

Intra- and Inter-Individual Variations of Blood and Urinary Water-Soluble Vitamins in Japanese Young Adults Consuming a Semi-Purified Diet for 7 Days

Katsumi SHIBATA¹, Tsutomu FUKUWATARI¹, Toshiaki WATANABE² and Mamoru NISHIMUTA³

¹*School of Human Cultures, The University of Shiga Prefecture, Hikone, Shiga 522–8533, Japan*

²*School of Humanities for Environmental Policy and Technology, University of Hyogo, Himeji, Hyogo 670–0092, Japan*

³*Division of Human Nutrition, National Institute of Health and Nutrition, Tokyo 162–8636, Japan*

(Received September 29, 2008)

Summary We have previously reported the levels of water-soluble vitamins in the blood and urine of Japanese young adults. In the present paper, to assess the variations in these water-soluble vitamin markers during the above experiment, we comprehensively determined the intra- and inter-individual variations of blood and urinary water-soluble vitamins to exactly the same amount of water-soluble vitamin intakes in the same experiment. The blood samples before breakfast and the 24-h urine samples were periodically collected from Japanese college male ($n=10$) and female ($n=10$) students consuming a semi-purified diet with water-soluble vitamins based on Japanese Dietary Reference Intakes for 7 d, and the intra- and inter-individual variations of blood and urinary water-soluble vitamins or their metabolites in blood and urine samples after adaptation were calculated. Although urinary excretion of vitamin B₁₂ and vitamin C showed high intra-individual variations in both males and females, other urinary vitamins and all blood vitamins showed less than 20% of within-subject coefficients of variance in either male or female. Those showing more than 20% of between-subject coefficients of variances in both male and female were blood vitamin B₆, vitamin B₁₂ and folate levels, and urinary vitamin B₁, vitamin B₂, vitamin B₁₂, nicotinamide metabolites, pantothenic acid, biotin and vitamin C. These results showed that oral administration of constant of water-soluble vitamins generally decreased intra-individual variation, while individual differences in urinary vitamin excretion were observed.

Key Words water-soluble vitamins, blood, urine, human study

A decrease of the vitamin contents in the body causes a vitamin deficiency, and vitamin levels in blood and urine are low in that state. The concentration of nutrients is well controlled in healthy people, and their blood and urinary water-soluble vitamin levels are kept constant. Therefore, water-soluble vitamin contents in blood and urine are used to assess their status, and these indicators have also been used for estimating dietary reference intakes (DRIs) in the USA and Japan (1, 2). The use of valid nutritional markers for water-soluble vitamins is helpful to evaluate one's nutritional status for the vitamins, and further assessment of water-soluble vitamin levels in blood and urine is important to use as more established nutritional markers. To determine water-soluble vitamin levels in the blood and urine of Japanese subjects, we have previously reported those values obtained from the last day of experiments in Japanese young adults consuming a semi-purified diet based on the Japanese Recommended Dietary Allowance for 7 d (3). Although the subjects took exactly the same amount of vitamins for consecutive 7 d and followed the same schedule through the experiment, their water-soluble vitamin contents in

blood and urine were different by individual (3). It is well known that these biomarkers show some individual differences; however, how individuals respond and adapt to a constant level of water-soluble vitamin intake remains to be clarified. One of methods giving us the answer to these questions is to investigate variability in water-soluble vitamin markers during the experiment, and new findings will also contribute to the understanding of those biomarkers for effective use. In particular, determination of the intra- and inter-individual variations of biomarkers will be required to estimate the representative values in individuals and the mean values in the group. We have shown only the data obtained on the last day of the experiment in the previous report (3), but we also periodically collected blood and 24-h urine samples from the subjects in the same experiment. In this report, thus, we comprehensively determined the intra- and inter-individual variations of blood and urinary water-soluble vitamins in Japanese young adult consuming a semi-purified diet for 7 d.

MATERIALS AND METHODS

Subjects. Healthy Japanese college students, consisting of 10 males and 10 females, participated in the experiment. This experiment was the same as shown in

E-mail: kshibata@shc.usp.ac.jp

the previous report (3). The mean (\pm SD) age, height, weight and BMI of the male subjects were 20.4 ± 1.3 y, 1.73 ± 0.07 m, 61.4 ± 7.5 kg, and 20.5 ± 2.2 kg/m², respectively, and those of the female subjects were 20.7 ± 0.7 y, 1.64 ± 0.04 m, 54.2 ± 3.4 kg, and 20.1 ± 1.3 kg/m², respectively. Prior to the experiment, they had physical checkups, and their hematological and blood biochemical analysis showed normal values. This study was reviewed and approved by the Ethical Committee of the National Institute of Health and Nutrition.

Diet and experimental design. All subjects were housed in the same facility during the experiment. The experimental design is described in a previous paper (3). The subjects took a semi-purified diet based on Japanese DRIs and a vitamin mixture during the experiment (4). The diet consisted of wheat flour, gluten, cornstarch, sucrose, soybean oil, rapeseed oil, lard, soluble dietary fiber, insoluble dietary fiber and mineral mixture, and contained 2,300 kcal/d of energy, 71 g/d of protein, 50 g/d of fat and 387 g/d of carbohydrate for male subjects, and 1,800 kcal/d of energy, 55 g/d of protein, 40 g/d of fat and 292 g/d of carbohydrate for female subjects. The vitamin mixture contained 1.2 mg/d ($3.6 \mu\text{mol/d}$) of thiamin hydrochloride, 1.2 ($3.2 \mu\text{mol/d}$) mg/d of riboflavin, 2.0 mg/d ($7.5 \mu\text{mol/d}$) of pyridoxine hydrochloride, $2.4 \mu\text{g/d}$ (1.8 nmol/d) of cyanocobalamin, 4.2 mg/d ($34 \mu\text{mol/d}$) of nicotinamide, 5.5 mg/d ($23 \mu\text{mol/d}$) of calcium pantothenate, 200 $\mu\text{g/d}$ (453 nmol/d) of pteroylmonoglutamic acid, 30 $\mu\text{g/d}$ (123 nmol/d) of biotin and 100 mg/d ($568 \mu\text{mol/d}$) of ascorbic acid for male subjects, and 0.9 mg/d ($2.7 \mu\text{mol/d}$) of thiamin hydrochloride, 1.0 mg/d ($2.7 \mu\text{mol/d}$) of riboflavin, 1.5 mg/d ($5.7 \mu\text{mol/d}$) of pyridoxine hydrochloride, $2.4 \mu\text{g/d}$ (1.8 nmol/d) of cyanocobalamin, 2.8 mg/d ($23 \mu\text{mol/d}$) of nicotinamide, 5.5 mg/d ($23 \mu\text{mol/d}$) of calcium pantothenate, 200 $\mu\text{g/d}$ (453 nmol/d) of pteroylmonoglutamic acid, 30 $\mu\text{g/d}$ (123 nmol/d) of biotin and 100 mg/d ($568 \mu\text{mol/d}$) of ascorbic acid for female subjects.

The urine samples named "day 1" were collected from the second urine on day 1 to the first urine on day 2, and "day 7" from the second urine on day 7 to the first urine on day 8. After the volumes of the urine samples had been measured, the collected urine samples were immediately treated to avoid destruction of water-soluble vitamins, and then stored at -20°C until needed. The blood was taken from a cubital vein at 08:30 on days 1, 3, 5 and 8 in males, and days 1, 3 and 8 in females before breakfast, treated immediately to avoid destruction of water-soluble vitamins, and stored at -20°C until needed.

Chemicals. Wheat flour (soft flour, first grade) was obtained from Nisshin Flour Milling Inc. (Tokyo). Wheat gluten, raw cornstarch, soybean oil, 13 kinds of vitamins (3), and minerals (3) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Rapeseed oil was purchased from Ajinomoto Co. Ltd. (Tokyo, Japan). Coconut oil and lard were both obtained from CLEA Japan, Inc. (Tokyo, Japan). Fibersol, used as a soluble dietary fiber, was obtained from Matsutani

Chemical Industry Co., Ltd. (Osaka, Japan) and Ramie powder, used as an insoluble dietary fiber, was from Tosco (Tokyo, Japan).

Thiamin hydrochloride ($\text{C}_{12}\text{H}_{17}\text{ClN}_4\text{OS}\cdot\text{HCl}=337.27$), riboflavin ($\text{C}_{17}\text{H}_{20}\text{N}_4\text{O}_6=376.37$), pyridoxal phosphate monohydrate ($\text{C}_8\text{H}_{10}\text{NO}_6\text{P}\cdot\text{H}_2\text{O}=265.16$), cyanocobalamin ($\text{C}_{63}\text{H}_{88}\text{CoN}_{14}\text{O}_{14}\text{P}=1,355.40$), nicotinamide (Nam; $\text{C}_6\text{H}_6\text{N}_2\text{O}=122.13$), calcium pantothenate ($\text{C}_{18}\text{H}_{32}\text{N}_2\text{O}_{10}\cdot\text{Ca}=476.54$), folic acid ($\text{C}_{19}\text{H}_{19}\text{N}_7\text{O}_6=441.40$), D(+)-biotin ($\text{C}_{10}\text{H}_{16}\text{N}_2\text{O}_3\text{S}=244.31$), and L(+)-ascorbic acid ($\text{C}_6\text{H}_8\text{O}_6=176.13$) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC, $\text{C}_8\text{H}_9\text{NO}_4=183.16$) was made by ICN Pharmaceuticals (Costa Mesa, California, USA) and obtained through Wako Pure Chemical Industries. N¹-Methylnicotinamide (MNA) chloride ($\text{C}_7\text{H}_9\text{N}_2\text{O}\cdot\text{HCl}=159.61$) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N¹-Methyl-2-pyridone-5-carboxamide (2-Py, $\text{C}_7\text{H}_8\text{N}_2\text{O}_2=152.15$) and N¹-methyl-4-pyridone-3-carboxamide (4-Py, $\text{C}_7\text{H}_8\text{N}_2\text{O}_2=152.15$) were synthesized by the methods of Pullman and Colowick (5) and Shibata et al. (6), respectively.

All other chemicals used were of the highest purity available from commercial sources.

Determination of vitamins and their metabolites in blood and urine.

Vitamin B₁: Vitamin B₁ content in whole blood was determined as the sum of thiamin, TMP and TDP. Urinary thiamin and blood vitamin B₁ were determined by the HPLC-post labeled fluorescence method (7).

Vitamin B₂: Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in whole blood were converted to lumiflavin by photolysis, and lumiflavin was determined as vitamin B₂ by the HPLC method (8). Urinary riboflavin was determined by the HPLC method (9).

Vitamin B₆: Plasma PLP was determined by the HPLC method (10). For analysis of 4-PIC, a metabolite of pyridoxal, 1 mL of 1 mol/L HCl was added to 9 mL urine. Urinary 4-PIC was determined by the HPLC method (11).

Vitamin B₁₂: Serum vitamin B₁₂ was determined by using a fully automated chemiluminescence analyzer. Urinary vitamin B₁₂ was converted to cyanocobalamin by boiling with potassium cyanide at acidic pH (12). Cyanocobalamin in urine was determined by the microbioassay method using *Lactobacillus leichmanii*, ATCC 7830 (12).

Niacin: NAD and NADP in whole blood were converted to nicotinamide by autoclave, and nicotinamide was determined by the HPLC method (6). Urinary 2-Py, 4-Py and MNA, nicotinamide metabolites, were determined by the HPLC method (6, 13), and the sum of these compounds was determined as nicotinamide metabolites.

Pantothenic acid: Bound pantothenic acid such as CoA and pantetheine in whole blood was digested to free form by alkaline phosphatase and liver amidase. Pantothenic acid in blood and urine were determined by the microbioassay method using *Lactobacillus plan-*

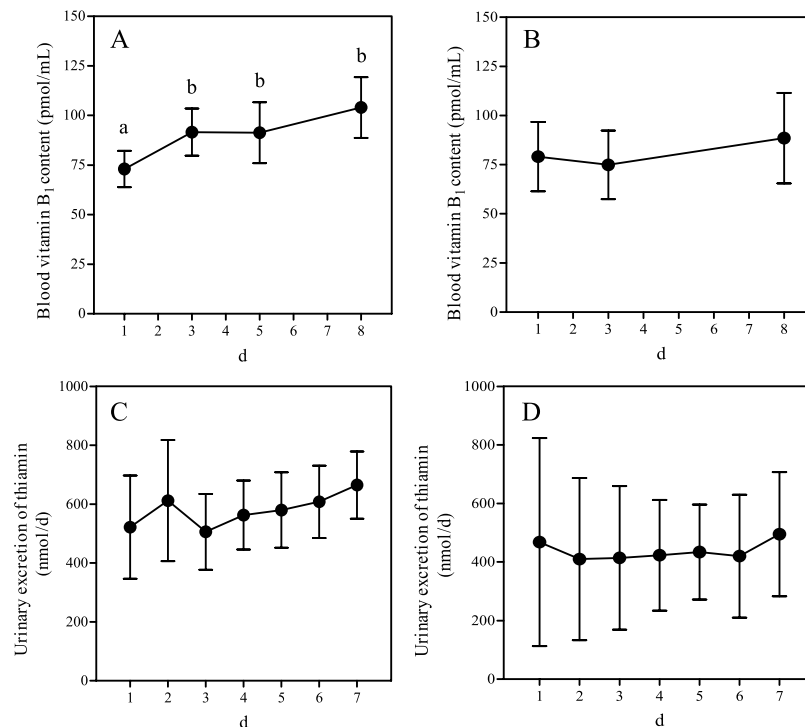


Fig. 1. Alterations of blood vitamin B₁ level in male (A) and female subjects (B), and of urinary thiamin in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

tarum ATCC 8014 (14).

Folate: Serum folate was determined by an automated method based on the competitive protein-binding assay using an automated chemiluminescence analyzer. Urinary folate was determined by the microbiassay method using *Lactobacillus casei* ATCC 2733 (15).

Biotin: Serum and urinary biotin were determined by the microbiassay method using *Lactobacillus plantarum* ATCC 8014 (16).

Vitamin C: Total ascorbic acid in serum was determined by an HPLC-UV method (17). Ascorbic acid in urine was determined by the 2,4-dinitrophenylhydrazine method (18).

Statistical analysis. All statistical analysis was performed using a computer program, GraphPad Prism version 4.03 (GraphPad Software, San Diego, CA, USA). The significant differences in the values from the first to last day of experiments were tested by using repeated one-way analysis of variance with Tukey-Kramer multiple-comparison tests. The differences of $p<0.05$ were considered to be statistically significant. Intra- and inter-individual variations were calculated with analysis of variance, using the data on the last 2 blood and last 3 urine collections. We also calculated the number of blood and urine sample collections required to estimate the true blood and urine levels within 10 and 20% of their true mean with a 95% confidence interval, using following formula (19):

$$n = (Z_{\alpha} CV_W / D_0)^2$$

where n = the number of the days needed per subject, Z_{α} = 1.96, CV_W = the within-subject coefficient of varia-

tion (%), and D_0 = the specific degree of error as a percentage of long-term true level (10% or 20%). Spearman's rank correlation coefficients were calculated to evaluate the relation between water-soluble vitamin contents in blood and urine collected on the last day of experiments. All analyses were conducted separately for males and females.

RESULTS

A total of 40 blood and 70 daily urine samples were collected from male subjects, 30 blood and 70 urine samples were from female subjects, and these samples were measured.

Vitamin B₁

The alteration of blood vitamin B₁ as the sum of thiamin, TMP and TDP, and urinary excretion of thiamin are shown in Fig. 1. Intake of 1.2 mg/d of thiamin chloride increased blood vitamin B₁ from the third day in male subjects, and those values were not changed in female subjects. Urinary excretion of thiamin did not alter during the experiment in either male or female subjects. Using the data on the last 2 blood and last 3 urine samples, the within-subject coefficient of variance (CV_W) and between-subject coefficient of variance (CV_B) in blood vitamin B₁ and urinary thiamin levels after adaptation were determined. CV_W in blood vitamin B₁ was 15.8 and 21.7% in male and female subjects, respectively, showing relative invariability (Table 1). These values in urinary excretion of thiamin were relatively invariable in male subjects (CV_W = 14.4%), and relatively variable in female subjects (CV_W = 29.4%, Table 2). CV_B in urinary thiamin was higher

Table 1. Variability estimates of blood water-soluble vitamins, and the number of the days of collecting blood samples required to estimate the true blood water-soluble vitamins within 10 and 20% of their true mean calculated from the data on the last 2 blood samples in male and female subjects consuming a semi-purified diet for 7 d.

	Male (n=10)					Female (n=10)				
	CV _{WS} ^a (%)	CV _{BS} ^b (%)	$\sigma_{WS}^2/\sigma_{BS}^2$ ^c	10% (d)	20% (d)	CV _{WS} ^a (%)	CV _{BS} ^b (%)	$\sigma_{WS}^2/\sigma_{BS}^2$	10% (d)	20% (d)
Blood vitamin B ₁	15.8	17.6	0.81	9.6	2.4	21.7	29.6	0.54	18.0	4.5
Blood vitamin B ₂	9.0	14.5	0.38	3.1	0.8	8.4	9.4	0.80	2.7	0.7
Plasma PLP	16.0	43.7	0.13	9.9	2.5	11.9	33.8	0.12	5.5	1.4
Serum vitamin B ₁₂	3.8	22.5	0.03	0.6	0.1	34.4	27.3	1.58	45.5	11.4
Blood nicotinamide	8.7	12.7	0.47	2.9	0.7	14.1	13.5	1.09	7.7	1.9
Blood pantothenic acid	14.9	14.2	1.10	8.5	2.1	13.7	8.1	2.91	7.3	1.8
Serum folate	15.3	31.7	0.23	9.0	2.3	28.6	25.8	1.23	31.5	7.9
Serum biotin	4.5	6.6	0.47	0.8	0.2	6.2	5.4	1.32	1.5	0.4
Plasma ascorbic acid	18.2	17.3	1.11	12.7	3.2	65.5	18.6	12.42	165.0	41.2

^a Within-subject coefficient of variance.

^b Between-subject coefficient of variance.

^c Ratio of within- and between-subject variance.

Table 2. Variability estimates of urinary water-soluble vitamins, and the number of the days of collecting urinary samples required to estimate the true urinary water-soluble vitamins within 10 and 20% of their true mean calculated from the data on the last 3 urine samples in male and female subjects consuming a semi-purified diet for 7 d.

	Male (n=10)					Female (n=10)				
	CV _{WS} ^a (%)	CV _{BS} ^b (%)	$\sigma_{WS}^2/\sigma_{BS}^2$ ^c	10% (d)	20% (d)	CV _{WS} ^a (%)	CV _{BS} ^b (%)	$\sigma_{WS}^2/\sigma_{BS}^2$	10% (d)	20% (d)
Thiamin	14.4	28.1	0.26	8.0	2.0	29.4	62.9	0.22	33.2	8.3
Riboflavin	10.6	100.6	0.01	4.3	1.1	38.7	40.5	0.91	57.5	14.4
4-Pyridoxic acid	17.4	26.5	0.43	11.6	2.9	12.4	9.0	1.92	5.9	1.5
Vitamin B ₁₂	23.8	51.1	0.22	21.7	5.4	24.0	46.4	0.27	22.2	5.5
Nicotinamide metabolites	21.3	50.1	0.18	17.4	4.3	19.8	40.2	0.24	15.0	3.8
Pantothenic acid	16.9	40.1	0.18	10.9	2.7	15.1	26.6	0.32	8.8	2.2
Folate	8.7	19.5	0.20	2.9	0.7	15.3	9.9	2.39	9.0	2.3
Biotin	15.6	30.2	0.27	9.3	2.3	21.5	34.8	0.38	17.8	4.4
Ascorbic acid	27.0	44.7	0.37	28.1	7.0	42.6	44.9	0.90	69.8	17.4

^a Within-subject coefficient of variance.

^b Between-subject coefficient of variance.

^c Ratio of within- and between-subject variance.

than 50% in female subjects (CV_{BS}=62.9%). This high variability is due to one subject whose urinary thiamin was highest during the experiment, twice higher than the mean value. We also determined the number of sample collections required to estimate true levels within errors of 10 and 20%. The number of blood sample collection within 10% was 10 and 19 d in males and females, respectively, and those of urine sample collections were 9 and 34 d. No correlation was observed between blood and urinary levels in vitamin B₁.

Vitamin B₂

Alterations of blood vitamin B₂ as the sum of riboflavin, FMN and FAD, and urinary excretion of riboflavin during the experiments are shown in Fig. 2. Blood vitamin B₂ and urinary riboflavin did not change throughout the experiments. CV_{WS} and the CV_{BS} in blood vitamin B₂ level were approximately 10% in male and

female subjects, showing invariability (Tables 1 and 2). The values in urinary excretion of riboflavin was invariable in males (CV_{WS}=10.6%), but was variable in female subjects (CV_{WS}=38.7%). Although CV_{BS} in blood vitamin B₂ was low, that in urinary riboflavin was high, especially in male subjects (CV_{BS}=100.6%). This high variability in male subjects is due to broad values; the highest urinary riboflavin was 8 times higher than the lowest. To estimate the true blood and urine levels within an error of 10%, 3–4 and 5–58 d were needed, respectively. No correlation was observed between blood and urinary levels in vitamin B₂.

Vitamin B₆

Alterations of plasma PLP and urinary excretion of vitamin B₆ metabolite 4-PIC during the experiments are shown in Fig. 3. Plasma PLP and urinary 4-PIC slightly increased in female subjects but not in male subjects.

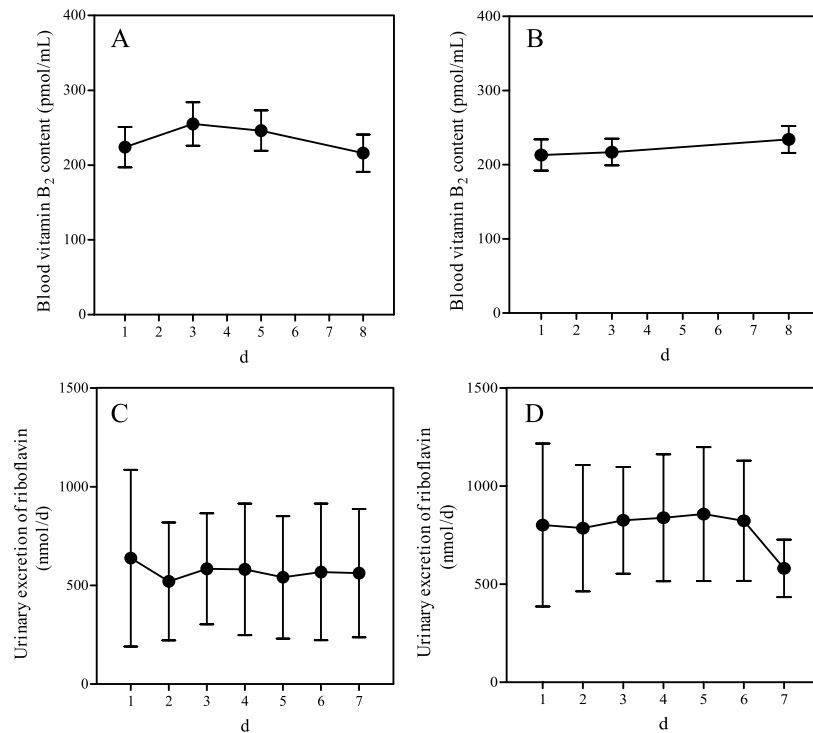


Fig. 2. Alterations of blood vitamin B₂ level in male (A) and female subjects (B), and of urinary riboflavin in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

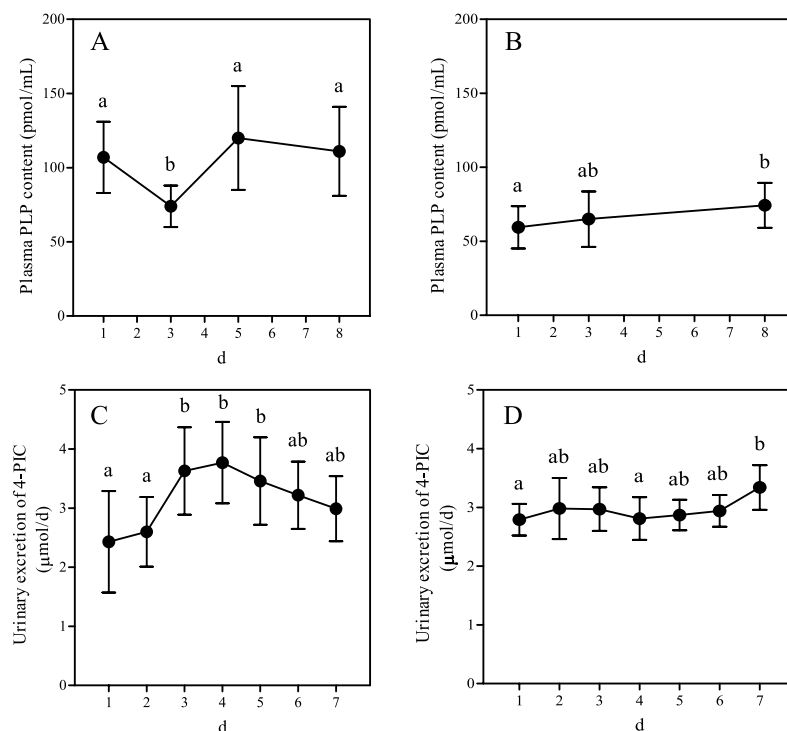


Fig. 3. Alterations of plasma PLP level in male (A) and female subjects (B), and of urinary vitamin B₆ metabolite 4-pyridoxic acid (4-PIC) in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

CV_{WS} in plasma PLP level and urinary 4-PIC was less than 20%, showing invariability (Tables 1 and 2). CV_{BS} in plasma PLP was high in both male and female subjects, and that in urinary 4-PIC was relatively high in male and low in female subjects (CV_{BS}=26.5 and 9.0%,

respectively). To estimate the true blood and urine levels within an error of 10%, 6–10 and 6–16 d were needed, respectively. No correlation was observed between plasma and urinary levels in vitamin B₆.

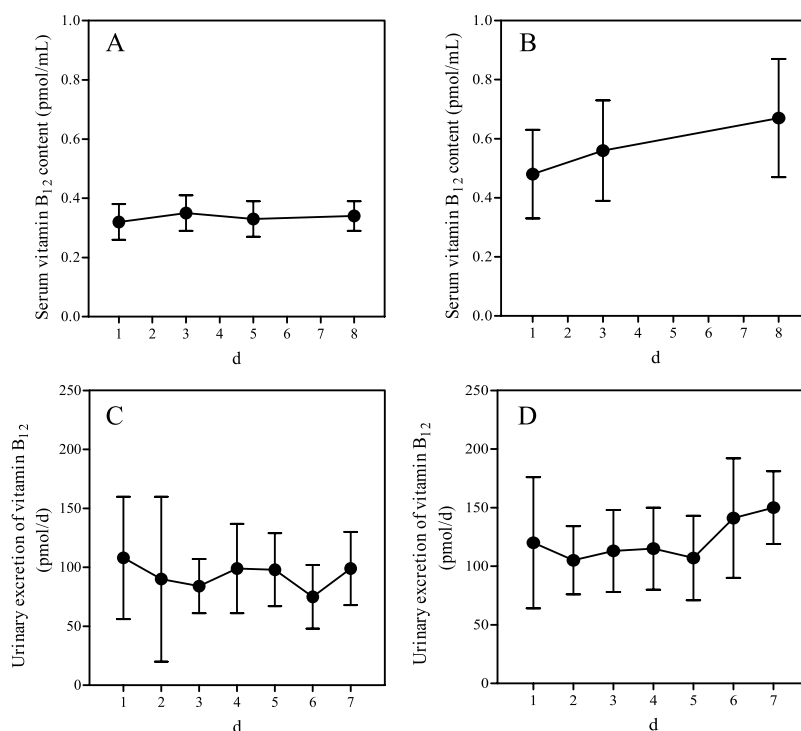


Fig. 4. Alterations of serum vitamin B₁₂ level in male (A) and female subjects (B), and of urinary vitamin B₁₂ in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

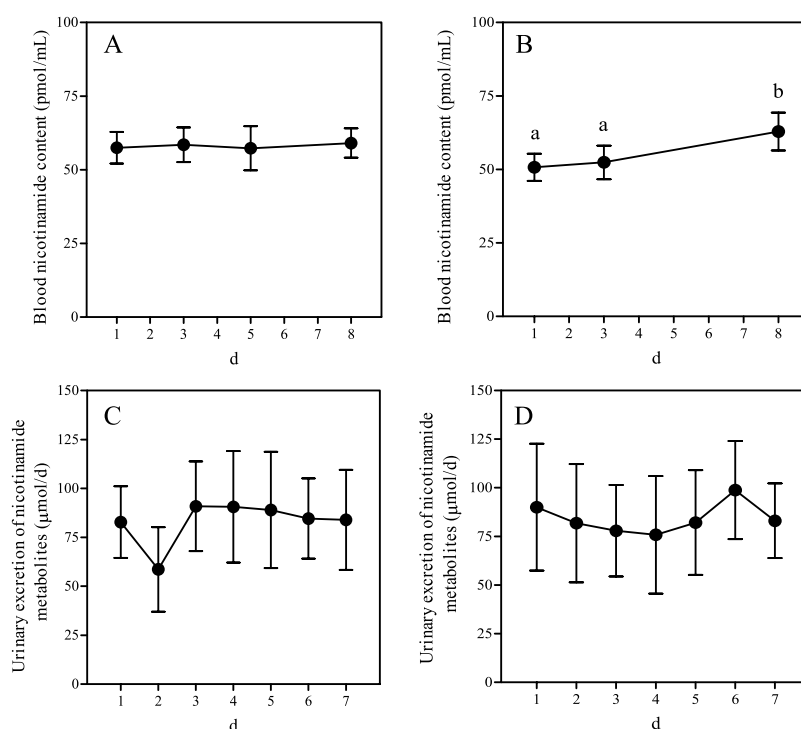


Fig. 5. Alterations of blood nicotinamide content in male (A) and female subjects (B), and of urinary nicotinamide metabolites in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

Vitamin B₁₂

Alterations of serum and urinary vitamin B₁₂ during the experiments are shown in Fig. 4. Serum and urinary vitamin B₁₂ did not change during the experiments. CV_{WS} in serum and urinary vitamin B₁₂ levels

was approximately 30–50% but not in serum vitamin B₁₂ levels of male subjects (Tables 1 and 2). CV_{BS} in serum vitamin B₁₂ was \sim 25%, and that in urinary vitamin B₁₂ was high, \sim 50%. To estimate the true blood and urine levels within an error of 10%, 1–49 and 22–

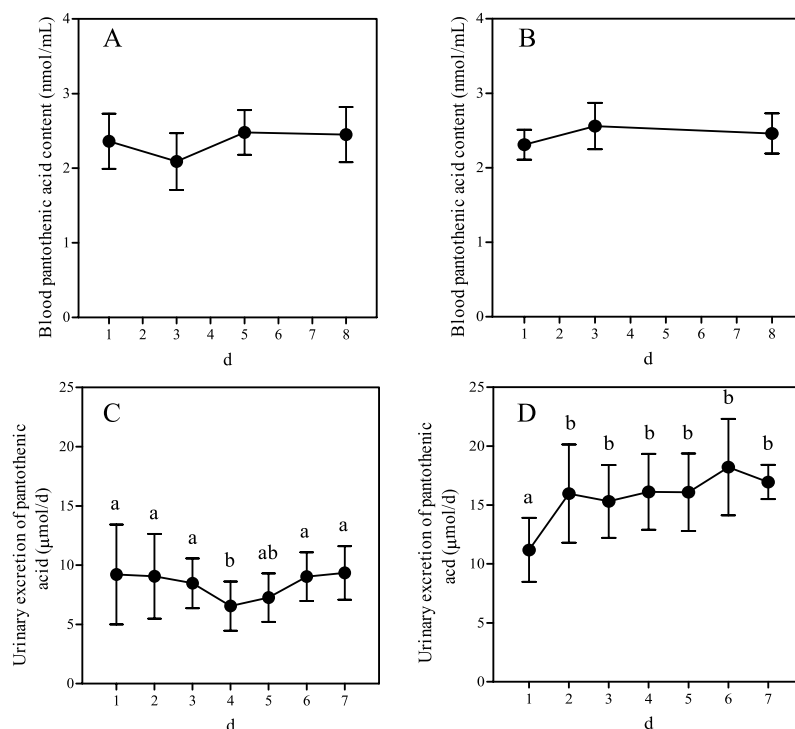


Fig. 6. Alterations of blood pantothenic acid level in male (A) and female subjects (B), and of urinary pantothenic acid in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

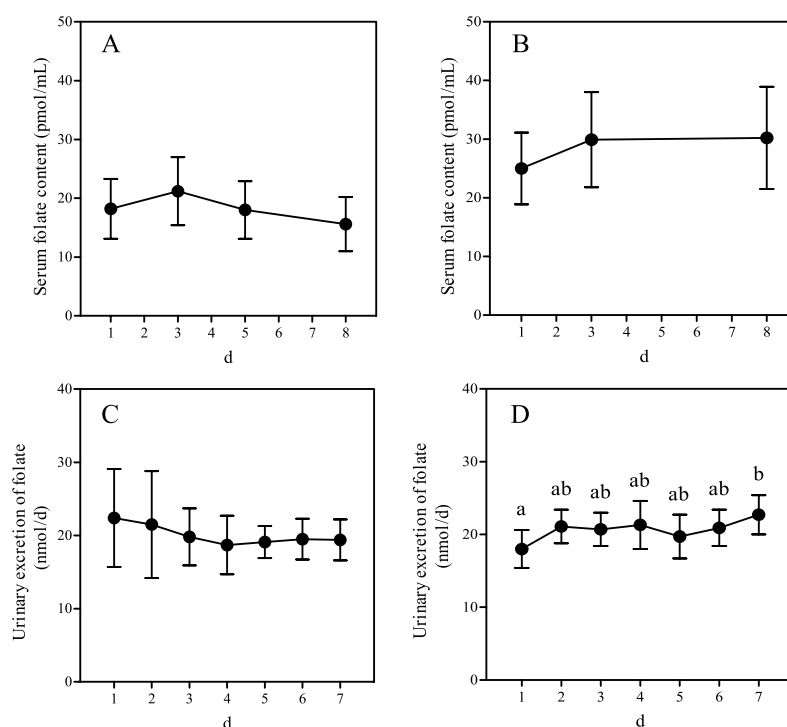


Fig. 7. Alterations of serum folate level in male (A) and female subjects (B), and of urinary folate in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

23 d were needed, respectively. No correlation was observed between serum and urinary levels in vitamin B₁₂.

Niacin

Alterations of blood nicotinamide and urinary excretion

of nicotinamide metabolites during the experiments are shown in Fig. 5. Blood nicotinamide levels in female subjects increased, while other indicators did not change during the experiments. CV_{WS} in blood nicotinamide level was 8.7 and 14.1% in male and female

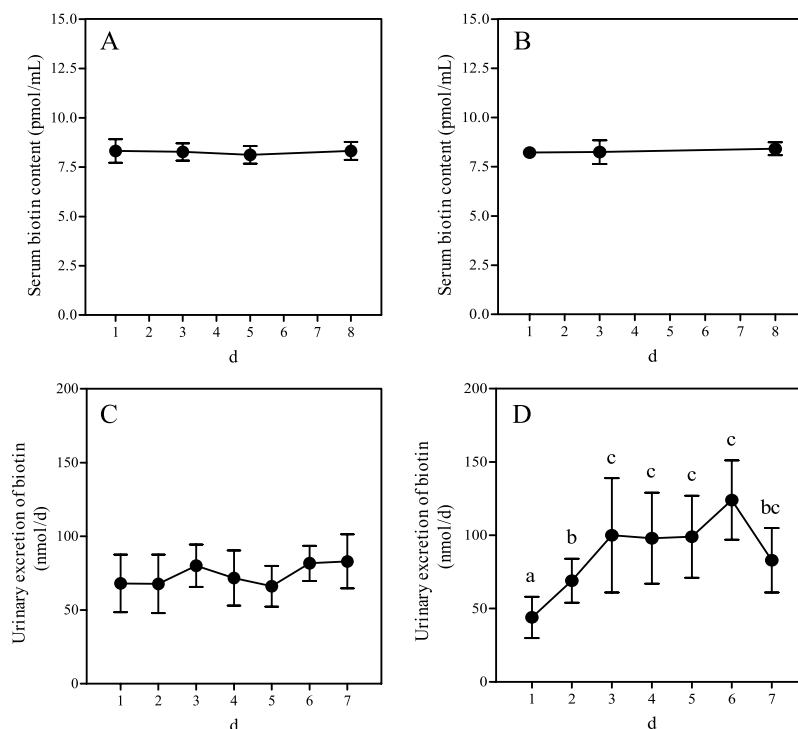


Fig. 8. Alterations of serum biotin level in male (A) and female subjects (B), and of urinary biotin in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

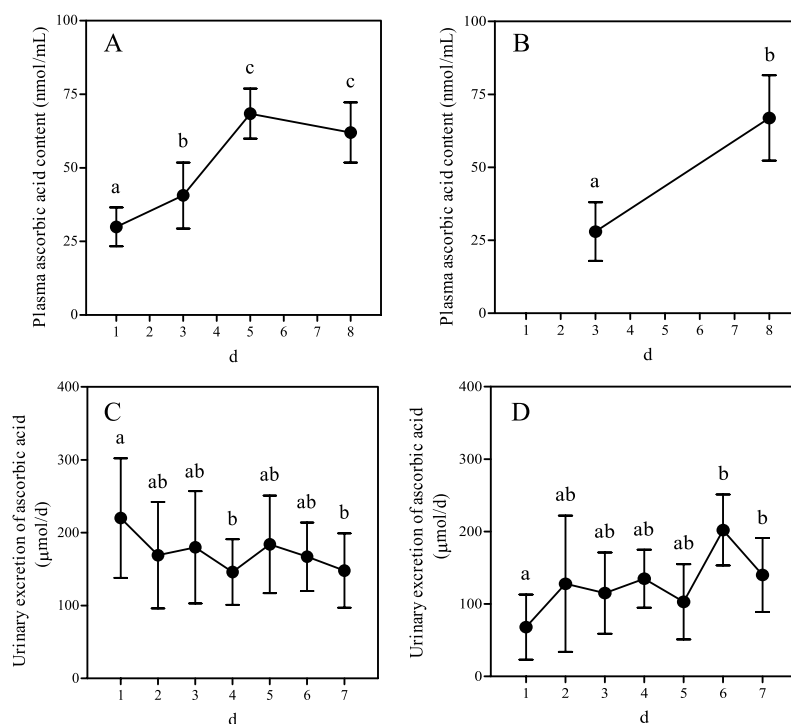


Fig. 9. Alterations of plasma ascorbic acid level in male (A) and female subjects (B), and of urinary ascorbic acid in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

subjects, respectively, showing invariability (Tables 1 and 2). These values in urinary excretion of nicotinamide metabolites were $\sim 20\%$. CV_{BS} in blood nicotinamide was low, $\sim 15\%$, and that in urinary nicotinamide metabolites was high, $\sim 50\%$. To estimate the

true blood and urine levels within an error of 10%, 3–8 and 16–18 d were needed, respectively. No correlation was observed between blood nicotinamide and urinary nicotinamide metabolites.

Pantothenic acid

Alterations of blood and urinary pantothenic acid during the experiments are shown in Fig. 6. Urinary pantothenic acid content in female subjects increased at day 2, and then came to the definite value during the experiments. Urinary pantothenic acid in male and blood pantothenic acid did not change. CV_{WS} in blood and urinary pantothenic acid levels was $\sim 15\%$, showing invariability. CV_{BS} was low in blood pantothenic acid, and was high in urinary pantothenic acid. To estimate the true blood and urine levels within an error of 10%, 8–9 and 9–11 d were needed, respectively. No correlation was observed between blood and urinary pantothenic acid levels.

Folate

Alterations of serum and urinary folate are shown in Fig. 7. Urinary folate content in female subjects increased, while urinary content in males and blood folate did not change during the experiments. CV_{WS} in serum folate was relatively invariable in male subjects ($CV_{WS}=15.3\%$), and relatively variable in female subjects ($CV_{WS}=28.6\%$, Tables 1 and 2). These values in urinary excretion of folate were relatively invariable. CV_{BS} was high in serum folate, and was low in urinary folate. To estimate the true blood and urine levels within an error of 10%, 10–32 and 3–9 d were needed, respectively. No correlation was observed between blood and urinary folate levels.

Biotin

Alterations of serum and urinary biotin are shown in Fig. 8. Urinary biotin content in female subjects increased, while urinary biotin in male and blood biotin did not change during the experiments. CV_{WS} in serum biotin was $\sim 5\%$ showing invariability, whereas that value in urinary excretion of biotin was 15–20%. CV_{BS} in serum biotin was very low, and that value in urinary biotin was high, $\sim 30\%$. To estimate the true blood and urine levels within an error of 10%, 1–2 and 10–18 d were needed, respectively. No correlation was observed between blood and urinary biotin levels.

Vitamin C

Alterations of plasma and urinary ascorbic acid are shown in Fig. 9. Unfortunately, the amount of blood samples was not enough for repeated measurement, and plasma ascorbic acid level in female subjects obtained on day 1 could not be determined. Furthermore, the feeding of the test diet for 3 d was not enough to come to the definite value for plasma ascorbic acid levels in female subjects, and the intra-individual variations of plasma ascorbic acid reflected adaptation to the test diet in female subjects. Taking 100 mg of ascorbic acid increased plasma ascorbic acid levels in both male and female subjects, and urinary excretion of ascorbic acid in female subjects but not in male subjects. CV_{WS} in plasma ascorbic acid was invariable in male subjects, but not in female subjects. That value in urinary ascorbic acid was variable both in male and female subjects. CV_{BS} in plasma ascorbic acid was $\sim 20\%$, and these values in urinary ascorbic acid were $\sim 45\%$. To estimate the true blood and urine levels within an error of 10%,

13–465 and 29–70 d were needed, respectively. No correlation was observed between blood and urinary ascorbic acid levels.

DISCUSSION

In this 7-d study of 10 male and 10 female subjects consuming a semi-purified diet with a vitamin mixture based on RDA, blood and 24-h urine samples were periodically collected from the subjects during the study. Some blood and urinary vitamin levels increased or decreased from the first day to the third or fifth day, and then showed stable values. These results showed that the feeding of the test diet for 3 to 5 d was enough to adapt to vitamin mixtures for blood and urinary vitamin levels. Therefore, the intra-individual variations of blood water-soluble vitamins were determined using blood samples at day 5 and 8 in male, and day 3 and 8 in female subjects (Table 1). Those variations of urinary levels were also determined using urine samples from day 5 to 7 (Table 2). Determination of CV_{WS} showed that all blood water-soluble vitamin levels were invariable in male subjects, whereas serum vitamin B_{12} and serum folate levels were variable in female subjects. CV_{WS} in urinary vitamin B_{12} and ascorbic acid were more than 20% in both male and female subjects, and those values in other urinary vitamins were relatively invariable in at least either male or female subjects. These results suggest that oral administration of a constant amount of water-soluble vitamins generally decreased intra-individual variations. We also determined the number of sample collections required to estimate the true values, and the results showed that the collection of urine samples for 1–5 d was enough to estimate those values within 20% of the true mean for most water-soluble vitamins.

In the present study, the inter-individual variations of blood water-soluble vitamins were also determined using blood samples at day 5 and 8 in males, and day 3 and 8 in female subjects. CV_{BS} in plasma PLP, serum vitamin B_{12} and serum folate was more than 20% in both male and female subjects, and those in other blood vitamins was less than 20%. On the other hand, CV_{BS} in most urinary vitamins except for vitamin B_6 metabolite and folate was 20–50% in both male and female subjects. Although the intra-individual variation was invariable in most blood and urine water-soluble vitamins, the inter-individual variation in urinary vitamins was generally higher than that in blood vitamins.

In the present study, CV_{WS} in most urinary water-soluble vitamins was approximately 10–30%, and CV_{BS} was more than 30%. To our knowledge, there is only a single report to assess CV_{WS} and CV_{BS} in urinary excretion of water-soluble vitamins (20). Tasevska et al. collected daily urine samples 30 consecutive days from 13 healthy participants consuming their usual diets, and their CV_{WS} , CV_{BS} and $\sigma_{WS}^2/\sigma_{BS}^2$ ratio in urinary thiamin were 32.5%, 36.7% and 0.72, respectively (20). In the present study, CV_{WS} in urinary thiamin obtained was lower than the reported value. Taking same amount of vitamin B_1 for 7 consecutive days might cause small

changes in individuals. CV_{BS} of male subjects was lower, but that of female subjects was higher than the reported value. Some female subjects showed a very high or low urinary excretion rate throughout the experiment, and these wide differences between individuals caused high between-subject variability in females. A similar phenomenon was observed in urinary excretion of other water-soluble vitamins. Tasevska et al. also investigated the inter-individual variations in percentage urinary recovery of thiamin, and their high variability implies that urinary thiamin can be used as a concentration biomarker rather than recovery and predictive biomarkers (20). The former biomarker can be used as a tool for ranking individuals according to dietary exposure, and the latter two can quantitatively reflect the balance between intake and output or predict dietary intake. Since the subjects took exactly the same amount of water-soluble vitamins for 7 consecutive days in the present study, the inter-individual variations in urinary recovery of each vitamin could not be determined. Further study will be needed to determine which type of dietary biomarker the urinary vitamins can be used as.

In the present study, intake of 100 mg/d of ascorbic acid increased plasma ascorbic acid levels in both male and female subjects, and urinary excretion of ascorbic acid in female subjects. The effect of oral vitamin C intakes on ascorbic acid levels in plasma and urine were precisely investigated (21, 22). The relationship between daily vitamin C intake and plasma ascorbic acid levels displays sigmoid kinetics, and the steep portion of the curve is observed between 30 and 100 mg of vitamin C intake (21, 22). Plasma ascorbic acid levels at the end of the experiment were 62.0 ± 10.2 and 66.9 ± 14.6 nmol/mL in male and female subjects, respectively, in the present study, and these values were consistent with the reported values of approximately 60 nmol/mL in healthy volunteers taking 100 mg of ascorbic acid daily (21, 22). In the present study, plasma ascorbic acid levels were 29.9 ± 6.6 and 28.0 ± 10.0 nmol/mL in male subjects at the start of the experiment and in female subjects on the 3rd day of the experiment, respectively. When these values were put to the steep portion of the curve previously reported, estimated vitamin C intake might be 50–60 mg/d prior to the experiment in the subjects. Mean dietary vitamin C intake in Japanese women college students is 73 ± 38 mg/d (23). Although we did not survey vitamin C intakes of the subjects in the few days prior to the experiment, the subjects might have taken less than 100 mg/d of vitamin C prior to the experiment, and intake of 100 mg/d of vitamin C might have improved their values. Since higher vitamin C intake also increases urinary excretion of ascorbic acid (20, 21, 24), increase of urinary ascorbic acid during the experiment in female subjects might have been due to higher vitamin C intake during the experiment than in the previous days.

Methylenetetrahydrofolate reductase (MTHFR) is an important enzyme in folate metabolism that catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate. A base change from C to T

at nucleotide position 677 of the MTHFR gene results in coding for valine (GTC) rather than alanine (GCC), and this single nucleotide polymorphism is associated with lower plasma folate concentrations and elevated plasma homocysteine concentrations (25, 26). The prevalence rate of this homozygous mutant among Japanese women was ~20%, and their serum folate levels were lower than in individuals with CC and CT alleles (27, 28). In the present study, CV_{BS} in serum folate level was relatively high, 31.7 and 25.8% in male and female subjects, respectively, and these values were higher than CV_{BS} in urinary excretion of folate. Since we did not determine genotype for MTHFR in the subjects, whether nucleotide polymorphism might cause this high CV_{BS} in serum folate level or not is unclear.

Vitamin B₆ status such as plasma PLP and urinary 4-PIC, and its relationship with dietary intake in free-living healthy subjects has been reported (29). Both plasma PLP and urinary 4-PIC highly correlate to vitamin B₆ intake, and plasma PLP also correlates to urinary 4-PIC (29). In the present study, no correlation was observed between blood and urinary water-soluble vitamin levels. The differences between the reported study and the present study were that the subjects took their diet freely in the reported study, and took exactly the same diet for 7 consecutive days in the present study. CV values in vitamin B₆ intake, plasma PLP and urinary 4-PIC were 30–40% in the previous study, and those in plasma PLP and urinary 4-PIC were 10–20% in the present study. Widely ranging values are generally needed to observe significant correlation. However in the present study, the blood and urinary water-soluble vitamin levels converged within a narrow range because of the oral administration of a constant amount of pyridoxine hydrochloride, and these restrictions might fail to show relationships between these indicators.

In conclusion, our human study showed how blood and urinary water-soluble vitamins varied even when the subjects were orally administered a constant amount of water-soluble vitamin for 7 consecutive days. All blood vitamins and most urinary vitamins showed small CV_{WS} in at least either male or female. CV_{BS} in blood vitamins was smaller than that in urinary vitamins except for folate. These high CV_{BS} in urinary vitamins was due to a small number of subjects showing a different urinary excretion rate from other subjects. Recent validation studies have developed urinary compounds such as urinary nitrogen (30), potassium (31) and sugars (32) as nutritional biomarkers for evaluating nutritional status. In future, it is important to determine which urinary water-soluble vitamins can be used as dietary biomarkers of such types such as recovery, predictive and concentration biomarkers in order to validate dietary assessment, quantitatively measure or predict vitamin intake in groups and individuals, or rank individuals to assess their vitamin status.

Acknowledgments

This investigation is a part of results in "Basic Studies on the Requirements of Water-soluble Vitamins for Japanese (principal investigator, Katsumi Shibata)," which was supported by the Ministry of Health, Labour and Welfare of Japan. The authors would like to thank Drs. Mieko Kimura (Takeda Research Institute of Life Science & Preventive Medicine), Nobuko Ohishi (Institute of Applied Biochemistry), Haruhito Tsuge (Emeritus Professor, Gifu University), Fumio Watanabe (Faculty of Agriculture, Tottori University), Shigeru Shigeoka (Faculty of Agriculture, Kinki University), and Naotaka Hashizume (School of Home Economics, Wako Women's University) for their technical assistance and advice.

REFERENCES

- Food and Nutrition Board, Institute of Medicine. 1998. Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin B₆, Folate, Vitamin B₁₂, Pantothenic Acid, Biotin, and Choline. National Academy Press, Washington DC.
- Ministry of Health, Labour, and Welfare. 2004. Dietary Reference Intakes for Japanese, 2005. Tokyo.
- Shibata K, Fukuwatari T, Ohta M, Okamoto H, Watanabe T, Fukui T, Nishimuta M, Totani M, Kimura M, Ohishi N, Nakashima M, Watanabe F, Miyamoto E, Shigeoka S, Takeda T, Murakami M, Ihara H, Hashizume N. 2005. Values of water-soluble vitamins in blood and urine of Japanese young men and women consuming a semi-purified diet based on the Japanese Dietary Reference Intakes. *J Nutr Sci Vitaminol* **51**: 319–328.
- Ministry of Health, Labor and Welfare. 1999. Recommended Dietary Allowances in the Japanese Population, 6th ed, Dietary Reference Intakes. Tokyo.
- Pullman ME, Colowick SP. 1954. Preparation of 2- and 6-pyridones of N¹-methylnicotinamide. *J Biol Chem* **206**: 121–127.
- Shibata K, Kawada T, Iwai K. 1988. Simultaneous micro-determination of nicotinamide and its major metabolites, N¹-methyl-2-pyridone-5-carboxamide and N¹-methyl-3-pyridone-4-carboxamide, by high-performance liquid chromatography. *J Chromatogr* **424**: 23–28.
- Kimura M, Fujita T, Itokawa Y. 1982. Liquid chromatographic determination of the total thiamin content of blood. *Clin Chem* **28**: 29–31.
- Ohkawa H, Ohishi N, Yagi K. 1982. A simple method for micro-determination of flavin in human serum and whole blood by high-performance liquid chromatography. *Biochem Int* **4**: 187–194.
- Ohkawa H, Ohishi N, Yagi K. 1983. New metabolites of riboflavin appear in human urine. *J Biol Chem* **258**: 5623–5628.
- Rybak ME, Pfeiffer CM. 2004. Clinical analysis of vitamin B₆: determination of pyridoxal 5'-phosphate and 4-pyridoxic acid in human serum by reversed-phase high-performance liquid chromatography with chlorite post-column derivatization. *Anal Biochem* **333**: 336–344.
- Gregory JF 3rd, Kirk JR. 1979. Determination of urinary 4-pyridoxic acid using high performance liquid chromatography. *Am J Clin Nutr* **32**: 879–883.
- Watanabe F, Katsura H, Takenaka S, Fujita T, Abe K, Tamura Y, Nakatsuka T, Nakano Y. 1999. Pseudovitamin B₁₂ is the predominant cobamide of an algal health food, spirulina tablets. *J Agric Food Chem* **47**: 4736–4741.
- Shibata K. 1987. Ultramicro-determination of N¹-methylnicotinamide in urine by high-performance liquid chromatography. *Vitamins* **61**: 599–604 (in Japanese).
- Skeggs HR, Wright LD. 1944. The use of *Lactobacillus arabinosus* in the microbiological determination of pantothenic acid. *J Biol Chem* **156**: 21–26.
- Aiso K, Tamura T. 1998. Trienzyme treatment for food folate analysis. Optimal pH and incubation time for α -amylase and protease treatment. *J Nutr Sci Vitaminol* **44**: 361–370.
- Fukui T, Iinuma K, Oizumi J, Izumi Y. 1994. Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. *J Nutr Sci Vitaminol* **40**: 491–498.
- Fujiwara Y, Otsuka M, Ihara H, Ito S, Fujisaki M, Inomata M, Tomabeche K, Kodama K, Igarashi O, Okuda K, Mino M, Chibata I, Hashizume N, Itokawa Y. 2001. Proposed standard for total ascorbic acid values in human plasma: HPLC procedure as a reference method. *J Jpn Soc Nutr Food Sci* **54**: 41–44.
- Shigeoka S, Yokota A, Nakano Y, Kitaoka S. 1979. The effect of illumination on the L-ascorbic acid content in *Euglena gracilis* z. *Agric Biol Chem* **43**: 2053–2058.
- Beaton GH, Milner J, Corey P. 1979. Source of variance in 24-hour dietary recall date: implications for nutrition study, design and interpretation. *Am J Clin Nutr* **32**: 2546–2549.
- Tasevska N, Runswick SA, McTaggart A, Bingham SA. 2008. Twenty-four-hour urinary thiamine as a biomarker for the assessment of thiamine intake. *Eur J Clin Nutr* **62**: 1139–1147.
- Levine M, Conry-Cantilena C, Wang Y, Welch RW, Washko PW, Dhariwal KR, Park JB, Lazarev A, Graumlich JF, King J, Cantilena LR. 1996. Vitamin C pharmacokinetics in healthy volunteers: evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* **93**: 3704–3709.
- Levine M, Wang Y, Padayatty SJ, Morrow J. 2001. A new recommended dietary allowance of vitamin C for healthy young women. *Proc Natl Acad Sci USA* **98**: 9842–9846.
- Kimura N, Fukuwatari T, Sasaki R, Hayakawa F, Shibata K. 2003. Vitamin intake in Japanese women college students. *J Nutr Sci Vitaminol* **49**: 149–155.
- Fukuwatari T, Shibata K. 2008. Urinary water-soluble vitamin and their metabolites contents as biomarkers for evaluating vitamin intakes in young Japanese women. *J Nutr Sci Vitaminol* **54**: 223–229.
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJH, den Heijer M, Kluijtmans LAJ, van den Heuvel LP, Rozen R. 1995. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* **10**: 111–113.
- Bagley PJ, Selhub J. 1998. A common mutation in the methylenetetrahydrofolate reductase gene is associated with an accumulation of formylated tetrahydrofolates in red blood cells. *Proc Natl Acad Sci USA* **95**: 13217–13220.
- Hiraoka M, Kato K, Saito Y, Yasuda K, Kagawa Y. 2004. Gene–nutrient and gene–gene interactions of controlled

- folate intake by Japanese women. *Biochem Biophys Res Commun* **316**: 1210–1216.
- 28) Hiraoka M. 2004. Folate intake, serum folate, serum total homocysteine levels and methylenetetrahydrofolate reductase C677T polymorphism in young Japanese women. *J Nutr Sci Vitaminol* **50**: 238–245.
- 29) Chang SJ, Hsiao LJ, Lee YC, Hsuen SY. 2007. Vitamin B₆ status assessment in relation to dietary intake in high school students aged 16–18 years. *Br J Nutr* **97**: 764–769.
- 30) Bingham SA. 2003. Urine nitrogen as a biomarker for the validation of dietary protein intake. *J Nutr* **133**: 921S–924S.
- 31) Tasevska N, Runswick SA, Bingham SA. 2006. Urinary potassium is as reliable as urinary nitrogen for use as a recovery biomarker in dietary studies of free living individuals. *J Nutr* **136**: 1334–1340.
- 32) Tasevska N, Runswick SA, McTaggart A, Bingham SA. 2005. Urinary sucrose and fructose as biomarkers for sugar consumption. *Cancer Epidemiol Biomarkers Prev* **14**: 1287–1294.