

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.

Acknowledgements

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REFERENCES

- 1) Bischof, M. G., Siu-Caldera, M. -L., Weiskopf, A. Vouros, P., Cross, H. S., Peterlik, M. and Reddy, G. S. (1998) Differentiation-related pathways of $1\alpha,25$ -dihydroxycholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells: production of $1\alpha,25$ -dihydroxy-3-epi-cholecalciferol. *Exp. Cell. Res.*, **241**, 194-201.
- 2) Masuda, S., Kamao, M., Schroeder, N. J., Makin, H. L. J., Jones, G., Kremer, R., Rhim, J. and Okano, T. (2000) Characterization of 3-epi- $1\alpha,25$ -dihydroxyvitamin D₃ involved in $1\alpha,25$ -dihydroxyvitamin D₃ metabolic pathway in cultured cell lines. *Biol. Pharm. Bull.*, **23**, 133-139.
- 3) Kamao, M., Tatematsu, S., Reddy G. S., Hatakeyama, S., Sugiura, M., Ohashi, N., Kubodera, N. and Okano, T. (2001) Isolation, identification and biological activity of 24R,25-dihydroxy-3-epi-vitamin D₃: a novel metabolite of 24R,25-dihydroxyvitamin D₃ produced in rat osteosarcoma cells (UMR 106). *J. Nutr. Sci. Vitaminol. (Tokyo)*, **47**, 108-115.
- 4) Higashi, T., Ogasawara, A. and Shimada, K. (2000) Investigation of C-3 epimerization mechanism of 24,25-dihydroxyvitamin D₃ in rat using liquid chromatography/mass spectrometry. *Anal. Sci.*, **16**, 477-482.

- 5) Kamao, M., Tatematsu, S., Hatakeyama, S., Sakaki, T., Sawada, N., Inouye, K., Ozono, K., Kubodera, N., Reddy, G. S. and Okano, T. (2004) C-3 epimerization of vitamin D₃ metabolites and further metabolism of C-3 epimers. *J. Biol. Chem.*, **279**, 15897-15907.
- 6) Napoli, J. L. and Horst, R. L. (1984) Vitamin D Metabolism. In *Vitamin D: Basic and Applied Aspects* (Kumar, R., Eds.). Martinus Nijhoff, Boston, pp.91-123.
- 7) Jones, G. (1978) Assay of vitamins D₂ and D₃, and 25-hydroxyvitamins D₂ and D₃ in human plasma by high-performance liquid chromatography. *Clin. Chem.*, **24**, 287-298.
- 8) Haddad, J. G. and Chyu, K. J. (1971) Competitive protein-binding radioassay for 25-hydroxycholecalciferol. *J. Clin. Endocrinol. Metab.*, **33**, 992-995.
- 9) Hollis, B. W. and Napoli, J. L. (1985) Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. *Clin. Chem.*, **31**, 1815-1819.
- 10) Lind, C., Chen, J. and Byrjalsen, I., (1997) Enzyme immunoassay for measuring 25-hydroxyvitamin D₃ in serum. *Clin. Chem.*, **43**, 943-949.
- 11) Carter, G. D., Carter, R., Jones, J. and Berry, J. (2004) How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. *Clin. Chem.*, **50**, 2195-2197.
- 12) Carter, G. D., Carter, C. R., Gunter, E., Jones, J., Jones, G., Makin, H. L. and Sufi, S. (2004) Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. *J. Steroid Biochem. Mol. Biol.*, **89-90**, 467-471.
- 13) Binkley, N., Krueger, D., Cowgill, C. S., Plum, L., Lake, E., Hansen, K. E., DeLuca, H. F. and Drezner, M. K. (2004) Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. *J. Clin. Endocrinol. Metab.*, **89**, 3152-3157.
- 14) Hollis, B. W. (2000) Comparison of commercially available ¹²⁵I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. *Clin. Chem.*, **46**, 1657-1661.
- 15) Collins, M. D. and Jones, D. (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbial. Rev.*, **45**, 316-354.
- 16) Sato, Y., Honda, Y., Kuno, H. and Oizumi, K. (1998) Menatetrenone ameliorates osteopenia in disuse-affected limbs of vitamin D- and K-deficient stroke patients. *Bone*, **23**, 291-296.

- 17) Craciun, A. M., Wolf, J., Knapen, M. H., Brouns, F. and Vermeer, C. (1998) Improved bone metabolism in female elite athletes after vitamin K supplementation. *Int. J. Sports Med.*, **19**, 479-484.
- 18) Feskanich, D., Weber, P., Willett, W. C., Rockett, H., Booth, S. L. and Colditz, G. A. (1999) Vitamin K intake and hip fractures in women: a prospective study. *Am. J. Clin. Nutr.*, **69**, 74-79.
- 19) Booth, S. L., Tucker, K. L., Chen, H., Hannan, M. T., Gagnon, D. R., Cupples, L. A., Wilson, P. W., Ordovas, J., Schaefer, E. J., Dawson-Hughes, B. and Kiel, D. P. (2000) Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. *Am. J. Clin. Nutr.*, **71**, 1201-1208.
- 20) Tsugawa, N., Suhara, Y., Kamao, M. and Okano, T. (2005) Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 3001-3007.
- 21) Krall, E. A., Sahyoun, N., Tannenbaum, S., Dallal, G. E., Dawson-Hughes, B. (1989) Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. *Engl. J. Med.*, **321**, 1777-1783.
- 22) Webb, A. R., Pilbeam, C., Hanafin, N., Holick, M. F. (1990) An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. *Am. J. Clin. Nutr.* **51**, 1075-1081.
- 23) Suhara, Y., Kamao, M., Tsugawa, N. and Okano, T. (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 757-763.
- 24) Kamao, M., Suhara, Y., Tsugawa, N. and Okano, T. (2005) Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. *J. Chromatogr. B*, **816**, 41-48.
- 25) Tsugawa, N., Shiraki, M., Suhara, Y., Kamao, M., Tanaka, K. and Okano, T. (2006) Vitamin K status of healthy Japanese women: age-related vitamin K requirement for γ -carboxylation of osteocalcin. *Am. J. Clin. Nutr.*, **83**, 380-386.
- 26) Report of the Subdivision on Resources, The Council for Science and Technology,

Ministry of Education, Culture, Sports, Science and Technology, JAPAN (2005)
Standard Tables of Food Composition in Japan, Fifth revised and enlarged edition.

- 27) Hollis, B. W., Roos, B. A., Draper H. H. and Lambert, P. W. (1981) Vitamin D and its metabolites in human and bovine milk. *J. Nutr.*, **111**, 1240-1248.
- 28) Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y., Kobayashi, T., Kodama, S. and Matsuo, T. (1988) The determination of vitamin D and its metabolites in human breast and cow's milk. *J. Micronutrient. Anal.*, **4**, 193-208.
- 29) Higashi, T., Awada, D. and Shimada, K. (2001) Simultaneous determination of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in human plasma by liquid chromatography-tandem mass spectrometry employing derivatization with a Cookson-type reagent. *Biol. Pharm. Bull.*, **24**, 738-743.

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- <i>trans</i> -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.

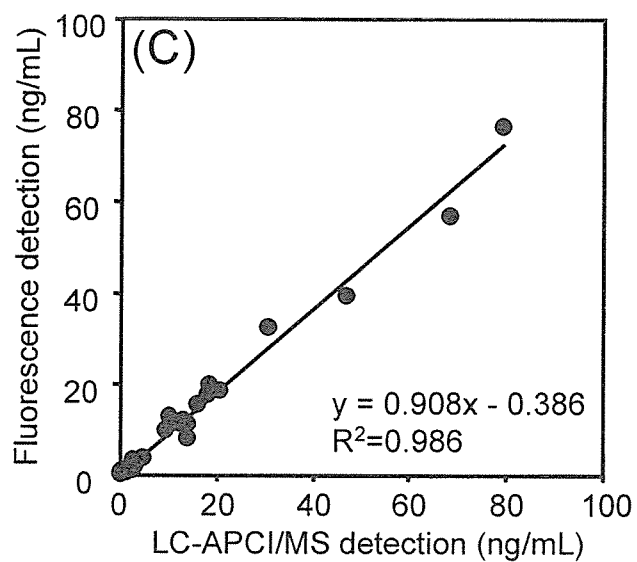
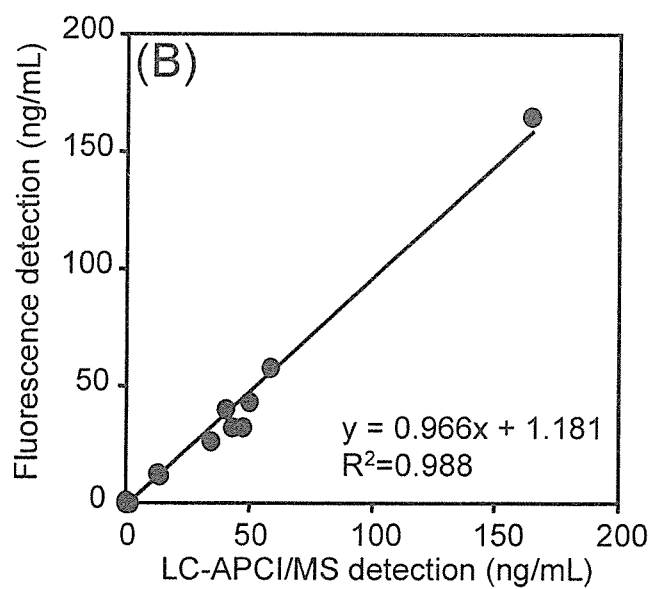
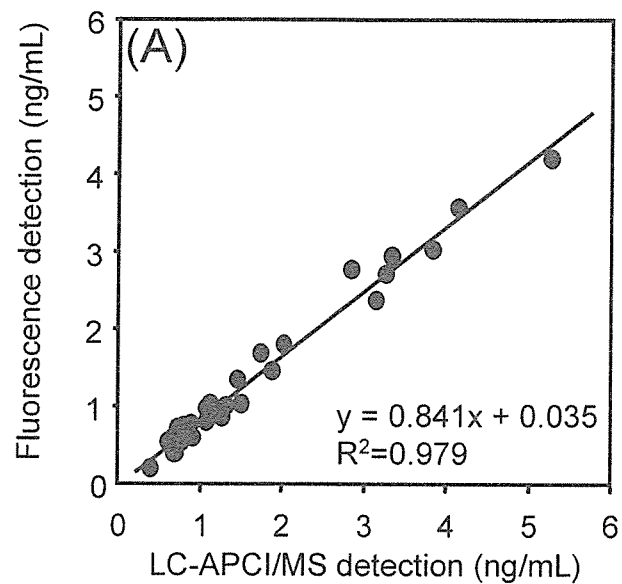
² The product of the concentrations of fat-soluble vitamins in human milk and infant's consumption of human milk (780 mL/day).

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



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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 D₃ [25(OH)D₃] and 25-hydroxyvitamin D₂ [25(OH)D₂] 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 D₃, 25(OH)D₃, 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, $1\alpha,25$ -dihydroxyvitamin D [$1\alpha,25(\text{OH})_2\text{D}$], or the inactive form of vitamin D, $24,25$ -dihydroxyvitamin D [$24,25(\text{OH})_2\text{D}$], in the kidney. In addition, it was demonstrated that vitamin D and its metabolites are also metabolized to their respective C-3 epimers¹⁻⁵⁾. Vitamin D₃, which is the form of vitamin D synthesized by vertebrates including humans, and vitamin D₂, 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 conditions⁶⁾. 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) detector⁷⁾, 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 results¹¹⁻¹⁵⁾. Moreover, conventional RIA measures 25(OH)D along with $24,25(\text{OH})_2\text{D}$ because their antibodies exhibit 100 % cross-reaction with $24,25(\text{OH})_2\text{D}$.

Meanwhile, one of the most common nutritional indicators of vitamin K is the plasma concentration of phylloquinone (PK, vitamin K₁). PK is produced by plants and algae, and the other vitamin K form, menaquinones (MKs, vitamin K₂), 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 repeats¹⁵). 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 -carboxyglutamyl (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)D₃, 25(OH)D₂ and 24,25(OH)₂D₃ in human plasma using LC-APCI/MS/MS to provide a gold standard²⁰). The method involves the use of deuterated 25(OH)D₃ 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 C₁₈ 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)D₃, 25(OH)D₂ and 24,25(OH)₂D₃ were 98-104 %. The average intraassay variation values (relative standard deviation) for 25(OH)D₃, 25(OH)D₂ and 24,25(OH)₂D₃ were 5.7, 4.5 and 11.4 %, respectively. The average interassay variation values for 25(OH)D₃, 25(OH)D₂ and 24,25(OH)₂D₃ were 2.5, 5.1 and 9.9 %, respectively. Mean plasma concentrations of 25(OH)D₃, 25(OH)D₂ and 24,25(OH)₂D₃ in healthy postmenopausal

women (n=98) were 20.5 ± 7.9 (mean \pm 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)₂D₃ 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/MS²³). As internal standard compounds, ¹⁸O-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 standards²⁴). 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

Japan²⁶). PK was widely distributed in green vegetables and algae, and high amounts were found in spinach and broccoli (raw, 498 and 307 $\mu\text{g}/100\text{g}$ 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 $\mu\text{g}/100\text{g}$) and the egg yolk of hen's eggs (raw, 64 $\mu\text{g}/100\text{g}$). MK-7 was observed characteristically in fermented soybean products such as natto (939 $\mu\text{g}/100\text{g}$). The mean total vitamin K intake of Japanese young women was about 230 $\mu\text{g}/\text{day}$ and 94 % of participants met the AI of vitamin K for women aged 18-29 y in Japan, 60 $\mu\text{g}/\text{day}$. 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 $\mu\text{g}/\text{day}$, 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 analysis²⁹). The contents of all-*trans*-retinol, vitamin D₃, 25(OH)D₃, α -tocopherol, PK, MK-4 and MK-7 in breast milk were 0.39 ± 0.14 $\mu\text{g}/\text{mL}$, 0.10 ± 0.15 ng/mL, 0.08 ± 0.04 ng/mL, 3.96 ± 1.84 $\mu\text{g}/\text{mL}$, 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 $\mu\text{g}/\text{day}$). The concentrations of all-*trans*-retinol, β -carotene, 25(OH)D₃, 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.

Acknowledgements

This work was supported in part by a Grant-in-aid-for Comprehensive Research on Cardiovascular Diseases and the Research on the Dietary Reference Intakes in Japanese from the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

- 1) Bischof, M. G., Siu-Caldera, M. -L., Weiskopf, A. Vouros, P., Cross, H. S., Peterlik, M. and Reddy, G. S. (1998) Differentiation-related pathways of $1\alpha,25$ -dihydroxycholecalciferol metabolism in human colon adenocarcinoma-derived Caco-2 cells: production of $1\alpha,25$ -dihydroxy-3-epi-cholecalciferol. *Exp. Cell. Res.*, **241**, 194-201.
- 2) Masuda, S., Kamao, M., Schroeder, N. J., Makin, H. L. J., Jones, G., Kremer, R., Rhim, J. and Okano, T. (2000) Characterization of 3-epi- $1\alpha,25$ -dihydroxyvitamin D₃ involved in $1\alpha,25$ -dihydroxyvitamin D₃ metabolic pathway in cultured cell lines. *Biol. Pharm. Bull.*, **23**, 133-139.
- 3) Kamao, M., Tatematsu, S., Reddy G. S., Hatakeyama, S., Sugiura, M., Ohashi, N., Kubodera, N. and Okano, T. (2001) Isolation, identification and biological activity of 24*R*,25-dihydroxy-3-epi-vitamin D₃: a novel metabolite of 24*R*,25-dihydroxyvitamin D₃ produced in rat osteosarcoma cells (UMR 106). *J. Nutr. Sci. Vitaminol.* (Tokyo), **47**, 108-115.
- 4) Higashi, T., Ogasawara, A. and Shimada, K. (2000) Investigation of C-3 epimerization mechanism of 24,25-dihydroxyvitamin D₃ in rat using liquid chromatography/mass spectrometry. *Anal. Sci.*, **16**, 477-482.

- 5) Kamao, M., Tatematsu, S., Hatakeyama, S., Sakaki, T., Sawada, N., Inouye, K., Ozono, K., Kubodera, N., Reddy, G. S. and Okano, T. (2004) C-3 epimerization of vitamin D₃ metabolites and further metabolism of C-3 epimers. *J. Biol. Chem.*, **279**, 15897-15907.
- 6) Napoli, J. L. and Horst, R. L. (1984) Vitamin D Metabolism. In *Vitamin D: Basic and Applied Aspects* (Kumar, R., Eds.). Martinus Nijhoff, Boston, pp.91-123.
- 7) Jones, G. (1978) Assay of vitamins D₂ and D₃, and 25-hydroxyvitamins D₂ and D₃ in human plasma by high-performance liquid chromatography. *Clin. Chem.*, **24**, 287-298.
- 8) Haddad, J. G. and Chyu, K. J. (1971) Competitive protein-binding radioassay for 25-hydroxycholecalciferol. *J. Clin. Endocrinol. Metab.*, **33**, 992-995.
- 9) Hollis, B. W. and Napoli, J. L. (1985) Improved radioimmunoassay for vitamin D and its use in assessing vitamin D status. *Clin. Chem.*, **31**, 1815-1819.
- 10) Lind, C., Chen, J. and Byrjalsen, I., (1997) Enzyme immunoassay for measuring 25-hydroxyvitamin D₃ in serum. *Clin. Chem.*, **43**, 943-949.
- 11) Carter, G. D., Carter, R., Jones, J. and Berry, J. (2004) How accurate are assays for 25-hydroxyvitamin D? Data from the international vitamin D external quality assessment scheme. *Clin. Chem.*, **50**, 2195-2197.
- 12) Carter, G. D., Carter, C. R., Gunter, E., Jones, J., Jones, G., Makin, H. L. and Sufi, S. (2004) Measurement of Vitamin D metabolites: an international perspective on methodology and clinical interpretation. *J. Steroid Biochem. Mol. Biol.*, **89-90**, 467-471.
- 13) Binkley, N., Krueger, D., Cowgill, C. S., Plum, L., Lake, E., Hansen, K. E., DeLuca, H. F. and Drezner, M. K. (2004) Assay variation confounds the diagnosis of hypovitaminosis D: a call for standardization. *J. Clin. Endocrinol. Metab.*, **89**, 3152-3157.
- 14) Hollis, B. W. (2000) Comparison of commercially available ¹²⁵I-based RIA methods for the determination of circulating 25-hydroxyvitamin D. *Clin. Chem.*, **46**, 1657-1661.
- 15) Collins, M. D. and Jones, D. (1981) Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbial. Rev.*, **45**, 316-354.
- 16) Sato, Y., Honda, Y., Kuno, H. and Oizumi, K. (1998) Menatetrenone ameliorates osteopenia in disuse-affected limbs of vitamin D- and K-deficient stroke patients. *Bone*, **23**, 291-296.

- 17) Craciun, A. M., Wolf, J., Knapen, M. H., Brouns, F. and Vermeer, C. (1998) Improved bone metabolism in female elite athletes after vitamin K supplementation. *Int. J. Sports Med.*, **19**, 479-484.
- 18) Feskanich, D., Weber, P., Willett, W. C., Rockett, H., Booth, S. L. and Colditz, G. A. (1999) Vitamin K intake and hip fractures in women: a prospective study. *Am. J. Clin. Nutr.*, **69**, 74-79.
- 19) Booth, S. L., Tucker, K. L., Chen, H., Hannan, M. T., Gagnon, D. R., Cupples, L. A., Wilson, P. W., Ordovas, J., Schaefer, E. J., Dawson-Hughes, B. and Kiel, D. P. (2000) Dietary vitamin K intakes are associated with hip fracture but not with bone mineral density in elderly men and women. *Am. J. Clin. Nutr.*, **71**, 1201-1208.
- 20) Tsugawa, N., Suhara, Y., Kamao, M. and Okano, T. (2005) Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 3001-3007.
- 21) Krall, E. A., Sahyoun, N., Tannenbaum, S., Dallal, G. E., Dawson-Hughes, B. (1989) Effect of vitamin D intake on seasonal variations in parathyroid hormone secretion in postmenopausal women. *Engl. J. Med.*, **321**, 1777-1783.
- 22) Webb, A. R., Pilbeam, C., Hanafin, N., Holick, M. F. (1990) An evaluation of the relative contributions of exposure to sunlight and of diet to the circulating concentrations of 25-hydroxyvitamin D in an elderly nursing home population in Boston. *Am. J. Clin. Nutr.* **51**, 1075-1081.
- 23) Suhara, Y., Kamao, M., Tsugawa, N. and Okano, T. (2005) Method for the determination of vitamin K homologues in human plasma using high-performance liquid chromatography-tandem mass spectrometry. *Anal. Chem.*, **77**, 757-763.
- 24) Kamao, M., Suhara, Y., Tsugawa, N. and Okano, T. (2005) Determination of plasma vitamin K by high-performance liquid chromatography with fluorescence detection using vitamin K analogs as internal standards. *J. Chromatogr. B*, **816**, 41-48.
- 25) Tsugawa, N., Shiraki, M., Suhara, Y., Kamao, M., Tanaka, K. and Okano, T. (2006) Vitamin K status of healthy Japanese women: age-related vitamin K requirement for γ -carboxylation of osteocalcin. *Am. J. Clin. Nutr.*, **83**, 380-386.
- 26) Report of the Subdivision on Resources, The Council for Science and Technology,

Ministry of Education, Culture, Sports, Science and Technology, JAPAN (2005)
Standard Tables of Food Composition in Japan, Fifth revised and enlarged edition.

- 27) Hollis, B. W., Roos, B. A., Draper H. H. and Lambert, P. W. (1981) Vitamin D and its metabolites in human and bovine milk. *J. Nutr.*, **111**, 1240-1248.
- 28) Takeuchi, A., Okano, T., Tsugawa, N., Katayama, M., Mimura, Y., Kobayashi, T., Kodama, S. and Matsuo, T. (1988) The determination of vitamin D and its metabolites in human breast and cow's milk. *J. Micronutrient. Anal.*, **4**, 193-208.
- 29) Higashi, T., Awada, D. and Shimada, K. (2001) Simultaneous determination of 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃ in human plasma by liquid chromatography-tandem mass spectrometry employing derivatization with a Cookson-type reagent. *Biol. Pharm. Bull.*, **24**, 738-743.

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- <i>trans</i> -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).