Fat-Soluble and Water-Soluble Vitamin Contents of Breast Milk from Japanese Women

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Summary To determine the concentrations of fat-soluble and water-soluble vitamins in the maternal milk of Japanese women, we collected human milk samples from more than 4,000 mothers living throughout Japan between December 1998 and September 1999, and defined as group A the 691 samples among these that met the following conditions: breast milk of mothers who were under 40 y of age, who did not smoke habitually and/or use vitamin supplements, and whose babies showed no symptoms of atopy and had birth weights of 2.5 kg or more. We then analyzed the contents of vitamins individually. Large differences were observed among the contents of individual human milk samples. The mean contents of each component were as follows: vitamin A, 159.0±95.2 IU/100 mL; vitamin E, 0.325±0.165 µg/mg/100 mL; vitamin D3 (cholecalciferol), 8.0±10.7 ng/100 mL; vitamin B1 (thiamin), 12.3±3.2 µg/100 mL; vitamin B2, 38.4±12.7 µg/100 mL; vitamin B6, 5.7±2.5 µg/100 mL; vitamin B12, 0.04±0.02 µg/100 mL; vitamin C, 5.1±1.9 mg/100 mL; biotin, 0.50±0.23 µg/100 mL; choline, 9.2±1.8 mg/100 mL; folic acid, 6.2±2.9 µg/100 mL; inositol, 12.0±3.6 mg/100 mL; nicinamide, 32.9±20.4 µg/100 mL; and pantothentic acid, 0.27±0.09 mg/100 mL. The concentrations of derivatives and/or related compounds of vitamin A (retinol, α-carotene), vitamin E (α-, γ-, and δ-tocopherol), and B2 (riboflavin, flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD)) were determined separately. The contents of each were found to vary greatly as the duration of lactation increased. The present results indicate that it is necessary to evaluate individual differences in human milk in order to perform valid research regarding infant formula.

Key Words human milk, fat-soluble vitamin, water-soluble vitamin, composition

The vitamin contents of human milk from well-nourished women and the respective intakes of their exclusively breast-fed infants provide a primary knowledge base for estimates of infant vitamin requirements and recommended levels of intake and for the formulation of human milk substitutes (1, 2). However, information is lacking regarding the normal changes in vitamin concentrations during lactation. One reason for this is that little data about “mother’s milk” is available that can be used for reference. Numerous excellent studies have measured the vitamin components of human milk (3-32), but some of the data that could be used for comparison of samples has been limited because researchers often used pooled milk samples and did not have a well-defined population. Therefore, data that allows reliable comparison studies is not available.

Furthermore, there have been a few comprehensive studies regarding the composition of breast milk from Japanese women, but all of these were performed before 1990 (33, 34). Recent and substantial changes in the eating habits of Japanese people, such as the increased eating of processed foods seems likely to have had a significant influence on the composition of breast milk of mothers.

Although we have already determined the concentration of vitamin K, including K1 (phyloquinone) and K2 (menaquinone-n; MK-n), in Japanese maternal milk (35), the present study aimed to clarify the lactational influences on the concentrations of vitamins other than vitamin K in human milk. Because vitamins have various physiological activities based on differences in their chemical structures, the concentrations of vitamins and their derivatives and/or related compounds were determined with as much precision as possible. Namely, vitamin A (retinol, α-carotene), vitamin D (cholecalciferol), vitamin E (α, β, γ, δ-tocopherol), vitamin C (L-ascorbic acid), vitamin B1 (thiamin), vitamin B2 (riboflavin, flavin mononucleotide (FMN), flavin adenine dinucleotide (FAD)), vitamin B6, vitamin B12, biotin, choline, folic acid (pteroylglutamic acid), inositol, niacinamide, and pantothentic acid concentrations were determined at 10-365 d postpartum in the breast milk of approximately 690 mothers living in various regions of Japan.
MATERIALS AND METHODS

Milk collection. The overall methods were as described previously (35). Briefly, human milk samples were randomly collected twice; in the summer (between July and September, 1998) and in the winter (between December, 1998 and March, 1999), from approximately 4,000 mothers who were at various stages of lactation (1–365 d postpartum) and were living in various regions of Japan. Informed consent was obtained orally from the subjects prior to enrollment in this study. This study was conducted in accordance with the recommendations of the Declaration of Helsinki for clinical trials in human subjects. Approximately 50 mL of human milk was obtained at an intermediate time during suckling, placed in a nylon bag (Kaneson, Osaka, Japan) and stored in a freezer. At the same time, a personal information sheet (date and time of milk collection, right and/or left breast, mother’s age, smoking habits and/or use of vitamin supplements, birth weight of the infant, atopic symptoms of the infant, etc.) was completed by the mother. Frozen milk samples and information sheets were collected periodically by employees of Meiji Dairies Corporation, and were transported by delivery service at about −20°C to the Nutrition Research Institute, and were stored at about −40°C in a Sanyo Medical Freezer Model U-442 (Osaka, Japan) until preliminary sample preparation.

Preliminary milk preparation. Frozen human milk samples were thawed in tap water for 30 min and sonicated in ice water with a Branson ultrasonic cleaner model 5510J-MTH (Branson Ultrasonics Co., Dranbury, CT, USA) twice for 15 min each. The milk was divided into aliquots in 5-mL polypropylene tubes (Assist Co., Tokyo, Japan), and then stored at about −40°C until analysis.

Classification of human milk. Human milk samples were classified into 4 groups ("A", "B", "C", and "D") according to background data from the mother (age, smoking habits and use of vitamin supplements) and/or of the infant (birth weight, symptoms of atopy). Samples used for our study comprised group A: namely, breast milk of mothers who were under 40 y old, not in the habit of smoking or using vitamin supplements, and whose babies showed no symptoms of atopy and had birth weights of 2.5 kg or more. The number of specimens that could be precisely analyzed for water-soluble and fat-soluble vitamins was approximately 700, and of these, 691 belonged to group A.

Human milk samples in group A were further classified into subgroups according to several conditions such as season (summer or winter) and lactation stage (6–10, 11–20, 21–89, 90–365 d), and were also classified into 17 subgroups according to the regions where the mothers lived in Japan. All of the mothers in this group were healthy and their average age was 29.17 ± 4.01 y and they gave birth to infants whose birth weight was 3.142 ± 4.25 g.

Chemicals and reagents. Retinol palmitate, α-tocopherol, cholecalciferol, thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, cyanocobalamin, t(+)-ascorbic acid, pteroylglutamic acid, and nicotinamide, which were used as quantitative standards, were of the Japanese Pharmacopoeia grade. Vitamin E reference standard (Esai Co., Ltd. Tokyo, Japan), β-carotene and FMN (Sigma-Aldrich Co., St. Louis, MO, USA), FAD, mecoinostiol, and (+)-pantothenic acid calcium salt (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were purchased from Wako Pure Chemical Industries, Ltd. t(-)-Biotin (Merk) and choline chloride were purchased from Kanto Kagaku, Ltd. (Tokyo, Japan). Acetonitrile, 1,4-dioxane, ethanol, ethyl acetate, methanol, n-hexane, and 2-propanol, which were used as the mobile phase, were of HPLC grade (Wako Pure Chemical Industries, Ltd.) and were used without further purification. Sep-Pak silica cartridges, Bond Elut® SAX, and qualitative filter paper (No. 2 & 6) were obtained from Waters Corporation (Milford, MA, USA), Varian, Inc. (Palo Alto, CA, USA), and Toyo Roshi Kaisya, Ltd. (Tokyo, Japan), respectively.

Apparatus. The HPLC system for vitamin analysis consisted of an appropriate combination of a pump (Hitachi model L-6000, Tokyo, Japan), fluorescence detector (Jasco model FP920S, Tokyo, Japan; Shimadzu models RF-530(S) and RF-550, Kyoto, Japan; Hitachi models F-1080 and L-7480), UV/VIS detector (Hitachi models L-4000 and L-4200H; Jasco model UV-970), electrochemical amperometric detector (Yamada model VMD-501, Yanagimoto MFG Co., Ltd., Kyoto, Japan; ESA model Coulomch II 5200A, Chelmsford, MA, USA), and recorder (Hitachi model 561). A Model 7125 manual injection valve (Rheodyne, Cotati, CA, USA) equipped with a 20-, 50-, 100-, and 200-μL sample loop was also used. Solvents were filtered using a Millipore (Milford) system with 0.20-μm membrane filters (47 mm).

The columns used for vitamin analysis were as follows: a Nucleosil 50-5 column (5 μm, 250×4.6 mm; Chemco Scientific Co., Ltd., Osaka, Japan) for analysis of vitamin A, vitamin E, and vitamin C and for fractionation of crude vitamin D; a Hitachi Gel 3056 (5 μm, 250×4.6 mm; Hitachi Co., Ltd.) for analysis of vitamin D and folic acid; a Carotenoid C30 column (5 μm, 150×4.6 mm; Yamada Co., Ltd., Kyoto, Japan) for β-carotene; an L-column ODS (5 μm, 150×4.6 mm; Communications Evaluation and Research Institute, Tokyo, Japan) for vitamin B1 and B2; a Shodex RS pak DE-613 column (6 μm, 150×6.0 mm; Showa Denko K.K., Tokyo, Japan) and an AC-Enzymap column (200 mesh, 4×5 mm; Eicom Corporation, Kyoto, Japan) for choline; an ULTRON CI column (10 μm, 200×4.6 mm; Shinwa Chemical Industries Ltd., Kyoto, Japan) for inositol; and a Chemosorb 7-ODS-H column (7 μm, 250×4.6 mm; Chemco Scientific Co., Ltd.) for nicotinamide.

Biochemical analyses of vitamins. All analyses of light-sensitive vitamins such as retinol, tocopherol, and riboflavin were conducted under limited lighting in order to avoid degradation. Details are given in the relevant section below.

Vitamin A (retinol) and E (tocopherols). Vitamin A and E concentrations in human milk were measured
chiefly according to the modified method of Hasegawa et al. (36). Namely, 0.25 g of pyrogallol, 0.1 g of hydroquinone, 4 g of KOH, and 50 mL of ethanol were added to 20 mL of human milk, and the samples were boiled for 1 h. After cooling to room temperature, the samples were extracted three times with diethyl ether and the combined extracts were washed and dried under nitrogen and reconstituted in 5 mL of hexane (solution A). Samples were analyzed using an HPLC system equipped with a fluorescence spectrometer. Samples were eluted isocratically with a mobile phase of hexane: 2-propanol (99.6:0.4, v/v). The detection of retinol absorbance was monitored using a fluorescence detector with a 325-nm excitation filter and a 470-nm emission filter. Absorbance was also monitored with a 298-nm excitation filter and a 328-nm emission filter for the detection of tocopherols. Concentrations were calculated by comparison to standard solutions of retinol palmitate and α-tocopherol. The concentration of vitamin A was expressed based on the concentration of retinol and β-carotene in equivalents of retinol (IU/100 mL) where 1 IU = 0.3 mg all-trans retinol.

**β-Carotene (all-trans form).** One milliliter of solution A was dried under nitrogen and reconstituted in 1 mL of 2,6-di-tert-butyl-p-cresol (1 mg/mL). Samples were analyzed using an HPLC system coupled to a UV/VIS detector. Samples were eluted isocratically with a mobile phase of methanol: t-butylmethylether: acetic acid buffer, pH 4.0 (63:35:2, v/v). β-Carotene was detected at 450 nm. We calculated the β-carotene concentration in samples based on a ratio of 6:1 for conversion of β-carotene to vitamin A, and thus 6 mg of β-carotene is considered equivalent to 1 mg of retinol or 1 mg of retinol equivalent (RE).

**Vitamin D₃ (cholecalciferol).** Vitamin D₃ concentrations in human milk were measured chiefly according to the combination methods of “Standard Methods of Analysis for Hygienic Chemists” (37) and the modified method of Hasegawa et al. (36). As the majority of vitamin D activity is known to be from ergocalciferol (vitamin D₂), cholecalciferol (vitamin D₃), and the hydroxylated metabolites, we estimated cholecalciferol concentration in human milk in this study. Solution A (3.5 mL) was loaded by means of a glass syringe into a Sep-Pak silica cartridge that had been previously rinsed with n-hexane. The cartridge was eluted with 10 mL of 0.4% (v/v) isopropyl alcohol in n-hexane to elute a fraction containing cholecalciferol. The sample was dried under nitrogen and reconstituted in 0.5 mL of n-hexane. Samples were analyzed using an HPLC system coupled to a UV detector. Samples were eluted isocratically with a mobile phase of hexane: 2-propanol (99.6:0.4, v/v). Cholecalciferol was detected at 265 nm. The fractions containing cholecalciferol were collected and evaporated to dryness with nitrogen, and redissolved in 100 μL of methanol. Thirty microliters of this solution was then injected into an HPLC system. The mobile phase consisted of a mixture of acetonitrile and methanol (87:13, v/v), and absorbance was monitored at 265 nm using a UV detector.

**Vitamin B₁₂.** Vitamin B₁₂ contents in human milk were measured chiefly according to the combination of “Standard Tables of Food Composition in Japan” (38) and the modified method of Hasegawa et al. (36). Sulfuric acid (0.6 mL, 0.05 mol/L) was added to 4 mL of human milk, which was then heated in a boiling water bath for 15 min. After cooling to room temperature, the pH of the sample was adjusted to 4.5 with 4 mol/L sodium acetate solution, and the sample was incubated for 16–17 h at 37°C with approximately 30 mg of Takadiastase and a few drops of toluene. After boiling in water for 10 min, the sample was adjusted to 25 mL with mobile phase, and subjected to HPLC analysis. Absorbance was monitored using a fluorescence detector with a 375-nm excitation filter and a 450-nm emission filter.

**Vitamin B₂ (riboflavin, FMN, FAD).** Vitamin B₂ concentrations in human milk were measured chiefly according to the modified method of Hasegawa et al. (36). Riboflavin activity is known to exist in several forms in human milk (3), and thus we estimated its content as the total concentration of riboflavin, FMD, and FAD. To precipitate proteins, 0.5 mL of 10% trichloroacetic acid (TCA) was added to 4 mL of milk, and the volume was adjusted to 25 mL with mobile phase. The resulting precipitate was removed by centrifugation and the supernatant was filtered through a 0.45-μm filter (Millipore, Mass, USA). Analytical HPLC was performed using a mobile phase of a mixture of 0.05 mol/L of 1-hexanesulfonic acid sodium salt in 0.02 mol/L phosphoric acid and acetonitrile (80:20, v/v), and absorbance was monitored at 530 nm using a fluorescence detector. The concentration of vitamin B₂ was calculated from the following formula based on molecular weights (riboflavin: 376.4, FMN: 456.3, and FAD: 785.5): vitamin B₂ = riboflavin + FMN x 0.825 + FAD x 0.479.

**Vitamin B₆.** Vitamin B₆ concentrations in human milk were measured chiefly according to the analytical methods of “Standard Tables of Food Composition in Japan” (38). Human milk (4 mL) was added to 175 mL of 0.05 M HCl solution, and autoclaved at 121°C for 30 min. The pH was adjusted to 4.9–5.1 with NaOH solution, and the mixture was transferred to a volumetric flask (250 mL) and diluted to volume with distilled water. The sample was filtered through qualitative filter paper No. 6, and vitamin B₆ in the filtrate was estimated by a microbiological assay using *Saccharomyces carlsbergensis* strain 4228 ATCC 9080. Growth of *S. carlsbergensis* was quantified in a spectrophotometer by determining absorbance at 600 nm.

**Vitamin B₁₂.** Vitamin B₁₂ contents in human milk were microbiologically measured using the modified method described in AOAC (39). Briefly, human milk (4 mL) was mixed with 10 mL of 0.6 M acetate buffer, 0.4 mL of 0.05% (w/v) potassium cyanide solution, and 10 mL of distilled water. The sample was boiled in a water bath for 30 min and cooled. The sample was then mixed with 0.6 mL of 10% (w/v) metaphosphoric acid and was adjusted to 50 mL with distilled water. Twenty-five mil-
lilliters of sample filtrate obtained using qualitative filter paper No. 2 was adjusted to pH 6.0 with sodium hydroxide solution, and the volume was adjusted to 50 mL with distilled water. Vitamin B12 in this solution was estimated by a microbiological assay using *Lactobacillus leichmannii* ATCC 7830. Growth of *L. leichmannii* was quantified in a spectrophotometer by determining absorbance at 630 nm.

**Vitamin C.** Vitamin concentrations in human milk were measured chiefly according to the analytical methods of “Standard Tables of Food Composition in Japan” (38). A human milk sample (1 mL) was added to 50 mL of 5% metaphosphoric acid solution, and mixed well. After centrifugation (3,000 rpm, 10 min), the supernatant was filtered through qualitative filter paper No. 2. Two milliliters of the filtrate was oxidized with a few drops of 0.2% indophenol solution, and mixed with 2 mL of 2% thiourea-5% metaphosphoric acid solution and 0.5 mL of 2% 2,4-dinitrophenylhydrazine-9 N sulfuric acid solution, and heated for 1 h at 50°C. After cooling to room temperature, the sample was supplemented with 2 mL of ethyl acetate and mixed for 20 min. The supernatant was used for HPLC analysis after dehydrogenation by addition of 0.5 g of anhydrous sodium sulfate. The mobile phase was a mixture of n-hexane : ethyl acetate : n-propanol : acetic acid (40 : 30 : 2 : 1, v/v/v/v), and absorbance was monitored at 495 nm with a UV/VIS detector.

**Biotin.** Biotin content was measured microbiologically with *Lactobacillus arabinosus* strain 17-5 ATCC 8014 according to the modified method of Hasegawa et al. (36). Briefly, human milk (4 mL) was added to 20 mL of 0.5 M sulfuric acid solution, and the mixture was autoclaved at 121°C for 15 min. The pH was adjusted to 4.4-4.6 with NaOH solution, and the solution was filtered with qualitative filter paper No. 2. The pH of the filtrate was adjusted again to 6.7-6.9 with NaOH solution, and the solution was transferred into a volumetric flask (100 mL) and diluted to volume with distilled water. Biotin content was estimated by microbiological assay using the growth of *L. arabinosus*, which was quantified in a spectrophotometer by determining absorbance at 640 nm.

**Choline.** Choline in human milk was detected using a modified method of Potter et al. (27). Briefly, 4 mL of human milk was added to 180 mL of 2.2 mol/L hydrochloric acid and was then heated for 6 h in an oil bath (110-130°C). After cooling to room temperature, the solution was transferred to a volumetric flask (200 mL) and diluted to 20 mL with distilled water. After 5 mL of this solution was supplemented with 0.5 mL of 10 µg/mL ethylhomocholine (N,N-dimethyl (N-ethyl)-3-amino-1-propanol) as an internal standard, the pH was adjusted to 6.8 with KOH solution. The mixture was transferred to a volumetric flask (20 mL) and the supernatant was applied to an HPLC system equipped with an electrochemical amperometric detector (E1 = 450 mV). The mobile phase was 0.085 M dipotassium hydrogenphosphate-phosphoric acid buffer (pH 8.0) supplemented with 50% tetramethylammonium chloride solution (130 mg/1,000 mL) and sodium n-octyl sulfate (600 mg/1,000 mL). Choline in human milk was electrochemically detected after enzyme reaction with choline oxidase using an immobilized enzyme column.

**Folic acid (pteroylglutamic acid).** The folic acid concentration in human milk was measured by HPLC according to the fluorimetric method based on the procedure described by Allfrey et al. (40). Briefly, human milk (8 mL) was transferred to a volumetric flask (50 mL) and diluted to volume with 0.1 mol/L of NaOH solution, and then shaken for about 30 min for alkaline extraction. The precipitate was removed by centrifugation (3,000 rpm for 10 min), and the pH of the supernatant was adjusted to 7.0-7.5 by adding phosphate solution. Four milliliters of the sample was loaded onto an anion exchange column (Bond Elut® SAX) previously rinsed with distilled water. The column was eluted with 4 mL of a mixture of 20% (w/v) sodium chloride solution and 200 mmol/L sodium acetate (1 : 1, v/v). Two milliliters of the elution was mixed with 20 µL of 2.5 mol/L sodium acetate and 40 µL of 4% (w/v) of potassium permanganate, and was then heated for 10 min in a water bath. After cooling to room temperature, the sample was transferred to a volumetric flask (5 mL) and diluted to volume with mobile phase after adding 20 µL of hydrogen peroxide. The sample was subjected to HPLC with a mobile phase of a mixture of phosphate buffer (pH 3.5) and acetonitrile (97 : 3, v/v). Absorbance was monitored at 450 nm with a fluorescence detector.

**Inositol.** Inositol in human milk was detected using a modified method of Wang et al. (41). Briefly, human milk (4 mL) was mixed with 180 mL of 2.2 mol/L hydrochloric acid and was then heated for 6 h in an oil bath (110-130°C). The solution was transferred to a volumetric flask (200 mL) and diluted to volume with distilled water. The sample was then applied to an HPLC system. The mobile phase was a mixture of acetonitrile : distilled water (60 : 40, v/v), and detection was performed with a pulsed amperometric detector. The parameters of the detector were set as follows: E1 = +100 mV, T1 = 300 ms, E2 = +650 mV, T2 = 120 ms, E3 = -950 mV, T3 = 120 ms.

**Nicin (nicotinamide).** Nicin in human milk is known to be a component of nicotinamide, nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), and so on. We estimated the niacin concentration in human milk as the concentration of unbound nicotinamide in this study. The nicotinamide concentration in human milk was measured by HPLC based on the method of Hasegawa et al. (36) after precipitation of protein by addition of acetonitrile. Briefly, human milk (6 mL) was added to 24 mL of acetonitrile and mixed well. After centrifugation (3,000 rpm, 10 min), the supernatant was used for HPLC analysis. The mobile phase used a mixture of 4 mmol/L of sodium diocyl sulfosuccinate in 2 mmol phosphate buffer (pH 3.0) and acetonitrile (6 : 4, v/v), and absorbance was monitored at 260 nm with a UV detector.
Table 1. Fat-soluble vitamin composition of milk samples.*

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Average±SD (n)</th>
<th>Summer (n)</th>
<th>Winter (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin A</td>
<td>159.0±95.2 (115)</td>
<td>153.7±73.5 (57)</td>
<td>164.2±113.1 (58)</td>
</tr>
<tr>
<td>Retinol</td>
<td>47.7±28.6 (115)</td>
<td>46.1±22.0 (57)</td>
<td>49.2±33.9 (58)</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>2.6±2.1 (115)</td>
<td>2.4±1.6 (57)</td>
<td>2.7±2.5 (58)</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.325±0.165 (115)</td>
<td>0.320±0.155 (57)</td>
<td>0.330±0.175 (58)</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>0.309±0.159 (115)</td>
<td>0.306±0.151 (57)</td>
<td>0.313±0.168 (58)</td>
</tr>
<tr>
<td>β-Tocopherol</td>
<td>0.012±0.004 (115)</td>
<td>0.011±0.004 (57)</td>
<td>0.013±0.005 (58)</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>0.106±0.057 (115)</td>
<td>0.096±0.041 (57)</td>
<td>0.117±0.069 (58)</td>
</tr>
<tr>
<td>δ-Tocopherol</td>
<td>0.027±0.023 (115)</td>
<td>0.027±0.030 (57)</td>
<td>0.027±0.015 (58)</td>
</tr>
<tr>
<td>Vitamin D</td>
<td>8.0±10.7 (114)</td>
<td>7.3±10.8 (57)</td>
<td>8.6±10.6 (57)</td>
</tr>
</tbody>
</table>

* Average±SD (n).

Table 2. Water-soluble vitamin composition of milk samples.*

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Average±SD (n)</th>
<th>Summer (n)</th>
<th>Winter (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin B₁</td>
<td>12.3±3.2 (114)</td>
<td>12.1±3.2 (57)</td>
<td>12.4±3.2 (57)</td>
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<tr>
<td>Vitamin B₂</td>
<td>38.4±12.7 (114)</td>
<td>40.2±13.4 (57)</td>
<td>36.7±11.9 (57)</td>
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<tr>
<td>Riboflavin</td>
<td>4.6±6.2 (114)</td>
<td>5.7±7.3 (57)</td>
<td>3.5±4.6 (57)</td>
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<tr>
<td>FMN</td>
<td>1.4±2.8 (114)</td>
<td>1.4±3.0 (57)</td>
<td>1.3±2.6 (57)</td>
</tr>
<tr>
<td>FAD</td>
<td>68.3±22.4 (114)</td>
<td>69.7±21.6 (57)</td>
<td>67.0±23.4 (57)</td>
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<tr>
<td>Vitamin B₆</td>
<td>5.7±2.5 (159)</td>
<td>5.6±2.4 (57)</td>
<td>5.8±2.6 (102)</td>
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<tr>
<td>Vitamin B₁₂</td>
<td>0.04±0.02 (115)</td>
<td>0.04±0.02 (57)</td>
<td>0.05±0.03 (58)</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>5.1±1.9 (117)</td>
<td>4.9±2.1 (57)</td>
<td>5.4±1.7 (60)</td>
</tr>
<tr>
<td>Biotin</td>
<td>0.50±0.23 (129)</td>
<td>0.47±0.22 (60)</td>
<td>0.52±0.23 (69)</td>
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<tr>
<td>Choline</td>
<td>9.2±1.8 (145)</td>
<td>9.6±1.7 (59b)</td>
<td>9.0±1.8 (86b)</td>
</tr>
<tr>
<td>Folic acid</td>
<td>6.2±2.9 (114)</td>
<td>6.3±2.4 (57)</td>
<td>6.1±3.3 (57)</td>
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<tr>
<td>Inositol</td>
<td>12.6±3.6 (145)</td>
<td>12.1±3.4 (59)</td>
<td>13.0±3.7 (86)</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>32.9±20.4 (119)</td>
<td>36.6±24.3 (59)</td>
<td>29.2±15.0 (60)</td>
</tr>
<tr>
<td>Pantothentic acid</td>
<td>0.27±0.09 (124)</td>
<td>0.26±0.09 (57)</td>
<td>0.28±0.09 (67)</td>
</tr>
</tbody>
</table>

* Average±SD (n).

Values with superscript letters differ significantly: p<0.05.

Pantothenic acid. Pantothenic acid concentration in human milk was estimated by microbiological assay based on the modified procedure described by Hasegawa et al. (36). Briefly, before enzymatic cleavage of bound pantothenic acid from milk protein, the sample (4 mL) was supplemented with 45 mL of distilled water and autoclaved at 121°C for 15 min. After cooling to room temperature and enzymatic treatment with amylase and papain at 55°C for 18 h, the pantothenic acid content in the sample was estimated by microbiological assay using the growth of L. arabinosus strain 17-5 ATCC 8014, which was quantified in a spectrophotometer by determining absorbance at 640 nm.

Statistical analysis. Values are expressed as means with SD. Effects of the different stages of lactation, season, and living district on the concentrations were tested using the Kruskal-Wallis test followed by the Mann-Whitney two-sample test.

Differences between means were considered significant at p<0.05. All statistical analyses were conducted using a statistical computer program (Stat View, Abacus Concepts, Inc., Berkeley, CA, USA) for Windows.

Ethical considerations. This study was approved by the Research and Ethics Committee of the Nutritional Science Research Institute of Meiji Dairies Corporation.

RESULTS

The average contents of fat-soluble and water-soluble vitamins in human milk samples are presented in Tables 1 and 2.

Although a large number of samples were obtained for group A (691 samples), and the contents of each sample were analyzed individually, the number of samples analyzed for each component was somewhat smaller due to various problems in the analytical procedures, such as insufficient sample volume.

We compared the data between milk samples collected in two different periods, namely summer and
winter. The contents of all vitamins in human milk had large standard deviations, and neither significant seasonal nor regional variations were noted in the average levels of any component except choline. Therefore, we focused on possible lactation period-dependent differences in vitamin concentrations in human milk. The concentrations of each nutrient according to lactation stage are shown in Tables 3 and 4. Although there were large variations in the sample numbers obtained at different lactation stages, the magnitude of the standard deviation of nutrient levels was almost the same at all stages.

The concentration of water-soluble vitamins in human milk showed variations due to stage of lactation, and accordingly these vitamins could be roughly classified into 3 groups: 1) levels increased as lactation stage increased: vitamin B1, vitamin B2 (riboflavin), and vitamin B6; 2) levels decreased as lactation stage...
increased: vitamin B$_{12}$, vitamin C, folic acid and inositol; and 3) levels remained almost constant as lactation stage increased: biotin, choline, niacin, and pantotheneic acid. In contrast, the levels of all of fat-soluble vitamins (vitamin A, retinol, β-carotene, vitamin E (α-, β-, γ-, and δ-tocopherol), and vitamin D) decreased as lactation stage increased.

On the whole, the present average concentrations of each vitamin, except riboflavin, niacin, and vitamin B$_{6}$, were very similar to previously reported values (3, 4). Although there was an apparent difference in MK-7 concentration depending on region where the mother lived according to a report by Kojima et al. (35), the vitamin D concentration was not affected by seasonal and/or regional variations (northern versus southern regions; data not shown).

**DISCUSSION**

The purpose of the present study was to identify determinants of human milk vitamin concentration among presumably well-nourished Japanese women. When compared with the previously reported results of investigations into Japanese mother's milk (33, 34), our results were generally comparable. However, our findings made it clear that there is a large variation in the composition of Japanese mother's milk.

To date, numerous well-designed studies have been performed to examine vitamin concentrations in human milk, and there were quantitative differences in reported mean values of vitamins among them, even when seemingly similar methodology was employed. Picciano (3) pointed out that this might have been due to genetic differences among donors, to sample handling procedures that inadvertently destroyed labile vitamins, or to interference in analytical schemes that are reproducible and therefore not controlled. We believe that the data in the present report are the first that can be evaluated statistically.

It is known that fat-soluble vitamins have numerous related compounds (4, 5). We estimated the total concentrations of retinol and β-carotene as vitamin A, and those of α-, β-, γ-, and δ-tocopherol as vitamin E in human milk. On the other hand, with regard to vitamin D, we were only able to estimate the concentration of vitamin D$_{3}$ (cholecalciferol) as vitamin D because of the difficulty of an accurate quantitation. It is well known that human milk contains metabolites other than vitamin D$_{3}$, such as vitamin D$_{2}$ (ergocalciferol), 25-OH cholecalciferol (25-OH-D) and 1,25-(OH)$_{2}$ cholecalciferol, Hollis (15), using a liquid binding assay coupled with HPLC, has quantified these metabolites in human milk, and thus we hope to study these related compounds in human milk in the future.

Our results concerning vitamin contents in human milk largely agree with previous reports about the composition of human milk (3–32), but differ with regard to a few points, for example, riboflavin, niacin, and vitamin B$_{6}$ contents.

With regard to riboflavin concentration, when FMN and FAD contents were converted into riboflavin activities based on molecular weights, vitamin B$_{2}$ content as “total riboflavin” was in agreement with reported values (3, 17, 18). The most noteworthy point with respect to vitamin B$_{2}$ content in human milk is the large concentration of FAD, and the difference between our results and previous reports might be due to the more accurate assessment of FAD content. Our results showed that a sizable fraction of riboflavin activity is furnished by FAD. Because it is well known that a sequential process in which hydrolysis of FMN and FAD at the brush border membrane in the small intestine is followed by absorption of free riboflavin, it is necessary to measure precisely nutrient content of human milk for assessment of infant and mother nutrition.

Concerning niacin, we estimated the unbound niacinamide concentration based on a method using HPLC, although niacin in human milk was reported to be constituted of nicotinamide, nicotinamide adenine dinucleotide (NAD), and nicotinamide adenine dinucleotide phosphate (NADP) (42). Therefore, the difference of our results compared with reported values might be due to the difference of analytical methods.

Tryptophan is also known to be a precursor of niacin and 60 mg of dietary tryptophan is equivalent to 1 mg of niacin. Actual niacin values for human milk would be higher owing to the possible contribution from conversion of tryptophan. Yamawaki et al. (43) estimated the tryptophan concentration in pooled human milk from Japanese women during various lactation stages as follows: 37.1±13.5 mg/100 mL (6–10 d), 26.6±2.8 mg/100 mL (11–20 d), 19.7±1.9 mg/100 mL (21–89 d), 16.7±1.5 mg/100 mL (90–180 d), and 15.5±2.0 mg/100 mL (181–365 d). Based on the combination of these reported values and our results, niacin concentration in human milk was re-estimated as follows (NE=niacin equivalent activity): 0.65 mg NE/100 mL (6–10 d), 0.49 mg NE/100 mL (11–20 d), 0.37 mg NE/100 mL (21–89 d), 0.32 mg NE/100 mL (90–180 d), and 0.30 mg NE/100 mL (180–365 d). As excess dietary tryptophan is converted to niacin, much of the niacin in human milk might be derived from tryptophan. But the contents of tryptophan in human milk don’t provide enough explanation for our niacin values reported here. We want to estimate contents of other niacin-related compounds detected in human milk, such as NAD and NADP in the future.

Our value for vitamin B$_{6}$ content (5.7±2.5 µg/100 mL) was considerably smaller than the reported values, which vary between 9 and 31 µg/100 mL (3, 19). Our method in this study was a microbiological assay, which is known to be generally nonspecific. We believe that a microbiological assay for vitamin B$_{6}$ content is not necessarily inferior to HPLC, but the contents of compounds related to vitamin B$_{6}$, such as pyridoxal and pyridoxamine, should have been considered in order to allow a more accurate assessment.

Picciano (3) reported that most water-soluble vitamin concentrations are lower in colostrum (1–5 d) when compared with mature milk (>1 mo), with the exception of vitamin B$_{12}$, but our results showed that
such variations could be roughly classified into 3 groups, namely 1) vitamins of which levels increased as lactation stage increased such as vitamin B₃, and so on, 2) vitamins of which levels decreased as lactation stage increased such as vitamin B₁₂ and so on, and 3) vitamins of which levels remained almost constant as lactation stage increased such as biotin and so on. Because the mammary gland cannot synthesize water-soluble vitamins, their origins are likely to be the maternal plasma and/or the maternal diet. Unfortunately, we do not have precise information about the dietary habits of the mothers in our study, but we believe that these variations in vitamin content may be related to the mechanisms of regulation of water-soluble vitamins.

Some researchers have pointed out that season and maternal sunlight exposure may affect milk vitamin D levels (44). We compared the results of vitamin D content with sampling information, but found that the concentration in mature milk was not affected by the season and/or region in Japan because of their large standard deviations (data not shown). Although the vitamin D content in mother’s milk is known to be extremely low, and quantification itself is very difficult, the large variations in vitamin D concentration that we observed may have been related to the mother’s condition.

We believe that our results are of importance in the setting of an Estimated Average Requirement of vitamins for infant nutrition in Japan. But knowledge of contents and forms of the vitamins secreted and factors capable of having an impact is still far from complete. For instance, little is known about the quantity of nutrients that infants take in because we were unable to acquire information about the volume of each milk sample in our research. Moreover, neither maternal plasma content nor vitamin content in the maternal diet was examined. We hope to further study these points in the future. Changes continue to be made to infant formulas, and these changes generally result in products with compositions and functions closer to those of human milk. At present we can only suggest that investigators be aware that the potential exists for variation in milk composition depending on lactation period.

CONCLUSION

In summary, our results largely agree with previous reports about the composition of human milk. We confirmed that the composition of human milk is affected by factors such as stage of lactation, and varies depending on the individual.

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