Contents of All Forms of Vitamin B₆, Pyridoxine-β-Glucoside and 4-Pyridoxic Acid in Mature Milk of Japanese Women According to 4-Pyridoxolactone-Conversion High Performance Liquid Chromatography

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Summary The contents of six vitamin B₆ forms, pyridoxine-β-glucoside, and 4-pyridoxic acid in mature milk of 20 Japanese lactating women consuming ordinary Japanese foods were determined by a 4-pyridoxolactone-conversion HPLC method. These compounds were determined with the average recovery rate of 83.9% or more. The average total content of vitamin B₆ forms was 1.01 ± 0.32 (μmol/L). Pyridoxal and pyridoxal 5′-phosphate were found in all of the samples, and their average contents were 0.71 ± 0.28 (μmol/L) and 0.16 ± 0.07 (μmol/L), respectively. Pyridoxamine, pyridoxine, pyridoxamine 5′-phosphate, pyridoxine 5′-phosphate, and pyridoxine-β-glucoside were found in 15, 14, 13, 9, and 7 samples, respectively. The presence of pyridoxine 5′-phosphate was for the first time found in human milk. A method for the determination of 4-pyridoxic acid, which is the excretion form of vitamin B₆o, was modified to quantitate it by isocratic HPLC. 4-Pyridoxic acid was found in all samples, and its average content was 0.094 ± 0.040 (μmol/L), which was only 12% of its content in cow (Holstein) milk. The total content of vitamin B₆ forms, and predominant presence of pyridoxal among other vitamin B₆ forms in the Japanese women’s milk samples shared similar characteristics with American women’s milk samples.

Key Words vitamin B₆, Japanese women’s milk, pyridoxal, pyridoxine-β-glucoside, 4-pyridoxic acid

Foods contain the family of vitamin B₆ compounds which consists of six forms, i.e., pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), pyridoxine 5′-phosphate (PNP), pyridoxal 5′-phosphate (PLP), and pyridoxamine 5′-phosphate (PMP) (Fig. 1). PLP is a coenzyme for many enzymes involved in amino acid and carbohydrate metabolism, and plays a key role in the nutritional function of vitamin B₆. The other forms show the same nutritional efficiency because they are converted into PLP in cells. Plants contain a storage form of vitamin B₆, pyridoxine-β-glucoside (PNG) (1), which is not counted as a vitamin B₆ form although its utilization is 58% relative to PN (2). 4-Pyridoxic acid (4-PA), which exhibits no vitamin B₆ activity (3), was found in some foods, such as milk.

The composition of vitamin B₆ and the related compounds in human milk has been extensively studied, because breast milk is the sole source of nutrients for almost all infants up to 4–6 mo. The total content of vitamin B₆ in the milk of American women have been investigated by high performance liquid chromatography (HPLC) in several laboratories (1, 4–7). The studies showed that the mature milk samples contained 1 μmol/L of total vitamin B₆ in which PL was the major (65.9–82.8% of the total content) form in the milk. PLP was the second (12.5–31%) in the mature milk although it was not found in the transitional milk (7). The contents of the other forms were very low: PM (0–5.2% and 11.1% in the mature and transitional milk, respectively), PMP (0–1.9% and 0%), and PN (0–4.4% and 11.8%). So far, PNP has not been found. 4-PA content was approximately 0.2 (μmol/L) (8).

In contrast to the detailed studies on the milk of American women, only one study has reported on the total content of vitamin B₆ and the distribution of vitamin B₆ forms in milk of Japanese women. Average total content of vitamin B₆ in 25 mature milk samples determined by HPLC was approximately 1.45 (μmol/L) (9). The contents of PLP and PL were 1.15 and 0.30 (μmol/L), respectively; the relative content of PLP and PL was the reverse of that found in the American wom-
en's milk. No other forms or vitamin B₆-related compounds were found. Recently, the total but not the individual content in Japanese women’s milk was reported to be 1.36 (µmol/L) (10). Thus, the distribution of vitamin B₆ forms, PNG and PA in Japanese women’s milk has not been analyzed.

Recently, we developed a new method (4-pyridoxolactone-conversion HPLC method) for determination of individual vitamin B₆ forms and PNG. In the method, all vitamin B₆ forms and PNG are specifically converted into 4-pyridoxolactone (PLA) (Fig. 1), a highly fluorescent derivative of vitamin B₆, by combinations of five enzymes, and then the PLA produced is quantitated by isocratic reversed-phase HPLC (11). Because of high substrate specificities of the enzymes used, and of the high fluorescence of PLA, the method needs only small-sized analytical samples, and all of the vitamin B₆ forms and PNG in several food samples were determined with high recoveries (12, 13).

4-PA in biological samples has been determined in several laboratories by HPLC. A highly sensitive method involves a conversion of 4-PA into PLA by treatment with HCl (14). Then, PLA produced is quantitated by cation-exchange HPLC. Here, we have modified the method to quantitate by isocratic reversed-phase HPLC.

In this report, we have determined all forms of vitamin B₆, PNG, and 4-PA in 20 mature milk samples from healthy Japanese women, who answered a brief diet history questionnaire (BDHQ) (15). PNG, which so far had not been detected in milk samples, were found in several Japanese women’s milk samples. 4-PA was found in all samples. Its contents were very low compared to that in cow milk.

**MATERIALS AND METHODS**

**Materials.** Six forms of standard vitamin B₆, PNG, five kinds of enzymes, and their substrates used in this study were obtained as described previously (11). PLA was prepared biotechnologically (16). 4-PA was purchased from Sigma-Aldrich Japan Inc. (Tokyo, Japan). All other chemicals used in this study were of the highest purity available. Cow (Holstein) milk was purchased from SunShine Co. (Kochi, Japan).

**Mature milk samples.** The milk samples were collected from 20 healthy Japanese women living in or near Kochi City: age, 26–37 (average ± SD, 32.1 ± 3.6); BMI, 18.1–26.2 (20.8 ± 2.4); and lactation stage, 60–188 d (134 ± 43) postpartum. Their heights and weights are shown in Table 1. Mothers participating in the study had no medical history and were not receiving any vitamin B₆ supplements during their pregnancy or postpartum. Informed consent was obtained in written form from the subjects prior to enrollment in this study. Ethical approval was obtained from the Kochi Gakuen College Ethics Committee and was conducted in compliance with the Helsinki Declaration.

Approximately 10 mL of the women’s milk was obtained at an intermediate time during suckling at 10:00–14:00 hours from May 26th to June 21th, 2011, and placed in a 500 mL sterilized plastic beaker (Sanplatec, Tokyo) without preservatives. The milk (1 mL, each) was immediately transferred into an ice-cold Eppendorf tube, and then stored at −30°C until use.

Dietary intakes of the women were assessed by a BDHQ system (15). This system gives the history of deduced diet intake of the previous 1 mo.

**Determination of vitamin B₆ forms and PNG.** The thawed milk sample was mixed thoroughly on a shaker to prevent separation of fat and aqueous phases. To the milk sample (100 µL) was added 1 mL 0.1 M HCl, and then the mixture was incubated at 100°C for 30 min in a SCINICS ALB-121 aluminum dry block bath (Tokyo, Japan). After cooling, 40 µL of 50% (w/v) trichloroacetic acid was added to the mixture. The mixture was then mixed thoroughly, and additionally incubated at 100°C for 5 min in the bath. After cooling, the pH of the
mixture was adjusted to 7.5 by adding 40 μL of 0.5 M Tris-HCl (pH 7.5) and 28 μL of 5.0 M NaOH. Water was then added to make the volume of the sample solution 2.0 mL. The mixture was centrifuged at 10,000 × g for 5 min at 4°C, and the supernatant (100 μL) was used for the enzymatic reaction. For recovery studies, definite amounts of standard vitamin B₆ forms and PNG were separately added to the thawed milk, and then the standard vitamin B₆-spiked milk samples were also subjected to the steps of the analytical sample preparation.

The enzyme reactions for conversion of vitamin B₆ forms and PNG into PLA were done with seven reaction mixtures as described previously (13) with the exception that 100 μL of the milk sample was used instead of 5–50 μL of the extract of foods: a decreased amount of water was added to make the volume of reaction
RESULTS

Contents of vitamin B₆ forms and PNG in the Japanese women’s milk samples

The contents of vitamin B₆ forms and PNG in 20 mature milk samples of Japanese women were determined (Table 1). The recoveries of each of the samples are also shown. The average recoveries±SD (%) of PL, PM, PN, PLP, PMP, PNP, and PNG were 90.1±6.6, 91.0±2.2, 89.1±6.5, 83.9±8.8, 90.4±8.4, 86.6±9.5, and 94.5±11.2, respectively. Thus, the compounds in milk samples could be determined without extraction of lipids. PL was found in all samples, and its average content in the 20 milk samples was 0.71±0.28 (µmol/L). PM was found in 15 samples, and its average content was 0.03±0.03 (µmol/L). PN was found in 14 samples, volume, 0.5 mL/min and 100 µL, respectively; and fluorescence detection, excitation at 360 nm and emission at 430 nm.

For the quantitation of PLA produced from 4-PA by the HCl treatment, the same HPLC conditions were used with exception that the flow rate was changed to 1.0 mL/min. Because the amount of 4-PA in the human milk was very low, a better shape of PLA elution peak was obtained when the flow rate was increased.

Enzyme and protein assay. The activities of five enzymes used were assayed every time just before usage for enzymatic conversion of vitamin B₆ forms and PNG into PLA as described previously (13).

Concentrations of protein in the petroleum-ether-extracted milk were determined by the Lowry method (17).

Statistical analysis. Values are expressed as average ±SD of three experiments. Correlation between two variables was examined with the Excel program.

RESULTS

Cow milk

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Human milk

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Fig. 2. HPLC chromatograms for the analysis of 4-PA in the cow (Holstein) and Japanese woman’s milk samples. Arrows show PLA peaks. The attenuation of the charts was 32 mA/full scale. The cow milk (5 µL) and Japanese woman’s milk (No. 9, 50 µL), with or without 2 pmol (amount applied to the HPLC column) of standard 4-PA, were subjected to the HCl treatment, and then applied to HPLC.
and its average content was 0.05±0.07 (μmol/L). PLP was also found in all samples, and its average content was 0.16±0.07 (μmol/L). PMP was found in 13 samples, and its average content was 0.03±0.05 (μmol/L). PNP was found in 9 samples, and its average content was 0.03±0.05 (μmol/L). PNG was found in 7 samples, and its average content was 0.03±0.05 (μmol/L). Thus, the average relative amount of vitamin B6 forms and PNG only showed a positive correlation coefficient of 0.4509 (r=−0.67, p<0.01) as shown in Fig. 3.

The protein concentration of the Japanese women’s milk samples are shown in Table 2. The average protein concentration of the Japanese women’s milk was 13.8±2.4 g/L (10.3±1.1 mg/d) of Japan (18).

**DISCUSSION**

The main forms (PL and PLP) and the total content originally contain PLA because the peak of PLA was only found in HCl-treated samples. 4-PA-spiked samples showed higher peaks, corresponding to the increase of PLA produced from the spiked 4-PA. Thus, the modified isocratic HPLC was applicable for quantitation of 4-PA in the cow and Japanese women’s milk samples. The contents and recoveries of the Japanese women’s milk samples are shown in Table 2. The contents of 4-PA in the Japanese women’s milk samples were 22.7±0.6–151.1±0.9 (nmol/L), and the average was 93.8±40.1. 4-PA contents in the American women’s milk samples were 212±200 (nmol/L) (8).

The 4-PA content of cow milk was 766.5±13.8 (nmol/L). It was much higher than that of the human milk. The high content of 4-PA (525.0±226.0 nmol/L) in cow (Holstein) milk has been reported (8).

The correlation between the content of 4-PA and those of vitamin B6 forms and PNG in the Japanese women’s milk was examined. PM contents only showed a negative correlation with 4-PA contents with the determination coefficient of 0.4509 (r=−0.67, p<0.01) as shown in Fig. 3.

The protein concentration of the Japanese women’s milk samples are shown in Table 2. The average protein concentration of the samples was 13.4±2.4 g/L (10.3±0.1–18.6±0.3 g/L). The protein concentration showed no correlation with the content of the vitamin B6 forms (PL: r=0.005, p>0.1; PM: r=0.35, p>0.1; PNG: r=0.39, p>0.05; PMP: r=0.09, p>0.1; PMP: r=0.06, p>0.1; PNG: r=0.16, p>0.1), PNG (r=0.19, p>0.1), or the total content of vitamin B6 (r=0.004, p>0.1).

**BDHQ analysis**

To examine the normality of milk samples used, dietary habits of the Japanese mothers were assessed using BDHQ. BDHQ analysis showed that the mothers consumed ordinary Japanese foods and no alcohol or supplement. The deduced average intake of energy, protein, fat, and vitamin B6 (pyridoxine equivalent determined by a microbiological method) were 1,884.9±463.7 kcal/d, 67.6±16.3 g/d, 44.4±14.4 g/d, and 1.17±0.33 mg/d, respectively. The deduced average intake of vitamin B6 was higher than the Recommended Daily Allowance (1.1 mg/d) of Japan (18).
(about 1 μmol/L) of vitamin B₆ in the Japanese women’s mature milk was quite similar to those in the American women’s mature milk so far reported (1, 4–7, 19). The American women’s milk samples were analyzed by reversed-phase HPLC (1, 7, 19), ion-exchange HPLC (4, 5) or a microbiological method (6). Here, PNP, which had not been found in American mothers’ mature milk samples, was found in 45% of Japanese women’s milk samples, although the contents were low. PNG, which had been reported to comprise a mean of 2.5% of the total vitamin B₆ contents (6), was found in 35% of Japanese women’s milk samples, and accounted for a mean of 4% of the total vitamin B₆ contents. Although there was a correlation between PNP and PNG contents, it may not be significant, because only five samples contained both of them, and five samples contained either one of them. The limited detection of PNG in the milk samples coincides with the results that there is little practical significance to the consumption of PNG, which is contained mainly in plant-derived foods, on the content of PNG in milk by free-living populations (6).

The average concentrations of PL and PLP in 12 American lactating women’s whole blood samples were 0.078 ± 0.033 and 0.042 ± 0.012 μmol/L, respectively (20). Because there are no practical differences in contents and kinds of vitamin B₆ forms in mature milk between the Japanese and American women, these values may be applicable to the Japanese women. Thus, the concentrations of PL and PLP in the milk may be 9-fold and 3.8-fold higher than those in the whole blood, respectively. The results suggested that PL and PLP were selectively excreted into the women’s milk. Although the excretion of PN (21, 22) and its transporter (23) have been reported, there is no information about PL or PLP excretion mechanisms.

There was a correlation between contents of PM and 4-PA, which is the excretion form of vitamin B₆. Although the graph of correlation (Fig. 3) showed the correlation is significant, the mechanism that caused the correlation is not known. The analysis of more milk samples is required to confirm the results and to elucidate the mechanism. There was no significant correlation between contents of each of the vitamin B₆ forms or total contents of free and phosphate forms. The results were in contrast to those obtained in analyses of sushi toppings (sashimi, raw meat of fishes) (12). In the toppings, the total contents of the free and phosphate forms exhibited strong correlation with a determination coefficient of 0.938; the total amounts of the phosphate forms were about 3-fold higher than those of the free forms. The contents of PM and PMP, PL and PLP, and PN and PLP moderately correlated with determination coefficients of 0.717, 0.689, and 0.556, respectively, while the contents of PN did not correlate with those of PNP. The correlations may be general characteristic in the cells. Thus, although we have no data about the distribution of vitamin B₆ forms in mammary gland cells, the distribution of vitamin B₆ forms in the milk may not reflect the intracellular distribution, PL being specifically excreted into the milk. The priority of PL for infants over other vitamin B₆ forms, especially PN, which is generally used as a supplement for formula milk, has not been examined.

Here, 20 milk samples were analyzed. There is a need to analyze a large number of milk samples from lactating women living in various regions in Japan to generalize the results obtained here.

Acknowledgments

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