

Table 1. Composition of the diet

Vitamin-free milk casein	20.0
L-Methionine	0.2
Gelatinized cornstarch	46.9
Sucrose	23.4
Corn oil	5.0
Mineral mixture (AIN-93-G)	3.5
Vitamin mixture (AIN-93)	1.0

Values are expressed as g per 100g of diet.

Table 2. Contents of vitamins in vitamin mixture (AIN-93)¹⁴⁾

Nicotinic acid	3.000
Ca Pantothenate	1.600
Pyridoxine-HCl	0.700
Thiamin-HCl	0.600
Riboflavin	0.600
Folic acid	0.200
D-Biotin	0.020
Vitamin B ₁₂ (cyanocobalamin) (0.1% in mannitol)	2.500
Vitamin E (all- <i>rac</i> - α -tocopheryl acetate) (500 IU/g)	15.000
Vitamin A (all- <i>trans</i> -retinyl palmitate) (500,000 IU/g)	0.800
Vitamin D ₃ (cholecalciferol) (400,000 IU/g)	0.250
Vitamin K (phyllquinone)	0.075
Powdered sucrose	974.655

Values are expressed as g per kg of vitamin mixture.

いものと交換した。その際に体重、飼料摂取量、飲水量を測定した。測定には動物計量用電子天秤(メトラー・トレード株)を用いた。ラットは飼育最終日の午前9時に断頭屠殺した。

母乳を採取するために、7～8週齢のWistar系雌性ラットを日本クレア株より購入し、Table 1に示した精製飼料で一週間飼育後、Wistar系雄性ラットと交配させた。約3週間の妊娠期間を経て、11～12週齢のラットの母乳を採集した。

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2. 試料の調製

2-1. 尿の採集

7～12週齢のラットの24時間尿(午前9時～翌日午前

9時)を集めた。尿は腐敗防止のために1Mの塩酸1mLを含む三角フラスコ中に集め、尿量を測定後、分析に用いるまで-20℃にて保存した。なお、尿中葉酸量を定量するために、採尿後の尿900 μ Lに1Mアスコルビン酸溶液100 μ Lを添加後、-20℃にて冷凍保存した。

2-2. 血液の採集

7～10週齢のラットを断頭屠殺し、血液をEDTA入り試験管に採集した。全血および遠心分離(1,700 \times g, 30分, 室温)によって得られた血漿を分析に供した。

2-3. 母乳の採集

出産した日を出産後1日目とし、出産後9, 13, 17, 21日目に実験動物用搾乳装置(WAT-2001, 有限会社リトルレオナルド)を用いて搾乳をおこなった。搾乳当日は母乳をためるために午前9時に母ラットを別のケージに移し、午後3時より開始した。計12ヶ所の乳房全てから均等に、2mL程度搾乳した。母ラットは搾乳後、仔ラットのもとに返し、通常通り仔ラットの世話をしていることを確認した。母乳は生理食塩水にて希釈し、マイクロチューブに小分けして-20℃にて冷凍保存した。母乳中ビタミンB₁、ビタミンB₁₂、ニコチンアミドの定量には、試料を遠心分離(20,400 \times g, 10分, 4℃)後、最上にある脂肪層を除去した上清を使用した。

3. 分析

3-1. ビタミンB₁の測定

尿中の遊離型チアミン量を測定するために、尿を直接HPLCによる分析に供した。定量方法は文献2)に示したポストカラム-HPLC法にしたがった。血中および母乳中の総ビタミンB₁濃度はチアミンとチアミン二リン酸の合計量とし、試料にトリクロロ酢酸溶液を加えて除タンパク後、尿と同様の方法で定量した²⁾。

3-2. ビタミンB₂の測定

尿中の遊離型リボフラビン量を測定するために、尿を直接HPLCによる分析に供した³⁾。血中および母乳中の総ビタミンB₂濃度は、リボフラビン、FMN、FADを光分解してルミフラビンとした後、HPLC法により定量した⁴⁾。

3-3. ビタミンB₆とその関連化合物の測定

尿中のビタミンB₆異化代謝産物である4-ピリドキシニン酸(4-PIC)量を測定するために、尿を直接HPLCによる分析に供した⁵⁾。血漿中および母乳中のピリドキサール、ピリドキサールリン酸濃度を測定するために、試料にメタリン酸を加えて除タンパク後、HPLCによる分析に供した⁶⁾。これらの合計量をビタミンB₆濃度として示した。

3-4. ビタミンB₁₂の測定

尿中ビタミンB₁₂量を測定するために、シアン化カリウム存在下で試料中のビタミンB₁₂をシアノコバラミンに変換し、安定化処理を行った。その処理液を*Lactobacillus leichmanii* ATCC 7830を用いた微生物学的定

量法に供した⁷⁾。血漿中、母乳中ビタミンB₁₂濃度の測定も同様におこなった⁷⁾。

3-5. ニコチンアミドとその関連化合物の測定

尿中のニコチンアミドおよびその異化代謝産物量は、ニコチンアミド、N¹-メチルニコチンアミド(MNA)、N¹-メチル-2-ピリドン-5-カルボキサミド(2-Py)、N¹-メチル-4-ピリドン-3-カルボキサミド(4-Py)の合計量として示した。尿中のニコチンアミド、2-Py、4-Pyは文献8に示したHPLC法で同時定量した。尿中MNA含量は文献9に示したHPLC法にて定量した。血中および母乳中総ニコチンアミド濃度は、文献10に示したHPLC法にて定量した。

3-6. パントテン酸の測定

尿中の遊離型パントテン酸量を測定するために、尿を*Lactobacillus plantarum* ATCC 8014を用いた微生物学的定量法に供した¹¹⁾。血漿中パントテン酸濃度は、加熱による除タンパク後の上清を微生物学的定量法に供して測定した¹¹⁾。母乳中総パントテン酸濃度を測定するために、ホスファターゼ・パンテテイナーゼ処理をおこない、結合型パントテン酸を遊離型に変換後、微生物学的定量法に供した¹¹⁾。

3-7. 葉酸の測定

尿中葉酸量を測定するために、尿を直接*Lactobacillus casei* ATCC 27773を用いた微生物学的定量法に供した¹²⁾。血漿中葉酸濃度も同様の方法で測定した¹²⁾。母乳中葉酸濃度を測定するために、プロテアーゼ・コンジュガーゼ処理をおこない、葉酸を遊離型に変換後、微生物学的定量法に供した¹²⁾。

3-8. ビオチンの測定

尿中の遊離型ビオチン量を測定するために、尿を直接*Lactobacillus plantarum* ATCC 8014を用いた微生物学的定量法に供した。血漿中、母乳中総ビオチン濃度を測定するために、試料に硫酸溶液を加えてオートクレーブし、結合型ビオチンを遊離型に変換後、微生物学的定量法に供した。測定は文献13を参考に、比濁法にて行った。

4. データの選別・解析

本資料には、2003年度～2008年度に本研究室で行われた研究のうち、対照群に通常の精製飼料(20%カゼイン・1%ビタミン混合)を与えて飼育した38実験、総計268匹のラットのデータを用いた。但し、測定したビタミンの種類は実験ごとにより異なるので、測定したn数は、「測定結果のまとめ」の値のうしろに括弧をつけて示した。

母乳中ビタミン含量については1実験、4匹のラットのデータを用いた。

体重変化量、飼料摂取量、飲水量の値はTable 1に示した精製飼料で一週間以上飼育したものである。値は週齢別に平均値±標準誤差で示した。統計解析にはGraphPad Software社(San Diego, CA, USA)のGraphPad Prism 5を使用し、非線形回帰分析の結果を実線で示した。

尿中B群ビタミン排泄量および母乳中B群ビタミン濃度について、同一個体から得られた値は平均化したのち、その平均値を一つの値として処理した。

測定結果のまとめ

1. 体重と飼料摂取量、水分摂取量の推移

離乳後の3週齢から12週齢までのラットの体重、飼料摂取量、飲水量の推移をFig. 1に示した。体重は3週齢から12週齢まで直線的に増加しつづけた(Fig. 1-A)。飼料摂取量は6週齢までは直線的に増加し、その後は概ね18g/日の値をとった(Fig. 1-B)。飲水量も飼料摂取量と同じく6週齢で一定値となり、6週齢以降の平均飲水量は概ね20mL/日の値をとった(Fig. 1-C)。なお、飲水量はS字型曲線を示した。

2. 尿中B群ビタミン排泄量

尿中B群ビタミン排泄量のヒストグラムをFig. 2に示した。各ビタミン排泄量の平均値±標準誤差は、チアミンは77.2±3.9nmol/日(n=139)、リボフラビンは108±3nmol/日(n=125)、4-PICは289±4nmol/日(n=136)、ビタミンB₁₂は31.7±1.9pmol/日(n=64)、ニコチンアミドとその異化代謝産物の合計量は6.48±0.18μmol/日(n=89)、パントテン酸は837±18nmol/日(n=133)、葉酸は8.75±0.56nmol/日(n=35)、ビオチンは4.45±0.15nmol/日(n=135)であった。

なお、アスコルビン酸溶液にて抗酸化処理をおこなわなかった尿をもちいた場合の葉酸排泄量は4.48±0.22nmol/日(n=101)であり、アスコルビン酸処理の必要性を示している。

3. 血中B群ビタミン濃度

血中B群ビタミン濃度のヒストグラムをFig. 3に示した。各ビタミン濃度の平均値±標準誤差は、ビタミンB₁は316±10pmol/mL全血(n=55)、ビタミンB₂は196±7pmol/mL全血(n=44)、ビタミンB₆は1.60±0.08nmol/mL血漿(n=37)、ビタミンB₁₂は3.33±0.12pmol/mL血漿(n=34)、ニコチンアミドは122±4nmol/mL全血(n=32)、パントテン酸は3.11±0.23nmol/mL血漿(n=32)、葉酸は198±10pmol/mL血漿(n=54)、ビオチンは43.1±1.9pmol/mL血漿(n=39)であった。

4. 母乳中B群ビタミン濃度

母乳中B群ビタミン濃度の搾乳日別推移をFig. 4に示した。全てのB群ビタミンについて、搾乳日による差は認められなかったため、一頭体ずつの平均値をとって処理した。母乳中ビタミン濃度の平均値±標準誤差(n=4)は、ビタミンB₁は0.60±0.11nmol/mL、ビタミンB₂は8.87±0.50nmol/mL、ビタミンB₆は8.83±0.37nmol/mL、ビタミンB₁₂は25.6±1.8pmol/mL、ニコチンアミドは58.8

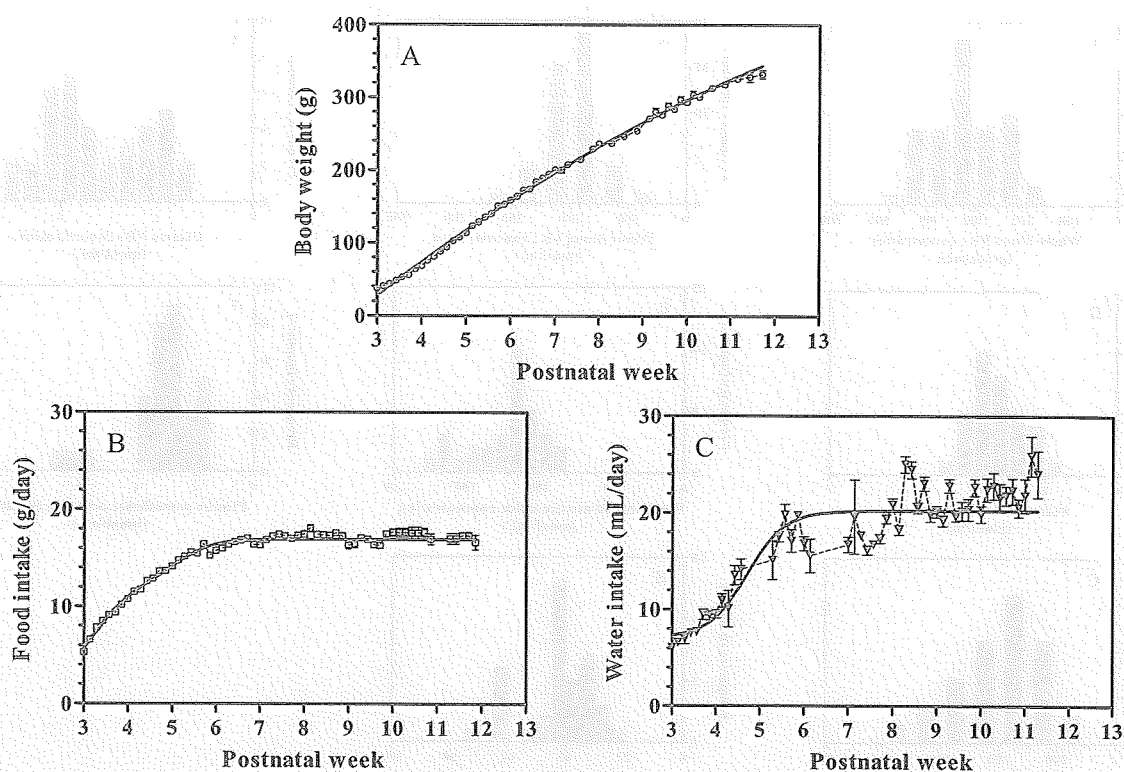


Fig. 1. Curves for body weight (A), food intake (B) and water intake (C) during growth in weaning male Wistar strain rats. Each value is expressed as mean \pm SEM (n = 6-119).

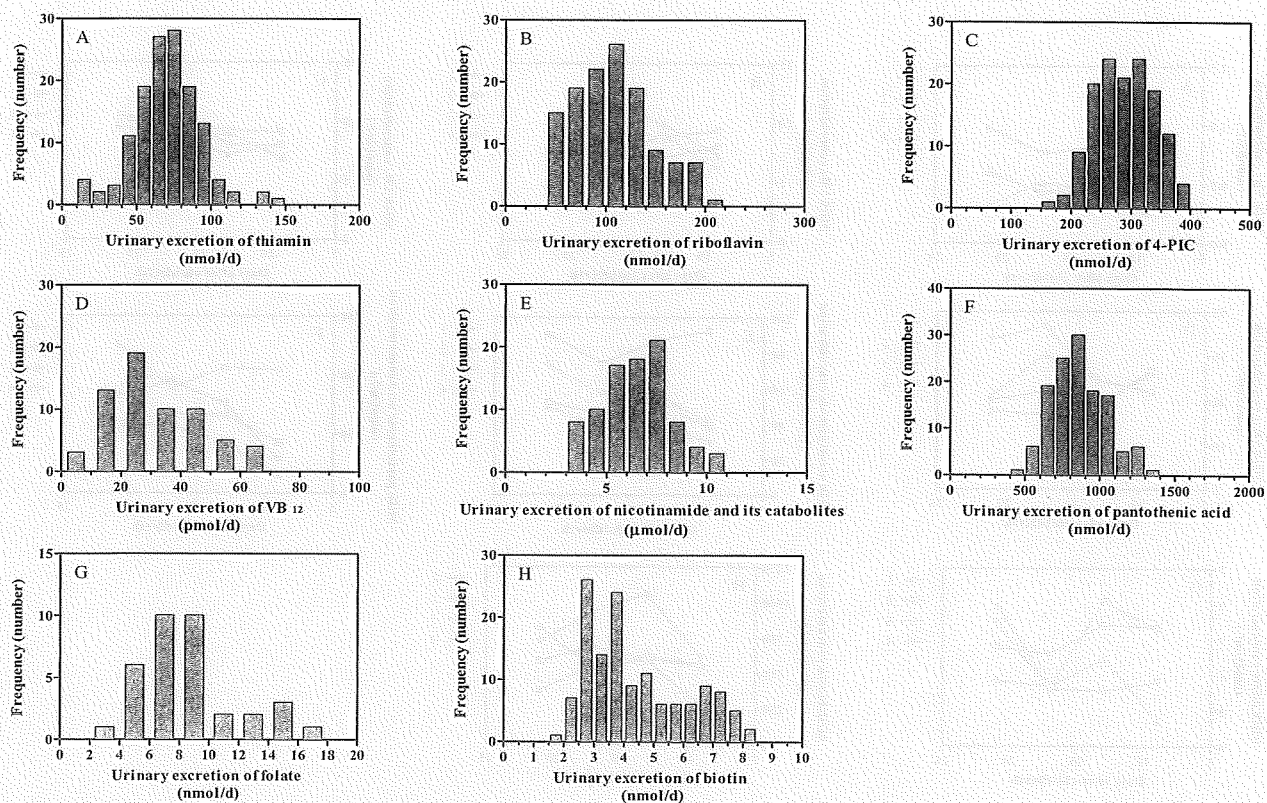


Fig. 2. Urinary excretion of thiamin (A), riboflavin (B), 4-pyridoxic acid (C), vitamin B₁₂ (D), nicotinamide and its catabolites (E), pantothenic acid (F), folate (G), and biotin (H) in male Wistar strain rats.

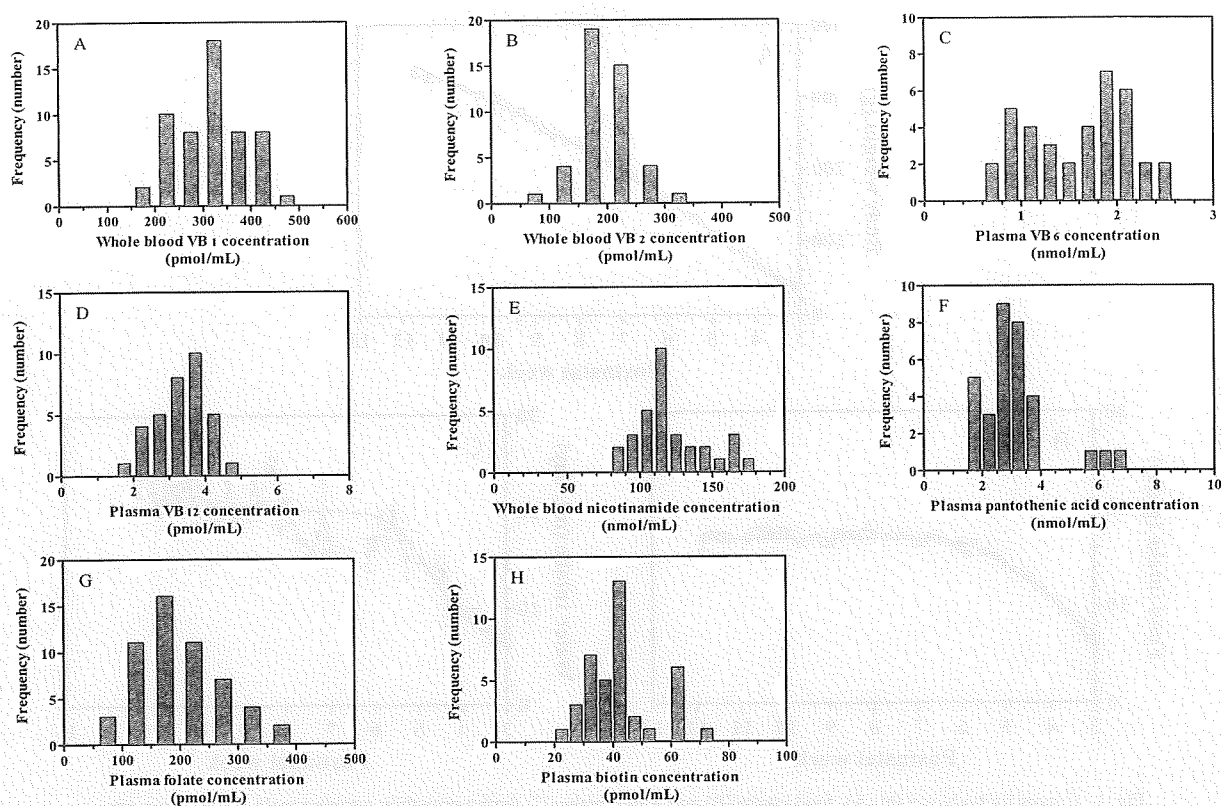


Fig.3 Concentrations of vitamin B₁ (A), vitamin B₂ (B), vitamin B₆ (C), vitamin B₁₂ (D), nicotinamide (E), pantothenic acid (F), folate (G), and biotin (H) in blood of male Wistar strain rats.

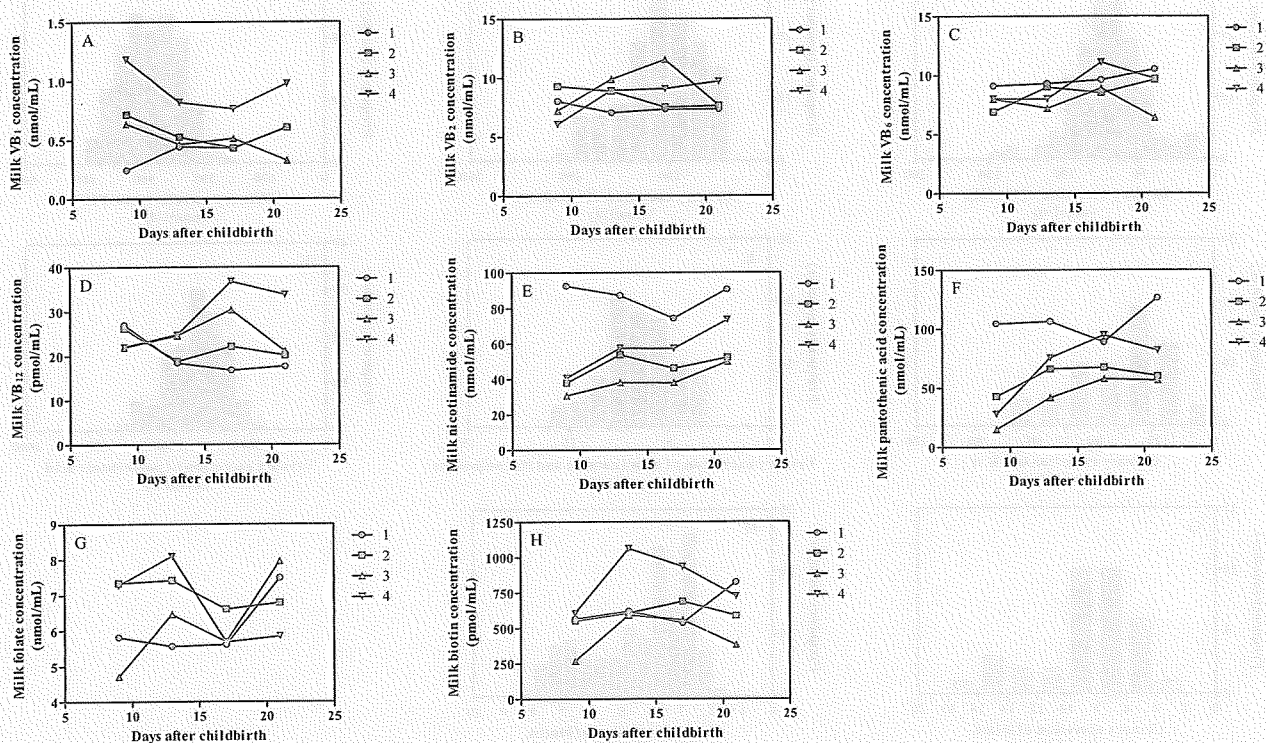


Fig.4 Concentrations of vitamin B₁ (A), vitamin B₂ (B), vitamin B₆ (C), vitamin B₁₂ (D), nicotinamide (E), pantothenic acid (F), folate (G), and biotin (H) in milk of female Wistar strain rats. Numbers "1, 2, 3, 4" indicate the number of the rat.

±10.0 nmol/mL, パントテン酸は 68.2±10.4 nmol/mL, 葉酸は 6.21±0.22 nmol/mL, ビオチンは 0.61±0.08 nmol/mL であった.

(平成 21.5.11 受付)

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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 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

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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 microbiassay 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 microbiassay method using *Lactobacillus plan-*

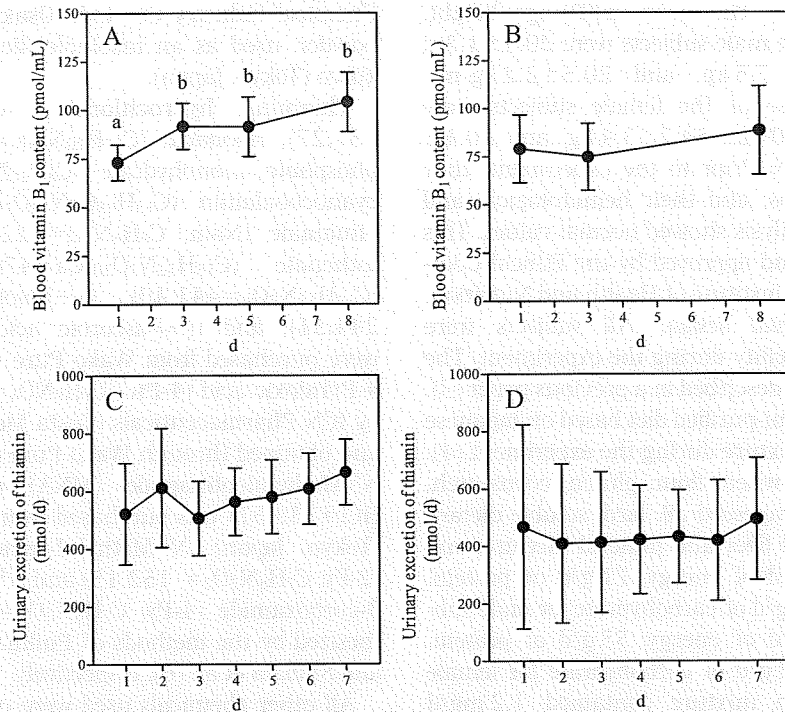


Fig. 1. Alterations of blood vitamin B₁ level in male (A) and female subjects (B), and of urinary thiamin in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

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 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 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_{WS}) and between-subject coefficient of variance (CV_{BS}) in blood vitamin B₁ and urinary thiamin levels after adaptation were determined. CV_{WS} 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 ($CV_{WS} = 14.4\%$), 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 (%)	$\sigma_{WS}^2/\sigma_{BS}^2$ ^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.

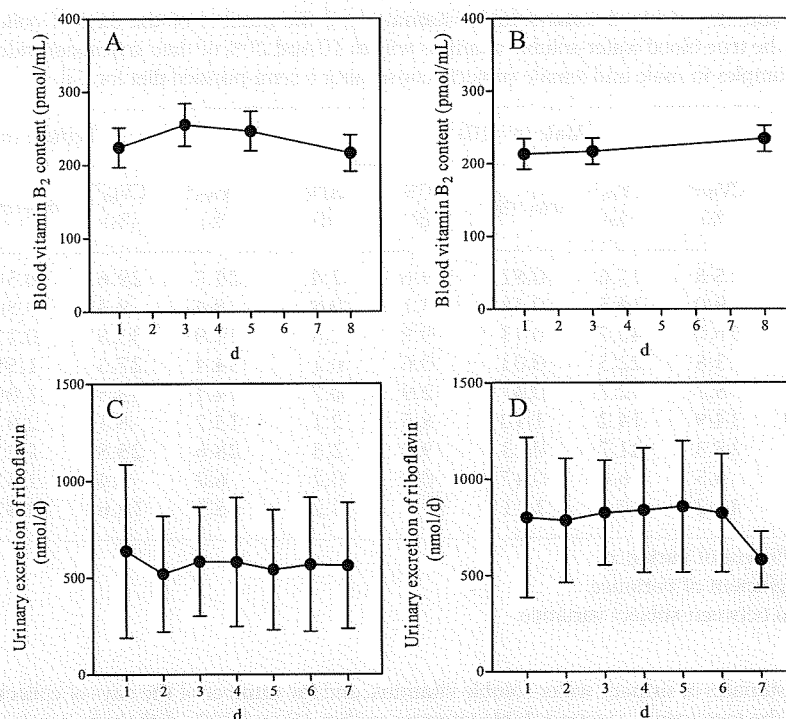


Fig. 2. Alterations of blood vitamin B₂ level in male (A) and female subjects (B), and of urinary riboflavin in male (C) and female subjects (D). Each value is expressed as the mean ± SD (n=10). A different superscript letter means significant difference at p < 0.05.

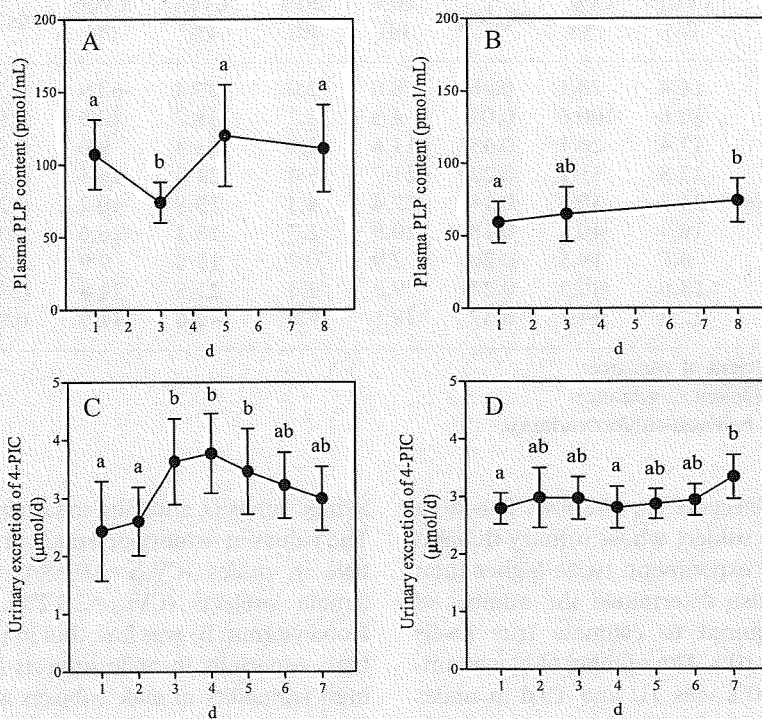


Fig. 3. Alterations of plasma PLP level in male (A) and female subjects (B), and of urinary vitamin B₆ metabolite 4-pyridoxic acid (4-PIC) in male (C) and female subjects (D). Each value is expressed as the mean ± SD (n=10). A different superscript letter means significant difference at p < 0.05.

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.

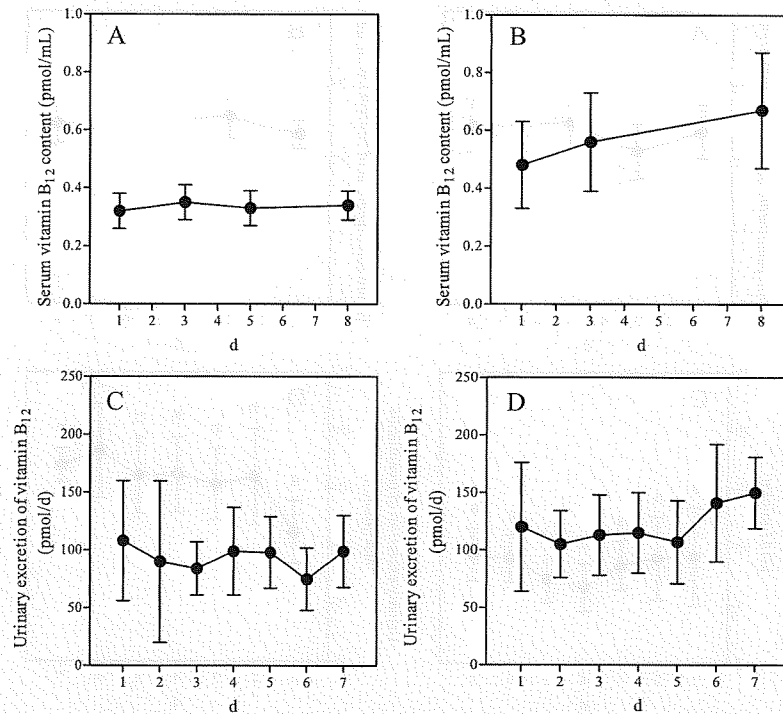


Fig. 4. Alterations of serum vitamin B₁₂ level in male (A) and female subjects (B), and of urinary vitamin B₁₂ in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

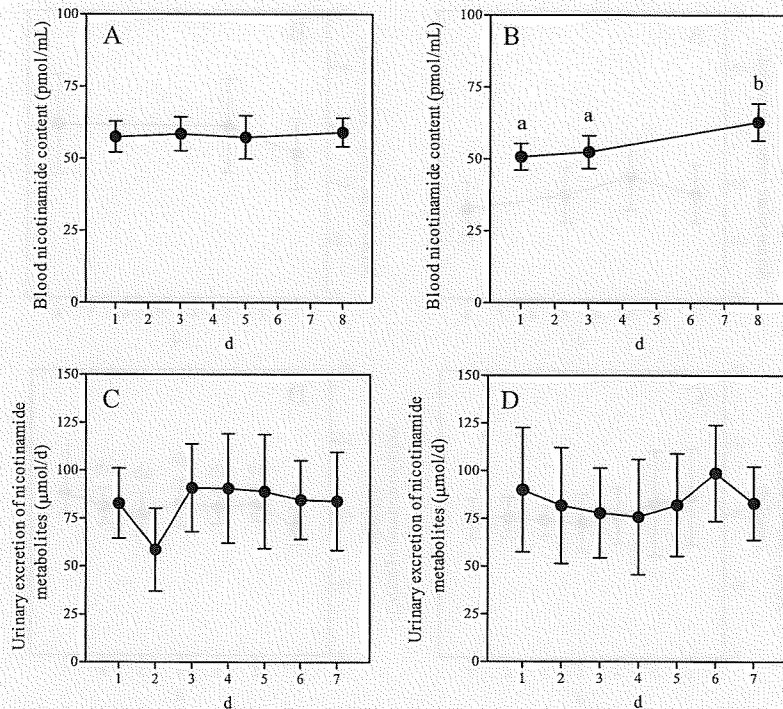


Fig. 5. Alterations of blood nicotinamide content in male (A) and female subjects (B), and of urinary nicotinamide metabolites in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p<0.05$.

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–

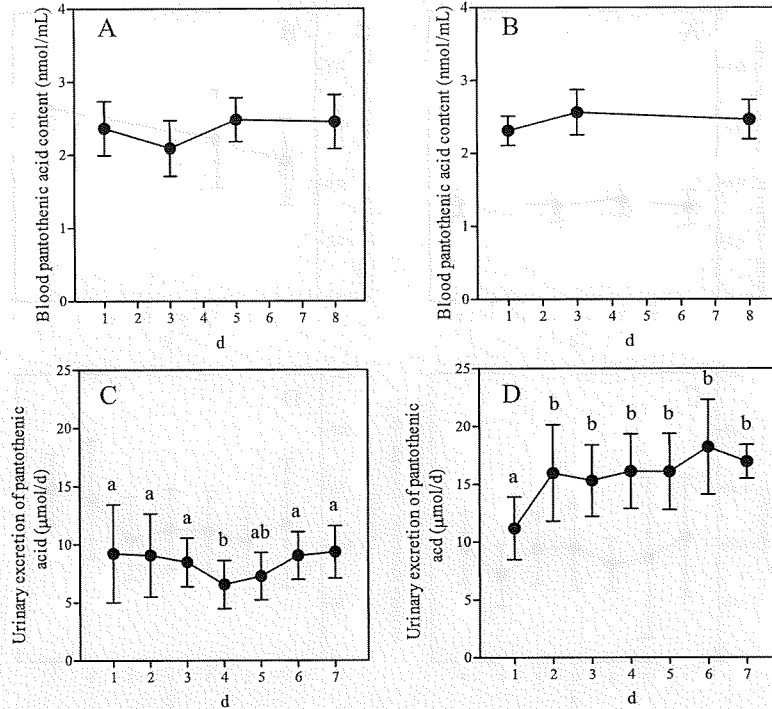


Fig. 6. Alterations of blood pantothenic acid level in male (A) and female subjects (B), and of urinary pantothenic acid in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

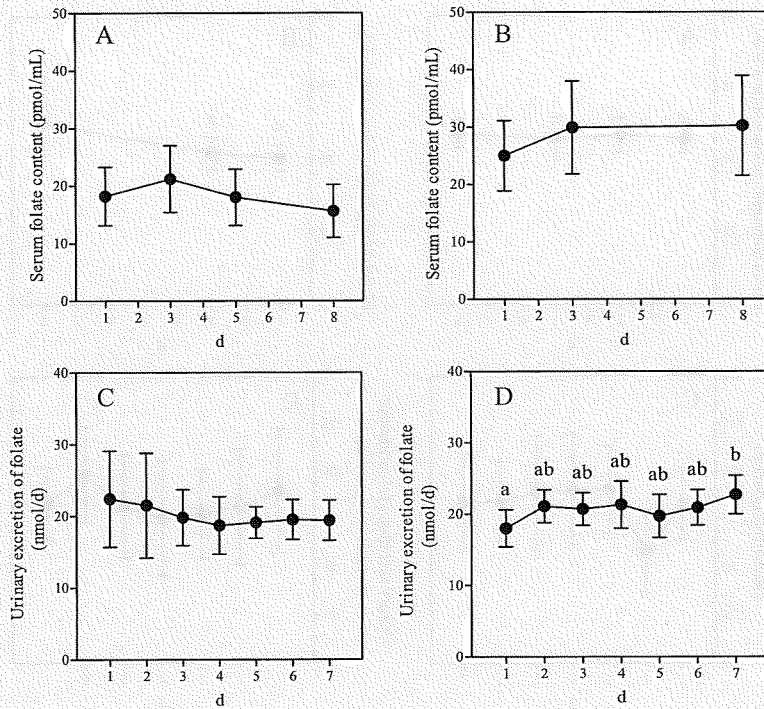


Fig. 7. Alterations of serum folate level in male (A) and female subjects (B), and of urinary folate in male (C) and female subjects (D). Each value is expressed as the mean±SD (n=10). A different superscript letter means significant difference at p<0.05.

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

Niacin

Alterations of blood nicotinamide and urinary excre-

tion 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

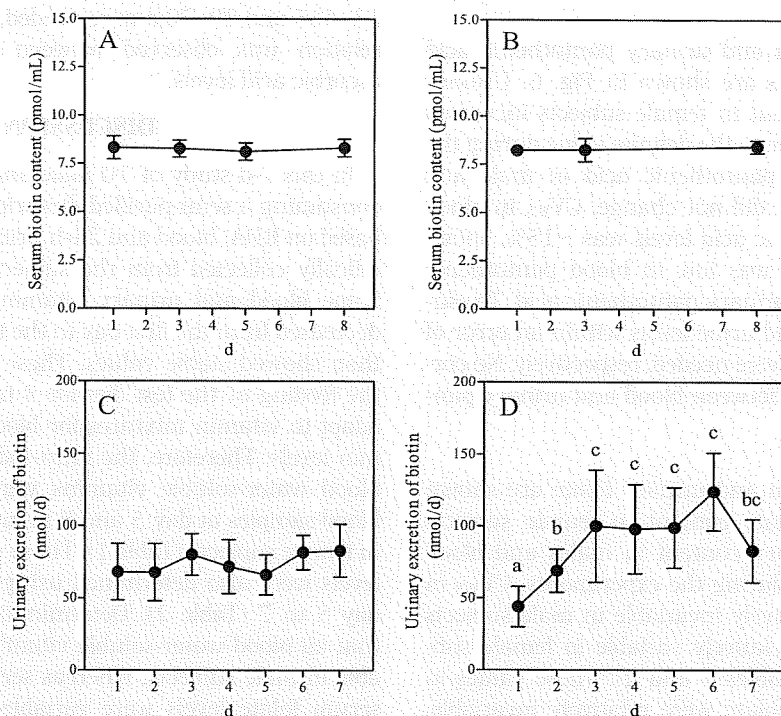


Fig. 8. Alterations of serum biotin level in male (A) and female subjects (B), and of urinary biotin in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

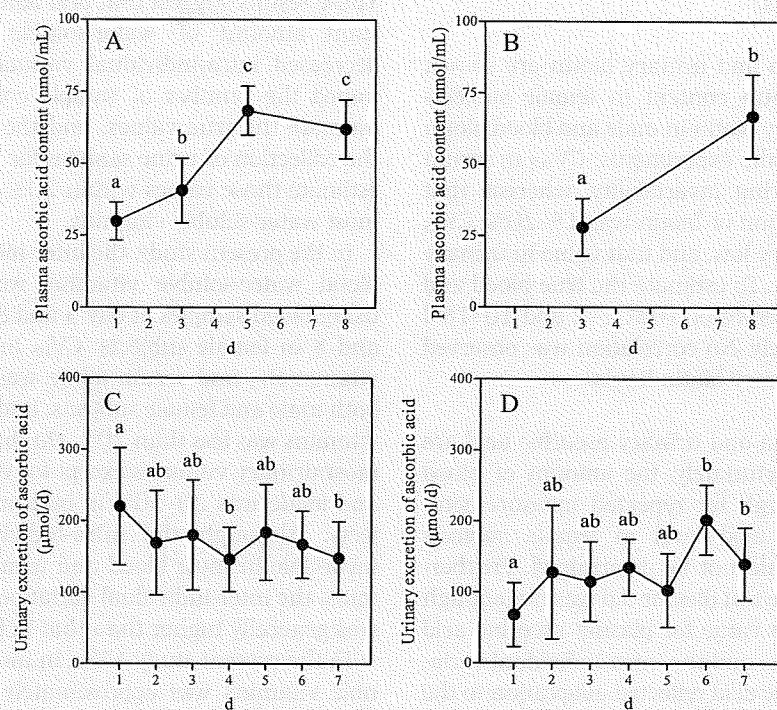


Fig. 9. Alterations of plasma ascorbic acid level in male (A) and female subjects (B), and of urinary ascorbic acid in male (C) and female subjects (D). Each value is expressed as the mean \pm SD ($n=10$). A different superscript letter means significant difference at $p < 0.05$.

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 B₁₂ and serum folate levels were variable in female subjects. CV_{WS} in urinary vitamin B₁₂ 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 B₁₂ 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 B₆ 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 B₁ 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.

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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 B₁₂ 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 B₁₂ and urine volume were determined. The load of cyanocobalamin increased vitamin B₁₂ content in the urine by only 1.3-fold. Urinary vitamin B₁₂ 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 B₁₂ is dependent upon urine volume, but not on intake of vitamin B₁₂. Physical characteristics and urine volume are less important for B-group vitamins except for vitamin B₁₂ 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 B₁ is highly correlated with mean 24-hour urine thiamin levels, which shows that urinary thiamin is a useful marker for intake of vitamin B₁ 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 B₁ 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 B₁₂ and urine volume, and showed that urinary vitamin B₁₂ 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 B₁₂ 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 ($\mu\text{mol}/\text{d}$)	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 ($\mu\text{mol}/\text{d}$)	82.0 ± 33.6	93.3 ± 44.6
Urinary pantothenic acid ($\mu\text{mol}/\text{d}$)	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 \pm 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/m², and 1.91 ± 0.19 m², respectively; and those of female subjects were 1.55 ± 0.05 m, 50.4 ± 6.4 kg, 20.9 ± 2.3 kg/m², and 1.54 ± 0.11 m², respectively. The experiment period was 3 days, and subjects consumed similar diets throughout the experiment. They took 1.5 mg of cyanocobalamin as vitamin B₁₂ 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. N¹-Methylnicotinamide (MNA) chloride was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N¹-Methyl-2-pyridone-5-carboxamide (2-Py) and N¹-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 B₁ 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 B₂ as free riboflavin was determined by HPLC [18]. For the analysis of 4-PIC (a metabolite of vitamin B₆), 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 B₁₂ 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 \pm 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 B₁₂ 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 B₁₂ 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 B₁₂, 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 B₁₂ intake on urinary excretion of vitamin B₁₂

Subjects were orally administered 1.5 mg cyanocobalamin after breakfast, and 24-hour urinary vitamin B₁₂ content was measured. Fig. 1A shows urinary concentration of