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

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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 B_{12} 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 B_6, vitamin B_{12} and folate levels, and urinary vitamin B_1, vitamin B_2, vitamin B_{12}, 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

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the previous report (*3*). The mean (±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/m^2, 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/m^2, 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 μmol/d) of thiamin hydrochloride, 1.2 (3.2 μmol/d) mg/d of riboflavin, 2.0 mg/d (7.5 μmol/d) of pyridoxine hydrochloride, 2.4 μg/d (1.8 nmol/d) of cyanocobalamin, 4.2 mg/d (34 μmol/d) of nicotinamide, 5.5 mg/d (23 μmol/d) of calcium pantothenate, 200 μg/d (453 nmol/d) of pteroylmonoglutamic acid, 30 μg/d (123 nmol/d) of biotin and 100 mg/d (568 μmol/d) of ascorbic acid for male subjects, and 0.9 mg/d (2.7 μmol/d) of thiamin hydrochloride, 1.0 mg/d (2.7 μmol/d) of riboflavin, 1.5 mg/d (5.7 μmol/d) of pyridoxine hydrochloride, 2.4 μg/d (1.8 nmol/d) of cyanocobalamin, 2.8 mg/d (23 μmol/d) of nicotinamide, 5.5 mg/d (23 μmol/d) of calcium pantothenate, 200 μg/d (453 nmol/d) of pteroylmonoglutamic acid, 30 μg/d (123 nmol/d) of biotin and 100 mg/d (568 μmol/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 ($C_{12}H_{17}ClN_4OS$-HCl=337.27), riboflavin ($C_{17}H_{20}N_4O_6$=376.37), pyridoxal phosphate monohydrate ($C_8H_{10}NO_6P$-H_2O=265.16), cyanocobalamin ($C_{63}H_{88}CoN_{14}O_{14}P$=1,355.40), nicotinamide (Nam; $C_6H_6N_2O$=122.13), calcium pantothenate ($C_{18}H_{32}N_2O_{10}$-Ca=476.54), folic acid ($C_{19}H_{19}N_7O_6$=441.40), D(+)-biotin ($C_{10}H_{16}N_2O_3S$=244.31), and L(+)-ascorbic acid ($C_6H_8O_6$=176.13) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC, $C_8H_9NO_4$=183.16) was made by ICN Pharmaceuticals (Costa Mesa, California, USA) and obtained through Wako Pure Chemical Industries. N^1-Methylnicotinamide (MNA) chloride ($C_7H_9N_2O$-HCl=159.61) was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-Methyl-2-pyridone-5-carboxamide (2-Py, $C_7H_8N_2O_2$= 152.15) and N^1-methyl-4-pyridone-3-carboxamide (4-Py, $C_7H_8N_2O_2$=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 B_1: Vitamin B_1 content in whole blood was determined as the sum of thiamin, TMP and TDP. Urinary thiamin and blood vitamin B_1 were determined by the HPLC-post labeled fluorescence method (*7*).

Vitamin B_2: Riboflavin, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in whole blood were converted to lumiflavin by photolysis, and lumiflavin was determined as vitamin B_2 by the HPLC method (*8*). Urinary riboflavin was determined by the HPLC method (*9*).

Vitamin B_6: 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 B_{12}: Serum vitamin B_{12} was determined by using a fully automated chemiluminescence analyzer. Urinary vitamin B_{12} 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-*

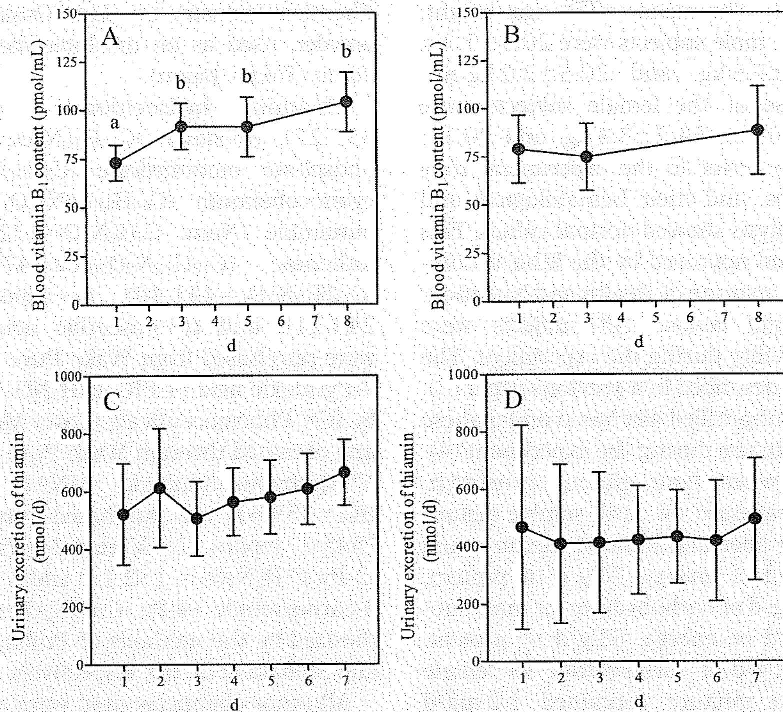

Fig. 1. Alterations of blood vitamin B_1 level in male (A) and female subjects (B), and of urinary thiamin in male (C) and female subjects (D). Each value is expressed as the mean±SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

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 microbioassay method using *Lactobacillus casei* ATCC 2733 (*15*).

Biotin: Serum and urinary biotin were determined by the microbioassay 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, $Z_\alpha=1.96$, CV_W=the within-subject coefficient of variation (%), 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 B_1

The alteration of blood vitamin B_1 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 B_1 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_{WS}) and between-subject coefficient of variance (CV_{BS}) in blood vitamin B_1 and urinary thiamin levels after adaptation were determined. CV_{WS} in blood vitamin B_1 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_{WS}=14.4%), and relatively variable in female subjects (CV_{WS}= 29.4%, Table 2). CV_{BS} 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] (%)	$\sigma^2_{WS}/\sigma^2_{BS}$[c]	10% (d)	20% (d)	CV_{WS}[a] (%)	CV_{BS}[b] (%)	$\sigma^2_{WS}/\sigma^2_{BS}$	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] (%)	$\sigma^2_{WS}/\sigma^2_{BS}$[c]	10% (d)	20% (d)	CV_{WS}[a] (%)	CV_{BS}[b] (%)	$\sigma^2_{WS}/\sigma^2_{BS}$	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 B_1.

Vitamin B_2

Alterations of blood vitamin B_2 as the sum of riboflavin, FMN and FAD, and urinary excretion of riboflavin during the experiments are shown in Fig. 2. Blood vitamin B_2 and urinary riboflavin did not change throughout the experiments. CV_{WS} and the CV_{BS} in blood vitamin B_2 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 B_2 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 B_2.

Vitamin B_6

Alterations of plasma PLP and urinary excretion of vitamin B_6 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.

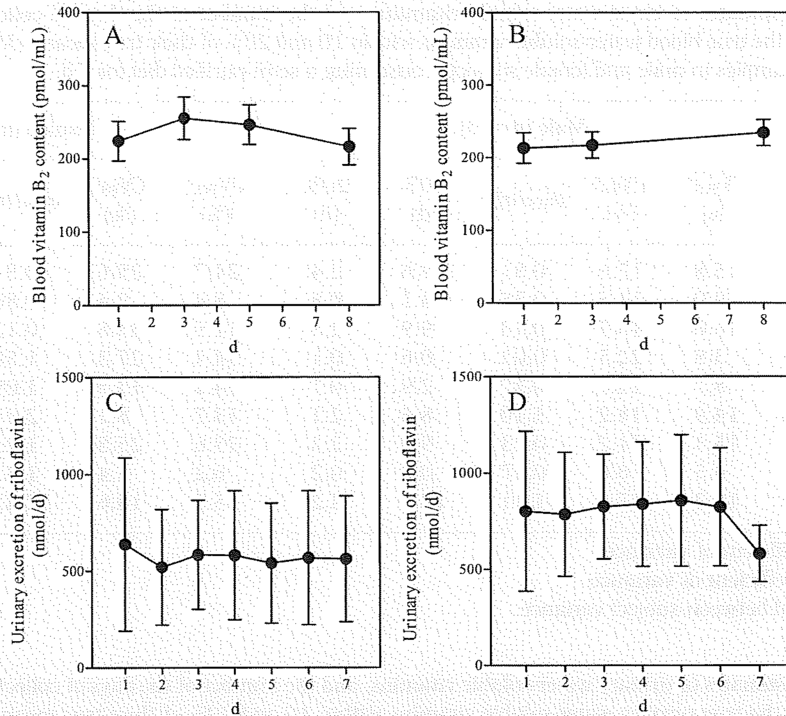

Fig. 2. Alterations of blood vitamin B_2 level in male (A) and female subjects (B), and of urinary riboflavin in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at $p<0.05$.

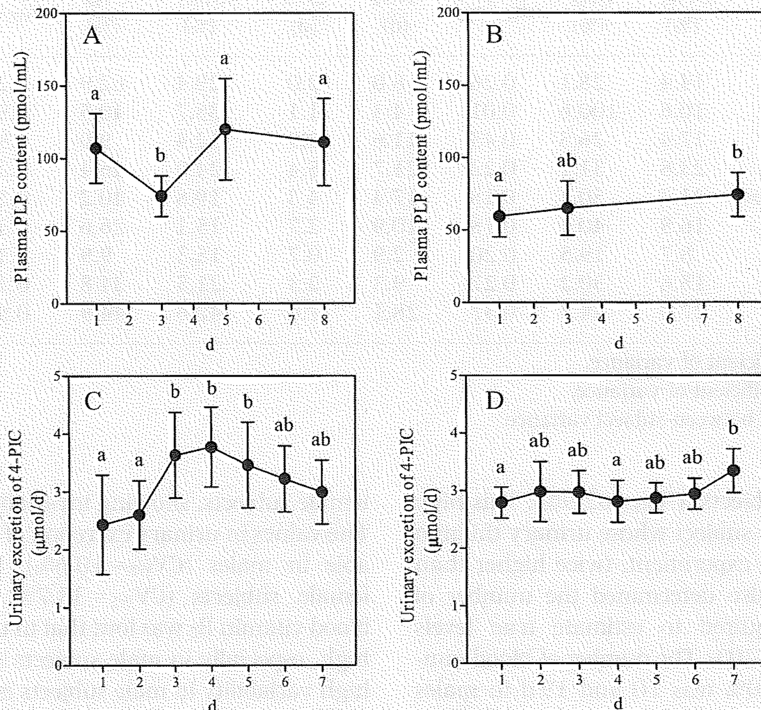

Fig. 3. Alterations of plasma PLP level in male (A) and female subjects (B), and of urinary vitamin B_6 metabolite 4-pyridoxic acid (4-PIC) in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at $p<0.05$.

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 B_6.

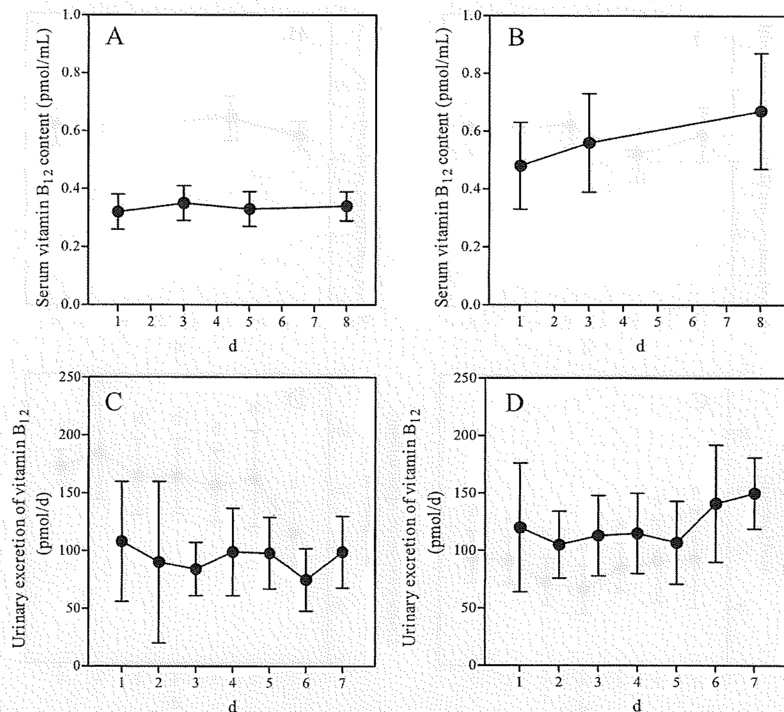

Fig. 4. Alterations of serum vitamin B_{12} level in male (A) and female subjects (B), and of urinary vitamin B_{12} in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at $p<0.05$.

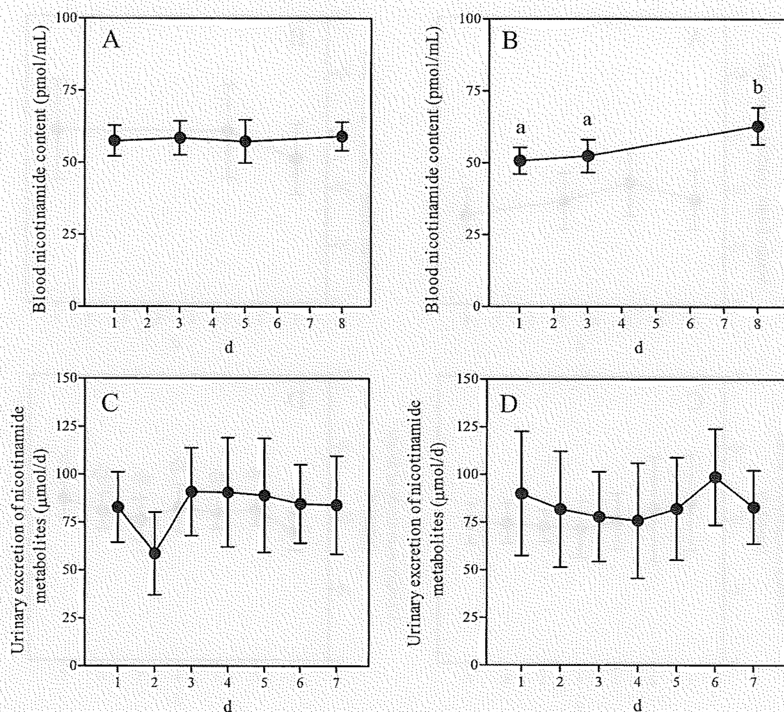

Fig. 5. Alterations of blood nicotinamide content in male (A) and female subjects (B), and of urinary nicotinamide metabolites in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at $p<0.05$.

Vitamin B_{12}

Alterations of serum and urinary vitamin B_{12} during the experiments are shown in Fig. 4. Serum and urinary vitamin B_{12} did not change during the experiments. CV_{WS} in serum and urinary vitamin B_{12} levels was approximately 30–50% but not in serum vitamin B_{12} levels of male subjects (Tables 1 and 2). CV_{BS} in serum vitamin B_{12} was ~25%, and that in urinary vitamin B_{12} was high, ~50%. To estimate the true blood and urine levels within an error of 10%, 1–49 and 22–

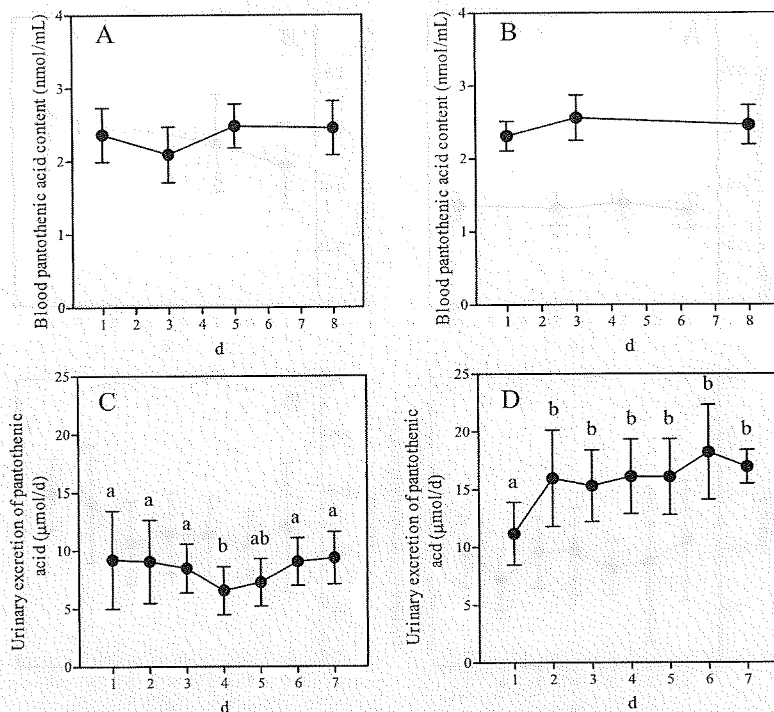

Fig. 6. Alterations of blood pantothenic acid level in male (A) and female subjects (B), and of urinary pantothenic acid in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

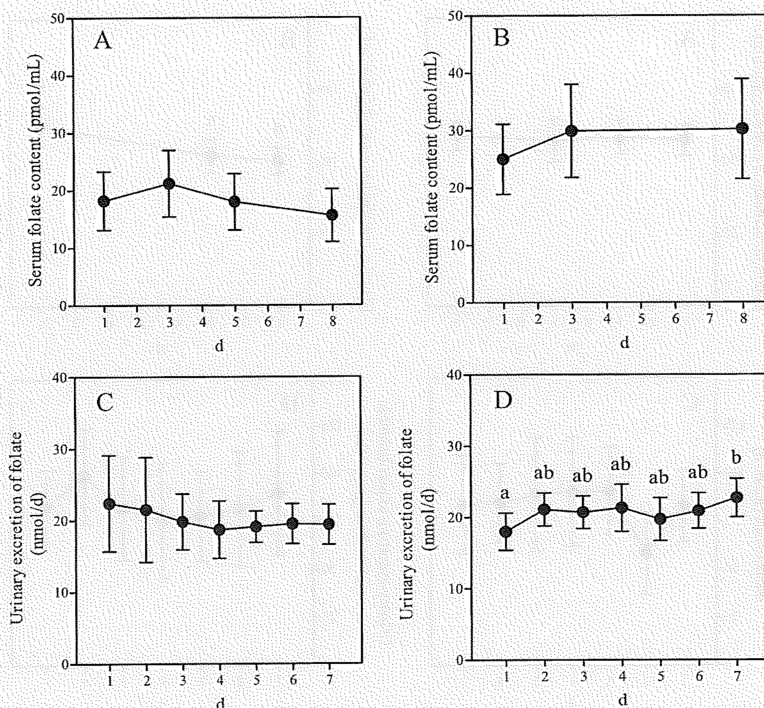

Fig. 7. Alterations of serum folate level in male (A) and female subjects (B), and of urinary folate in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

23 d were needed, respectively. No correlation was observed between serum and urinary levels in vitamin B_{12}.

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

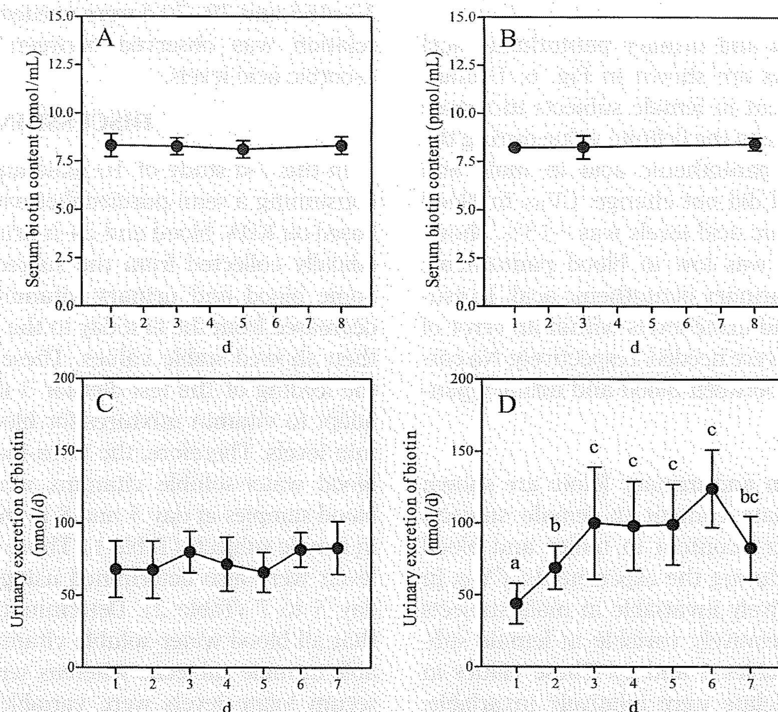

Fig. 8. Alterations of serum biotin level in male (A) and female subjects (B), and of urinary biotin in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

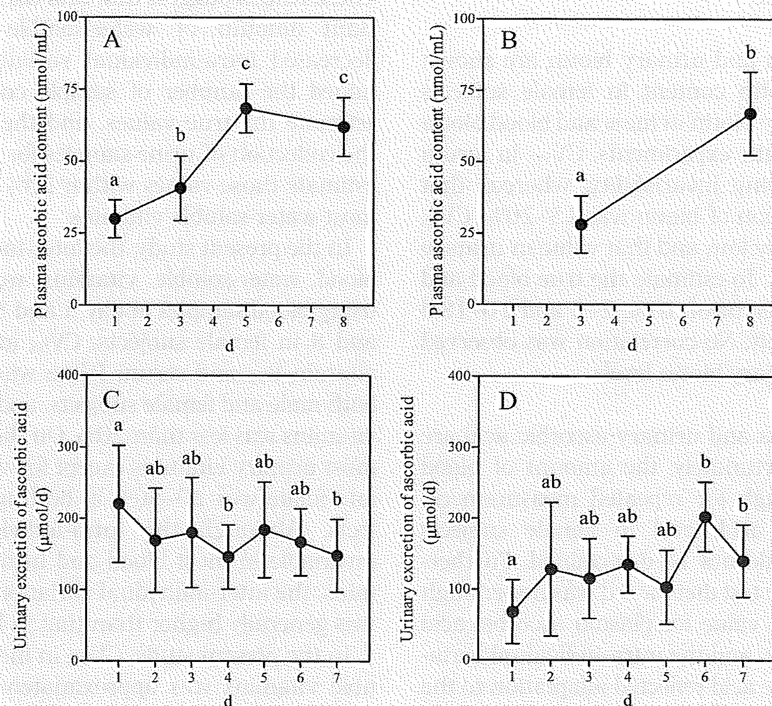

Fig. 9. Alterations of plasma ascorbic acid level in male (A) and female subjects (B), and of urinary ascorbic acid in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

subjects, respectively, showing invariability (Tables 1 and 2). These values in urinary excretion of nicotinamide metabolites were ~20%. CV_{BS} in blood nicotinamide was low, ~15%, and that in urinary nicotinamide metabolites was high, ~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 ~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 ~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, ~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 ~20%, and these values in urinary ascorbic acid were ~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 $\sigma^2_{WS}/\sigma^2_{BS}$ 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 B_6 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 B_6 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 B_6 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.

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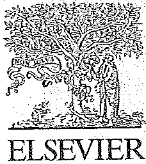

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Urinary excretion of vitamin B12 depends on urine volume in Japanese female university students and elderly

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Abstract

Recent studies have shown that urinary excretion of water-soluble vitamins reflects their intake in humans. However, some have reported that physical characteristics and urine volume may affect the amount of vitamin compounds found in urine. We hypothesized that physical characteristics and urine volume could affect urinary excretion of B-group vitamins. Twenty-four–hour urine samples were collected from 186 free-living Japanese women aged 19 to 21 years and 104 free-living Japanese subjects aged 70 to 84 years. Correlations between urinary output of each B-group vitamin and body height, body weight, body mass index, body surface area, urine volume, and urinary creatinine were determined. Only urinary vitamin B_{12} was strongly correlated to urine volume in young ($r = 0.683$, $P < .001$) and elderly ($r = 0.523$, $P < .001$) subjects. To confirm this finding, 20 Japanese adults were orally administered 1.5 mg of cyanocobalamin (500-fold higher daily intake); and correlations between urinary vitamin B_{12} and urine volume were determined. The load of cyanocobalamin increased vitamin B_{12} content in the urine by only 1.3-fold. Urinary vitamin B_{12} was strongly correlated with urine volume on the day before taking, the day of taking, and the day after taking cyanocobalamin ($r = 0.745$, $P < .001$; $r = 0.897$, $P < .0001$; and $r = 0.855$, $P < .0001$, respectively). We conclude that urinary excretion of vitamin B_{12} is dependent upon urine volume, but not on intake of vitamin B_{12}. Physical characteristics and urine volume are less important for B-group vitamins except for vitamin B_{12} as biomarker.

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Keywords: Biomarker; Human; Urine; Nutritional status; Assessment; B-group vitamins

Abbreviations: 2-Py, N^1-methyl-2-pyridone-5-carboxamide; 4-PIC, 4-pyridoxic acid; 4-Py, N^1-methyl-4-pyridone-3-carboxamide; BMI, body mass index; BSA, body surface area; IF, intrinsic factor; MNA, N^1-methylnicotinamide; TC, transcobalamin.

1. Introduction

A nutritional biomarker can be an indicator of nutritional status with respect to the intake or metabolism of dietary constituents. Such biomarkers can be designated into one or more of 3 categories: (1) a means of validation of dietary instruments, (2) surrogate indicators of dietary intake, or (3) integrated measures of nutritional status of a nutrient [1]. Recent validation studies have developed urinary compounds as nutritional biomarkers for estimating nutrient intake. For example, 24-hour urinary nitrogen has been established as a marker for protein intake [2]; urinary potassium, for intake of energy and potassium [3]; and urinary sugars, for sugar intake [4].

Water-soluble vitamins are absorbed from the digestive tract after ingestion, stored in the liver, delivered to peripheral tissues, and then excreted in the urine. The levels of urinary

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water-soluble vitamins or their metabolites decrease markedly as vitamin status declines. Urinary vitamins are affected by recent dietary intake. When sufficient water-soluble vitamins are absorbed to store in body tissues, the excess is excreted in the urine. Urinary excretions of water-soluble vitamins or their metabolites such as thiamin, riboflavin, and nicotinamide are indicators for estimating dietary reference intake in the United States and Japan [5,6]. We reported that the level of each water-soluble vitamin or its metabolite in urine collected after 24 hours was strongly correlated with its intake if young women consumed a standard Japanese diet containing several water-soluble vitamins [7]. Thirty-day mean intake of vitamin B_1 is highly correlated with mean 24-hour urine thiamin levels, which shows that urinary thiamin is a useful marker for intake of vitamin B_1 under strictly controlled conditions [8]. These results suggest that the level of urinary water-soluble vitamins or their metabolites is a good nutritional marker for assessing vitamin intake. We also reported on the levels of water-soluble vitamins in the blood and urine of young people consuming a semipurified diet with water-soluble vitamins for 7 days [9]. Urinary excretion of these vitamins varied between subjects more than blood levels did. One possible explanation for this variation is that factors such as nutrient requirements, energy expenditure, tissue turnover, kidney reabsorption, and physical characteristics differ between individuals.

In fact, urinary excretion of vitamin B_1 is varied with the urine volume [10]; and furosemide-induced diuresis increases the thiamin excretion rate [11]. Physical characteristics also affect the amount of urinary compounds. For example, individuals excreting higher urinary nitrogen had greater weight and body mass index (BMI) than those excreting average or lower nitrogen [12]; and creatinine clearance is positively correlated with BMI [13]. In this context, we hypothesized that physical characteristics and urine volume could affect urinary excretion of B-group vitamins. Therefore, we measured urinary excretion of these vitamins in free-living, healthy human subjects and determined the correlations between each of the urinary B-group vitamins and these factors. We further focused on the relationship between the level of urinary vitamin B_{12} and urine volume, and showed that urinary vitamin B_{12} content was dependent upon urine volume. Our findings may contribute to the establishment and effective use of urinary B-group vitamins as biomarkers for assessment of nutrition of water-soluble vitamins.

2. Methods and materials

2.1. Subjects

A total of 227 healthy, free-living, female dietetic students aged 18 to 23 years were recruited for this study from a university in Japan. A total of 136 elderly Japanese subjects were also recruited through local advertisements. The purpose and protocol were explained to all participants before joining the study, and written informed consent was obtained from each participant and from the parents of participants younger than 20 years. We excluded the participants with cold or influenza and those who had taken a multivitamin supplement at least once during the previous month. We also used the strict International Collaborative Study of Macronutrients, Micronutrients and Blood Pressure (INTERMAP) criteria for completeness of urine sampling [14] that included a collection time outside of the 22- to 26-hour range and subject response where collection was not complete and total volume was less than 250 mL. After this screening, 186 young female and 104 elderly subjects were eligible. The age, body weight, body height, BMI, and body surface area (BSA) of subjects are shown in Table 1. This study was approved by the Ethical Committee of The University of Shiga Prefecture (Shiga, Japan).

2.2. Collection of 24-hour urine samples from free-living Japanese subjects

A single 24-hour urine sample was collected to measure urinary water-soluble vitamins and their metabolites. In the morning, participants were asked to discard the first specimen and to record the time on a sheet. The next morning, participants were asked to collect the last specimen at the same time as when the specimen was discarded the previous morning and to record the time on the sheet. After the urine sample was collected, the volume of the sample was measured. Aliquots of urine were stabilized to avoid destruction of water-soluble vitamins and their metabolites and then stored at −20°C until analysis.

2.3. Experimental design for vitamin B_{12} loading

Healthy Japanese adults (10 men; mean age, 25.9 ± 1.0 years; 10 women; mean age, 23.5 ± 6.4 years) participated in

Table 1
Characteristics of the subjects

	Young	Elderly
No. of subjects	186	104
No. of female subjects	186	59
Age (y)	20.1 ± 1.0	73.0 ± 3.0
Height (m)	1.58 ± 0.05	1.55 ± 0.09
Weight (kg)	51.7 ± 6.0	56.2 ± 9.0
BMI (kg/m^2)	20.6 ± 2.1	23.2 ± 2.9
BSA (m^2)	1.58 ± 0.10	1.61 ± 0.16
Urine volume (mL/d)	881 ± 431	1589 ± 637
Urinary creatinine (g/d)	1.02 ± 0.16	0.860 ± 0.255
Urinary thiamin (nmol/d)	495 ± 377	419 ± 426
Urinary riboflavin (nmol/d)	358 ± 315	542 ± 411
Urinary 4-PIC (μmol/d)	3.72 ± 1.51	4.82 ± 2.45
Urinary vitamin B_{12} (pmol/d)	44.9 ± 24.8	90.8 ± 50.3
Urinary nicotinamide metabolites (μmol/d)	82.0 ± 33.6	93.3 ± 44.6
Urinary pantothenic acid (μmol/d)	15.8 ± 5.7	15.7 ± 6.3
Urinary folic acid (nmol/d)	24.0 ± 9.9	27.2 ± 15.5
Urinary biotin (nmol/d)	65.7 ± 25.7	70.2 ± 33.4

Each number except for numbers of subjects and female subjects was expressed as mean ± SD.

the experiment to determine the effect of cyanocobalamin intake on urinary excretion. The mean body height, body weight, BMI, and BSA of male subjects were 1.72 ± 0.05 m, 70.8 ± 15.3 kg, 23.8 ± 4.8 kg/m^2, and 1.91 ± 0.19 m^2, respectively; and those of female subjects were 1.55 ± 0.05 m, 50.4 ± 6.4 kg, 20.9 ± 2.3 kg/m^2, and 1.54 ± 0.11 m^2, respectively. The experiment period was 3 days, and subjects consumed similar diets throughout the experiment. They took 1.5 mg of cyanocobalamin as vitamin B_{12} after breakfast on the second day. The 24-hour urine samples were collected for 3 successive days. The volume of urine samples was measured; the samples were immediately treated as described below and then stored at −20°C until required. None of the subjects took regular medication or dietary supplements and did not habitually consume alcohol or tobacco.

2.4. Laboratory measurements

Thiamin hydrochloride, riboflavin, pyridoxine-HCl, cyanocobalamin, nicotinamide, calcium pantothenate, pteroylmonoglutamic acid, and D(+)-biotin were purchased from Wako Pure Chemical Industries (Osaka, Japan). 4-Pyridoxic acid (4-PIC) was made by ICN Pharmaceuticals (Costa Mesa, CA) and obtained through Wako. N^1-Methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N^1-Methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized following the methods of Pullman and Colowick [15] and Shibata et al [16], respectively. All other chemicals were of the highest purity available from commercial sources.

For the analysis of thiamin, 1 mL of 1 mol/L HCl was added to 9 mL urine. Urinary content of vitamin B_1 as free thiamin was determined by the high-performance liquid chromatography (HPLC)–postlabeled fluorescence method [17]. For the analysis of riboflavin, 1 mL of 1 mol/L HCl was added to 9 mL urine. Urinary content of vitamin B_2 as free riboflavin was determined by HPLC [18]. For the analysis of 4-PIC (a metabolite of vitamin B_6), 1 mL of 1 mol/L HCl was added to 9 mL urine. Urinary content of 4-PIC was determined by HPLC [19]. The concentration of vitamin B_{12} in urine was assayed by a microbiological method with *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 [20]. For the analysis of MNA, 2-Py and 4-Py, and nicotinamide metabolites, 1 mL of 1 mol/L HCl was added to 9 mL urine. Urinary content of nicotinamide metabolites as the sum of 2-Py, 4-Py, and MNA was determined by HPLC [16,21]. For the analysis of pantothenic acid, urine samples were not treated; and urinary content was determined by the microbioassay method using *L plantarum* ATCC 8014 [22]. For the analysis of folate, 1 mL of 1 mol/L ascorbic acid was added to 9 mL urine. Urinary content of free folate was determined by a microbioassay method using *L casei* ATCC 2733 [23]. For the analysis of biotin, urine samples were not treated; and urinary content of free biotin was determined by a microbioassay method using *L plantarum* ATCC 8014 [24]. Urinary creatinine was determined by colorimetric method [25].

2.5. Statistical analyses

Results are presented as means ± SD. Linear regression was determined using the computer program GraphPad Prism version 5.01 (GraphPad Software, San Diego, CA). To determine the relationship between urinary vitamins and factors such as physical characteristics, urine volume, and urinary creatinine in young and elderly subjects (n = 186 and 104, respectively), normality was tested by means of Kolmogorov-Smirnov and Shapiro-Wilk tests; and then correlation coefficients were calculated using Spearman rank correlation coefficients. To determine the relationship between urinary vitamin B_{12} and urine volume, correlation coefficients were calculated using Pearson coefficients (n = 20). The significance of the linear correlation coefficient was tested using Fisher transformation test. The level of significance was set to $P < .05$.

3. Results

3.1. Relationship of urinary B-group vitamin contents with physical characteristics, urine volume, and urinary creatinine in free-living Japanese subjects

Twenty-four–hour urine samples were collected from free-living young women and elderly Japanese subjects. The outputs of urinary B-group vitamins were measured. The results obtained from male elderly subjects were similar to those from female elderly subjects, so correlations were determined in the group as a whole. Their urine volume and urinary excretion of B-group vitamins and creatinine are shown in Table 1. Their urinary B-group vitamins divided by physical characteristics, urine volume, or urinary creatinine in young female and elderly subjects are shown in Tables 2 and 3, respectively. Correlations (r) between urinary B-group vitamin outputs and physical characteristics, urine volume, or urinary creatinine were determined.

Urinary excretion of vitamin B_{12} was strongly correlated with urine volume in young female and elderly subjects (r = 0.683, $P < .001$ and r = 0.523, $P < .001$, respectively). Urinary volume was weakly correlated with urinary riboflavin, 4-PIC, nicotinamide metabolites, pantothenic acid, folate, and biotin in young female subjects, and with pantothenic acid in elderly subjects. Relationships between body height and urinary vitamin B_{12}, body weight and urinary nicotinamide metabolites, and BSA and nicotinamide metabolites were examined in young female and elderly subjects; but their correlations were weak.

3.2. Effects of vitamin B_{12} intake on urinary excretion of vitamin B_{12}

Subjects were orally administered 1.5 mg cyanocobalamin after breakfast, and 24-hour urinary vitamin B_{12} content was measured. Fig. 1A shows urinary concentration of

Table 2
Urinary excretion of B-group vitamin expressed as per physical characteristics, urine volume, or urinary creatinine in Japanese female university students

	Urinary outputs per body height	Urinary outputs per body weight	Urinary outputs per BMI	Urinary outputs per BSA	Urinary outputs per urine volume	Urinary outputs per urinary creatinine
Thiamin	313 ± 238 (nmol/m)	9.73 ± 7.71 (nmol/kg)	24.4 ± 19.5 (nmol/[kg m^2])	315 ± 243 (nmol/m^2)	672 ± 622 (nmol/L)	490 ± 372 (nmol/g)
Riboflavin	225 ± 200 (nmol/m)	6.97 ± 6.14 (nmol/m)	17.5 ± 15.2 (nmol/[kg m^2])	227 ± 200 (nmol/m^2)	434 ± 363* (nmol/L)	354 ± 320* (nmol/g)
4-PIC	2.35 ± 0.95 (μmol/m)	72.4 ± 30.4† (nmol/kg)	181 ± 76* (nmol/[kg m^2])	2.36 ± 0.96† (μmol/m^2)	4.94 ± 2.62* (μmol/L)	3.73 ± 1.77 (μmol/g)
Vitamin B_{12}	28.3 ± 15.5* (pmol/m)	0.870 ± 0.474* (pmol/kg)	2.19 ± 1.21 (pmol/kg/m^2)	28.4 ± 15.4* (pmol/m^2)	52.7 ± 20.0‡ (pmol/L)	44.5 ± 24.4 (pmol/g)
Nam met	51.8 ± 21.0 (μmol/m)	1.59 ± 0.61* (μmol/kg)	3.99 ± 1.56 (μmol/kg/m^2)	52.0 ± 20.4* (μmol/m^2)	111 ± 70* (μmol/L)	82.7 ± 37.5 (μmol/g)
Pantothenic acid	10.0 ± 3.6 (μmol/m)	308 ± 113 (nmol/kg)	771 ± 279 (nmol/[kg m^2])	10.0 ± 3.6 (μmol/m^2)	21.3 ± 11.3* (μmol/L)	15.9 ± 6.4 (μmol/g)
Folic acid	15.1 ± 6.2 (nmol/m)	468 ± 199 (pmol/kg)	1.17 ± 0.50 (nmol/[kg m^2])	15.2 ± 6.3 (nmol/m^2)	30.4 ± 12.5* (nmol/L)	23.9 ± 9.8* (nmol/g)
Biotin	41.4 ± 15.9 (nmol/m)	1.28 ± 0.50 (nmol/kg)	3.22 ± 1.31 (nmol/[kg m^2])	41.6 ± 16.0* (nmol/m^2)	87.5 ± 43.5* (nmol/L)	65.1 ± 25.2† (nmol/g)

Each number was expressed as mean ± SD, n = 186. The 24-hour urinary excretion of B-group vitamins was divided by body height (meters), body weight (kilograms), BMI (kilograms per square meter), BSA (square meters), urine volume (liters per day), and urinary creatinine (grams per day). Spearman correlation coefficients of each 24-hour urinary B-group vitamin outputs with physical characteristics, urine volume, or urinary creatinine were calculated. Nam met indicates nicotinamide metabolites.
Statistical differences were expressed as:
* $P < .05$.
† $P < .01$.
‡ $P < .001$.

Table 3
Urinary excretion of B-group vitamin expressed as per physical characteristics, urine volume, or urinary creatinine in Japanese elderly

	Urinary outputs per body height	Urinary outputs per body weight	Urinary outputs per BMI	Urinary outputs per BSA	Urinary outputs per urine volume	Urinary outputs per urinary creatinine
Thiamin	270 ± 273 (nmol/m)	7.85 ± 8.22 (nmol/kg)	18.8 ± 19.7 (nmol/[kg m^2])	266 ± 271 (nmol/m^2)	306 ± 349 (nmol/L)	531 ± 573 (nmol/g)
Riboflavin	351 ± 268 (nmol/m)	9.89 ± 7.81 (nmol/m)	23.5 ± 17.8 (nmol/[kg m^2])	341 ± 26 (nmol/m^2)	399 ± 385 (nmol/L)	706 ± 598 (nmol/g)
4-PIC	3.09 ± 1.50* (μmol/m)	86.6 ± 42.0 (nmol/kg)	210 ± 109 (nmol/[kg m^2])	2.99 ± 1.43 (μmol/m^2)	3.50 ± 2.07 (μmol/L)	5.87 ± 2.90 (μmol/g)
Vitamin B_{12}	58.3 ± 32.3* (pmol/m)	1.65 ± 0.94 (pmol/kg)	3.98 ± 2.24 (pmol/kg/m^2)	56.5 ± 31.5 (pmol/m^2)	61.3 ± 33.1‡ (pmol/L)	109 ± 66* (pmol/g)
Nam met	59.9 ± 27.8 (μmol/m)	1.67 ± 0.74* (μmol/kg)	4.03 ± 1.87* (μmol/kg/m^2)	57.8 ± 26.0* (μmol/m^2)	68.4 ± 40.5 (μmol/L)	114 ± 54* (μmol/g)
Pantothenic acid	10.1 ± 4.1 (μmol/m)	286 ± 122 (nmol/kg)	683 ± 285 (nmol/[kg m^2])	9.83 ± 4.03 (μmol/m^2)	10.9 ± 4.7* (μmol/L)	19.1 ± 7.6* (μmol/g)
Folic acid	17.6 ± 10.3 (nmol/m)	499 ± 304 (pmol/kg)	1.18 ± 0.69 (nmol/[kg m^2])	17.1 ± 10.1 (nmol/m^2)	19.8 ± 12.5 (nmol/L)	34.6 ± 21.6 (nmol/g)
Biotin	45.3 ± 21.5 (nmol/m)	1.28 ± 0.63 (nmol/kg)	3.06 ± 1.47 (nmol/[kg m^2])	44.0 ± 21.0 (nmol/m^2)	50.0 ± 28.4 (nmol/L)	85.0 ± 38.0 (nmol/g)

Each number was expressed as mean ± SD, n = 104. The 24-hour urinary excretion of B-group vitamins was divided by body height (meters), body weight (kilograms), BMI (kilograms per square meter), BSA (square meters), urine volume (liters per day), and urinary creatinine (grams per day). Spearman correlation coefficients of each 24-hour urinary B-group vitamin outputs with physical characteristics, urine volume, or urinary creatinine were calculated. Nam met indicates nicotinamide metabolites.
Statistical differences were expressed as:
* $P < .05$.
‡ $P < .001$.

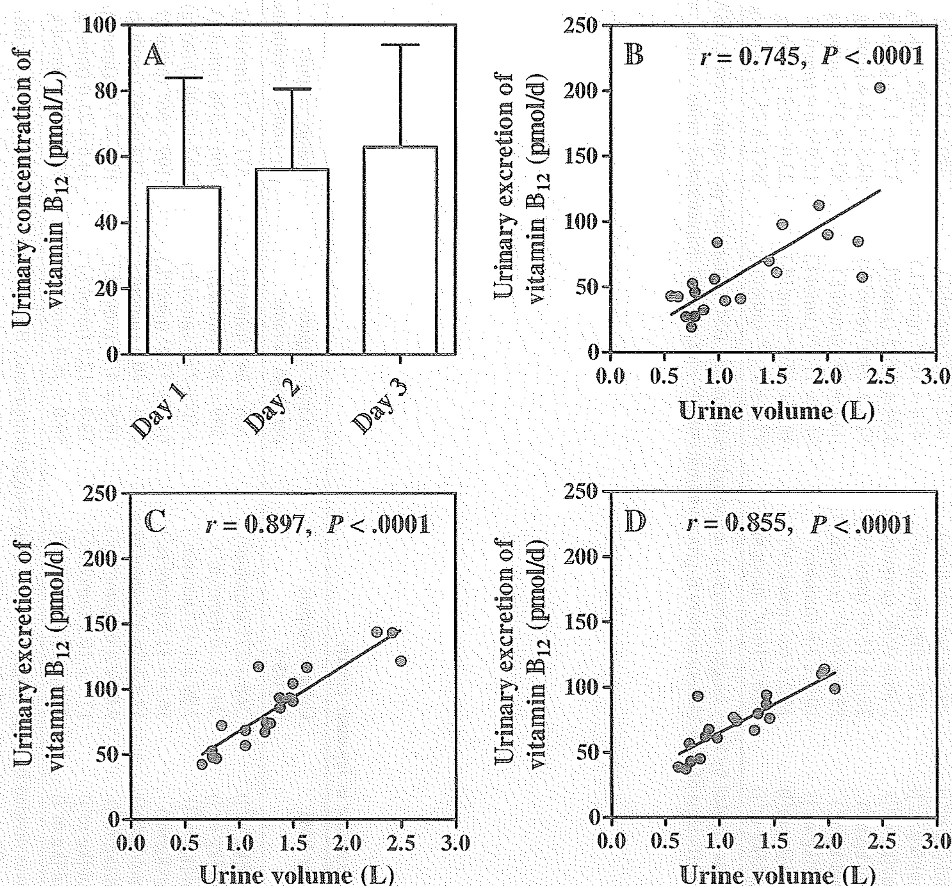

Fig. 1. Effect of administration of a pharmacologic dose of cyanocobalamin on urinary concentration of vitamin B_{12} (A) and the correlations between urinary vitamin B_{12} and urine volume on the day before cyanocobalamin intake (B), the day of intake (C) and the day after intake (D). Twenty Japanese adults consumed similar foods for 3 days and took a 1.5-mg cyanocobalamin tablet after breakfast on day 2. The 24-hour urine sample was collected for 3 successive days, and Pearson correlation coefficients between urinary vitamin B_{12} and urine volume on each day were determined.

vitamin B_{12} on the day before taking, the day of taking, and the day after taking vitamin B_{12}. Urinary concentration of vitamin B_{12} did not change during the 3 days. Intake of 1.5 mg vitamin B_{12} slightly increased urinary vitamin B_{12} from 64.3 ± 41.4 to 85.6 ± 31.1 pmol/d ($P < .05$) and was 72.7 ± 23.0 pmol/d on the day after intake. Urinary excretion of vitamin B_{12} was strongly correlated with urine volume on each day ($r = 0.745$, $P < .001$; $r = 0.897$, $P < .0001$; and $r = 0.855$, $P < .0001$, respectively) (Fig. 1B-D).

4. Discussion

To investigate if physical characteristics, urinary creatinine, and urine volume were related to the amount of B-group vitamins in urine, 24-hour urine samples were collected from 186 young women and 104 elderly Japanese subjects. Except for vitamin B_{12}, urinary B-group vitamins showed weak or no correlations with urine volume, urinary creatinine, and physical characteristics such as body height, body weight, BMI, and BSA. Urinary excretion of vitamin B_{12} was strongly correlated with urine volume in young and elderly subjects (Tables 2 and 3). To determine how urinary vitamin B_{12} is affected by its intake and urine volume, subjects took a pharmacologic dose of cyanocobalamin. Urinary vitamin B_{12} increased only 1.3-fold by its intake, and its concentration was not affected (Fig. 1A). Urinary vitamin B_{12} was always strongly correlated with urine volume even on the day before, the day of, and the day after intake (Fig. 1B-D). These results clearly showed that urinary excretion of vitamin B_{12} was dependent upon urine volume, but not on intake of vitamin B_{12}.

Recent studies suggest that urinary excretion of water-soluble vitamins is a useful marker to evaluate their intake. An investigation to assess urinary thiamin as a potential biomarker for thiamin intake reported a highly significant correlation between individuals' 30-day mean value of thiamin intake and mean excretion levels, and most subjects showed a high correlation between daily urinary thiamin and dietary thiamin [8]. Alcohol intake, carbohydrate intake, and physical activity were expected to affect vitamin B_1 metabolism [26-28]; but multiple regression models showed that only thiamin intake—not body weight, physical activity, alcohol intake, or carbohydrate intake—was the

predictor of thiamin excretion in the 30-day study [8]. These findings suggest that thiamin intake largely contributes to urinary thiamin content and that other factors affect it only slightly. Increasing the intake of water-soluble vitamins linearly increases their urinary excretion in a dose-dependent manner and is highly correlated with their excretion [7]. In the present study, urinary B-group vitamins except vitamin B_{12} showed weak or no correlations with physical characteristics, urine volume, and urinary creatinine. These findings suggest that intake of B-group vitamin mostly affects their urinary excretion. Physical characteristics, urine volume, and urinary creatinine do not affect excretion of urinary B-group vitamins.

Vitamin B_{12} is different from other B-group vitamins with respect to its mechanism of absorption and main excretion route [29]. Free vitamin B_{12} binds with haptocorrin and gastric intrinsic factor (IF) in the stomach and the duodenum, respectively. This IF–vitamin B_{12} complex attaches to the intestinal IF–vitamin B_{12} receptor in the ileal mucosa, and vitamin B_{12} enters to the enterocyte [30]. The excretion of IF is limited, so only 1% of the pharmacologic dose of vitamin B_{12} is absorbed [31]. The main excretion route of vitamin B_{12} is through the bile, as less than 10% of the total loss of vitamin B_{12} from the body is through urine [29]. Several studies have investigated the change in urinary vitamin B_{12} levels after administration of vitamin B_{12}. Oral administration of 1 mg of vitamin B_{12} increases the level of urinary vitamin B_{12} by 1.5- to 2.0-fold [32], whereas an intramuscular injection of 0.45 mg vitamin B_{12} increases it by 2- to 3-fold [33]. In the present study, oral intake of 1.5 mg vitamin B_{12} (500-fold higher than daily intake) increased the level of urinary vitamin B_{12} by 1.3-fold. These results suggest that the change in the level of urinary vitamin B_{12} is too small to evaluate intake of vitamin B_{12} and that measurement of the level of urinary vitamin B_{12} is an inadequate assessment of vitamin B_{12} intake.

Urine volume was not correlated with urinary B-group vitamin content (except for vitamin B_{12}). This suggests that urine volume does not affect urinary excretion of B-group vitamins. Although most urinary B-group vitamins were correlated with urine volume in free-living young women, their correlations were weak ($r < .2$). The level of urinary vitamin B_{12} was strongly correlated with urine volume in all investigations and experiments. The mean urinary vitamin B_{12} concentration was 52.7 ± 20.0 and 61.3 ± 33.1 pmol/L in young female and elderly subjects, respectively, and 51.1 ± 18.4, 64.8 ± 11.3, and 64.0 ± 15.0 pmol/L the day before, the day of, and the day after vitamin B_{12} intake, respectively. These results suggest that urinary vitamin B_{12} concentration is always constant at about 60 pmol/L. Vitamin B_{12} released from the liver or absorbed from the ileum binds to carrier protein transcobalamin (TC) in serum [34]. The TC–vitamin B_{12} complex is filtered in the glomeruli, and the proximal convoluted tubule reabsorbs this complex via a receptor-mediated system [30]. Megalin is an essential receptor for reabsorption of the TC–vitamin B_{12} complex in the proximal tubule because megalin-deficient mice showed 4-fold higher urinary excretion of vitamin B_{12} and 28-fold higher renal vitamin B_{12} clearance compared with control mice [35]. Megalin binds to the TC–vitamin B_{12} complex with an estimated affinity (K_d) of about 183 nmol/L [36]. This high affinity may explain why urinary loss of vitamin B_{12} is very low. Little is known about how water regulation, mediated by regulatory factors such as aquaporin, vasopressin, and angiotensin, is linked to reabsorption of vitamin B_{12}.

The major limitation of this study was that we were unable to assess the true nutrition status of the subjects. In the present study, we determined no more than the correlations between urinary output of each B-group vitamin and physical characteristics, urine volume, and urinary creatinine in the free-living subjects and did not investigate B-group vitamin intakes in the subjects. The factors affecting urinary excretion of B-group vitamins may involve the intestinal mucosal interaction, absorption, in-blood transport, tissue enzyme degradation, host nutritional status, and a diversity of physiologic conditions such as serum pH, kidney function, protein interference, and fat codigestion. To clarify the problems mentioned above, subjects would be required to stay in an institution under strictly controlled conditions such as diet, physical activity, and lifestyle. Although the present investigation could not assess the degree of contribution to urinary B-vitamins by physical characteristics and urine volume, we could determine that urinary vitamin B_{12} was dependent upon urine volume. This finding was clearly confirmed by the present intervention study showing a strong correlation between urinary vitamin B_{12} and urine volume. Another limitation is that the subjects were restricted to female dietetic students and the elderly. Our subjects may have been highly health conscious and wellness motivated. Thus, our results may not be extrapolated to general populations but only to populations with similar characteristics.

We evaluated B-group vitamin intake from urinary levels of B-group vitamins and their metabolites in healthy subjects. The findings of the present study showed that urinary excretion of B-group vitamins, except for vitamin B_{12}, was not affected by physical characteristics, urine volume, and urinary creatinine in healthy subjects. Only urinary excretion of vitamin B_{12} was affected by urine volume, and oral administration of pharmacologic doses of vitamin B_{12} did not change levels of urinary vitamin B_{12}. These results suggest that the parameters assessed in the present study cannot be used to estimate B-group vitamin intake from urinary B-group vitamins and that urinary vitamin B_{12} is not a marker to estimate its intake. Further studies may contribute to the establishment and effective use of these biomarkers for assessment of water-soluble vitamin nutrition.

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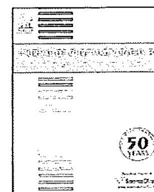

Short communication

Fluorometric determination of pantothenic acid in human urine by isocratic reversed-phase ion-pair high-performance liquid chromatography with post-column derivatization

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ABSTRACT

We describe here a method for the determination of pantothenic acid, vitamin B_5, in human urine by isocratic reversed-phase ion-pair HPLC with post-column derivatization. Pantothenic acid in urine was separated using a Tosoh ODS-80Ts (4.6 i.d. × 250 mm) column with phosphate–sodium hydroxide buffer (pH 7.0) containing dodecyltrimethylammonium chloride. Following the isolation of pantothenic acid it was decomposed to pantoic acid and β-alanine by alkali treatment. The product β-alanine was post-derivatized to the fluorescent 1-alkylthio-2-alkylisoindole with orthophthaldialdehyde in the presence of 3-mercaptopropionic acid. In the proposed method, a urine sample can be directly injected into a HPLC system without any pre-clean up treatment. The limit of detection was 3 pmol (*ca.* 650 pg) per 20 μL of urine at a signal-to-noise ratio of 5:1 and the limit of quantification was 5 pmol (*ca.* 1000 pg) per 20 μL of urine, which was sufficiently sensitive for the determination of pantothenic acid in human urine. The total time required for the analysis was *ca.* 25 min. The proposed method can be used to assess the pantothenic acid content of human urine as an alternative to the standard microbioassay for pantothenic acid.

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1. Introduction

Recent studies have shown that the analysis of urinary compounds can provide a great deal of useful nutritional information. For example, urinary excretion of protein, potassium and sugar [1–3] can be used as biomarkers to estimate the intake of the respective nutrients.

Pantothenic acid (PaA) is a water-soluble vitamin, and there are reports of a correlation between urinary excretion and intake [4]. Urinary excretion of PaA is therefore a suitable surrogate indicator of PaA intake. Several methods for the measurements of PaA have been reported. The most reliable and common method is a microbioassay which uses *Lactobacillus plantarum* ATCC 8014 [5]. The merits of the assay include its sensitivity and the fact that expensive instruments and reagents are not required. The disadvantage of the microbioassay is that it requires specialist knowledge and technique. Alternative chemical assay methods have therefore been developed. For example, the use of radioimmunoassay [6,7] and indirect enzyme immunoassay [8–10] have been reported. Radioisotopes and scintillation counting are required for the former, and non-commercially available antisera are needed for the latter. Methods using gas chromatography–mass spectrometry with multiple ion detection [11]; liquid chromatography–mass spectrometry [12–14]; and liquid chromatography–tandem mass spectrometry [15] have also been reported. Whilst these methods may be associated with high precision, the analytical systems required are expensive and difficult to maintain.

High-performance liquid chromatography (HPLC) assays using ultraviolet (UV) [16–19] or fluorometric detection [20,21] to measure PaA have been reported. The assay using UV detection cannot be applied to urinary PaA because the PaA molecule absorbs very weakly in the UV region. Although the fluorometric determination of PaA as reported by Pakin et al. [21] would be suitable for the rapid determination of the compound in urine, the sample requires pre-treatment and the HPLC separation can be problematic. In this assay, the sample must be purified and the pH adjusted several times which leads to dilution of the sample. We applied this method to the measurement of PaA in human urine; however, we were unable to detect PaA in some samples because of its low concentration in human urine.

In this study, we describe a novel assay for measuring PaA in urine by isocratic reversed-phase ion-pair HPLC with post-column derivatization.

2. Experimental

2.1. Chemicals and reagents

Calcium pantothenate, orthophthalaldehyde (OPA), 3-mercaptopropionic acid (3-MPA), sodium hydroxide, potassium

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