fluorescence-labeling reagent, is highly sensitive and stable for conjugated dienes. Recently, DMEQ-TAD has been used for the derivatization of vitamin D metabolites to improve ionization efficiency of LC–MS/MS with APCI [27]. In this study, DMEQ-TAD derivatization enhanced the measurement sensitivity of D and 25(OH)D by about 40 times.

3.2. Sensitivity, recovery and reproducibility

The detection limits of RE, β-carotene, α-Toc, PK, MK-4, MK-7, DMEQ-TAD-D_3, DMEQ-TAD-D_2, DMEQ-TAD-25(OH)D_3 and DMEQ-TAD-25(OH)D_2 based on a signal-to-noise ratio of 3:1, were 1–250 pg per injection (Table 2). The recoveries of fat-soluble vitamins were about 91–105%. Inter-assay CV values of each vitamin calculated by measurements of pooled human breast milk were 1.9–11.9%. The sensitivity and overall recovery combined with reproducibility allowed the measurement of fat-soluble vitamins containing vitamins A, D, E and K with 10 mL of breast milk.

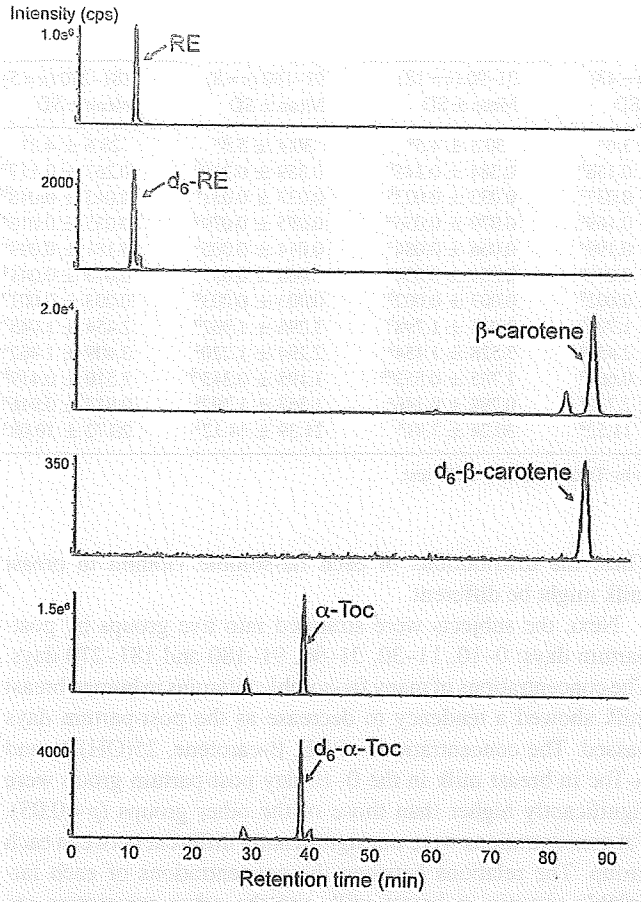

Fig. 2. LC–MS/MS MRM chromatograms of human breast milk sample for determination of RE, β-carotene and α-Toc in Condition 1. The concentrations of RE, β-carotene and α-Toc in this sample are 0.244, 0.055 and 2.131 μg/mL, respectively.

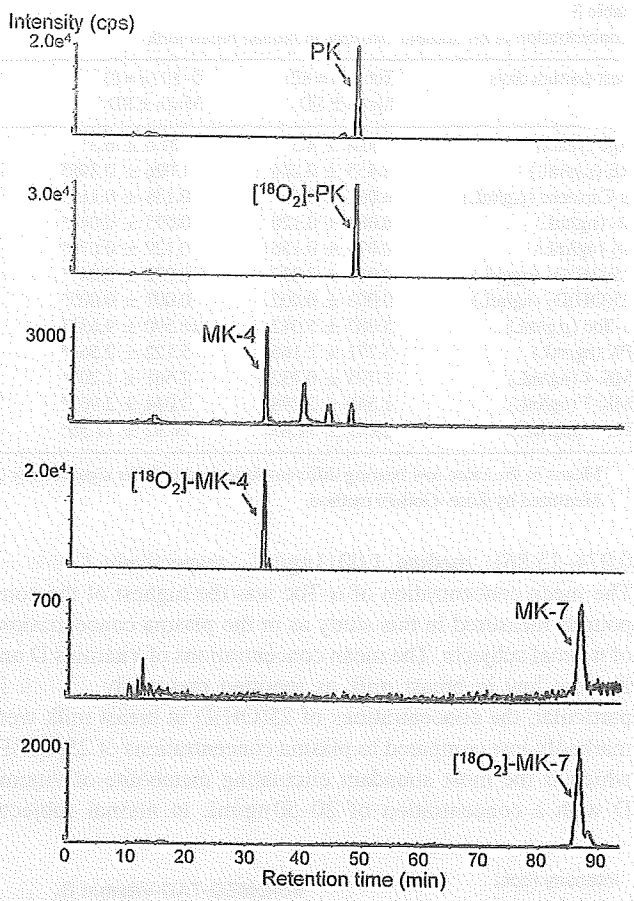

Fig. 3. LC–MS/MS MRM chromatograms of human breast milk sample for determination PK, MK-4 and MK-7 in Condition 1. The concentrations of PK, MK-4 and MK-7 in this sample are 1.628, 1.462 and 0.344 ng/mL, respectively.

3.3. Concentration of fat-soluble vitamins in human breast milk

This method was applied to breast milk samples obtained from 82 Japanese lactating mothers. The mean concentration of RE, β-carotene, D_3, D_2, 25(OH)D_3, 25(OH)D_2, α-Toc, PK, MK-4 and MK-7 of 82 lactating mothers were 0.455 (range, 0.097–1.783; median, 0.406) μg/mL, 0.062 (range, 0.002–0.375; median, 0.045) μg/mL, 0.088 (range, 0.010–1.116; median 0.061) ng/mL, 0.078 (range, 0–1.300; median, 0.021) ng/mL, 0.081 (range, 0.023–0.172; median, 0.078) ng/mL, 0.003 (range, 0–0.012; median 0.003) ng/mL, 5.087 (range, 0.387–35.664; median 3.590) μg/mL, 3.771 (range, 0.953–12.382; median 3.481) ng/mL, 1.795 (range, 0.720–4.750; median 1.611) ng/mL and 1.540 (range,

Table 2
Accuracy of measurement of fat-soluble vitamins

	Detection limit (pg)	Recovery[a]		Inter-assay	
		Mean ± SD (%)	CV (%)	Mean ± SD (mL^{-1})	CV (%)
RE	50	104.7 ± 7.5	7.2	0.489 ± 0.029 μg	7.2
β-Carotene	250	97.4 ± 7.0	8.2	0.027 ± 0.001 μg	3.8
α-Toc	100	96.3 ± 5.5	5.7	2.839 ± 0.058 μg	2.1
PK	10	97.5 ± 8.3	8.6	0.383 ± 0.014 ng	3.7
MK-4	10	99.4 ± 7.9	8.0	0.206 ± 0.004 ng	1.9
MK-7	80	97.1 ± 5.6	5.7	0.117 ± 0.011 ng	9.6
DMEQ-TAD-D_3	1	97.5 ± 3.8	3.9	0.040 ± 0.002 ng	5.4
DMEQ-TAD-D_2	1	105.0 ± 4.7	4.5	0.014 ± 0.002 ng	11.9
DMEQ-TAD-25(OH)D_3	2	93.9 ± 3.0	3.1	0.117 ± 0.005 ng	4.0
DMEQ-TAD-25(OH)D_2	1	90.9 ± 8.8	9.7	0.006 ± 0.001 ng	9.8

[a] Calculated by measurements of pooled human breast milk spiked with fat-soluble vitamins: RE, 20 μg; β-carotene, 0.6 μg; D_3, 20 ng; D_2, 20 ng; 25(OH)D_3, 20 ng; 25(OH)D_2, 20 ng; PK, 30 ng; MK-4, 20 ng; MK-7, 10 ng/20 mL of human breast milk.

Table 3
Concentration of fat-soluble vitamins in human breast milk

Post-partum days	Total ($n=82$) Mean ± SD	0–10 ($n=8$) Mean ± SD	11–30 ($n=43$) Mean ± SD	31–90 ($n=18$) Mean ± SD	91–180 ($n=8$) Mean ± SD	181–270 ($n=5$) Mean ± SD
Age (years)	30.8 ± 4.5	27.6 ± 6.3[a]	32.0 ± 3.6[a]	30.3 ± 4.5[a]	30.4 ± 5.6[a]	28.6 ± 4.3[a]
RE (μg/mL)	0.455 ± 0.264	1.026 ± 0.398[a]	0.418 ± 0.138[b]	0.384 ± 0.145[b]	0.359 ± 0.219[b]	0.267 ± 0.117[b]
β-Carotene (μg/mL)	0.062 ± 0.063	0.188 ± 0.112[a]	0.059 ± 0.037[b]	0.033 ± 0.023[b]	0.033 ± 0.031[b]	0.043 ± 0.048[b]
D_3 (ng/mL)	0.088 ± 0.128	0.075 ± 0.046[a]	0.103 ± 0.169[a]	0.079 ± 0.056[a]	0.075 ± 0.079[a]	0.035 ± 0.016[a]
D_2 (ng/mL)	0.078 ± 0.156	0.129 ± 0.076[a]	0.073 ± 0.199[a]	0.066 ± 0.084[a]	0.014 ± 0.005[a]	0.181 ± 0.099[a]
25(OH)D_3 (ng/mL)	0.081 ± 0.037	0.072 ± 0.047[a]	0.085 ± 0.038[a]	0.084 ± 0.034[a]	0.068 ± 0.037[a]	0.073 ± 0.041[a]
25(OH)D_2 (ng/mL)	0.003 ± 0.002	0.007 ± 0.003[a]	0.003 ± 0.002[b]	0.003 ± 0.002[b]	0.003 ± 0.003[b]	0.003 ± 0.001[b]
α-Toc (μg/mL)	5.087 ± 5.042	16.590 ± 9.635[a]	4.079 ± 1.795[b]	3.911 ± 1.798[b]	3.296 ± 1.962[b]	2.454 ± 1.045[b]
PK (ng/mL)	3.771 ± 2.166	5.122 ± 2.561[a]	3.938 ± 2.450[a]	3.528 ± 1.454[a]	2.294 ± 1.220[a]	3.409 ± 1.462[a]
MK-4 (ng/mL)	1.795 ± 0.732	2.561 ± 1.207[a]	1.802 ± 0.664[b]	1.785 ± 0.553[ab]	1.195 ± 0.343[ab]	1.510 ± 0.419[b]
MK-7 (ng/mL)	1.540 ± 2.298	3.044 ± 2.901[a]	1.675 ± 2.732[a]	0.798 ± 0.746[a]	1.363 ± 1.292[a]	0.917 ± 0.916[a]
Fat** (mg/mL)	28.89 ± 11.65	24.92 ± 11.55[a]	32.64 ± 11.52[a]	30.24 ± 7.91[a]	21.39 ± 14.12[a]	20.72 ± 10.08[a]

* Means in the same row bearing different superscripts differ significantly ($p<0.05$) by Tukey–Kramer HSD test.
** Measured by Röse–Gotlieb method.

0.074–15.861; median 1.001) ng/mL, respectively (Table 3). The mean concentration of α-Toc was the highest of the compounds measured in this study as in the plasma concentrations of normal subjects. The mean concentrations of vitamins D and K were low in breast milk as reported previously [7–12]. In particular, the concentrations of 25(OH)D in breast milk were markedly low compared to plasma concentrations of 25(OH)D, which is the most abundant circulating metabolite of vitamin D with a concentration of 20–50 ng/mL in normal subjects.

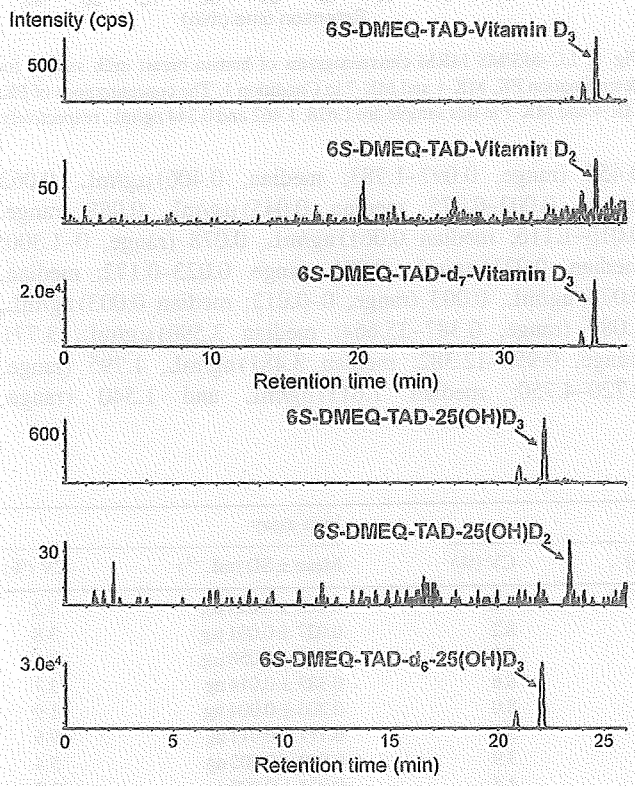

Fig. 4. LC–MS/MS MRM chromatograms of human breast milk sample after DMEQ-TAD derivatization for determination of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 in Condition 2. The concentrations of D_3, D_2, 25(OH)D_3 and 25(OH)D_2 in this sample are 0.058, 0.005, 0.103 and 0.002 ng/mL, respectively.

Thus, the distributions of each fat-soluble vitamin in breast milk might be different.

Next, the subjects were stratified into five groups by post-partum days: 0–10, 11–30, 31–90, 91–180 and 181–270 days. The concentrations of many fat-soluble vitamins in human breast milk showed a tendency to decrease as the post-partum days passed. The concentrations of RE, β-carotene, 25(OH)D_2 and α-Toc in breast milk in the 0–10 day post-partum group were significantly higher than those of the other groups ($p<0.05$). Fat concentrations were not significantly different between each group. The relations between the concentrations of each fat-soluble vitamin in breast milk and the other parameters are shown in Table 4. Post-partum days correlated significantly and negatively with the concentrations of RE, β-carotene, α-Toc and MK-4 in breast milk. Generally, vitamins A and E are high in colostrum, and decreased and stable in mature breast milk. Sakurai et al. [32] reported that the concentrations of RE, β-carotene and α-Toc in breast milk decreased as the duration of lactation increased; however, clear correlations between the concentration of D_3 and the stage of lactation were not observed. Kojima et al. [13] demonstrated that PK and MK-4 concentrations in breast milk were high in colostrum and decreased during the course of lactation. Taken together, these results suggest that the concentrations of MK-4 out of vitamin K derivatives in breast milk were influenced by the stage of lactation as well as vitamins A and E.

The concentrations of RE correlated significantly and positively with those of β-carotene, 25(OH)D_2, α-Toc, PK, MK-4 and MK-7. The concentrations of β-carotene and α-Toc were also positively correlated with 25(OH)D_2 and vitamin K derivatives. It should be noted that correlations between the concentrations of D_3 and D_2 or 25(OH)D_3 and 25(OH)D_2 were observed; however, correlations between D and 25(OH)D were not found. Therefore, the rate of secretion of vitamin D compounds from plasma to breast milk might be different depending on their polarity. Positive correlations were observed among the concentrations of vitamin K derivatives. In addition, fat concentrations also correlated significantly and positively with 25(OH)D_3, α-Toc, PK and MK-4. These results suggest that the

Table 4
Relation between concentrations of each fat-soluble vitamin in human breast milk[a]

	RE		β-Carotene		D_3		D_2		25(OH)D_3	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	−0.1528	0.1706	−0.0996	0.3735	0.2188	**0.0483**	0.1941	0.0806	0.2004	0.0710
Post-partum days	−0.3472	**0.0014**	−0.2530	**0.0218**	−0.1187	0.2881	0.0745	0.5058	−0.1021	0.3615
RE	–	–	–	–	–	–	–	–	–	–
β-Carotene	0.7588	**<.0001**	–	–	–	–	–	–	–	–
D_3	0.0294	0.7931	0.0349	0.7554	–	–	–	–	–	–
D_2	−0.0017	0.9879	0.0602	0.5908	0.7984	**<.0001**	–	–	–	–
25(OH)D_3	0.0548	0.6248	0.2208	**0.0462**	0.1490	0.1815	−0.0967	0.3875	–	–
25(OH)D_2	0.2794	**0.0110**	0.4132	**0.0001**	−0.0889	0.4269	−0.0824	0.4617	0.2748	**0.0125**
α-Toc	0.7957	**<.0001**	0.7702	**<.0001**	0.0659	0.5562	0.0401	0.7205	0.1146	0.3054
PK	0.4081	**0.0001**	0.4711	**<.0001**	0.0273	0.8075	−0.0218	0.8458	0.3324	**0.0023**
MK-4	0.5541	**<.0001**	0.5142	**<.0001**	0.2725	**0.0132**	0.1882	0.0905	0.2022	0.0685
MK-7	0.3264	**0.0026**	0.4773	**<.0001**	−0.0463	0.6795	−0.0039	0.9725	0.1025	0.3596
Fat	0.1442	0.2481	0.0929	0.4583	0.0840	0.5027	−0.1460	0.2422	0.4596	**0.0001**

	25(OH)D_2		α-Toc		PK		MK-4		MK-7	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Age	−0.2127	0.0550	−0.1433	0.1990	0.0599	0.5927	−0.0196	0.8615	−0.0700	0.5320
Post-partum days	−0.0462	0.6806	−0.2819	**0.0103**	−0.1803	0.1051	−0.2853	**0.0094**	−0.1110	0.3210
α-Toc	0.3989	**0.0002**	–	–	–	–	–	–	–	–
PK	0.2838	**0.0098**	0.5050	**<.0001**	–	–	–	–	–	–
MK-4	0.2059	0.0634	0.6545	**<.0001**	0.5751	**<.0001**	–	–	–	–
MK-7	0.1338	0.2307	0.4036	**0.0002**	0.3722	**0.0006**	0.2460	**0.0259**	–	–
Fat	0.1565	0.2095	0.2638	**0.0323**	0.5514	**<.0001**	0.5095	**<.0001**	0.0870	0.4873

[a] Values listed in bold face showed significant correlation.

concentrations of fat may have a considerable effect on those of vitamins E and K in breast milk. In contrast, factors other than fat, such as binding protein, may contribute to the concentrations of vitamins A and D in breast milk.

4. Conclusions

Here, we show a quantification method for fat-soluble vitamins in breast milk by LC–MS/MS. The present method maximizes the sensitivity and selectivity of the latest generation of tandem mass spectrometry and derivatization technique for the measurement of representative compounds of fat-soluble vitamins in human breast milk. The assay includes vitamins with a wide range of polarity, and this method has the advantage of low sample volume requirement. This method can apply the measurement of fat-soluble vitamins in other biological samples such as plasma, and may be useful for large-scale studies and the setting of Dietary Reference Intakes of fat-soluble vitamins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2007.09.023.

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Vitamin D status, bone mass, and bone metabolism in home-dwelling postmenopausal Japanese women: Yokogoshi Study

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Abstract

Little has been understood about vitamin D status in relation to bone health in Asian women. The purpose of this study was to identify how the serum 25-hydroxyvitamin D (25[OH]D) concentration is associated with bone mass and bone metabolism. This cross-sectional, community-based epidemiologic study was conducted among 600 ambulatory postmenopausal women. The serum 25(OH)D concentration was measured with radioimmunoassay. Other blood biochemical measurements were intact parathyroid hormone and markers of bone turnover, including osteocalcin and type I collagen cross-linked N-telopeptides. Bone mineral density (BMD) of the lumbar spine and right femoral neck were measured with the dual-energy X-ray absorptiometry method using a QDR4500a. The mean serum 25(OH)D concentration was 55.6 nmol/L (SD 14.6). Serum 25(OH)D concentration was linearly associated with BMD of the femoral neck (R^2=0.020, P=0.003), but not with BMD of the lumbar spine. Odds ratios (ORs) for low BMD (defined as t score ≤ −2.5 SD) were calculated for strata defined by 25(OH)D concentration. The prevalence of low BMD of the lumbar spine was significantly higher in the 40- to 50-nmol/L 25(OH)D group (adjusted OR=3.0, 95% CI: 1.3–7.0) compared to the reference group (≥ 70 nmol/L). Prevalence of low BMD for the femoral neck was significantly higher in the 30- to 40-nmol/L (adjusted OR=3.6, 95% CI: 1.1–12.1) and the 40- to 50-nmol/L (adjusted OR=7.6, 95% CI: 2.5–23.2) groups compared to the reference group (≥70 nmol/L). The mean serum concentration of intact PTH was significantly higher in subjects with serum 25(OH)D <50 nmol/L compared to those with serum 25(OH)D ≥ 50 nmol/L. The present study suggests that higher serum 25(OH)D concentrations are associated with increased BMD of the femoral neck, and that a serum 25(OH)D concentration of at least 70 nmol/L is needed to obtain high BMD of the femoral neck, and that of at least 50 nmol/L is needed to achieve normal PTH levels and prevent low BMD in home-dwelling postmenopausal Japanese women.

Keywords: Bone density; Epidemiology; Japanese; Vitamin D; Postmenopause

Introduction

Vitamin D insufficiency is an important risk factor for the development of osteoporosis and osteoporotic fractures in the elderly. One mechanism by which this excess risk is conferred is through an increase in parathyroid hormone production [1]. Moreover, vitamin D insufficiency may cause decreased muscle function and standing balance [2], leading to an increased frequency of falls. Supplementation with vitamin D, particularly among the elderly and among women, is recommended in many European and North American countries.

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Table 1
Demographic and physical characteristics, nutritional intakes, bone mineral density (BMD), and serum biochemical profiles of study subjects

	N	Mean	SD
Age (years)	600	63.5	5.8
Menopausal age (years)	598	51.0	8.3
Number of children	599	2.3	0.8
Height (cm)	600	150.7	5.5
Weight (kg)	600	53.1	8.3
Body mass index (kg/m^2)	600	23.4	3.5
Thigh muscle strength (kg)	584	36.0	7.6
Grip strength (kg)	599	23.2	3.9
Timed Up & Go test (s)	593	5.0	1.0
Calcium intake[a] (mg/day)	600	527	160
Vitamin D intake (μg/day)	600	11.7	2.7
BMD at lumbar spine (g/cm^2)	599	0.846	0.147
BMD at femoral neck (g/cm^2)	598	0.668	0.094
Serum 25-hydroxyvitamin D (nmol/L)	600	55.6	14.6
Serum 1,25-dihydroxyvitamin D (pmol/L)	598	130.5	44.5
Serum intact parathyroid hormone (pmol/L)	600	4.24	1.40
Serum osteocalcin (mg/ml)	600	9.93	3.95
Serum type I collagen cross-linked N-telopeptides (nmol BCE/L)	595	21.0	6.5

[a] Calcium intake from dietary source was 518 mg (SD 147).

There may be ethnic differences in the effects of low vitamin D status on bone mass or bone metabolism. African Americans typically have lower vitamin D levels than Caucasian Americans, yet they have a lower prevalence of osteoporosis . Furthermore, the relationship between serum 25-hydroxyvitamin D (25[OH]D, an index of vitamin D status) concentrations and bone mineral density (BMD) may differ between blacks and whites . These findings demonstrate the importance of studies aimed at understanding the effect of vitamin D status on bone in non-white populations.

There have been only a few studies on the association between vitamin D status and bone parameters in Asians; those that have been conducted have typically had small sample sizes. One large population-based study among Japanese elderly women reported a cutoff level of serum 25(OH)D concentration in relation to elevated serum parathyroid hormone (PTH) as low as 40 nmol/L. This is lower than cutoff levels reported recently by several studies among Caucasian patients and is less than current recommended levels (75–80 nmol/L or higher) of serum 25(OH)D . These results suggested a possible ethnic difference between Asians and whites.

The primary aim of this study is to investigate the association between the serum 25(OH)D concentration and bone mass or bone metabolism among Japanese postmenopausal women. Results from this study may inform the appropriate levels of serum 25(OH)D to aim for in preventive vitamin D supplementation programs for these women.

Subjects and methods

Subjects

All 1310 women who lived in Yokogoshi area (Niigata City, Japan) aged between 55 and 74 years on March 31, 2006, were invited to participate in the Yokogoshi Study, a cross-sectional, epidemiologic, community-based investigation of bone health for postmenopausal women. The study was conducted in November 2005. Of the 1310 women, 674 (51.5%) agreed to participate in the study. All participants were non-institutionalized and ambulatory. The following women who had medical histories that may have affected their bone metabolism were excluded from analysis: (1) 13 women with a history of bilateral oophorectomy, (2) 7 women who had undergone corticosteroid therapy, and (3) 54 women treated with bisphosphonates, selective estrogen receptor modulators, active vitamin D analogues, vitamin K (menatetrenone), estrogen, or calcitonin for suspected osteoporosis. Ultimately, 600 of 674 (89%) women agreeing to participate in the study formed the group analyzed. Written informed consent was obtained from all subjects. The protocol of this study was approved by the Ethics Committee of Niigata University School of Medicine.

BMD measurement

BMDs of the lumbar spine (L2–4) and right femoral neck were measured through the dual-energy X-ray absorptiometry (DXA) method using a

Table 2
Results of simple linear regression analyses with bone mineral density (BMD) as the dependent variable

Predictor variable	BMD of the lumbar spine			BMD of the femoral neck		
	Regression coefficient (β)	R^2	P value	Regression coefficient (β)	R^2	P value
Age (years)	−0.00611	0.057	<0.0001	−0.00473	0.084	<0.0001
Years since menopause	−0.00289	0.039	<0.0001	−0.00193	0.042	<0.0001
Number of children	−0.00158	0.000	0.8312	−0.00216	0.000	0.6519
Height (cm)	0.00481	0.033	<0.0001	0.00322	0.036	<0.0001
Weight (kg)	0.00610	0.119	<0.0001	0.00440	0.151	<0.0001
Body mass index (kg/m^2)	0.0118	0.078	<0.0001	0.00854	0.099	<0.0001
Thigh muscle strength (kg)	0.00464	0.058	<0.0001	0.00341	0.076	<0.0001
Grip strength (kg)	0.00914	0.059	<0.0001	0.00541	0.050	<0.0001
TUG test[a] (s)	−0.101	0.013	0.0050	−0.0876	0.024	0.0002
Engage in housework (No, 0; yes, 1)	−0.00392	0.000	0.8908	−0.0179	0.002	0.3289
Engage in light exercise (No, 0; yes, 1)	−0.0110	0.001	0.3705	−0.00537	0.001	0.4936
Engage in farmwork (No, 0; yes, 1)	−0.0159	0.003	0.1864	−0.00164	0.000	0.8323
Calcium intake (mg/day)	0.0000631	0.005	0.0946	0.0000225	0.001	0.3532
Serum 25(OH)D (nmol/L)	0.000622	0.004	0.1322	0.000914	0.020	0.0005
Serum $1,25(OH)_2D$ (pmol/L)	−0.000405	0.015	0.0028	−0.000246	0.013	0.0046
Serum intact PTH[a] (pmol/L)	−0.0183	0.002	0.3298	−0.0357	0.015	0.0029
Serum osteocalcin (ng/ml)	−0.00868	0.054	<0.0001	−0.00532	0.050	<0.0001
Serum NTX (nmol BCE/L)	−0.113	0.043	<0.0001	−0.0655	0.035	<0.0001

Abbreviations: TUG, Timed "Up & Go"; 25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D$, 1,25-dihydroxyvitamin D; PTH, parathyroid hormone; NTX, type I collagen cross-linked N-telopeptides.
[a] Logarithmically transformed.

QDR4500a (Hologic Inc., Bedford, MA, USA) by a single, trained X-ray technician. The in vivo coefficients of variation (CVs) of the BMD measurements were 0.3% for the lumbar spine and 0.6% for the femoral neck.

Physical examination

The grip strength of each hand was measured once with a digital hand dynamometer, and the average value of both hands was adopted. Isometric thigh muscle strength of both legs together was measured with a leg muscle dynamometer (T.K.K.5710g, Takei Scientific Instruments, Co., Ltd., Niigata, Japan). Walking ability (walking time) was assessed by the timed "Up & Go" (TUG) test [7]. Body height and weight of the subjects in light underwear were measured to the nearest 1 mm and 100 g, respectively. The body mass index (BMI) was calculated by dividing body weight (kg) by the square of body height (m^2).

Biochemical measurements

A 6-h-fasting blood specimen was drawn in the daytime. The specimen was immediately maintained at 4 °C. The serum was obtained within 1 day of collection by centrifugation at 1613×*g* for 10 min and stored at −80 °C until the biochemical analysis. The serum 25(OH)D concentration was determined by radioimmunoassay (DiaSorin, Stillwater, MN, USA) with an inter-assay CV value of 9.9%. The serum 1,25-dihydroxyvitamin D (1,25[OH]$_2$D) concentration was determined by radioimmunoassay (IDS Ltd., Boldon, England, UK), which has an inter-assay CV value of 12.8%. The serum intact PTH concentration was measured with a two-site immunoradiometric assay (Nichols Institute Diagnostics, San Clemente, CA, USA), which has an inter-assay CV value of 1.5%. The serum osteocalcin (OC) concentration was determined by an immunoradiometric assay (Mitsubishi Kagaku Medical, Inc., Tokyo, Japan) with an inter-assay CV value of 6.6%. The serum type I collagen cross-linked N-telopeptides (NTX) concentration was determined by an enzyme-linked immunosorbent assay (Osteomark NTX Serum, Ostex International, Inc., Seattle, WA, USA), which had an inter-assay CV value of 2.8%.

Interview

Demographic, lifestyle, and nutritional information was obtained through interview. Age, reproductive history, medical history, and current medications were recorded. Current calcium intake was assessed with a previously validated food frequency questionnaire [8]. The correlation coefficient between values measured by this method and the conventional 3-day diet record was 0.668. Physical activity levels were assessed based on whether subjects engaged in the following three activities at least once a week: (1) housework, (2) light exercise, such as gate ball (or croquet), taking walks, and so on, as light activity, and (3) farmwork (or gardening), as moderate activity.

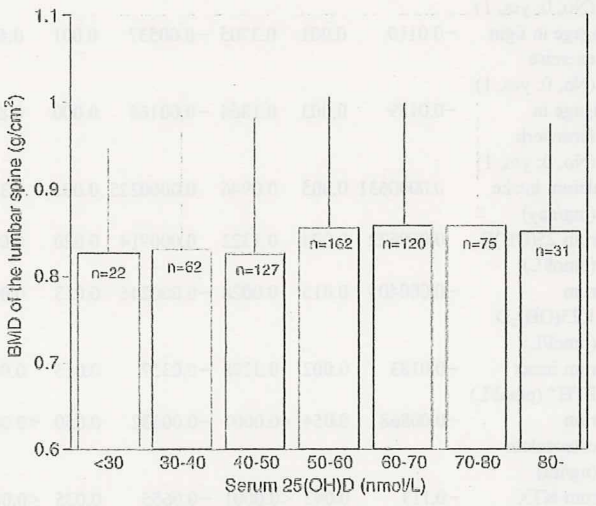

Fig. 1. Mean (plus SD) values of bone mineral density (BMD) of the lumbar spine for each 10-nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. The serum 25(OH)D concentration was not linearly associated with BMD at the lumbar spine (P=0.1322), although 50 nmol/L may be an inflection point.

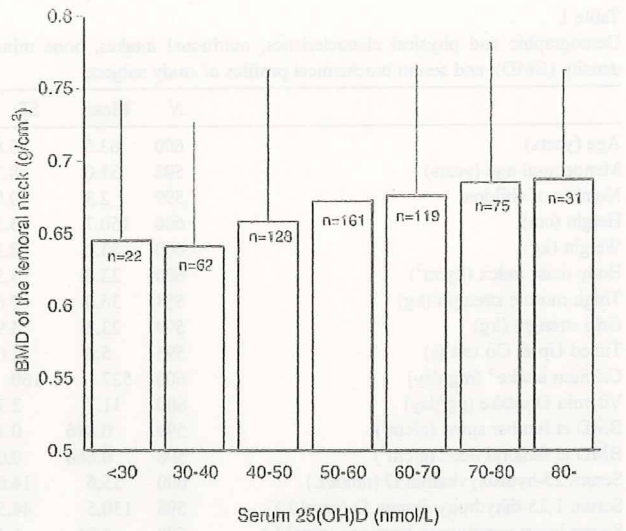

Fig. 2. Mean (plus SD) values of bone mineral density (BMD) of the femoral neck for each 10-nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. BMD becomes higher as the 25(OH)D level becomes higher beginning from the 40- to 50-nmol/L group of serum 25(OH)D.

Statistical analysis

All continuous variables were checked for normality. TUG test, serum intact PTH, and NTX concentrations were skewed to higher values and were transformed logarithmically prior to conducting statistical tests. Categorical variables, such as "housework", "light exercise", and "farmwork" were coded as 0 for "no" and 1 for "yes". Student's *t*-test was used to test a difference in two mean values. Analysis of variance (ANOVA) was used to test differences among multiple mean values. ANOVA with Dunnett's multiple comparison was used to compare

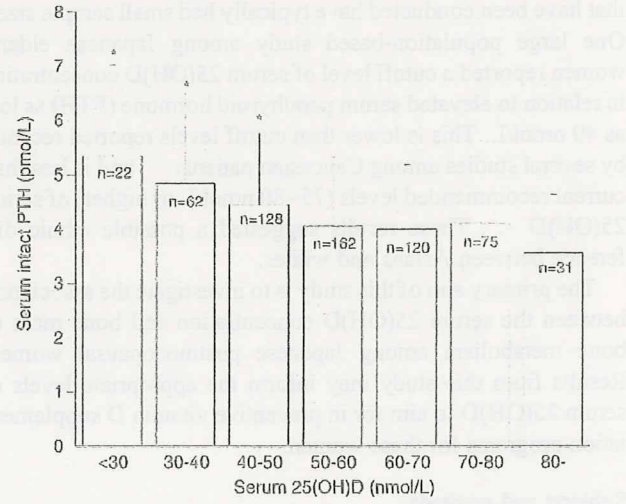

Fig. 3. Mean (plus SD) values of the serum intact parathyroid hormone (PTH) concentration for each 10 nmol/L increment in the serum 25-hydroxyvitamin D (25[OH]D) concentration. Mean serum intact PTH concentrations for 25(OH)D <30 nmol/L, 30–39 nmol/L, and 40–49 nmol/L, indicated with an asterisk (*), are significantly higher than those for serum 25(OH)D concentrations ≥50 nmol/L, as assessed by ANOVA with the Dunnett multiple comparison.

Table 3
Results of a stepwise multiple linear regression analysis predicting bone mineral density (BMD)

Independent variable	Regression coefficient (β)	Standard error	R^2	P value
BMD of the lumbar spine				
BMI (kg/m^2)	0.0115	0.0016	0.099	<0.0001
Age (years)	−0.00487	0.00117	0.058	<0.0001
Serum osteocalcin (ng/ml)	−0.00633	0.00152	0.041	<0.0001
Grip strength (kg)	0.00482	0.00146	0.016	0.0011
Calcium intake (mg/day)	0.0000904	0.0000344	0.012	0.0089
Years since menopause	−0.00142	0.00065	0.005	0.0279
Serum NTX[a] (nmol BCE/L)	−0.0434	0.0224	0.005	0.0535
BMD of the femoral neck				
BMI (kg/m^2)	0.00825	0.00010	0.112	<0.0001
Age (years)	−0.00521	0.00061	0.084	<0.0001
Serum osteocalcin (ng/ml)	−0.00422	0.00085	0.031	<0.0001
Serum 25(OH)D (nmol/L)	0.000705	0.000235	0.020	0.0029
Serum intact PTH[a] (pmol/L)	−0.0292	0.0107	0.013	0.0065
Grip strength (kg)	0.00215	0.00090	0.009	0.0167
Calcium intake (mg/day)	0.0000449	0.0000211	0.008	0.0336

Abbreviations: 25(OH)D, 25-hydroxyvitamin D; PTH, parathyroid hormone; NTX, type I collagen cross-linked N-telopeptides.
[a] Log-transformed values.

one mean value with other mean values. Simple linear regression analysis was used to identify predictors of BMD, indices of bone metabolism, including the log-transformed serum intact PTH, OC, and log-transformed NTX, and physical tests, including muscle strength and log-transformed TUG test as outcome variables. A stepwise multiple linear regression analysis was used to identify independent predictors of BMD. Candidate independent variables for the stepwise method were age, menopausal age, BMI, physical tests, lifestyle variables, calcium intake, the 25(OH)D, log-transformed serum intact PTH, OC, and log-transformed NTX concentrations. The serum 1,25(OH)$_2$D concentration was not included in the model because a negative association between serum 1,25(OH)$_2$D concentrations and BMDs was considered to be due to a compensatory increase of serum 1,25(OH)$_2$D concentrations for low bone mass [9,10]. Multiple logistic regression analyses were used to calculate adjusted odds ratios (ORs) of vitamin D insufficiency for "low BMD (t score ≤−2.5 SD)". Test for linear trend was performed by using the logistic regression technique. Computations were performed by using the SAS statistical package (release 8.02, SAS Institute Inc., Cary, NC, USA). A P value less than 0.05 was considered statistically significant.

Results

The demographic and physical characteristics, nutritional intake, bone mass, and serum biochemical profiles are shown in Table 1. The proportion of subjects who had the serum 25(OH)D concentration less than 30 nmol/L and 50 nmol/L were 22/600 (3.7%) and 212/600 (35.3%), respectively. On the physical activity measure, 572 (95.3%) subjects did housework, 250 (41.7%) engaged in light activity, and 298 (49.7%) engaged in farmwork (moderate activity). "Low BMDs" (t score ≤−2.5 SD) were observed in 106/599 (17.7%) of lumbar spines and 82/598 (13.7%) of femoral necks.

The results of the simple linear regression analyses with BMD as the outcome are shown in Table 2. The serum 25(OH)D concentration was not significantly associated with BMD of the lumbar spine but was positively associated with BMD of the femoral neck. Mean BMD at the lumbar spine for each 10-nmol/L increment in the serum 25(OH)D concentration is shown in Fig. 1. The serum 25(OH)D concentration was not linearly associated with BMD at the lumbar spine (P=0.1322). Mean BMDs at the femoral neck for each 10-nmol/L increment in the serum 25(OH)D concentration are shown in Fig. 2. BMD increases as the 25(OH)D concentration increases beginning from the 40- to 50-nmol/L group of serum 25(OH)D.

The serum 25(OH)D concentration was negatively associated with the log-transformed serum intact PTH concentration (β=−0.00543, R^2=0.061, P<0.0001). Mean serum intact PTH concentrations for each 10 nmol/L increment in the serum 25(OH)D concentration are shown in Fig. 3. Mean serum intact PTH concentrations for serum 25(OH)D <30 nmol/L, 30–39 nmol/L, and 40–49 nmol/L were significantly higher than a probable baseline intact PTH concentration, i.e., the mean intact PTH concentration for serum 25(OH)D concentrations ≥50 nmol/L. A linear association between calcium intake and the log-transformed serum intact PTH concentration was of borderline significance (P=0.0611). The log-transformed serum intact PTH concentration was significantly associated with both serum OC (β=1.29, R^2=0.011, P=0.0102) and log-transformed NTX (β=0.0749, R^2=0.008, P=0.0302) concentrations.

Table 4
Odds ratios (OR) and 95% confidence intervals (CI) for "low bone mineral density (BMD) (t score ≤−2.5 SD)" according to levels of serum 25(OH)D

	Levels of serum 25(OH)D (nmol/L)						P for trend
	<30 (n=22)	30–40 (n=62)	40–50 (n=127)	50–60 (n=162)	60–70 (n=120)	≥70 (n=106)	
Lumbar spine							
Prevalence of low BMD (%)	18.2	22.6	25.2	16.1	15.0	11.3	
Unadjusted OR	1.61	1.99	2.23	1.42	1.33	1 (ref.)	0.0109
95% CI	0.57–4.52	0.99–4.03	1.21–4.10	0.75–2.69	0.67–2.62		
Adjusted[a] OR	3.03	2.44	3.02	1.32	1.48	1 (ref.)	0.0173
95% CI	0.57–16.02	0.84–7.12	1.31–6.97	0.59– 2.99	0.61–3.59		
Femoral neck							
Prevalence of low BMD (%)	18.2	21.0	23.4	11.2	9.2	5.7	
Unadjusted OR	3.21	3.70	4.14	1.98	1.63	1 (ref.)	<0.0001
95% CI	0.99–10.44	1.48–9.25	1.79–9.57	0.81–4.81	0.63–4.26		
Adjusted[a] OR	2.86	3.59	7.55	2.07	1.40	1 (ref.)	0.0017
95% CI	0.28–29.03	1.06–12.11	2.45–23.24	0.74–5.80	0.45–4.35		

[a] Adjusted for age, menopausal age, BMI, calcium intake, grip strength, log-transformed intact PTH, OC, and log-transformed NTX.

The results of the stepwise multiple regression analysis are shown in . BMI was the predominant independent variable, followed by age and serum OC concentration for both BMDs of the lumbar spine and femoral neck. The serum 25(OH)D concentration was independently associated with BMD of the femoral neck, although its R^2 was smaller than those of BMI, age and serum OC concentration.

shows ORs for "low BMD (t score ≤ -2.5 SD)" by level of serum 25(OH)D. After adjustment for model covariates, prevalence of low BMD for the lumbar spine was significantly higher in the 40- to 50-nmol/L group compared to the reference group (≥ 70 nmol/L). Similarly, a significantly higher prevalence of low BMD of the femoral neck was observed in the 30- to 40-nmol/L and 40- to 50-nmol/L groups compared to the reference group (≥ 70 nmol/L). The serum 25(OH)D concentration was not significantly associated with the serum OC concentration ($P=0.1715$) or the serum NTX concentration ($P=0.2355$). The lack of these associations remained after subjects were restricted to those with serum 25(OH)D concentrations <50 nmol/L ($P=0.4839$ for serum OC and $P=0.9574$ for serum NTX).

The serum 25(OH)D concentration is generally believed to be associated with physical strength. However, the serum 25(OH)D concentration was significantly associated with neither thigh muscle strength ($P=0.1144$), grip strength ($P=0.3131$), nor the TUG test ($P=0.6140$). Even when comparing in these three physical variables between lower and higher subgroups by using any thresholds, there were no significant differences in any variables between them.

Discussion

This is the first large-scale epidemiologic study exploring a possible association between vitamin D status and bone mass, bone metabolism, or physical strength in postmenopausal Asian women. The mean serum 25(OH)D concentration (55.6 nmol/L) and prevalence of vitamin D insufficiency observed in this population were similar to those of other populations of ambulant Japanese elderly women . The vitamin D status of ambulant elderly Japanese, including this study population, is well maintained even in winter, due in part to high dietary intake of vitamin D from fish . This study demonstrated that the serum 25(OH)D concentration was linearly associated with BMD of the femoral neck in subjects with a serum 25(OH)D concentration of 30 nmol/L or higher. This finding is in accordance with the result of a large epidemiologic study recently conducted and supports a rationale that the serum 25(OH)D levels should be maintained 75–80 nmol/L or higher . By contrast, an association between the serum 25(OH)D concentration and BMD of the lumbar spine was not significant. This discrepancy has not been frequently reported in the literature, but may be explained by the fact that vitamin D status affects cortical bone more than spongy bone. This hypothesis is supported by Stone et al.'s finding that lower 25(OH)D levels are associated with hip but not calcaneal bone loss. Regarding the association between the serum 25(OH)D concentration and BMD of the lumbar spine, 50 nmol/L appears to be an inflection point ( ). This study may have failed to detect a true association due to the relatively small number of subjects at high 25(OH)D levels. Further studies should address this issue.

The present study showed that the serum 25(OH)D concentration of 50 nmol/L or lower was associated with low BMD (t score ≤ -2.5 SD) of both the lumbar spine and femoral neck (no significant increase in the prevalence of low BMD was observed in the <30 nmol/L group due to limited sample size). Study findings also suggest that vitamin D insufficiency is more strongly associated with low BMD in the femoral neck than in the lumbar spine.

Despite the significant associations observed between serum 25(OH)D concentration and BMD, the low R^2 values associated with vitamin D status in multivariate analysis indicate that it accounted for only a small proportion of the variance in BMD in the study population. Results of the present study are in line with the findings of two recent population-based investigations targeting postmenopausal women. The Rancho Bernardo Study showed a slight but significant association between serum 25(OH)D and femoral BMD, and the OFELY Study showed serum 25(OH)D not to be a significant determinant of bone loss. On the other hand, there have been two clinic-based studies in which the serum 25(OH)D concentration was correlated moderately with both spinal and femoral BMDs in postmenopausal women . As such, the strength of the association between vitamin D status and BMD seems to depend on which population is targeted.

Numerous studies have shown an inverse association between the serum 25(OH)D and intact PTH serum concentrations . The present study confirmed such an association with a threshold of 50 nmol/L of the serum 25(OH)D concentration for elevated serum intact PTH concentrations. This finding suggest that maintenance of serum 25(OH)D concentrations of at least 50 nmol/L is essential for maintaining bone health in postmenopausal Japanese women.

This study failed to confirm an association between serum 25(OH)D concentration and markers of bone turnover. Gallagher et al. also reported no or only a slight association between the serum 25(OH)D concentration and markers on bone turnover in a healthy elderly population. On the other hand, Jesudanson et al. showed a negative association between serum 25(OH)D concentration and serum bone resorption markers and alkaline phosphatase levels in postmenopausal women attending an osteoporosis clinic. Furthermore, an inverse relationship between serum 25(OH)D and markers of bone turnover was found in postmenopausal women with established osteoporosis . Taken together, these studies suggest an association between the serum 25(OH)D and markers of bone turnover may be observed in frail populations, such as osteoporotic women, but not in the general population of postmenopausal women.

Our study also demonstrated that serum intact PTH is associated with BMD of the femoral neck, but not with BMD of the lumbar spine. The lack of the association with the lumbar spine may be due to the fact that PTH affects cortical bone mass to a greater extent than spongy bone mass or because PTH does not have as great of an effect on bone mass in elderly Asian

populations compared to their European counterparts . Moreover, BMD of the femoral neck was independently associated with serum PTH and 25(OH)D, which suggests that each plays an independent role in bone metabolism and bone mass. PTH may affect BMD partly via increased bone turnover because high serum PTH was associated with both serum OC and NTX in this study. On the other hand, serum 25(OH)D may affect BMD not via increased bone turnover, as serum 25(OH)D did not link to bone turnover markers in this study but probably via increased calcium absorption in the intestine. The cross-sectional nature of this study has limitations in its ability to make causal relationships, and this hypothesis should be confirmed by a longitudinal study.

Low levels of vitamin D have been reported to be associated with impaired physical functions . To the contrary, the present study failed to demonstrate such an association between vitamin D status and muscle strength or the TUG test. The lack of the associations in this study may be due to relatively good vitamin D status (mean serum 25[OH]D concentration, 55.6 nmol/L), the study population being relatively young (mean age, 64.5 years), or ethnicity .

The elderly Japanese population has some characteristics in terms of diet and bone health that make them different from other general populations. They have lower calcium intake and higher vitamin D intake than elderly whites . In the present population, 95% of the subjects had total calcium intake of less than 800 mg/day, a daily calcium requirement in Japan . Their low calcium intake (527 mg/day) might diminish an effect of vitamin D on bone, and increase of calcium intake is hypothesized to alter strength of the association between vitamin D status and bone mass.

This study had some limitations. This study employed a cross-sectional design, which is limited in its ability to detect causal relationships. An intervention trial is needed in order to establish causality. In addition, subjects' participation rate of this study was approximately 50%, and thus selection bias may have occurred. For example, it is likely that healthier or more active women tended to participate in this study. Generalizations of our results to other populations should thus be made with caution.

In summary, the present study was the largest study to date to examine the relationship between vitamin D levels and bone health among Asian postmenopausal women. Our results suggest that higher serum 25(OH)D concentrations are associated with increased BMD of the femoral neck, and that a serum 25(OH)D concentration of at least 70 nmol/L is needed to obtain high BMD of the femoral neck, and that of at least 50 nmol/L is needed to achieve normal PTH levels and prevent low BMD. While significant associations were observed between vitamin D status and BMD of the femoral neck, the contribution of vitamin D status to BMD is relatively small, suggesting a role for other factors in low bone mass.

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ORIGINAL ARTICLE

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Low plasma phylloquinone concentration is associated with high incidence of vertebral fracture in Japanese women

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Abstract It has been reported that vitamin K supplementation effectively prevents fractures and sustains bone mineral density in osteoporosis. However, there are only limited reported data concerning the association between vitamin K nutritional status and bone mineral density (BMD) or fractures in Japan. The objectives were to evaluate the association between plasma phylloquinone (K_1) or menaquinone (MK-4 and MK-7) concentration and BMD or fracture in Japanese women prospectively. A total of 379 healthy women aged 30–88 years (mean age, 63.0 years) were consecutively enrolled. Plasma K_1, MK-4, MK-7, and serum undercarboxylated osteocalcin (ucOC) concentrations, BMD, and incidence of vertebral fractures were evaluated. In stepwise multiple linear regression analyses, L_{2-4} BMD and a bone turnover marker, log K_1, concentrations were independently correlated with vertebral fracture incidence. When subjects were divided into low and high K_1 groups by plasma K_1 concentration, the incidence of vertebral fracture in the low K_1 group (14.4%) was significantly higher than that in the high K_1 group (4.2%), and its age-adjusted RR was 3.58 (95% CI, 3.26–3.93). L_{2-4} BMD was not different between the two groups. These results suggest that subjects with vitamin K_1 insufficiency in bone have increased susceptibility for vertebral fracture independently from BMD.

Key words vitamin K · undercarboxylated osteocalcin · vertebral fracture · bone mineral density (BMD) · Japanese women · phylloquinone

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Introduction

Vitamin K is well known for its role in the synthesis of a number of blood coagulation factors. Vitamin K is also an important factor for bone metabolism via γ-carboxylation of vitamin K-dependent proteins such as osteocalcin (OC), matrix Gla protein, and protein S [1,2]. Low dietary phylloquinone (K_1) intake has been shown to be associated with increased hip fracture risk, notably among postmenopausal women [3,4]. Low dietary K_1 intake is also associated with low bone mineral density (BMD) at the hip and spine in pre- and postmenopausal women [5,6], and circulating levels of vitamin K_1 or K_2 were reported to be decreased in patients with hip fracture [7–10]. Those studies were mainly performed in Caucasians. There is only a limited amount of data concerning the association between vitamin K nutritional status and BMD or fractures in Japan. It has been reported that the intake of *natto*, which contains a high concentration of menaquinone-7 (MK-7), prevents hip fractures in Japanese [11] or promotes bone formation in premenopausal women [12]. However, another report showed that no differences in plasma K_1, menaquinone-4 (MK-4), and MK-7 were observed between patients with vertebral or hip fracture and normal subjects [13]. In animal models of osteoporosis, the effects of vitamin K_2 supplementation on bone mass, strength, and structure has been reported to be effective [14–17], or to be negative in ovariectomized rats [18–20], and the evidence is still equivocal. Although a relationship between vitamin K status and fracture risk has been reported, the relationship between BMD or fracture and vitamin K status is still controversial. Recently, it has been reported that vitamin K stimulates the differentiation of osteoblasts via not only γ-carboxylation but also steroid or xenobiotics receptors (SXR) [21].

Therefore, in the present study, we evaluated the association between plasma vitamin K (K_1, MK-4, and MK-7) concentrations and incidence of fracture or BMD in Japanese women prospectively, and assessed the importance of vitamin K status or γ-carboxylation of OC in reduction of fracture risk and increase of BMD.

Subjects and methods

Subjects

Japanese women in their thirties to eighties were consecutively enrolled in this study (2002–2003), and followed up by 2006. Women with metabolic bone diseases other than primary osteoporosis and women who were taking medicine related to bone metabolism such as active vitamin D, vitamin K, vitamin K antagonists, estrogen, bisphosphonates, or steroids were excluded. Women who had extremely low body mass index (BMI) (lower than 16) were also excluded. A total of 379 women (mean age, 63.0 ± 10.8 years; range, from 30 to 88 years) met the selection criteria for this study. The subjects consisted of 48 women aged 30–49 years, 202 women aged 50–69 years, and 129 women aged 70 years or older (70– years). Subjects were living in a rural area of Nagano. Most subjects have a backyard with their house, and they had the habit of frequently eating vegetables that they cultivated in their backyard.

Measurements

Plasma, serum and urine samples were collected from the subjects in the morning and stored immediately at –30°C until measurement. Plasma vitamin K (K_1, MK-4, and MK-7) was determined by the high-performance liquid chromatography-tandem mass spectrometry (LC-APCI-MS/MS) method [22]. ucOC as a sensitive marker for vitamin K insufficiency was measured by electrochemiluminesence immunoassay (ECLIA) (Sanko Junyaku, Japan). The antibody used in this ECLIA method is the same antibody used in the "Takara assay." However, the ucOC concentrations measured using this novel method were higher than those obtained using other methods, including the Takara assay. Intact OC was determined by immunoradiometric assay (IRMA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Japan).

Serum concentrations of 25-hydroxyvitamin D [25-OH-D; radioimmunoassay (RIA); DiaSorin, Stillwater, MN, USA], and intact (1-84, 7-84) parathyroid hormone [intact PTH, immunoradiometric assay (IRMA); Scantibodies Laboratory, Santee, CA, USA] were determined. A bone resorption marker, urinary excretion of *N*-telopeptide (NTX; as measured by enzyme-linked immunosorbent assay (ELISA; Osteomark, Ostex International Seattle, WA, USA), and a bone formation marker, bone-derived alkaline phosphatase (BAP; EIA; DS Pharma Biomedical, Japan), were measured. For the evaluation of calcium metabolism, serum concentrations of calcium (Ca) and phosphorus (P) were measured. Body mass index (BMI) was calculated as the weight in kilograms divided by the square of the height in meters.

Lumbar spine (L_{2-4}) and femoral neck (FN) BMD was measured by dual-energy X-ray absorptiometry (DXA) using a Lunar DPX-IQ (Lunar, Radisson, WI, USA). The interassay variance of this method in our laboratory was 0.5% ± 0.5% [coefficient of variation (CV) ± SD] [23]. Incident vertebral fracture was first defined by the semiquantitative method reported by Genant et al. [24]. When a marginal fracture was obtained, we performed quantitative measurements of vertebral body heights at the posterior, central, and anterior margins in both baseline and follow-up vertebral films. We then redefined the presence or absence of incident vertebral fractures in accordance with the criteria proposed by Fukunaga et al. [25]. Fractures were evaluated by one of the coauthors who had contributed to development of the method of Fukunaga et al. [25]. Incident fractures with apparent major trauma were excluded from the present study because we wanted to examine the relationship between vitamin K nutrition and fragility fracture occurrence.

Statistical analysis

All statistical analyses were performed by using statistical software JMP 6.0 J (SAS Institute, Cary, NC, USA). Logistic regression analysis was used to test univariable associations between the incidence of vertebral fracture and anthropometric parameters, bone metabolic parameters, or plasma vitamin K concentrations. Stepwise multiple linear regression analyses were performed to explore determinants of incident vertebral fractures. The following plausible predictors were included in the original model: (1) age, BAP, and K_1 concentration, and (2) L_{2-4} BMD, BAP, and K_1 concentration. Variables that correlated strongly with each other, such as age and L_{2-4} BMD, were not entered simultaneously in the original model. Forward stepwise regression was performed, and $P < 0.25$ was used to enter variables. Values of vitamin K concentrations were logarithmically transformed to improve normality in this analysis because plasma vitamin K concentrations were not normally distributed. A Cox proportional hazards model was used to assess the relationship between plasma K_1 concentration and vertebral fracture. Hazard ratios and 95% confidence intervals are evaluated by no adjusted model or adjusted model for BMD or BMI.

In the second analysis, subjects were divided into low and high K_1 groups by median K_1 concentration (2.67 nmol/l). Parametric comparisons used Student's *t* test. The incidence of vertebral fracture in the two groups was evaluated by the chi square test and crude or age-adjusted relative risks (RRs). Moreover, the age and L_{2-4} BMD values at which 25% of subjects would suffer fractures in the four groups were inversely predicted by logistic regression analysis.

Ethical considerations

The comprehensive study protocol including nutritional evaluation was reviewed by the ethics committee of Research Institute and Practice for Involutional Diseases (RIPID), and comprehensive written informed consent was obtained from all participants.