さらに，各群における骨折と骨密度あるいは年齢の関係をロジスティック解析し，骨折有病率が25%となる年齢とL_{2-4}BMDを逆推定した。I群の平均年齢は4群のなかで最も高かったものの，ロジスティック解析の結果，逆推定された年齢はI群で最も若く(68.5歳，95%CI：60.9～73.7)，IV群で最も高かった(74.2歳，95%CI：67.8～92.0)。一方，逆推定されたL_{2-4} BMDはI群で最も高く(0.8964，95%CI：0.7851～1.0482)，IV群で最も低かった(0.7197，95%CI：0.2289～0.8640)。対象者を低PK濃度群(I & II群)と高PK濃度群(III & IV群)に分けると，逆推定年齢は低PK濃度群で68.2歳(95%CI：63.8～71.9)，高PK濃度群で72.4歳(95%CI：68.2～79.8)となり，これらの推定値は互いの95%信頼限界の範囲を超える値となった。また，逆推定L_{2-4} BMDは，低PK濃度群で0.8929(95%CI：0.8104～0.9828)，高PK濃度群で0.7707(95%CI：0.5969～0.8561)となり，これらの推定値もまた互いの95%信頼限界の範囲を超える値となった。

これらの結果より，低PK群では高PK群に比べて高骨密度，低年齢で骨折が起こりやすいことが示唆され，VKの栄養状態の低下により骨折の危険性が増加する可能性が示唆された。

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論文類別： Minireview

Determination of Fat-Soluble Vitamins in Human Plasma, Breast Milk and Food Samples – Application in Nutrition Survey for Establishment of "Dietary Reference Intakes for Japanese" –

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SUMMARY

Dietary habits are an important risk factor for lifestyle-related diseases. To carry out a nutrition survey of fat-soluble vitamins, we developed determination methods of fat-soluble vitamins using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry or high-performance liquid chromatography with fluorescence detection. In these methods, stable isotope-labeled compounds or vitamin K analogs with a saturated side-chain were used as internal standards. These methods have high sensitivity and sufficient accuracy, and we applied them in a nutrition survey about the status of fat-soluble vitamins in Japanese women. Plasma concentrations of 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] in healthy postmenopausal women (n=98) were 20.5 ± 7.9 and 0.4 ± 1.4 ng/mL, respectively. A significant negative correlation in plasma levels between 25(OH)D and parathyroid hormone was observed. For vitamin K homologs, plasma levels of phylloquinone (PK), menaquinone-4 (MK-4) and menaquinone-7 (MK-7) in Japanese women of various ages (n=1409) were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The mean total vitamin K intake of Japanese young women was about 230 μg/day, and 94 % of participants met the Adequate Intake of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Moreover, we determined fat-soluble vitamins in breast milk collected from Japanese lactating women and revealed that the contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19 and 1.77 ± 0.68 ng/mL, respectively.

Key words—fat-soluble vitamins, vitamin D, vitamin K, nutrition survey

INTRODUCTION

In Japan, lifestyle-related diseases have been increasing with the advent of the aging society and it is acknowledged that dietary habits are an important risk factor for these diseases. Thus, a nutrition survey aimed at humans is needed as well as a study of the bioavailability, physiological function and metabolism of nutrients to obtain scientific information for the primary prevention of lifestyle-related diseases through the improvement of dietary habits and nutrition. We especially focused on vitamins D and K which are important fat-soluble vitamins for the prevention of osteoporosis.

It is well recognized that plasma or serum levels of 25-hydroxyvitamin D [25(OH)D] reflect the nutritional status of vitamin D in humans. Vitamin D is metabolized to 25(OH)D in the liver and subsequently to the active form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], or the inactive form of vitamin D, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], in the kidney. In addition, it was demonstrated that vitamin D and its metabolites are also metabolized to their respective C-3 epimers [1-5]. Vitamin D_3, which is the form of vitamin D synthesized by vertebrates including humans, and vitamin D_2, which is the major naturally occurring form in plants, are both metabolized in a similar fashion. 25(OH)D binds to vitamin D-binding protein (DBP) in the blood and is the most abundant circulating metabolite of vitamin D with a concentration of 20-50 ng/mL under normal conditions [6]. Thus, the plasma or serum concentration of 25(OH)D is considered to be a good indicator of the cumulative effects of exposure to sunlight and dietary intake of vitamin D. Plasma or serum 25(OH)D concentration can be measured by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector [7], competitive protein-binding assay (CPBA) [8], radioimmunoassay (RIA) [9] and enzyme immunoassay (EIA) [10]. In recent years, RIA and EIA have been widely used in many laboratories and hospitals because of their superior simplicity, rapidity and accuracy; however, these methods require high-quality control to ensure reliable results [11-15]. Moreover, conventional RIA measures 25(OH)D along with 24,25$(OH)_2$D because their antibodies exhibit 100 % cross-reaction with 24,25$(OH)_2$D.

Meanwhile, one of the most common nutritional indicators of vitamin K is the plasma concentration of phylloquinone (PK, vitamin K_1). PK is produced by plants and algae, and the other vitamin K form, menaquinones (MKs, vitamin K_2), is synthesized by bacteria. MKs

comprise a family of molecules distinguished from PK by unsaturated side-chains of isoprenoid units varying in length from 1 to 14 repeats [15]. Vitamin K is a cofactor for an enzyme that converts specific glutamyl residues in several proteins such as plasma clotting factors II (prothrombin), osteocalcin (bone Gla protein) and matrix Gla protein to γ-calboxyglutamyl (Gla) residues. These vitamin K-dependent proteins play crucial roles in blood coagulation and calcification. Several reports indicate an important role for vitamin K in bone health. The administration of vitamin K results in increased bone-mineral density (BMD) and reduced bone resorption in humans [16, 17]. In epidemiological studies, low dietary vitamin K intake was associated with an increased incidence of hip fracture [18, 19]; however, no large-scale nutrition survey of vitamin K has been conducted due to the low plasma concentration of vitamin K. There is still the problem with the accuracy of HPLC with fluorescence detection, which is usually used for the quantitation of plasma vitamin K.

Based on this background, we developed precise assay methods for vitamins D and K using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry (LC-APCI/MS/MS) and HPLC with a fluorescence detector. Then, we applied these methods in a nutrition survey of Japanese women.

Development of Determination Method for Vitamin D

We established a precise and sensitive assay method to determine $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ in human plasma using LC-APCI/MS/MS to provide a gold standard [20]. The method involves the use of deuterated $25(OH)D_3$ as an internal standard, which was synthesized in our laboratory. After the addition of the internal standard to 0.1 mL of plasma samples, methanol was added for protein removal. Vitamin D compounds were purified by C_{18} silicagel mini-column and detected by the MS/MS multiple reaction monitoring (MRM) method. The average spiked recoveries from authentic compounds added to normal human plasma samples for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 98-104 %. The average intraassay variation values (relative standard deviation) for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 5.7, 4.5 and 11.4 %, respectively. The average interassay variation values for $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ were 2.5, 5.1 and 9.9 %, respectively. Mean plasma concentrations of $25(OH)D_3$, $25(OH)D_2$ and $24,25(OH)_2D_3$ in healthy postmenopausal

women (n=98) were 20.5 ± 7.9 (mean ± S.D.), 0.4 ± 1.4 and 0.5 ± 0.7 ng/mL, respectively. The concentrations of 25(OH)D measured by the RIA method using a DiaSorin RIA kit were well correlated with the concentrations of 25(OH)D plus $24,25(OH)_2D_3$ measured by the proposed method, although the RIA method gave slightly higher concentrations than the LC-APCI/MS/MS method. In addition, a significant negative correlation was observed between plasma levels of 25(OH)D and parathyroid hormone (PTH) with the LC-APCI/MS/MS method. In contrast, no significant correlation was observed in plasma levels between 25(OH)D and PTH with the RIA method. Plasma PTH level is an important indicator of vitamin D deficiency or insufficiency. Recently, a negative correlation between plasma 25(OH)D and PTH levels was reported from some cohort studies of healthy subjects [21, 22]. These results suggest that this LC-APCI/MS/MS method would be useful for the evaluation of vitamin D status and provide useful information in the diagnosis of vitamin D insufficiency/deficiency, as well as for the treatment and prevention of osteoporosis with vitamin D.

Development of Determination Method for Vitamin K

We also developed a determination method for vitamin K homologs including PK, MK-4 and MK-7 in human plasma using LC-APCI/MS/MS [23]. As internal standard compounds, ^{18}O-labeled PK, MK-4 and MK-7 were used. After the addition of internal standards to 0.5 mL of plasma samples, vitamin K compounds were extracted with ethanol and hexane. The average spiked recoveries from authentic compounds added to normal human plasma samples for PK, MK-4 and MK-7 were 98-102 %. The average intraassay and interassay variation values for PK, MK-4 and MK-7 were less than 10 %. The quantitation limits for PK, MK-4 and MK-7 were less than 3 pg per injection. Thus, we conclude that this novel LC-APCI/MS/MS method has enough reproducibility and sensitivity to measure vitamin K in human plasma; however, this method does not establish a universal routine assay as it uses an expensive measuring instrument. Therefore, we developed an improved HPLC fluorescence determination method for vitamin K homologs using post-column reduction and synthetic vitamin K analogs with different lengths of the saturated alkyl side-chain as internal standards [24]. Selectivity and reproducibility were increased by optimizing chromatographic conditions

including the mobile phase and excitation wavelength for MK-4 or less polar derivatives, PK and MK-7. The detection limits for PK, MK-4 and MK-7 were less than 4 pg per injection. The recoveries of PK, MK-4 and MK-7 were 93-105 % and the inter- and intraassay variation values of normal human plasma for PK, MK-4 and MK-7 were less than 10 %. The data showed good correlation between the proposed HPLC fluorescence determination method and the LC-APCI/MS/MS method for PK (r^2=0.979), MK-4 (r^2=0.988) and MK-7 (r^2=0.986) (Fig. 1). These results suggest that the improved HPLC fluorescence detection method allows the determination of vitamin K to evaluate the clinical and nutritional status as well as the LC-APCI/MS/MS method. Thus, this method was applied to plasma samples from Japanese women of various ages (n=1409). Plasma levels of PK, MK-4 and MK-7 were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The plasma levels of PK in elderly women (62.7 ± 10.9 y) were significantly higher than those of high school and junior high school girls. The plasma concentrations of MK-4 have a tendency to increase during periods of growth. In addition, plasma PK and MK-7 concentrations correlated inversely with undercarboxylated osteocalcin (ucOC) in elderly women [25]. The plasma PK or MK-7 concentration required to minimize the ucOC concentration was higher in the group over 70 y, and it decreased progressively for each of the younger age groups. Thus, circulating vitamin K concentrations in elderly people should be kept higher than those in young people.

Vitamin K Content of Foods and Dietary Vitamin K Intake in Japanese Young Women

In the current "Dietary Reference Intakes (DRIs) for Japanese", the Adequate Intake (AI) of vitamin K is set at 75 μg for adult men, 60 μg for women aged 18-29 y, and 65 μg for women 30 y and over as a probable sufficient quantity for the maintenance of normal blood clotting. However, the current AI might not be sufficient to maintain bone health. In addition, the assessment of dietary intake of both PK and MKs is incomplete in regions where people habitually eat fermented food, such as Japan. To obtain a closer estimate of dietary intake of PK and MKs in Japanese young women, PK, MK-4 and MK-7 contents in food samples (58 food items) were determined using an improved HPLC method with fluorescence detection. Next, we assessed dietary vitamin K intake in Japanese young women aged 20-23 y (n=125), using the vitamin K contents measured here and the Standard Tables of Food Composition in

Japan [26]. PK was widely distributed in green vegetables and algae, and high amounts were found in spinach and broccoli (raw, 498 and 307 μg/100g wet weight, respectively, unpublished data). Although MK-4 was widely distributed in animal products, overall MK-4 content was lower than PK. Relatively high amounts of MK-4 were found in chicken meat (raw, 27 μg/100 g) and the egg yolk of hen's eggs (raw, 64 μg/100 g). MK-7 was observed characteristically in fermented soybean products such as natto (939 μg/100 g). The mean total vitamin K intake of Japanese young women was about 230 μg/day and 94 % of participants met the AI of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Mean daily intakes of PK, MK-4 and MK-7 (MK-4 equivalent value) were estimated as 155.9 ± 91.1, 16.9 ± 10.4 and 57.4 ± 83.7 μg/day, respectively. The contributions of PK, MK-4 and MK-7 (MK-4 equivalent value) to total vitamin K intake were 67.7, 7.3 and 24.9 %, respectively; therefore, PK from vegetables and algae, and MK-7 from pulses (including fermented soybean foods) were the major contributors to the total vitamin K intake of Japanese young women.

Nutrition Survey on Fat-Soluble Vitamins of Japanese Lactating Women

To estimate an infant's intake of fat-soluble vitamins, we determined their levels in breast milk collected from Japanese lactating women (n=51, age: 30.8 ± 4.4 y, post-partum day: 1.5 ± 1.2 m) by the LC-APCI/MS/MS method using stable isotope-labeled compounds as internal standards. It was reported that the concentrations of vitamin D and its metabolites in human breast milk were very low [27, 28]. Therefore, we used a derivatization method with a Cookson-Type reagent to improve ionization efficiency for the determination of vitamin D and its metabolites in LC-APCI/MS/MS analysis [29]. The contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK, MK-4 and MK-7 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19, 1.77 ± 0.68 ng/mL and 1.19 ± 1.54 ng/mL, respectively (Table 1). Daily intake of vitamin D calculated from an infant's consumption of breast milk, 780 mL/day was 0.47 μg, which did not meet current DRIs (AI, 2.5 μg/day). The concentrations of all-*trans*-retinol, β-carotene, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were positively correlated with lipid content; thus, the secretion of fat-soluble vitamins in breast milk is thought to be highly influenced by lipids.

Summary

We developed reliable determination methods for fat-soluble vitamins and applied them to a nutritional epidemiology study of Japanese. Further large-scale studies will be needed and the obtained data may be useful to maintain and improve health, and to establish DRIs for Japanese.

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FIGURE LEGEND

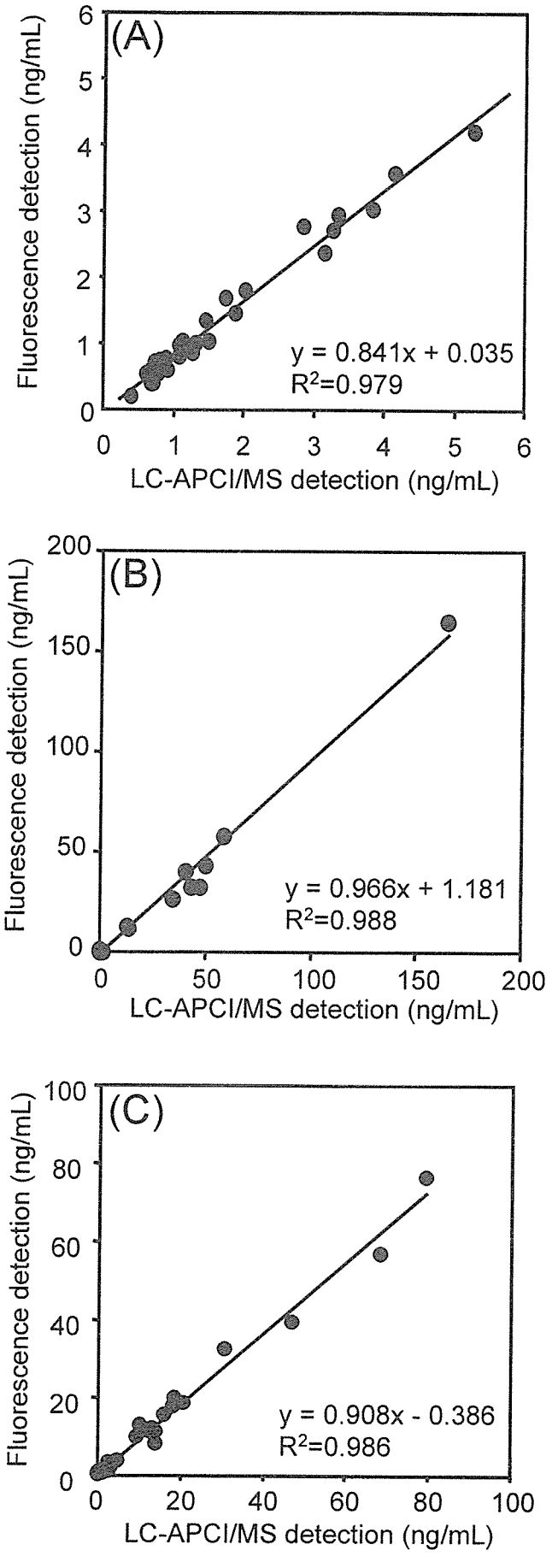
Figure 1. Correlation between the values obtained by HPLC with fluorescence detection using internal standards and those obtained by LC-APCI/MS. (A) PK; (B) MK-4; (C) MK-7.

Table 1. Concentrations of fat-soluble vitamins in human milk and estimated infant's intake

Vitamin	Compound	Concentration in human milk[1]	Estimated infant's intake[2]
A	all-*trans*-retinol	0.39 ± 0.14 (μg/mL)	335 μg RE/day[3]
	β-carotene	0.05 ± 0.04 (μg/mL)	
D	vitamin D_3	0.10 ± 0.15 (ng/mL)	0.47 μg/day[4]
	vitamin D_2	0.09 ± 0.19 (ng/mL)	
	25(OH)D_3	0.08 ± 0.04 (ng/mL)	
	25(OH)D_2	0.003 ± 0.002 (ng/mL)	
E	α-tocopherol	3.96 ± 1.84 (μg/mL)	3.09 mg/day
K	PK	3.56 ± 2.19 (ng/mL)	4.79 μg/day[5]
	MK-4	1.77 ± 0.68 (ng/mL)	
	MK-7	1.19 ± 1.54 (ng/mL)	

[1] Values are the means ± S.D., n=51.
[2] The product of the concentrations of fat-soluble vitamins in human milk and infant's consumption of human milk (780 mL/day).
[3] The sum of all-*trans*-retinol and β-carotene expressed as retinol equivalent (RE) value.
[4] The sum of vitamin D and vitamin D equivalent 25(OH)D [25(OH)D x 5, vitamin D conversion factor of 25(OH)D=5].
[5] The sum of PK, MK-4 and MK-4 equivalent MK-7 (MK-7 content x 444.7/649).

Fig. 1


(A)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.841x + 0.035
R²=0.979
0
1
2
3
4
5
6
(B)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.966x + 1.181
R²=0.988
0
50
100
150
200
(C)
Fluorescence detection (ng/mL)
LC-APCI/MS detection (ng/mL)
y = 0.908x - 0.386
R²=0.986
0
20
40
60
80
100

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SUMMARY

Dietary habits are an important risk factor for lifestyle-related diseases. To carry out a nutrition survey of fat-soluble vitamins, we developed determination methods of fat-soluble vitamins using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry or high-performance liquid chromatography with fluorescence detection. In these methods, stable isotope-labeled compounds or vitamin K analogs with a saturated side-chain were used as internal standards. These methods have high sensitivity and sufficient accuracy, and we applied them in a nutrition survey about the status of fat-soluble vitamins in Japanese women. Plasma concentrations of 25-hydroxyvitamin D_3 [25(OH)D_3] and 25-hydroxyvitamin D_2 [25(OH)D_2] in healthy postmenopausal women (n=98) were 20.5 ± 7.9 and 0.4 ± 1.4 ng/mL, respectively. A significant negative correlation in plasma levels between 25(OH)D and parathyroid hormone was observed. For vitamin K homologs, plasma levels of phylloquinone (PK), menaquinone-4 (MK-4) and menaquinone-7 (MK-7) in Japanese women of various ages (n=1409) were 1.03 ± 0.90, 0.12 ± 0.28 and 6.71 ± 13.6 ng/mL, respectively. The mean total vitamin K intake of Japanese young women was about 230 μg/day, and 94 % of participants met the Adequate Intake of vitamin K for women aged 18-29 y in Japan, 60 μg/day. Moreover, we determined fat-soluble vitamins in breast milk collected from Japanese lactating women and revealed that the contents of all-*trans*-retinol, vitamin D_3, 25(OH)D_3, α-tocopherol, PK and MK-4 in breast milk were 0.39 ± 0.14 μg/mL, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 μg/mL, 3.56 ± 2.19 and 1.77 ± 0.68 ng/mL, respectively.

Key words—fat-soluble vitamins, vitamin D, vitamin K, nutrition survey

INTRODUCTION

In Japan, lifestyle-related diseases have been increasing with the advent of the aging society and it is acknowledged that dietary habits are an important risk factor for these diseases. Thus, a nutrition survey aimed at humans is needed as well as a study of the bioavailability, physiological function and metabolism of nutrients to obtain scientific information for the primary prevention of lifestyle-related diseases through the improvement of dietary habits and nutrition. We especially focused on vitamins D and K which are important fat-soluble vitamins for the prevention of osteoporosis.

It is well recognized that plasma or serum levels of 25-hydroxyvitamin D [25(OH)D] reflect the nutritional status of vitamin D in humans. Vitamin D is metabolized to 25(OH)D in the liver and subsequently to the active form of vitamin D, 1α,25-dihydroxyvitamin D [1α,25$(OH)_2$D], or the inactive form of vitamin D, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D], in the kidney. In addition, it was demonstrated that vitamin D and its metabolites are also metabolized to their respective C-3 epimers [1-5]. Vitamin D_3, which is the form of vitamin D synthesized by vertebrates including humans, and vitamin D_2, which is the major naturally occurring form in plants, are both metabolized in a similar fashion. 25(OH)D binds to vitamin D-binding protein (DBP) in the blood and is the most abundant circulating metabolite of vitamin D with a concentration of 20-50 ng/mL under normal conditions [6]. Thus, the plasma or serum concentration of 25(OH)D is considered to be a good indicator of the cumulative effects of exposure to sunlight and dietary intake of vitamin D. Plasma or serum 25(OH)D concentration can be measured by high-performance liquid chromatography (HPLC) with an ultraviolet (UV) detector [7], competitive protein-binding assay (CPBA) [8], radioimmunoassay (RIA) [9] and enzyme immunoassay (EIA) [10]. In recent years, RIA and EIA have been widely used in many laboratories and hospitals because of their superior simplicity, rapidity and accuracy; however, these methods require high-quality control to ensure reliable results [11-15]. Moreover, conventional RIA measures 25(OH)D along with 24,25$(OH)_2$D because their antibodies exhibit 100 % cross-reaction with 24,25$(OH)_2$D.

Meanwhile, one of the most common nutritional indicators of vitamin K is the plasma concentration of phylloquinone (PK, vitamin K_1). PK is produced by plants and algae, and the other vitamin K form, menaquinones (MKs, vitamin K_2), is synthesized by bacteria. MKs

comprise a family of molecules distinguished from PK by unsaturated side-chains of isoprenoid units varying in length from 1 to 14 repeats [15]. Vitamin K is a cofactor for an enzyme that converts specific glutamyl residues in several proteins such as plasma clotting factors II (prothrombin), osteocalcin (bone Gla protein) and matrix Gla protein to γ-calboxyglutamyl (Gla) residues. These vitamin K-dependent proteins play crucial roles in blood coagulation and calcification. Several reports indicate an important role for vitamin K in bone health. The administration of vitamin K results in increased bone-mineral density (BMD) and reduced bone resorption in humans [16, 17]. In epidemiological studies, low dietary vitamin K intake was associated with an increased incidence of hip fracture [18, 19]; however, no large-scale nutrition survey of vitamin K has been conducted due to the low plasma concentration of vitamin K. There is still the problem with the accuracy of HPLC with fluorescence detection, which is usually used for the quantitation of plasma vitamin K.

Based on this background, we developed precise assay methods for vitamins D and K using liquid chromatography-atmospheric pressure chemical ionization/tandem mass spectrometry (LC-APCI/MS/MS) and HPLC with a fluorescence detector. Then, we applied these methods in a nutrition survey of Japanese women.

Development of Determination Method for Vitamin D

We established a precise and sensitive assay method to determine 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ in human plasma using LC-APCI/MS/MS to provide a gold standard [20]. The method involves the use of deuterated 25(OH)D_3 as an internal standard, which was synthesized in our laboratory. After the addition of the internal standard to 0.1 mL of plasma samples, methanol was added for protein removal. Vitamin D compounds were purified by C_{18} silicagel mini-column and detected by the MS/MS multiple reaction monitoring (MRM) method. The average spiked recoveries from authentic compounds added to normal human plasma samples for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 98-104 %. The average intraassay variation values (relative standard deviation) for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 5.7, 4.5 and 11.4 %, respectively. The average interassay variation values for 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ were 2.5, 5.1 and 9.9 %, respectively. Mean plasma concentrations of 25(OH)D_3, 25(OH)D_2 and 24,25$(OH)_2D_3$ in healthy postmenopausal

women (n=98) were 20.5 ± 7.9 (mean ± S.D.), 0.4 ± 1.4 and 0.5 ± 0.7 ng/mL, respectively. The concentrations of 25(OH)D measured by the RIA method using a DiaSorin RIA kit were well correlated with the concentrations of 25(OH)D plus $24,25(OH)_2D_3$ measured by the proposed method, although the RIA method gave slightly higher concentrations than the LC-APCI/MS/MS method. In addition, a significant negative correlation was observed between plasma levels of 25(OH)D and parathyroid hormone (PTH) with the LC-APCI/MS/MS method. In contrast, no significant correlation was observed in plasma levels between 25(OH)D and PTH with the RIA method. Plasma PTH level is an important indicator of vitamin D deficiency or insufficiency. Recently, a negative correlation between plasma 25(OH)D and PTH levels was reported from some cohort studies of healthy subjects [21, 22]. These results suggest that this LC-APCI/MS/MS method would be useful for the evaluation of vitamin D status and provide useful information in the diagnosis of vitamin D insufficiency/deficiency, as well as for the treatment and prevention of osteoporosis with vitamin D.

Development of Determination Method for Vitamin K

We also developed a determination method for vitamin K homologs including PK, MK-4 and MK-7 in human plasma using LC-APCI/MS/MS [23]. As internal standard compounds, ^{18}O-labeled PK, MK-4 and MK-7 were used. After the addition of internal standards to 0.5 mL of plasma samples, vitamin K compounds were extracted with ethanol and hexane. The average spiked recoveries from authentic compounds added to normal human plasma samples for PK, MK-4 and MK-7 were 98-102 %. The average intraassay and interassay variation values for PK, MK-4 and MK-7 were less than 10 %. The quantitation limits for PK, MK-4 and MK-7 were less than 3 pg per injection. Thus, we conclude that this novel LC-APCI/MS/MS method has enough reproducibility and sensitivity to measure vitamin K in human plasma; however, this method does not establish a universal routine assay as it uses an expensive measuring instrument. Therefore, we developed an improved HPLC fluorescence determination method for vitamin K homologs using post-column reduction and synthetic vitamin K analogs with different lengths of the saturated alkyl side-chain as internal standards [24]. Selectivity and reproducibility were increased by optimizing chromatographic conditions