Urinary Water-Soluble Vitamins and Their Metabolite Contents as Nutritional Markers for Evaluating Vitamin Intakes in Young Japanese Women

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Summary  Little information is available to estimate water-soluble vitamin intakes from urinary vitamins and their metabolite contents as possible nutritional markers. Determination of the relationships between the oral dose and urinary excretion of water-soluble vitamins in human subjects contributes to finding valid nutrition markers of water-soluble vitamin intakes. Six female Japanese college students were given a standard Japanese diet in the first week, the same diet with a synthesized water-soluble vitamin mixture as a diet with approximately onefold vitamin mixture based on Dietary Reference Intakes (DRIs) for Japanese in the second week, with a threefold vitamin mixture in the third week, and a sixfold mixture in the fourth week. Water-soluble vitamins and their metabolites were measured in the 24-h urine collected each week. All urinary vitamins and their metabolite levels except vitamin B₁₂ increased linearly in a dose-dependent manner, and highly correlated with vitamin intake (r=0.959 for vitamin B₁, r=0.927 for vitamin B₂, r=0.965 for vitamin B₆, r=0.957 for niacin, r=0.934 for pantothenic acid, r=0.907 for folic acid, r=0.962 for biotin, and r=0.952 for vitamin C). These results suggest that measuring urinary water-soluble vitamins and their metabolite levels can be used as good nutritional markers for assessing vitamin intakes.

Key Words  biomarker, human, urine, vitamin

A nutritional marker can be an indicator of nutritional status with respect to intake or metabolism of dietary constituents. Nutritional markers can be designated into one or more of three categories, 1) a means of validation of dietary instruments, 2) surrogate indicators of dietary intakes, or 3) integrated measures of nutritional status for a nutrient (1). Nutritional markers may be interpreted more broadly as a biological consequence of dietary intake or dietary patterns, and contribute to setting recommendations, tolerable levels and guidelines. Recent validation studies have developed the urinary compounds as nutritional markers to estimate nutrient intakes. For example, 24-h urinary nitrogen has been established as a marker for protein intake (2), the same as urinary potassium for energy and potassium intake (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 sites and then excreted to urine. Urinary water-soluble vitamins or their metabolites decrease markedly as vitamin status declines, and they are affected by recent dietary intake. Urinary excretion of water-soluble vitamins such as thiamin, riboflavin and niacin has been used for setting Dietary Reference Intakes (DRIs) in the USA and Japan (5, 6). However, only a single study investigated urinary vitamins as a possible marker for intake. Individuals’ 30-d means of thiamin intake are highly correlated with their mean 24-h urine thiamin levels under strictly controlled conditions, showing 24-h urinary thiamin as a useful marker for thiamin intake under strictly controlled conditions (7). Although pharmacological doses of water-soluble vitamin intake such as vitamin B₂ (8), nicotinamide (9) and biotin (10) dramatically increase urinary vitamin levels, few studies have investigated the relationship between several oral doses and dietary intake and urinary excretion of vitamin C, to the best of our knowledge (11, 12).

To determine whether urinary levels of water-soluble vitamins and their metabolites can be used as possible markers for estimating their intakes, six female Japanese college students were given a standard Japanese diet with or without a 1-, 3- and 6-fold vitamin mixture based on Dietary Reference Intakes (DRIs) for Japanese. The 24-h urinary excretion of water-soluble vitamins and their metabolites was measured, and the relationships between vitamin oral dose and urinary excretion were determined. This is the first report clearly to show that 24-h urinary vitamins and their metabolite levels were correlated to their intakes, and can be used as nutritional markers for their intakes.

SUBJECTS AND METHODS

Subjects. Six healthy female Japanese college students participated in the present experiment. They did not have regular use of medications or dietary supple-
Table 1. The composition of the diets.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Diet 2</th>
<th>Average</th>
<th>RDA²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy (kcal)</td>
<td>1,708</td>
<td>1,618</td>
<td>1,663</td>
</tr>
<tr>
<td>Protein (g)</td>
<td>68.5</td>
<td>61.5</td>
<td>65</td>
</tr>
<tr>
<td>Fat (g)</td>
<td>50.8</td>
<td>45.1</td>
<td>48.0</td>
</tr>
<tr>
<td>Carbohydrates (g)</td>
<td>236</td>
<td>237</td>
<td>237</td>
</tr>
<tr>
<td>Water-soluble vitamins¹</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin B₁ (mg as thiamin)</td>
<td>0.59</td>
<td>0.46</td>
<td>0.53</td>
</tr>
<tr>
<td>Vitamin B₂ (mg as riboflavin)</td>
<td>0.92</td>
<td>0.82</td>
<td>0.87</td>
</tr>
<tr>
<td>Vitamin B₆ (mg as pyridoxine)</td>
<td>1.24</td>
<td>0.86</td>
<td>1.05</td>
</tr>
<tr>
<td>Vitamin B₁₂ (μg as cyanocobalamin)</td>
<td>7.4</td>
<td>11.3</td>
<td>2.4</td>
</tr>
<tr>
<td>Niacin equivalent¹ (mg)</td>
<td>30.4</td>
<td>24.8</td>
<td>27.6</td>
</tr>
<tr>
<td>Pantothenic acid (mg)</td>
<td>9.3</td>
<td>9.3</td>
<td>9.3</td>
</tr>
<tr>
<td>Folic acid (μg as pteroylmonoglutamic acid)</td>
<td>230</td>
<td>282</td>
<td>256</td>
</tr>
<tr>
<td>Biotin (μg)</td>
<td>67</td>
<td>53</td>
<td>60</td>
</tr>
<tr>
<td>Vitamin C (mg as L-ascorbic acid)</td>
<td>118</td>
<td>112</td>
<td>115</td>
</tr>
</tbody>
</table>

¹ Water-soluble vitamins except for vitamin B₁₂ are measured. Other nutrients are calculated by using the Standard Tables of Food Composition in Japan (15).
² The niacin equivalent intake was calculated as follows: the average tryptophan content in food protein is 1.1% and the 1/60 (on a weight basis) of tryptophan taken was converted into niacin in the body.
³ The Recommended Dietary Allowance (RDA) for vitamin B₁ is 0.42 mg/1,000 kcal as thiamin, vitamin B₂ is 0.60 mg/1,000 kcal, vitamin B₆ is 0.023 mg/g protein, niacin is 5.8 mg NE/1,000 kcal, folic acid is 240 μg/d and vitamin C is 100 mg/d for Japanese adults, and the Adequate Intake for pantothenic acid is 5 mg/d and biotin is 45 μg/d for Japanese adult women (6).

The subjects consumed Diet 1 on days 1 and 3 each week, and Diet 2 on days 2 and 4.

ments, or habitual alcohol or cigarette consumption. Their age, body weight, height and body mass index (mean±SD) are 21.0±0.0 years old, 161.7±1.7 cm, 51.2±2.8 kg and 19.6±1.2, respectively. This study was reviewed and approved by The Ethical Committee of the National Institute of Health and Nutrition (Tokyo, Japan).

Chemicals. Thiamin hydrochloride, riboflavine, pyridoxine hydrochloride, nicotinamide, calcium pantothenate, pteroylmonoglutamic acid (folic acid), D(+)-biotin, L(+)-ascorbic acid were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). 4-Pyradoxic acid (4-PIC) was manufactured by ICN Pharmaceuticals (Costa Mesa, CA, USA) and obtained through Wako Pure Chemical Industries. N⁴-Methyl nicotinamide (NMA) chloride was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N⁴-Methyl-2-pyridone-5-carboxamide (2-Py) and N⁴-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized (13, 14). All the other chemicals used were of the highest purity available from commercial sources.

Diet. Two kinds of meals were given to the subjects. Diet 1 consisted of bread, margarine, ham, tomato, jelly and milk as breakfast; rice, miso-soup, Hamburg steak, cabbage, boiled spinach and Japanese tea as lunch; and rice, raw skipjack, laver, pan-fried vegetables and Japanese tea as dinner. Diet 2 consisted of bread, margarine, ham, tomato, jelly and milk as breakfast; rice, miso-soup, broiled chicken, cabbage, simmered hijiki and Japanese tea as lunch; and rice, raw scallop, laver, pan-fried vegetables and Japanese tea as dinner. The nutrient elements are shown in Table 1. The subjects consumed Diet 1 on days 1 and 3 each week, and Diet 2 on days 2 and 4.
Diet 1 and 2 were homogenized in water. Vitamin B1 was determined by the HPLC method (converted to lumiflavin by photolysis, and then determined by the HPLC method). Reduced and oxidized ascorbic acid, and 2,3-diketogluconic acid, were determined by the microbioassay method using \textit{Lactobacillus} \textit{plantarum} strain 4228 ATCC 9080 (24). NAD and NADP in the diets were converted to nicotinamide by autoclave, and total nicotinamide was determined by the HPLC method (13). Bound pantothenic acid such as CoA and pantetheine in the diets was digested to free form by alkaline phosphatase and pigeon liver amidase, and total pantothenic acid was determined by the microbioassay method using \textit{Lactobacillus plantarum} ATCC 8014 (20). Folates in the diets were digested to pteroylmonoglutamic acid by conjugase and protease, and pteroylmonoglutamic acid as total folic acid was determined by the microbioassay method using \textit{Lactobacillus casei} ATCC 2733 (21). Bound biotin in the diet was converted to free form by autoclave under acidic conditions, and total biotin was determined by the microbioassay method using \textit{Lactobacillus plantarum} ATCC 8014 (22). Reduced and oxidized ascorbic acid, and 2,3-diketogluconic acid in the diets were determined by the HPLC method (23).

For analysis of water-soluble vitamins in the diets, Diet 1 and 2 were homogenized in water. Vitamin B1, as sum of thiamin, TMP, TDP and TTP in the diets was determined by the HPLC-post labeled fluorescence method (16). Riboflavin, FMN and FAD in the diets were converted to lumiflavin by photolysis, and then determined by the HPLC method (17). Vitamin B6, vitamer in \textit{V. mix. A} and \textit{V. mix. B}, was determined by the microbioassay method using \textit{Lactobacillus casei} ATCC 8014 (24). Reduced and oxidized ascorbic acid, and 2,3-diketogluconic acid in the diets were determined by the HPLC method (23).

**RESULTS**

Vitamin B1

The urinary excretion of thiamin in the first week was $0.288 \pm 0.074 \mu\text{mol/d}$ to 0.53 mg/d (2.0 \mu\text{mol/d}) of thiamin intake (mean $\pm$ SD, $n=6$), and the level increased linearly until the fourth week taking 4.42 mg/d (22.4 \mu\text{mol/d}) (Fig. 1A). The correlation between urinary and oral thiamin was significantly high ($y=0.281x-0.514$, $r=0.959$; $p=0.0001$). The urinary recovery of thiamin (mean $\pm$ SD, $n=6$) was...
oral niacin was significantly high (relation between urinary nicotinamide metabolites and taking 95.0 mg NE/d (779 and the level increased linearly until the fourth week 5.5, 49.9/H11006. The urinary recovery of 4-PIC was 55.4/H11006. 1.05 mg/d (6.2/H11006 of  riboflavin intake, and the level increased linearly until the fourth week taking 6.61 mg/d (17.6/H11006 until the fourth week taking 14.6/H11006 and fourth week, respectively. Vitamin B2
The urinary excretion of riboflavin in the first week was 0.283±0.073 μmol/d to 0.87 mg/d (2.3 μmol/d) of riboflavin intake, and the level increased linearly until the fourth week taking 6.61 mg/d (17.6 μmol/d) (Fig. 1B). The correlation between urinary and oral riboflavin was significantly high (y=0.342x−0.901, r=0.926; p<0.0001). The urinary recovery of riboflavin was 12.3±3.2, 16.1±3.5, 16.4±5.0 and 31.6±6.9% in the first, second, third and fourth week, respectively. Vitamin B6
The urinary excretion of 4-PIC, a metabolite of vitamin B6, in the first week was 3.44±0.41 μmol/d to 1.05 mg/d (6.2 μmol/d) of pyridoxine intake, and the level increased linearly until the fourth week taking 7.66 mg/d (45.2 μmol/d) (Fig. 1C). The correlation between urinary 4-PIC and oral pyridoxine was significantly high (y=0.611x−0.59, r=0.966; p<0.0001). The urinary recovery of 4-PIC was 55.4±6.6, 65.1±5.5, 49.9±14.3 and 61.9±5.9% in the first, second, third and fourth week, respectively. Niacin
The urinary excretion of nicotinamide metabolites in the first week was 85.6±10.8 μmol/d to 27.6 mg niacin equivalents (NE)/d (226 μmol/d) of niacin intake, and the level increased linearly until the fourth week taking 95.0 mg NE/d (779 μmol/d) (Fig. 1D). The correlation between urinary nicotinamide metabolites and oral niacin was significantly high (y=0.852x−125.9, r= 0.957; p<0.0001). The urinary recovery of nicotinamide metabolites was 37.9±4.8, 43.6±6.2, 53.4±13.6 and 71.9±10.1% in the first, second, third and fourth week, respectively. Pantothenic acid
The urinary excretion of pantothenic acid in the first week was 14.6±2.0 μmol/d to 9.3 mg/d (42 μmol/d) of pantothenic acid intake, and the level increased linearly until the fourth week taking 40.7 mg/d (186 μmol/d) (Fig. 1E). The correlation between urinary and oral pantothenic acid was significantly high (y=0.378x−1.6, r=0.951; p<0.0001). The urinary recovery of pantothenic acid was 34.4±4.8, 39.1±6.1, 30.5±6.7 and 38.4±5.9% in the first, second, third and fourth week, respectively. Folate
The urinary excretion of folic acid in the first week was 0.022±0.009 μmol/d to 256 μg/d (0.58 μmol/d) of folate intake, and the level increased linearly until the fourth week taking 1.60 mg/d (3.62 μmol/d) (Fig. 1F). The correlation between urinary folic acid and oral folate was significantly high (y=0.277x−0.235, r= 0.907; p<0.0001). The urinary recovery of folic acid was 3.8±1.5, 5.1±1.5, 5.5±3.3 and 22.9±6.5% in the first, second, third and fourth week, respectively. Biotin
The urinary excretion of biotin in the first week was 74.5±12.0 nmol/d to 60 μg/d (246 nmol/d) of biotin intake, and the level increased linearly until the fourth week taking 242 μg/d (990 nmol/d) (Fig. 1G). The correlation between urinary and oral biotin was significantly high (y=0.316x+8.2, r=0.962; p<0.0001). The urinary recovery of biotin was 30.3±4.9, 35.6±4.8, 35.1±6.4 and 31.8±3.0% in the first, second,
third and fourth week, respectively.

**Vitamin C**

The urinary excretion of ascorbic acid and 2,3-diketogluconic acid in the first week was 0.29±0.08 mmol/d to 115 mg/d (0.65 mmol/d) of ascorbic acid intake, and the level increased linearly until the fourth week taking 715 mg/d (4.06 mmol/d) (Fig. 1H). The correlation between urinary ascorbic acid and 2,3-diketogluconic acid and oral ascorbic acid was significantly high ($r=1.26x-0.73, p<0.0001$). The urinary recovery of ascorbic acid and 2,3-diketogluconic acid was 45.2±12.6, 57.3±9.6, 83.6±20.4 and 111.2±23.5% in the first, second, third and fourth week, respectively.

**DISCUSSION**

To investigate the relationship between oral dose and urinary excretion of water-soluble vitamins and their metabolites, young Japanese women were administered a diet with or without varying amounts of the vitamins for 1 wk. Amount of the nutrients including water-soluble vitamins in the diets were close to RDA in DRIs (5, 6) and previous dietary assessment in free-living Japanese young women (25). The concentrations of all eight water-soluble vitamins and their metabolites in 24-h urine samples increased linearly in a dose-dependent manner, and strongly correlated with their intakes. These findings show that water-soluble vitamins and their metabolite levels in 24-h urine reflect the vitamin intakes under strictly controlled conditions, and suggest that vitamin intakes can be estimated from 24-h urinary vitamins and their metabolite contents.

In the present study, the correlations between urinary levels and their intakes for vitamin B$_2$ and folic acid were lower than those for other vitamins tested. The urinary riboflavin level linearly increased in a dose-dependent manner at 0.256 to 0.786 mg (0.58 to 2.7 μmol) vitamin B$_2$ intake, and then the level dramatically increased when the subjects took 6.61 mg (17.6 μmol) vitamin B$_2$. The urinary folic acid contents also showed a similar pattern to riboflavin: the contents linearly increased at 0.256 to 0.786 mg (0.58 to 1.78 μmol) folate intakes, and then dramatically increased at 1.60 mg (3.62 μmol) intake. The urinary vitamin levels may be affected by several factors such as absorption in the digestive tract, storage in the tissue, energy expenditure, tissue turnover and reabsorption in the kidney. However, no report has disclosed whether these factors change the urinary excretions of vitamins when humans take vitamins at the range used in the present study. Investigation of relationships for oral dose to urinary, blood and stored vitamin levels may explain what the dramatic increases in urinary riboflavin and folic acid mean.

We previously reported the levels of water-soluble vitamins and their metabolites in 24-h urine samples from young Japanese women consuming a semi-purified diet with a vitamin mixture for 7 d (26). The levels were 0.665±0.114 μmol thiamin/d to 0.71 mg/d (2.7 μmol/d) thiamin intake; 0.580±0.145 μmol ribo-
Some vitamin-vitamin interactions are well known for accumulating homocysteiny by a folate, vitamin B\textsubscript{6}, or vitamin B\textsubscript{12} deficiency, and requiring vitamin B\textsubscript{3} and vitamin B\textsubscript{6} for conversion of nicotinamide from tryptophan (36). These vitamin-vitamin interactions can be seen in some vitamin deficiencies, and little is known about how administrations of large amounts of water-soluble vitamins affect other vitamins’ metabolism. However, 1 g of ascorbic acid administration for 7 d does not alter plasma pyridoxal 5’-phosphate level or urinary excretion of 4-PI (37). We previously reported that 150 mg (1.22 mmol) of nicotinamide administration increased nicotinamide metabolites approximately 800 \textmu mol in 24 h urine (9). Chronic administration of a multivitamin supplement containing 150 mg of nicotinamide (1.22 mmol/d), 5.45 mg of fursulthiamine hydrochloride (12.5 \textmu mol/d), 3.5 mg of riboflavin (9.3 \textmu mol/d), 4.5 mg of pyridoxine hydrochloride (22 \textmu mol/d), 6.5 \mu g of cyanocobalamin (4.8 mmol/d), 15 mg of calcium pantothenate (63 \textmu mol/d as pantothenic acid) and 125 mg of ascorbic acid (0.71 mmol/d) increased nicotinamide metabolites approximately 700 \textmu mol in 24 h urine, showing that these doses of vitamin intake did not affect nicotinamide metabolism (38). Intestinal cells transport biotin, pantothentic acid and lipoate via a sodium-dependent multivitamin transporter (SMVT), and biotin uptake is inhibited by pantothentic acid at a micromolar range in vitro (39). This SMVT system is the major biotin uptake system in the intestinal cells, and physiological (nanomolar) concentrations of pantothenic acid have no effect on the biotin uptake in vitro (40). These reports and the present results that urinary excretions of biotin and pantothentic acid linearly or more increased with administration of vitamins mixtures suggest that biotin and pantothentic acid do not inhibit their absorption in the present study. Moreover, urinary excretions of other vitamins or their metabolites increased linearly in a dose-dependent manner, suggesting no major effect on water soluble vitamin metabolism or absorption because of vitamin administration.

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