*Biosci. Biotechnol. Biochem.*, **75** (1), 185–187, 2011

*Communication*

# Fluorometric Determination of 2-Oxoadipic Acid, a Common Metabolite of Tryptophan and Lysine, by High-Performance Liquid Chromatography with Pre-Chemical Derivatization

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Received October 8, 2010; Accepted December 8, 2010; Online Publication, January 7, 2011
[doi:10.1271/bbb.100723]

**2-Oxoadipic acid, a key metabolite of tryptophan and lysine, reacted with 1,2-diamino-4,5-methylenebenzene in an acidic solution to produce a fluorescent derivative. The reaction product was separated using a Tosoh ODS-80Ts column with 20 mmol/L of $KH_2PO_4$–$K_2HPO_4$ buffer (pH 7.0) containing 26% methanol at a flow rate 0.8 mL/min. The excitation wavelength of detection was 367 nm, and the emission wavelength was 446 nm. The limit of quantification was 1 pmol per injection, sufficiently sensitive for the determination of 2-oxoadipic acid in human and experimental animal urine.**

**Key words:** 2-oxoadipic acid; 1,2-diamino-4,5-methylenebenzene; tryptophan; lysine; urine

The nutritional factors affecting the metabolism of tryptophan-niacin have been studied,[1–3] and we have confirmed that the reaction of ACMS to α-aminomuconate-ε-semialdehyde, the branch point of tryptophan catabolism that leads to niacin production, is inversely related to the amount of niacin synthesis that occurs in the liver cell.[4,5]

2-OAA is a common catabolic metabolite of the essential amino acids tryptophan and lysine. We are interested in 2-OAA because excess dietary lysine might lead to a build-up of 2-OAA, which in turn would inhibit the catabolism of ACMS to 2-OAA, thus shunting ACMS (from tryptophan) toward niacin biosynthesis. In addition, we are interested in the relationship between dietary intake of tryptophan and/or lysine and the formation of 2-OAA. We have confirmed that urinary excretion of tryptophan catabolites such as kynurenic acid and xanthurenic acid reflect the intake of tryptophan.[6] Hence, we did quantitative analyses of 2-OAA, and learned that 2-oxoadipic aciduria, a rare congenital condition, was present, as first reported in 1975 by Przyrember *et al.*[7] Since that report, there have been seven reported cases,[8] found mainly by employing organic solvent extraction and GC-MS techniques,[9] but the GC-MS technique is not a practical measurement method for the detection of 2-OAA.

Nakamura *et al.*[10] reported an attractive chemical derivatization method for 2-oxo acids with DMB. Although no examination of the reaction between 2-OAA and DMB was described in their report, we succeeded in producing a fluorescent compound by reacting 2-OAA with DMB, and we developed a separation method based on this fluorescent compound by HPLC.

Here, we describe a new assay for measuring 2-OAA in the urine by HPLC with pre-chemical derivatization.

2-OAA was purchased from Sigma-Aldrich Chemicals (St. Louis, MO). DMB was purchased from Dojinkagaku Labs (Kumamoto, Japan). All the other chemicals and solvents used were of reagent grade.

A DMB solution was made by mixing the following in order: 8.7 mL of $H_2O$, 0.049 g of sodium hydrosulfite, 0.7 mL of 2-mercaptoethanol, 0.58 mL of concentrated HCl, and 0.016 g of DMB. The DMB solution was usable for at least 1 month when stored in a refrigerator. 2-Mercaptoethanol and sodium hydrosulfite were added to stabilize DMB during the reaction.

The 2-OAA was derivatized by the method of Nakamura *et al.*[10] The reaction is shown in Fig. 1. A total of 0.1 mL of the DMB solution was added to 0.1 mL of the 2-OAA solution or to a urine sample suitably diluted in a microtube with a sealed cap. The reaction was carried out by immersing the microtube in a boiling water bath for 45 min, and the microtube was cooled in ice water for at least 5 min. The resulting reaction mixture was filtered through a 0.45-μm filter (Millipore, Bedford, MA), and the filtrate (5 μL) was injected directly into the HPLC system. The fluorescent compound in the final mixtures was stable for at least 24 h when exposed to room light at room temperature.

Separation of the fluorescent product of 2-OAA in urine was carried out using a Tosoh ODS-80Ts (4.6 i.d. × 250 mm) column (Tosoh, Tokyo). The mobile phase consisted of a 20-mmol/L $KH_2PO_4$-$K_2HPO_4$ buffer (pH 7.0) containing 26% methanol. A flow rate of 0.8 mL/min was used, and the column temperature was maintained at 40 °C. Fluorometric detection was done at an excitation wavelength of 367 nm and an emission wavelength of 446 nm.

Stock solutions of 2-OAA were made of concentrations of up to 100 μmol/L with water, and were stored at −20 °C. Working standard solutions were diluted from the stock solutions to produce a series of concentrations (0.2 μmol/L, 0.3 μmol/L, 0.5 μmol/L, 1.0 μmol/L, 3.0 μmol/L, and 5.0 μmol/L). A total of 0.1 mL of each

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*Abbreviations*: 2-OAA, 2-oxoadipic acid; DMB, 1,2-diamino-4,5-methylenebenzene; ACMS, α-amino-β-carboxymuconate-ε-semialdehyde

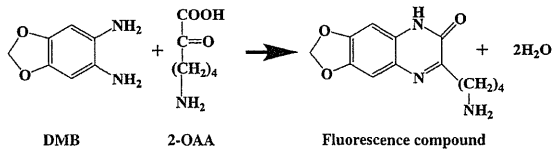


**Fig. 1.** Reaction of 2-OAA with DMB.

concentration was then reacted with 0.1 mL of the DMB solution, 5 μL of the reacted mixture containing 0.5, 0.75, 1.25, 2.5, 7.5, and 12.5 pmol of 2-OAA was injected into the HPLC system, and fluorescent intensities corresponding to 2-OAA were measured.

The linearity of the calibration curve was determined by plotting the peak areas ($y$) of the fluorescent intensity against the standard 2-OAA concentrations ($x$). The correlation coefficient was greater than 0.99, confirming that the calibration curve was linear over a concentration range of 0.5 to 12.5 pmol per injection for the 2-OAA standard. The typical standard curve can be represented by $y$ (fmol) $= 21 + 118x$ ($r = 0.999$). The limit of detection was 0.5 pmol (approximately 80 pg) per injection at a signal-to-noise ratio of 5:1. The limit of quantification was 1 pmol (approximately 160 pg) per injection, sufficiently sensitive for the determination of 2-OAA in human, rat, and mouse urine.

Spot urine samples collected from three healthy young Japanese women was used for validation of the method. The three urine samples were mixed. We removed 9 mL from the mixed urine sample, and then 1 mL of 1 mol/L HCl was added to the urine samples to stabilize 2-OAA. The acidified sample was compared with the optimal basal conditions in the HPLC system, which served as the quality control (QC) sample.

Short-term stability was determined by maintaining the QC urine at room temperature for 24 h, middle-term stability was evaluated by storing the QC urine at 4 °C for 7 d, and the long-term stability of 2-OAA was assessed at −20 °C for 30 d. The freeze-thaw stability of 2-OAA was determined over three cycles of thawing at 4 °C for 12 h and refreezing for 12 h. For each storage condition, five replicates were analyzed in each batch. The 2-OAA concentration after each storage period was related to the initial concentration determined for the samples, which were freshly prepared and processed immediately. The range of change was calculated by the following equation:

$$\text{Range of change (\%)} = (\text{concentration under each condition} / \text{concentration of fresh preparation}) \times 100.$$

The stability of 2-OAA over the short-term, the long-term, and the freeze-thaw cycles was found to be +2, −3, and −2% change respectively as compared to the value for fresh urine, which was taken to be 100%. Under all conditions, 2-OAA in urine was stable.

Within-run precision was calculated by analyzing five replicates of the QC urine on the same day. Between-run precision was determined by triplicate analysis of the QC urine on three separate occasions, and the value on each occasion was calculated by analyzing five replicates. The coefficient of variation (CV) was used to measure the precision:

$$\text{CV (\%)} = \{\text{standard deviation (SD)/mean}\} \times 100.$$

The CVs of the within- and between-run precision were 0.73% and 0.94% respectively.

Within-run accuracy was measured in different experiments to calculate precision, and was evaluated in the same experiment to ascertain the recovery percentages. Accuracy was expressed as relative error (RE) and determined by the following equation:

$$\text{RE (\%)} = \{(\text{observed concentration} - \text{added concentration}) / \text{added concentration}\} \times 100.$$

The accuracies as shown by RE were 1.4%, −2.1%, and 2.6% at concentrations of 1.25, 3.75, and 6.25 pmol per 5 μL of sample respectively.

These data indicate that the assay was reproducible, accurate, and reliable.

Recovery was calculated using the following formula:

$$\text{Recovery (\%)} = (\text{observed concentration} / \text{added concentration}) \times 100.$$

Three additional concentrations (1.0 μmol/L, 3.0 μmol/L, and 5.0 μmol/L of 2-OAA, which contained 50, 150, and 250 pmol/0.05 mL of standard 2-OAA), were added to 0.05 mL of the QC urine and then reacted with 0.1 mL of the DMB solution. All analyses were performed in triplicate. Recovery was 101 ± 3, 97 ± 1, and 96 ± 3% respectively.

A typical chromatogram of the reference 2-OAA derivative is shown in Fig. 2A, and the 2-OAA derivative eluted at approximately 14.5 min. When standard 2-OAA was reacted with the DMB-free solution (removing only DMB of the DMB solution), the peak was not detected, as shown in Fig. 2E.

Healthy young Japanese women (21–23 years old, n = 14) were recruited for this experiment. A 24-h urine sample was collected from the second passage of urine on the first day to the first passage on the next day. The urine sample volumes were measured, and 1 mL of 1 mol/L HCl was added to 9-mL urine samples to stabilize 2-OAA. The acidified urine samples were stored at −20 °C until needed. This study was reviewed and approved by The Ethical Committee of The University of Shiga Prefecture.

Male rats of the Wistar strain (6 weeks old) and female mice of the ICR strain (6 weeks old) were obtained from CLEA Japan (Tokyo) and immediately placed in individual metabolic cages. The animals were fed *ad libitum* for 21 d on a niacin-free 20% casein diet.[11] Urine samples (24 h; 10:00 AM–10:00 AM) on the last day were collected in amber bottles containing 1 mL of 1 mol/L HCl, and were stored at −20 °C until needed. The care and treatment of the experimental animals conformed to the University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

The chromatograms of derivatized urine sample from a human, a rat, and a mouse are shown in Fig. 2B, C, and D respectively. The 2-OAA derivative in the sample was characterized on the basis of its retention time and the entire excitation and emission spectra between 320 and 500 nm. Figure 2F, G, and H are chromatograms of

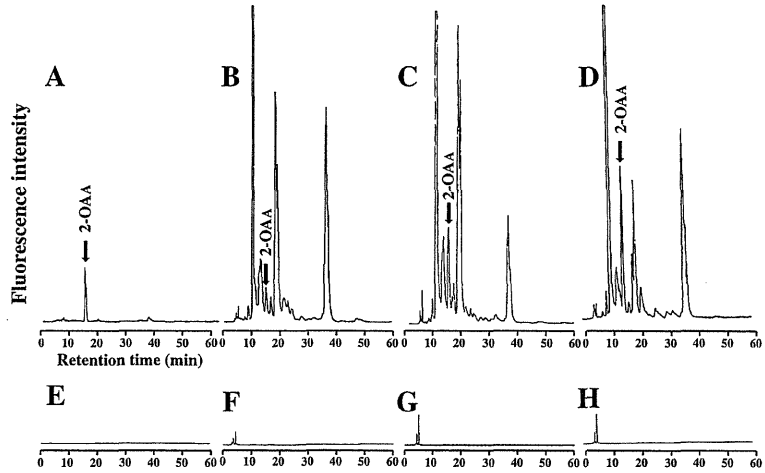


**Fig. 2.** Chromatograms of the 2-OAA Derivatives.

Conditions: column, Tosoh ODS 80 Ts (4.6 i.d. × 250 mm); mobile phase, 20 mmol/L $KH_2PO_4$–$K_2HPO_4$ buffer (pH 7.0) containing 26% methanol; flow rate, 0.8 mL/min; excitation wavelength, 367 nm; emission wavelength, 446 nm; column temperature, 40 °C. The chromatograms shown in upper level are of standard 2-OAA (A) (2.5 pmol/5 μL), a human urine sample (B) (1.1 pmol/5 μL), a rat urine sample (C) (3.6 pmol/5 μL), and a mouse urine sample (D) (6.6 pmol/5 μL) reacted with the DMB solution. The chromatograms in lower level are those of standard 2-OAA (E), a human urine sample (F), a rat urine sample (G), and a mouse urine sample (H) reacted with the DMB-free solution.

a human urine sample (F), a rat urine sample (G), and a mouse urine sample (H) reacted with DMB-free solution. The total HPLC analysis time was approximately 60 min.

There were many reactive compounds in the urine, indicating that the urine samples also contained several measurable 2-oxo acids. In future, we intend to develop simultaneous determination of the other 2-oxo acids in the urine.

The daily urinary excretion levels of 2-OAA in humans, rats, and mice were $14.6 \pm 2.8$ μmol/d (mean ± SEM, $n = 14$), $2.9 \pm 0.8$ μmol/d (mean ± SEM, $n = 5$), and $0.7 \pm 0.1$ μmol/d (mean ± SEM, $n = 5$) respectively. This is the first report on the urinary excretion of 2-OAA in healthy (non 2-OAA aciduria) people, rats, and mice.

The present method can be applied to study tryptophan and lysine metabolism.

## Acknowledgments

This report is part of a larger project: Development of an index of "metabolic upper intake level" instead of "tolerable upper intake level" of tryptophan for humans (principal investigator, Katsumi Shibata). The investigation was supported by a grant from the International Council on Amino Acid Science Research Funding.

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*Public Health Nutrition: 14(2), 327–333* doi:10.1017/S1368980010001904

# Twenty-four-hour urinary water-soluble vitamin levels correlate with their intakes in free-living Japanese schoolchildren

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**Submitted 15 December 2009: Accepted 10 May 2010: First published online 25 June 2010**

## Abstract

*Objective:* To examine the association between 24 h urinary water-soluble vitamin levels and their intakes in free-living Japanese schoolchildren.
*Design:* All foods consumed for four consecutive days were recorded accurately by a weighed food record. A single 24 h urine sample was collected on the fourth day, and the urinary levels of water-soluble vitamins were measured.
*Setting:* An elementary school in Inazawa City, Japan.
*Subjects:* A total of 114 healthy, free-living, Japanese elementary-school children aged 10–12 years.
*Results:* The urinary level of each water-soluble vitamin was correlated positively to its mean intake in the past 2–4 d (vitamin $B_1$: $r = 0{\cdot}42$, $P < 0{\cdot}001$; vitamin $B_2$: $r = 0{\cdot}43$, $P < 0{\cdot}001$; vitamin $B_6$: $r = 0{\cdot}49$, $P < 0{\cdot}001$; niacin: $r = 0{\cdot}32$, $P < 0{\cdot}001$; niacin equivalents: $r = 0{\cdot}32$, $P < 0{\cdot}001$; pantothenic acid: $r = 0{\cdot}32$, $P < 0{\cdot}001$; folic acid: $r = 0{\cdot}27$, $P < 0{\cdot}01$; vitamin C: $r = 0{\cdot}39$, $P < 0.001$), except for vitamin $B_{12}$ ($r = 0{\cdot}10$, $P =$ NS). Estimated mean intakes of water-soluble vitamins calculated using urinary levels and recovery rates were 97–102 % of their 3 d mean intake, except for vitamin $B_{12}$ (79 %).
*Conclusions:* The results show that urinary levels of water-soluble vitamins, except for vitamin $B_{12}$, reflected their recent intakes in free-living Japanese schoolchildren and could be used as a potential biomarker to estimate mean vitamin intake.

**Keywords**
Urinary water-soluble vitamin
Biomarker
Free-living
Japanese schoolchildren

Since vitamin deficiencies cause various disorders in the growth of schoolchildren, a method to evaluate vitamin status easily and accurately is desired for early screening at a primary preventive stage. Methods using biomarkers for assessing vitamin intakes offer an effective approach to evaluate vitamin status in individuals. Many preceding studies have investigated urinary excretion as a biomarker for vitamin intake[1–3]. We have also reported recently that 24 h urinary levels of water-soluble vitamins correlate highly with their intakes for Japanese college students in a strictly controlled environment[4,5]. Performing a study under a free-living environment without any interventions is the next step to confirm the applicability of the biomarker method. In the present study, we examined the association between 24 h urinary excretion of water-soluble vitamins and their dietary intakes for free-living schoolchildren to confirm the validity of the findings obtained in the controlled environment.

To capture dietary intake and calculate nutrients under a free-living environment, we used a weighed food record for four consecutive days. Although a weighed food record can provide relatively precise information regarding dietary intake compared with other dietary assessment methods[6], it is difficult for schoolchildren to complete a weighed food record without support. Few studies have reported this kind of assessment for free-living schoolchildren[7], while many studies have reported using a 24 h recall[8], a dietary diary[9] or an FFQ[10]. To overcome the difficulty of using a weighed food record for schoolchildren, we formed a close and cooperative relationship not only with the children but also their parents and teachers in the target elementary school before starting the study, through supporting the prolonged dietary education programme provided by the school board.

## Methods

### *Participants*

A total of 132 healthy, free-living schoolchildren aged 10–12 years voluntarily participated in the present study.

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The purpose and protocol were explained to all participants, as well as their parents, before joining the study, and written informed consent was obtained from each parent because all participants were less than 20 years old. We excluded participants diagnosed with the common cold or influenza, and those who had taken multivitamin supplements at least once during the previous month. In addition, we excluded participants whose 24 h urine collection or dietary records were considered incomplete, with a collection time outside the range of 22–26 h, urine volume <250 ml, creatinine excretion in relation to body weight outside the range of 10·8–25·2 mg/kg[11,12] or extremely low or high energy intake (<2092 or >16 736 kJ/d)[13]. After these screenings, 114 schoolchildren (sixty-seven boys and forty-seven girls) were found to be eligible. The study was reviewed and approved by The Ethical Committee of The University of Shiga Prefecture.

### *Dietary records*

This was a 4 d dietary assessment in which the participants were living freely and consuming their normal diet. The assessment was performed at one of the elementary schools in Inazawa City (population >130 000) in Aichi Prefecture, Japan, in June 2007 and June 2008. The first day (Monday) of the experimental period was defined as day 1, the second day as day 2, the third day as day 3, and the fourth day as day 4. All foods consumed during the 4 d period were recorded using a weighed food record[14]. A digital cooking scale (1 g unit; Tanita Inc., Tokyo, Japan), a set of dietary record forms, a dietary record manual and a disposable camera were distributed to the participants in advance. Upon entry in the dietary record, the status of food at oral intake was identified as 'raw', 'boiled', 'cooked', 'the presence of skin', 'a part of cooking ingredients' or 'with or without seasoning', and coded according to the fifth revised and enlarged edition of the *Standard Tables of Food Composition in Japan*[15]. The participants with support from their parents took photographs with the disposable camera of the dishes before and after eating. Several experienced dietitians used the photographs to check the records, asking participants or their parents to resolve any discrepancies or to give further information when needed. The food that remained after eating was measured with a digital scale and was deducted from the dietary record. For school meals, the registered dietitians completed the records on behalf of the participants. Nutrient and energy intakes were calculated using the SAS statistical software package version 6·12 (SAS Institute Inc., Cary, NC, USA), based on the current *Standard Tables of Food Composition in Japan*[15]. For vitamins, the intakes of eight water-soluble vitamins –vitamins $B_1$, $B_2$, $B_6$, $B_{12}$, niacin, pantothenic acid, folic acid and vitamin C – were calculated, except for biotin which is not designated in the current *Standard Tables of Food Composition in Japan*. Since niacin is synthesized from tryptophan, the amount of niacin equivalents was handled separately from niacin. Since 1 mg nicotinamide is synthesized from 60 mg tryptophan[16], niacin equivalents was calculated as the sum of niacin and 1/60 tryptophan intakes. For calculating mean vitamin intakes, the 2 d mean intake corresponds to average intakes on days 3 and 4. Similarly, the 3 d mean intake corresponds to average intakes on days 2–4, and the 4 d mean intake corresponds to average intakes on days 1–4.

### *24 h urine sampling*

A single 24 h urine sample was collected on the fourth day to measure urinary levels of water-soluble vitamins and their metabolites. It was collected from the second passage of urine on the fourth day to the first passage on the fifth day. The participants were asked to record all the times of urination on the sheet. After the total urine sample was collected, the volume was measured. Aliquots of the urine were stabilized to avoid destruction of water-soluble vitamins and their metabolites, and then stored at −20°C until analysis.

### *Urinalysis*

Urinary thiamine was determined by post-HPLC labelled fluorescence[17]. Urinary riboflavin was determined by HPLC[18]. Urinary vitamin $B_6$ metabolite, 4-pyridoxic acid, was determined by HPLC[19]. To measure urinary vitamin $B_{12}$, urine samples were added to 0·2-mM acetate buffer (pH 4·8), vitamin $B_{12}$ was converted to cyanocobalamin by boiling for 30 min with 0·0006 % w/w potassium cyanide at acidic pH, and cyanocobalamin was determined by a microbioassay using *Lactobacillus leichmanii* ATCC 7830[20]. Urinary $N^1$-methyl-2-pyridone-5-carboxamide and $N^1$-methyl-4-pyridone-3-carboxamide[21] and $N^1$-methylnicotinamide[22] were determined by HPLC, and the sum of these compounds was determined as nicotinamide metabolites. Urinary pantothenic acid was determined by a microbioassay using *Lactobacillus plantarum* ATCC 8014[23]. Urinary folic acid was determined by a microbioassay using *Lactobacillus casei* ATCC 2733[24]. Urinary reduced and oxidized ascorbic acid and 2,3-diketogluconic acid were determined by HPLC[25].

### *Statistical analysis*

To exclude extraordinarily abnormal urinary vitamin levels which might be caused by taking unexpected fortified foods, participants in the upper 5 % limit in terms of urinary excretion for each vitamin were removed from the 114 eligible participants, and a total of 108 samples were identified to be valid for data analysis for each water-soluble vitamin. Similar to a previous free-living study[2], males and females were not separated for analysis. The SPSS for Windows statistical software package version 16 (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. Values are presented as means and standard deviations. Since measurements of urinary and dietary water-soluble vitamins were not distributed normally, the data were converted logarithmically. Pearson correlation

**Table 1** Characteristics of the participants: 114 eligible Japanese elementary-school children aged 10–12 years

| | Total (*n* 114) | | Boys (*n* 67) | | Girls (*n* 47) | |
|---|---|---|---|---|---|---|
| Variable | Mean | SD | Mean | SD | Mean | SD |
| Anthropometric variables | | | | | | |
| Age (years) | 10·8 | 0·7 | 10·7 | 0·7 | 11·0 | 0·7 |
| Body height (cm) | 144·0 | 7·7 | 142·2 | 7·7 | 146·5 | 7·0 |
| Body weight (kg) | 36·7 | 8·3 | 34·6 | 7·2 | 39·8 | 8·9 |
| Rohrer index ($kg/cm^3 \times 10^7$) | 122·0 | 17·9 | 119·3 | 15·9 | 125·7 | 20·1 |
| Obesity index (%) | −4·01 | 3·8 | −6·5 | 13·3 | 0·4 | 13·8 |
| Dietary intake† | | | | | | |
| Total energy (kJ/d) | 8489 | 1298 | 8665 | 1409 | 8238 | 1086 |
| Protein (% of energy) | 14·9 | 2·5 | 14·9 | 2·6 | 14·8 | 2·1 |
| Fat (% of energy) | 29·0 | 5·8 | 29·1 | 6·0 | 28·8 | 5·5 |
| Carbohydrate (% of energy) | 54·8 | 8·7 | 54·7 | 9·3 | 55·1 | 7·7 |
| % Energy intake‡ | | | | | | |
| Breakfast | 21·3 | | 21·7 | | 20·8 | |
| Lunch | 32·7 | | 32·1 | | 33·6 | |
| Supper | 31·1 | | 31·4 | | 30·8 | |
| Snacks | 14·8 | | 14·8 | | 14·9 | |

†Dietary intake assessed from the consecutive 4 d dietary records.
‡Average starting time of each meal: breakfast, 06.50 hours; lunch, 12.30 hours; supper, 18.40 hours.

coefficients were calculated to determine the association between urinary and dietary measurements, and between dietary and estimated water-soluble vitamin intakes. $P < 0{\cdot}05$ was considered statistically significant. An ANOVA random-effects model was used to quantify inter- and intra-individual CV (%CV), which was used to estimate variability in vitamin intake.

## Results

The characteristics of the 114 eligible participants are presented in Table 1. Since each value was almost the same as those reported for children aged 10–11 years in the *Dietary Reference Intakes for Japanese* in 2005[13], the participants were considered as typical elementary-school children in Japan. During the experimental period, all participants were living freely. Inter- and intra-individual variations in dietary intake of water-soluble vitamins for the consecutive 4 d period are shown in Table 2. For intra-individual variations, %CV was 25–45 %, except for vitamin $B_{12}$ and vitamin C. For inter-individual variations, vitamin $B_1$, vitamin $B_{12}$, folic acid and vitamin C exceeded 50 %.

The correlations between 24 h urinary excretion of water-soluble vitamins and their intakes are shown in Table 3. For all vitamins except for vitamin $B_{12}$, a significant positive correlation was found between urinary excretion and dietary intake on day 4. For all vitamins except for pantothenic acid, the correlations on day 4 were higher than those on other days.

To examine the influence of dietary intake during the past few days on 24 h urinary excretion, we calculated the correlations between 24 h urinary excretions and mean dietary intakes, which are shown in Table 4. For all vitamins except for $B_{12}$, niacin equivalents and folic acid, the correlations between the urinary excretion (column 2 in Table 3) and the 3 d mean intake (column 5 in Table 4) were higher than those based on daily intake shown in Table 3 (columns 6, 9, 12 and 15). Because the most significant correlations were found between the urinary excretion and the 3 d mean intake, recovery rates (column 11 in Table 4) were derived from the urinary excretions (column 2 in Table 3) and the 3 d mean intakes (column 5 in Table 4), which are also shown in Table 4. Estimated mean intakes of water-soluble vitamins (column 13 in Table 4) were calculated using these recovery rates and urinary excretions. Estimated mean intakes, except for vitamin $B_{12}$, niacin equivalents and folic acid, correlated with 3 d mean intakes and were 97–102 % of the 3 d mean intake, except for vitamin $B_{12}$ (79 %).

**Table 2** Inter- and intra-individual variations in the dietary intake of water-soluble vitamins measured for the consecutive 4 d experimental period: eligible Japanese elementary-school children aged 10–12 years

| | %CV (*n* 108)† | |
|---|---|---|
| Vitamin | Inter-individual variations | Intra-individual variations |
| Vitamin $B_1$ | 71·0 | 31·1 |
| Vitamin $B_2$ | 28·8 | 29·5 |
| Vitamin $B_6$ | 5·7 | 32·1 |
| Vitamin $B_{12}$ | 166·8 | 95·0 |
| Niacin | 30·4 | 33·1 |
| Niacin equivalents | 8·8 | 25·2 |
| Pantothenic acid | 42·7 | 25·0 |
| Folic acid | 87·4 | 45·0 |
| Vitamin C | 62·2 | 65·5 |

†A total of 108 samples were valid for data analysis after removing the upper 5 % limit in terms of urinary excretion for each vitamin.

## Discussion

In the present study we found a significant positive correlation between the urinary excretion and the dietary

**Table 3** Measured values for 24 h urinary excretion collected on day 4 and daily vitamin intake for each water-soluble vitamin, and correlation between 24 h urinary excretion and daily vitamin intake (*n* 108), among eligible Japanese elementary-school children aged 10–12 years

| | 24 h urinary vitamin excretion† | | Vitamin intake at day 4 | | | Vitamin intake at day 3 | | | Vitamin intake at day 2 | | | Vitamin intake at day 1 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vitamin | Mean | SD | Mean | SD | *r*‡ | Mean | SD | *r*‡ | Mean | SD | *r*‡ | Mean | SD | *r*‡ |
| Vitamin $B_1$ (μmol/d) | 0·766 | 0·383 | 3·13 | 1·01 | 0·41*** | 2·90 | 0·85 | 0·25** | 2·60 | 0·74 | 0·22* | 2·75 | 0·92 | 0·07 |
| Vitamin $B_2$ (μmol/d) | 0·290 | 0·209 | 3·47 | 0·94 | 0·36*** | 3·75 | 1·13 | 0·36*** | 3·59 | 1·00 | 0·33*** | 3·60 | 1·17 | 0·23* |
| Vitamin $B_6$ (μmol/d) | 2·36 | 0·92 | 5·93 | 1·86 | 0·42*** | 5·96 | 1·65 | 0·32*** | 5·97 | 1·69 | 0·36*** | 6·00 | 2·41 | 0·17 |
| Vitamin $B_{12}$ (nmol/d) | 0·0256 | 0·0147 | 3·15 | 1·97 | 0·18 | 4·85 | 5·93 | 0·14 | 4·76 | 4·29 | −0·02 | 4·64 | 3·37 | 0·11 |
| Niacin (μmol/d) | – | | 97·0 | 32·3 | 0·28*** | 101·7 | 38·2 | 0·11 | 105·3 | 31·3 | 0·21* | 101·4 | 32·5 | 0·23* |
| Niacin equivalents (μmol/d) | 65·6 | 27·6 | 214 | 56 | 0·28** | 218 | 56 | 0·23** | 218 | 52 | 0·16 | 218 | 56 | 0·25** |
| Pantothenic acid (μmol/d) | 11·6 | 5·5 | 27·6 | 6·9 | 0·23* | 30·1 | 7·4 | 0·20* | 27·0 | 6·3 | 0·31*** | 28·7 | 7·8 | 0·25** |
| Folic acid (nmol/d) | 16·8 | 6·6 | 575 | 170 | 0·27** | 615 | 423 | 0·12 | 491 | 123 | 0·18 | 532 | 164 | 0·24* |
| Vitamin C (μmol/d) | 161 | 221 | 477 | 225 | 0·35*** | 448 | 313 | 0·23* | 403 | 289 | 0·26** | 445 | 328 | 0·18 |

†Urinary excretion for each vitamin corresponds to: thiamin for vitamin $B_1$; riboflavin for vitamin $B_2$; 4-pyridoxic acid for vitamin $B_6$; the sum of nicotinamide, $N^1$-methylnicotinamide, $N^1$-methyl-2-pyridone-5-carboxamide and $N^1$-methyl-4-pyridone-3-carboxamide for niacin equivalents; the sum of reduced and oxidized ascorbic acid and 2,3-diketogluconic acid for vitamin C.
‡*r* indicates the correlation between urinary excretion and dietary intake of the vitamin; significance of the correlation: *$P<0·05$, **$P<0·01$, ***$P<0·001$.



**Table 4** Summary of values derived from measured values (daily vitamin intake and 24 h urinary excretion in Table 3), i.e. mean dietary intakes and their correlations with 24 h urinary excretion, recovery rates and estimated mean intakes (*n* 108), among eligible Japanese elementary-school children aged 10–12 years

| | 2 d mean vitamin intake† (day 3–day 4) | | | 3 d mean vitamin intake (day 2–day 4) | | | 4 d mean vitamin intake (day 1–day 4) | | | % Recovery‡ | | Estimated mean vitamin intake§ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vitamin | Mean | SD | *r*‖ | Mean | SD | *r*‖ | Mean | SD | *r*‖ | Mean | SD | Mean | SD | *r*¶‖ | % Ratio†† |
| Vitamin $B_1$ (μmol/d) | 3·02 | 0·77 | 0·42*** | 2·88 | 0·63 | 0·42*** | 2·85 | 0·58 | 0·35*** | 27·6 | 12·2 | 2·83 | 1·42 | 0·37*** | 100 |
| Vitamin $B_2$ (μmol/d) | 3·61 | 0·85 | 0·41*** | 3·60 | 0·79 | 0·43*** | 3·60 | 0·78 | 0·42*** | 7·9 | 5·2 | 3·66 | 2·63 | 0·26** | 102 |
| Vitamin $B_6$ (μmol/d) | 5·94 | 1·41 | 0·45*** | 5·95 | 1·29 | 0·49*** | 5·96 | 1·35 | 0·43*** | 39·8 | 14·0 | 5·90 | 2·30 | 0·41*** | 100 |
| Vitamin $B_{12}$ (nmol/d) | 4·00 | 3·14 | 0·19* | 4·25 | 2·55 | 0·10 | 4·35 | 2·10 | 0·10 | 0·7 | 0·6 | 3·72 | 2·14 | 0·06 | 79 |
| Niacin (μmol/d) | 99·4 | 26·0 | 0·24* | 101·3 | 21·7 | 0·29** | 101·4 | 20·4 | 0·32*** | – | | – | | – | – |
| Niacin equivalents (μmol/d) | 216 | 48 | 0·29** | 217 | 43 | 0·29** | 217 | 39 | 0·32*** | 30·7 | 12·6 | 215 | 91 | 0·20* | 99 |
| Pantothenic acid (μmol/d) | 28·8 | 6·0 | 0·26** | 28·2 | 5·6 | 0·32*** | 28·3 | 5·7 | 0·32*** | 41·4 | 19·5 | 28·1 | 13·3 | 0·27** | 99 |
| Folic acid (nmol/d) | 595 | 236 | 0·23* | 560 | 174 | 0·24* | 553 | 147 | 0·27** | 3·1 | 1·3 | 536 | 211 | 0·09 | 97 |
| Vitamin C (μmol/d) | 462 | 200 | 0·39*** | 442 | 183 | 0·39*** | 443 | 170 | 0·39*** | 36·4 | 50·3 | 447 | 613 | 0·39*** | 100 |

†Mean dietary intake was calculated using daily dietary intake (Table 3).
‡% Recovery rate was derived from 24 h urinary excretion (Table 3)/3 d mean intake ×100.
§Estimated mean intake was calculated using 24 h urinary excretion (Table 3) and recovery rate.
‖*r* indicates the correlation between 24 h urinary excretion (Table 3) and mean intake; significance of the correlation: *$P<0·05$, **$P<0·01$, ***$P<0·001$.
¶*r* indicates the correlation between 3 d mean dietary intake and estimated intake; significance of the correlation: *$P<0·05$, **$P<0·01$, ***$P<0·001$.
††% Ratio indicates the ratio between 3 d mean intake and mean estimated intake.

intake of seven water-soluble vitamins, except for vitamin $B_{12}$, in free-living Japanese schoolchildren aged 10–12 years. The correlation between the urinary excretion and the dietary intake on the same day as urine collection was highest, except for pantothenic acid, compared with the correlations on other days. Moreover, the correlations between the urinary excretion and the mean dietary intakes during the past 2–4 d showed higher correlations, except for vitamin $B_{12}$ and folic acid, than those for daily intakes. These findings show that urinary levels of water-soluble vitamins are affected by not only their dietary intakes on the same day as urine collection, but also their intakes over the past few days.

The earlier intervention study showed extremely high positive correlations between urinary levels of water-soluble vitamins and their intakes[4]. In the earlier study, participants comprised college students and they consumed exactly the same defined diets, with or without synthesized water-soluble vitamin mixtures, for 4 weeks. In the present study, the dietary assessment for schoolchildren using a weighed food record was performed for four consecutive days without intervention. Assuming the dietary assessment protocol in the present study contributed best to reduce the errors in the dietary records, the similar results from the different groups and protocols indicate that the urinary levels of water-soluble vitamins are closely associated with vitamin intakes, and that this is true even for free-living schoolchildren.

Correlation coefficients between the urinary excretions and the 3 d mean intakes ranged from 0·24 to 0·49 with a mean of 0·36, except for vitamin $B_{12}$, which showed a lower level than reported in our earlier study[4]. The considerable inter- and intra-individual variability for vitamin intakes in a free-living environment might affect these modest correlations. In addition, several factors are also known to affect water-soluble vitamin metabolism. For example, carbohydrate and physical activity are known to affect vitamin $B_1$ metabolism[26–28], the bioavailability of pantothenic acid in food is half that of free pantothenic acid[29], and the single-nucleotide polymorphism of the methylenetetrahydrofolate reductase gene affects folic acid metabolism[30]. These factors might also affect the modest correlations.

The dietary habits of the schoolchildren who participated in this study were well disciplined. They had regular breakfast (before 07.00 hours), school lunch (around 12.30 hours) and supper (around 18.40 hours), with few snacks. The daily distributions of energy intakes were 21% at breakfast, 33% at lunch, 31% at supper and 15% for snacks, which is thought to be well balanced compared with that reported in a previous study: 24% at breakfast, 30% at lunch, 23% at supper and 23% for snacks[31]. Fifty-five per cent of energy intake was obtained from carbohydrates, 30% from fats and 15% from protein, which fits with the *Dietary Reference Intakes for Japanese*[13]. These data show that the participants had regular dietary habits with well-balanced nutrition.

In terms of the completeness of the dietary assessment in the present study, there are several limitations of using a weighed food record method. One of the limitations is the reliance on self-report. In the present study, to reduce errors associated with self-report, several dietitians reviewed the collated records along with the photos. Another limitation exists in the present food composition table in Japan. In a dietary assessment for free-living people, potential errors caused by the quality of the food composition table are inevitable, such as defects in food composition. For example, the composition of Japanese tea may vary depending on whether the extract of tea was made personally or whether it was a bottled tea beverage, because the present Japanese food composition table cannot differentiate such products. Such restrictions may lower the accuracy of the data obtained from a weighed food record. However, identifying the food status at oral intake and coding the intake according to the food composition table should contribute to increase the accuracy of the records.

In terms of completeness of 24 h urine collection, we used the INTERMAP criteria[11] as already described. Because the *p*-aminobenzoic acid (PABA) method requires intervention by taking PABA tablets orally and would be difficult for schoolchildren, we did not use that method to avoid any interventions. Because the participants in the present study were well motivated for the study, the proportion of them with incomplete urine samples was presumed to be small[32].

We have recently reported the intra-individual variations of urinary water-soluble vitamins in young Japanese, and our intervention study showed that the collection of 24 h urine samples for 1–5 d was required to estimate those values within 20% of the true mean[33]. Indeed, correlation between the 30 d mean urinary thiamin excretion and 30 d mean thiamin intake was higher than that between daily excretion and daily intake[1]. In the present study, urinary water-soluble vitamins were measured based on a single 24 h urine sample. Thus the urinary vitamin contents have potential for data inaccuracy from variability, and the results should be interpreted cautiously. However, recent findings also suggest that using several days of 24 h urine sample would improve the relationships between urinary excretion and intake of water-soluble vitamins.

A significant correlation was not found between urinary vitamin $B_{12}$ and dietary intake in this or a previous study[4]. This is consistent with studies showing that urinary vitamin $B_{12}$ increased by only 1·5 to 2 times when 1 mg of vitamin $B_{12}$, which is 300 times higher than usual intake, was administered orally, and by 2–3 times when 0·45 mg was injected intramuscularly[34,35]. Foods including vitamin $B_{12}$ were so limited that its intake showed an extremely high inter- and intra-individual variation in the present study.

Estimated mean intakes of water-soluble vitamins calculated using the urinary levels and recovery rates correlated

well with the 3 d mean intakes, except for vitamin $B_{12}$ and folic acid, and the estimated mean intakes agreed exactly with the 3 d mean intakes. These findings suggest that urinary levels of water-soluble vitamins can be used as a biomarker to assess their estimated mean intakes. As training schoolchildren to collect urine samples is easier than completing weighed food records, a nutritional assessment for water-soluble vitamins using urine samples and recovery rates is expected to be one of the applications of the present study.

In conclusion, for free-living Japanese schoolchildren aged 10–12 years, we found that 24 h urinary levels of water-soluble vitamins, except for vitamin $B_{12}$, correlated with their recent intakes, and can be used as a biomarker to assess, compare and validate estimated mean intakes of water-soluble vitamins.

## Acknowledgements

*Source of funding:* This study represents the results of 'Studies on the construction of evidence to revise the Dietary Reference Intake for Japanese people – Elucidation of the balance of micronutrients and major elements' (Principal Investigator: Katsumi Shibata), which was supported by a research grant for Comprehensive Related Diseases from the Ministry of Health, Labour and Welfare of Japan. *Conflict of interest:* The authors have no conflict of interest to declare. *Author responsibilities:* T.T. designed the study, performed experiments, completed the statistical analysis and prepared the manuscript. T.F. helped design the study, performed experiments and assisted with data analysis. S.S. reviewed the study and assisted with data analysis. K.S. contributed to the study design and supervised the study. All authors critically reviewed the manuscript. *Acknowledgements:* We thank all the schoolchildren and their families who supported this assessment. We also thank the teachers in Orizu Elementary School and the staff of the school board in Inazawa City, who expressed understanding and cooperated with this assessment.

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Vol.3, No.11, 672-676 (2011)
doi:10.4236/health.2011.311113



# Estimation of mineral and trace element intake in vegans living in Japan by chemical analysis of duplicate diets

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Received 10 September 2011; Revised 5 October 2011; Accepted 21 October 2011.

## ABSTRACT

**Thirty-six daily duplicate diet samples were collected from 12 healthy female Japanese vegans and sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese, iodine, selenium, chromium and molybdenum in the diets were measured to estimate mineral and trace element intake by Japanese vegans. Significantly higher intake of potassium, magnesium, phosphorus, iron, copper, manganese and molybdenum was observed in vegans than in general Japanese women, but no difference was observed in sodium, iodine, selenium and chromium intake. Vegan calcium intake tended to be low compared to that of general women but the difference was not significant. Since high potassium, magnesium and iron intakes cannot be achieved by general Japanese diets and high intake of potassium and magnesium may prevent hyperextension and cardiovascular disease in vegans, there are few problems with Japanese vegan diets regarding mineral and trace element intake, except for calcium intake, which is low as it is in the general Japanese people.**

**Keywords:** Vegan; Mineral intake; Trace Element Intake; Duplicate Diets; Japan

## 1. INTRODUCTION

Vegetarian diets, essentially excluding animal foods, have become increasingly popular in developed countries [1]. These diets are classified according to the types of animal foods consumed, and strict vegetarians consuming no foods of animal origin are known as vegans. Although vegan diets cause lower serum cholesterol, lower blood pressure and a reduced risk of cardiovascular diseases, eliminating all animal foods from the diet increases the risk of several micronutrient deficiencies, including vitamin $B_{12}$, vitamin D and n-3 fatty acids [2]. Regarding the intake of minerals and trace elements, vegetarians, including vegans, show low intakes of calcium, zinc and selenium because the main sources of these micronutrients are animal foods in Western diets [3,4].

Traditional Asian diets are predominately plant-based, differing from Western diets. In Japan, although the consumption of meat and dairy products has increased along with the Westernization of society, more than three quarters of the energy intake still depends on plant foods [5]. Accordingly, it is thought that the effect of adopting a vegan diet on the nutrient intake pattern is different between the West and Japan. However, little research has examined the nutrient intake of vegetarians in Japan [6], and research on the intake of minerals and trace elements by Japanese vegans is scarce. In the present study, to evaluate mineral and trace element intake by Japanese vegans, duplicate diet samples were collected from Japanese vegans, and concentrations of sodium, potassium, calcium, magnesium, phosphorus, iron, zinc, copper, manganese, iodine, selenium, chromium and molybdenum were measured.

## 2. SUBJECTS AND METHODS

### 2.1. Subjects and Duplicate Diet Sampling

In the present study, vegans were defined as people eating food of plant origin only. Twelve healthy female vegans were recruited through a vegetarian food shop located in Chiba Prefecture, Japan. The characteristics of the subjects are described in **Table 1**. Duplicate meals, beverages and between-meal snacks were collected over 24 h period; 36 duplicate diets from 12 subjects were sampled for 3 consecutive days between September and November 2010. All subjects gave informed consent for the use of their personal information in this study.

**Table 1.** Characteristics of vegan subjects (n = 12).

| | Mean ± SD | Median |
|---|---|---|
| Age (y) | 48.4 ± 12.9 | 47.5 |
| Duration of vegan diet (y) | 20.7 ± 14.5 | 12.0 |
| Height (cm) | 156.4 ± 7.7 | 157.0 |
| Weight (kg) | 49.1 ± 8.9 | 48.5 |
| Body mass index (kg/m$^2$) | 19.9 ± 2.4 | 19.7 |

## 2.2. Treatment of Samples

The daily duplicate diet sample was freeze-dried, homogenized and milled. Approximately 1 g of the dried sample was mixed with 200 mL of 1% HCl, shaken for 30 min and centrifuged. The supernatant was filtrated with 0.45-μm membrane filter. Filtrate thus obtained was used for the determination of sodium and potassium. Another 1 g of the dried sample was heated with 10 mL metal-free $HNO_3$ until the disappearance of insoluble components, and then, 2 mL metal-free $HClO_4$ was added to the digestion mixture, which was further heated until the appearance of white vapor of $HClO_4$. The volume of the digest was made up to 10 mL with pure water. Diluted digest thus obtained was used for the determination of calcium, magnesium, phosphorus, iron, zinc, copper, manganese, selenium and molybdenum. For the analysis of chromium, approximately 1 g of the dried sample was heated in an electric furnace (F-B1414M; As One, Osaka, Japan) at 550˚C for 16 h [7]. After dry incineration, the remaining ash was dissolved in 10 mL of 0.1 M $HNO_3$. Iodine in the dried samples was extracted with 0.5% tetramethylammonium hydroxide (TMAH) [8]. Two hundred milligrams of the dried samples was mixed with 40 mL of 0.5% TMAH and left overnight. The mixture was heated at 60˚C for 6 h and centrifuged. The supernatant was filtrated through a 0.45-μm membrane filter.

## 2.3. Analysis

Sodium, potassium, calcium, magnesium, iron, zinc, copper and manganese were measured using atomic absorption spectrometer (AA-6300; Shimadzu, Kyoto, Japan). Iodine, selenium, chromium and molybdenum were determined by inductively coupled plasma mass spectrometry (ICPMS) with direct nebulization. The ICPMS operating conditions were as follows: instrument, ICPM-8500 (Shimadzu); forward power, 1200 W; coolant gas flow rate, 7.0 L/min; auxiliary gas flow rate, 1.5 L/min; nebulizer gas flow rate, 0.58 L/min; sampling depth, 5.0 mm; integration time, 2.0 s; number of run, 20; mode of analysis, pulse; isotopes monitored, $^{52}$Cr, $^{82}$Se, $^{95}$Mo, $^{97}$Mo, $^{98}$Mo and $^{127}$I. Rhodium ($^{103}$Rh) and tellurium ($^{126}$Te, $^{128}$Te and $^{130}$Te) were used as internal standards. Phosphorus was determined with vanadomolybdate absorption spectrometry [9]. Protein, total lipid and energy were analyzed by a commercial service system (Japan Functional Food Analysis and Research Center, Fukuoka, Japan).

## 2.4. Statistical Analysis

For each subject, mean daily intake was calculated from the analytical results of duplicate diet samples from 3 consecutive days. The mean and median of the daily intake for 12 subjects were then calculated. For iodine, the mean and median were also calculated when each value was logarithmically transformed because values highly varied. Mean daily intake for 12 subjects was statistically compared with the mean daily intake by general Japanese women aged 30 to 49 y described in the National Health and Nutritional Survey in Japan (NHNSJ) [10] by calculation of the *Z*-score; in which women aged 30 to 49 y in NHNSJ, 2008 (n = 1053) were regarded as a population.

# 3. RESULTS AND DISCUSSION

In **Table 2**, daily intake of major nutrients, minerals and trace elements by 12 Japanese female vegans was summarized and compared with those by general Japanese women and several criteria in the Dietary Reference Intakes for Japanese (DRIJ) [11]. For the intake of energy, protein and total lipids, no difference was observed between vegans and general women.

Among major mineral intake, calcium intake by vegans was below the estimated average requirement (EAR) and tended to be low compared to that by the general population. In several Western researches, calcium intake by vegans was markedly lower than that by omnivores [12] and lacto-vegetarians [13]. In the present analysis, vegan calcium intake was somewhat low but was not significantly lower than in the general Japanese calcium intake. Since calcium intake by general Japanese people is always low due to the low consumption of dairy products, the low calcium intake of Japanese vegans may be inconspicuous.

Phosphorus intake by vegans was markedly higher than by general women. In Western research, a vegan diet contains low phosphorus and is appropriate for patients with renal failure [14]. In the West, because the major source of phosphorus in general diets is dairy products, vegan phosphorus intake is comparatively low; however, Japanese people ingest phosphorus mainly from plant foods [5]. The difference in the source of

 

**Table 2.** Intake of energy, protein, lipids, minerals and trace elements in Japanese vegans.

| | Vegans (n = 12) | | NHNSJ, 2008[1)] | | DRIJ, 2010[2)] | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean ± SD | Median | Mean ± SD | Median | EAR | RDA | AI | DG | UL |
| Energy (kcal) | 1847 ± 141 | 1840 | 1682 ± 469 | 1645 | 1750 | - | - | - | - |
| Protein (g) | 56.2 ± 8.1 | 58.4 | 60.2 ± 19.0 | 58.7 | 40 | 50 | - | - | - |
| Lipids (% energy) | 20.8 ± 7.3 | 21.0 | 24.5 ± 14.1 | 22.6 | - | - | - | 20 - 25 | - |
| Sodium (mg) | 3649 ± 1719 | 3029 | 3696 ± 1415[3)] | 3538 [3)] | 590 [3)] | - | - | <2950[3)] | - |
| Potassium (mg) | 3610 ± 1272* | 3217 | 1983 ± 777 | 1891 | - | - | 2000 | 2800 | - |
| Calcium (mg) | 361 ± 122 | 389 | 440 ± 224 | 406 | 550 | 650 | - | - | 2300 |
| Magnesium (mg) | 494 ± 112* | 462 | 214 ± 80 | 204 | 240 | 290 | - | - | - |
| Phosphorus (mg) | 1225 ± 311* | 1197 | 854 ± 284 | 830 | - | - | - | - | 3000 |
| Iron (mg) | 13.0 ± 2.4* | 12.2 | 6.9 ± 3.0 | 6.5 | 9.0 | 11.0 | 900 | - | 40 |
| Zinc (mg) | 8.3 ± 1.6 | 9.1 | 7.1 ± 2.4 | 6.9 | 8 | 9 | - | - | 35 |
| Copper (mg) | 1.75 ± 0.37* | 1.66 | 1.00 ± 0.35 | 0.96 | 0.6 | 0.7 | - | - | 10 |
| Manganese (mg) | 7.5 ± 2.2 | 7.9 | - | - | - | - | 3.5 | - | 11 |
| Iodine (μg) | 1865 ± 1934 | 1158 | - | - | 95 | 130 | - | - | 2200 |
| | 788 (255 - 2441)[4)] | 746[5)] | | | | | | | |
| Selenium (μg) | 87 ± 34 | 76 | - | - | 20 | 25 | - | - | 230 |
| Chromium (μg) | 27 ± 8 | 28 | - | - | 25 | 30 | - | - | - |
| Molybdenum (μg) | 540 ± 207 | 563 | - | - | 20 | 25 | - | - | 500 |

*Significant difference from NHNSJ data was observed at $p < 0.001$ by calculation of *Z*-score; [1)]Values for general Japanese women aged 30 to 49 y (n = 1053) quoted from the National Health and Nutrition Survey in Japan, 2008 [10]; [2)]Criteria for Japanese women aged 30 to 49 y in Dietary Reference Intakes for Japanese, 2010 [11]; EAR, estimated average requirement; RDA, recommended dietary allowance; AI, adequate intake; DG, tentative dietary goal for preventing lifestyle-related diseases; UL, tolerable upper intake level; [3)]Calculated from the values for salt; [4)]Geometrical mean with SD range in parentheses; [5)]Median calculated after logarithmic transformation of data for each daily duplicate diet sample.

phosphorus may contribute to the difference in phosphorus intake between Western and Japanese vegans. In addition, phytate may contribute to the high phosphorus intake in vegans because whole grains and beans contain it at a high level.

No difference was observed between vegans and general women in sodium intake. On the other hand, vegan potassium intake was markedly higher than by general women and far exceeded the tentative dietary goal for preventing lifestyle-related diseases (DG) in DRIJ. Similarly, markedly higher magnesium intake was observed in vegans than in general women. This high intake of potassium and magnesium is probably due to the high consumption of vegetables and fruit.

Among trace element intake, significantly higher iron and copper intake was observed in vegans than in general women. Similarly, manganese and molybdenum intake by vegans was markedly higher than by general Japanese, as described in several reports [15,16]. Intake of these four trace elements far exceeded the recommended dietary allowance (RDA) or the adequate intake (AI) in DRIJ. High intake of copper and manganese is also reported in Western researches [17], probably, because the high consumption of whole grains and beans results in high intake of these trace elements. The mean and median of vegan molybdenum intake exceeded the tolerable upper intake level of this element in DRIJ. This is also caused by high consumption of cereals and beans since they particularly soybean, contain molybdenum at a high level [16].

Although vegan zinc intake has been reported to be low [12], there was no difference between vegans and general women; however, because it has been reported that the serum zinc level in Japanese vegetarians tends to be low [18], it is necessary to examine whether phytate and/or dietary fiber, which are contained in whole grains and beans at a high level, decrease the bioavailability of zinc in Japanese vegan diets.

Since the main sources of selenium in general Japanese diets are fish, meats and eggs [19], the low sele-

 

nium intake by Japanese vegans is concern; however, selenium intake by Japanese vegans was comparable to that by general Japanese described in several previous reports [19-21]. Japanese vegans may ingest selenium from imported wheat and soybeans, which contain selenium at a high level [22]. Similarly to selenium intake, iodine and chromium intake by vegans was also comparable to general Japanese people described in the literature [20,23].

In conclusion, Japanese vegans are estimated to ingest high potassium, magnesium, phosphorus, iron, copper, manganese and molybdenum compared to general Japanese people. In particular, high potassium, magnesium and iron intake cannot be achieved by ingesting general Japanese diets. High intake of potassium and magnesium may lead to the preventing of hyperextension and cardiovascular disease in vegans [24]. Accordingly, there are few problems with Japanese vegan diets regarding mineral and trace element intake, except for calcium intake, which is low as it is in general Japanese people.

## 4. ACKNOWLEDGEMENTS

This study was supported by a grant for comprehensive research on cardiovascular and lifestyle disease from the Ministry of Health, Labour and Welfare of Japan.

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Original Article

# High prevalence of hypovitaminosis D and K in patients with hip fracture

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Although hip fracture is considered to be associated with hypovitaminosis D and K, few reports have previously studied both of them. We have studied the vitamin D- and K-status as well as the general nutritional status in ninety-nine patients with hip fracture. Mean serum concentration of 25hydroxy-vitamin D (25OH-D) in female fractured patients was only approximately 9 ng/mL, suggesting severe vitamin D deficiency. There was no significant difference between the two groups in serum concentration of intact parathyroid hormone in both genders and serum 25OH-D levels in the male subjects. Plasma concentrations of phylloquinone (vitamin $K_1$; PK) and menaquinone-7 (MK-7) were significantly lower in the fractured group than in the control group in both genders. Logistic regression analysis indicated that circulating concentrations of albumin, PK and 25OH-D were the significant and independent determinants of fracture risk, with their higher concentrations associated with decreased fracture risk. Finally, principal component analysis (PCA) was performed to summarize the clinical parameters into smaller numbers of independent components. Three components were obtained, each representing the overall nutritional status, the vitamin D status, and the vitamin K status. In conclusion, our study has shown that patients with hip fracture have vitamin D and K deficiency independent of general malnutrition.

**Key Words: hypovitaminosis D, hypovitaminosis K, patients with hip fracture, general malnutrition, principal component analysis**

## INTRODUCTION

Hip fracture is the most serious consequence of osteoporosis. In addition to the high mortality rates after fracture, even the survivors suffer from functional impairment and limited daily activities.[1] With increased percentage of the elderly in the society, the incidence of hip fracture is constantly increasing in Japan, as in other countries.[2] Hip fracture is also considered to be a great burden to the society because of costly medical expenditure.[3]

Among the various risk factors of hip fracture so far reported are the nutritional ones including poor vitamin D and K status. "Vitamin deficiency" causes various disorders with phenotypic abnormalities, such as osteomalacia and rickets by vitamin D deficiency, and clotting abnormality by vitamin K deficiency. Recently, however, it is known that inadequate supply of vitamins, even in the milder form, causes increased susceptibility to various diseases, and is called vitamin insufficiency.[4] For example, vitamin D insufficiency, through decreased calcium absorption and negative calcium balance, is associated with decreased bone mineral density (BMD) and increased risk of fracture. The prevalence of hypovitaminosis D has been reported to be quite high in patients with hip fracture in various countries.[5-7]

The most essential role of vitamin K is to act as the coenzyme in the γ-carboxylation of glutamic acid residue (glu) to γ-carboxyglutamin acid (gla) residue, through which four of the clotting factors acquire calcium binding capacity. It has long been held that the sole physiological action of vitamin K is the γ-carboxylation of these clotting factors in the liver. Recently, however, extrahepatic action of vitamin K has come to receive much attention.[8] For example, mice devoid of the matrix gla protein (MGP) gene, which is a gla-containing protein present in the skeleton and vasculature, died of severe arterial calcification.[9] Although mice lacking the osteocalcin gene had apparently higher bone mineral density than the control ones, they were more susceptible to bone loss after ovariectomy than their normal littermates, suggesting the compromised bone quality in these mice.[10] There also have been clinical observations to show the association between vitamin K inadequacy and hip fracture. For example, high intake of vitamin K was associated with

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Manuscript received 2 August 2010. Initial review completed 8 November 2010. Revision accepted 27 January 2011.

decreased risk of hip fracture,[11] and high serum concentration of undercarboxyled osteocalcin (ucOC), which is a sensitive indicator of insufficient vitamin K action in the skeleton, was a significant risk factor of hip fracture independent of BMD.[12]

Despite these observations, there have been few reports to evaluate the status of these two bone-active vitamins in hip fractured patients.[13] Thus in the current study, we have studied the serum concentration of these two bone-active vitamins in patients with hip fracture and age-matched controls.

## MATERIALS AND METHODS

### *Subjects*

Consecutive patients with hip fracture transferred to Tamana Central Hospital were studied. The duration of the enrollment was 6 months. Written informed consent was obtained in 99 cases from the patients or a family member when obtaining the patients' approval was practically impossible because of their poor general condition. Age-matched nursing home residents in close proximity to the hospital in Tamana City served as the control. Those without severe liver or kidney dysfunction, or those receiving bone-active drugs or supplementation with vitamin D or K, were encouraged to participate in the study, and the consent was obtained in 48 cases.

Informed consent was similarly obtained in 48 cases. Their background profiles are shown in Table 1. The study protocol was approved by the Ethical Committee of Tamana Central Hospital.

### *Laboratory data*

Blood was drawn within 24 hours following the fracture. After centrifugation, plasma and serum were stored under dark condition at –30C until assay. Serum concentration of 25 hydroxy-vitamin D (25OH-D) was measured by radioimmunoassay (RIA) (DiaSorin, Stillwater, MN, USA). Serum level of intact parathyroid hormone (PTH) was measured by electro chemiluminescent immunoassay (ECLIA) (Roche Diagnostics, Mannheim, Germany). Plasma vitamin $K_1$ (phylloquinone; PK), and $K_2$ (menaquinone-7; MK-7) levels were determined by high-performance liquid chromatography-tandem mass-mass spectrometry with atmospheric pressure chemical ionization (LC-APCI-MS/MS) using a HPLC system (Shimadzu, Kyoto, Japan) and API3000 LC-MS/MS System (Applied Biosystems, Foster City, CA) with $^{18}$O-labeled vitamin K as the internal standard.[14]

### *Statistical analyses*

Statistical analyses were done with SPSS 17.0J. Comparison of two independent groups was done with Student's t-test or Mann-Whitney test depending on normality. The association between vitamin status and the occurrence of hip fracture was analyzed by logistic regression analysis. The relationship between various nutritional indices and circulating vitamin D- and K-levels was analyzed with principal component analysis (PCA) as previously described.[15]

## RESULTS

### *Blood tests*

Baseline characteristics and data from blood examination are shown in Table 1. Serum albumin concentration was significantly lower in the fractured group in both genders, and serum cholesterol concentration and blood hemoglobin level were significantly lower in female patients with fracture. In Table 2 shows the blood concentrations of vitamin D, vitamin K and related molecules. Mean serum concentration of 25OH-D, which most reliably represents

**Table 1.** Patients' profiles

| | Male | | Female | |
|---|---|---|---|---|
| | Control (n=13) | Fracture (n=27) | Control (n=35) | Fracture (n=72) |
| Age | 82.2±9.3 | 82.6±7.6 | 84.1±7.8 | 85.5±7.0 |
| Serum albumin (g/dL) | 4.3±0.5 | 3.5±0.5** | 4.4±0.2 | 3.6±0.4** |
| Serum cholesterol (mg/dL) | 175.4±41.9 | 156.1±36.6 | 232.3±37.0 | 179.4±39.4** |
| Serum BUN (mg/dL) | 24.1±2.2 | 29.5±26.1 | 20.6±7.4 | 20.6±10.2 |
| Hemoglobin (g/dL) | 12.4±2.2 | 11.9±1.9 | 12.5±1.1 | 10.8±1.8** |
| Serum GOT (U/L) | 26.2±20.4 | 32.1±9.4 | 23.9±7.2 | 20.9±7.5 |
| Serum GPT (U/L) | 19.3±16.2 | 22.8±21.1 | 13.7±8.6 | 14.0±8.4 |

Data are shown as mean ± SD. The asterisk (**) denotes that the value in fracture group is significantly different from that in control group ($p<0.01$) by Student's t-test. BUN, GOT, and GPT are abbreviations for blood urea nitrogen, glutamyl oxaloacetic transaminase, glutamyl pyruvate transaminase, respectively.

**Table 2.** Serum concentrations of vitamin D, vitamin K and related molecules

| | Male | | Female | |
|---|---|---|---|---|
| | Control (n=13) | Fracture (n=27) | Control (n=35) | Fracture (n=72) |
| Serum 25OH-D (ng/mL) | 20.7±7.3 | 19.0±13.0 | 18.6±6.3 | 9.1±4.6** |
| Serum intact PTH (pg/mL) | 64.3±53.7 | 61.4±34.4 | 56.0±23.2 | 67.8±33.9 |
| Plasma PK (ng/mL) | 0.55±0.31 | 0.31±0.24* | 0.77±0.36 | 0.46±0.36** |
| Plasma MK-7 (ng/mL) | 4.28±3.75 | 1.60±1.60** | 10.8±7.01 | 2.67±4.13** |

Data are shown as mean ± SD. The asterisk denotes that the value in fracture group is significantly different from that in control group (*; $p<0.05$, **; $p<0.01$) by Student's t-test. 25OH-D, PK , and MK-7 are the abbreviations for 25 hydroxy-vitamin D, phylloquinone, and menaquinone-7, respectively.

**Table 3.** Logistic regression analysis

| | Odds ratio (95%CI) | *p* value |
|---|---|---|
| Serum 25OH-D (per 10ng/mL increase) | 0.246 (0.090-0.673) | <0.001 |
| Plasma PK (per 1ng/mL increase) | 0.072 (0.009-0.612) | 0.016 |
| Albumin (per 1g/dL increase) | 0.003 (0.000-0.054) | <0.001 |
| MK-7 (per 1ng/mL increase) | 0.867 (0.747-1.006) | 0.061 |
| Hemoglobin (per 1g/dL increase) | 1.482 (0.891-2.465) | 0.129 |
| Sex (1; Male, 2; Female) | 2.464 (0.381-15.95) | 0.344 |

Logistic regression analysis with stepwise method was done. Sex, circulating concentrations of albumin, hemoglobin, 25OH-D, PK, and MK-7 were included for analysis.

**Table 4.** Principal component analysis of nutrition indices

| | Component 1 | Component 2 | Component 3 |
|---|---|---|---|
| Serum Albumin | 0.744† | 0.481† | -0.028 |
| Serum total Cholesterol | 0.824† | 0.098 | 0.157 |
| Hemoglobin | 0.538† | 0.589† | -0.269 |
| Serum 25OH-D | 0.035 | 0.902† | 0.228 |
| Plasma PK | 0.191 | 0.109 | 0.922† |
| Plasma MK-7 | 0.773† | 0.009 | 0.210 |

Factor loadings to three components after varimax rotation are shown. †Loadings greater than 0.35

the vitamin D status, was approximately 20 ng/mL in all groups, except for the female fracture group where it was approximately 9 ng/mL. In both genders, serum 25OH-D levels were lower than 20 ng/mL in 90% and 61% of subjects, in the fracture and control groups, respectively. It was below 10 ng/mL in 50% and 7% of subjects in the fracture and control group, respectively. Serum concentration of intact PTH, which is a sensitive indicator of vitamin D insufficiency; hence secondary hyperparathyroidism, was not different between control and fracture groups in males. It was slightly higher in the fractured group than in the control group in female, which, however, did not reach statistical significance ($p$=0.07).

Serum concentrations of PK and MK-7 were significantly lower in the fracture group than in the control group in both genders.

***Logistic regression analysis for variables associated with hip fracture***

In order to evaluate whether the above-mentioned vitamin insufficiency is related to the occurrence of hip fracture, logistic regression analysis was performed. Of the factors subjected for analysis, circulating concentrations of albumin, PK and 25OH-D were the significant determinants, whereas MK-7, gender or hemoglobin level was not (Table 3). The odds ratio for fracture markedly decreased in accordance with increased concentrations of albumin, PK and 25OH-D.

***Principal component analysis (PCA)***

Since patients with hip fracture are generally malnourished, we considered it to be important whether the low vitamin D- and K-status as described above simply reflects overall malnutrition. Then PCA was performed with parameters included for analysis being: serum albumin and cholesterol concentrations, blood hemoglobin levels, and plasma 25OH-D, PK and MK-7. Three components were obtained as shown in Table 4. The first component was contributed by high serum albumin, total cholesterol, blood hemoglobin and plasma MK-7. The second component consisted of high serum albumin, blood hemoglobin and serum 25OH-D. The third component was composite of high plasma PK. Each component was interpreted as follows; the first, second, and third component representing overall nutritional status, vitamin D status, and vitamin $K_1$ status, respectively.

## DISCUSSION

In the present study, we have studied the blood concentration of 25OH-D, PTH, PK, MK-7 and other nutritional indices. In 90% of patients with hip fracture, serum 25OH-D level was lower than 20 ng/mL which is a generally accepted cut-off for hypovitaminosis D. In half of the patients, serum 25OH-D concentration fell into the severe hypovitaminosis D range of below 10 ng/mL. Nurmi *et al.* reported that serum 25OH-D level was lower than 15 ng/mL and 8 ng/mL in 53% and 9%, respectively, of the patients with hip fracture in Finland.[16] In a study on Japanese patients with hip fracture, Sakuma *et al.* reported that 62% of the patients had their serum 25OH-D level below 20 ng/ml.[7] Thus, the prevalence of hypovitaminosis D in the present study was compatible with the previous studies, but was even higher.

Serum concentration of 25OH-D in the fracture group was significantly lower than that in the control group in women, but not in men. There have been some reports to show that elderly women are more prone to vitamin D deficiency than elderly men. Hirani *et al.* reported that hypovitaminosis D was more prevalent in women than men with a odds ratio of 2.1.[17] Maggio *et al.* reported that age-related decline of serum 25OH-D was already evident shortly after age 50 in women, whereas in men it started only after age 70.[18] Thus there seems to be a gender dif-

ference that women are more prone to vitamin D inadequacy, for which there is no clear explanation at present.

Lack of significant difference in serum PTH level between fracture and control groups is most likely due to the large standard deviation in serum PTH concentration. However, there still can be alternative explanations. There have been some reports describing the absence of PTH elevation in face of hypovitaminosis D in patients with hip fracture.[19-22] Sahota *et al.* studied the vitamin D status in the post-hip fracture patients. They found that only half of them had elevated serum PTH levels, the rest had normal to low serum PTH levels in face of hypovitaminosis D.[19] As an explanation for this apparently paradoxical observation, they postulated magnesium deficiency as the underlying cause since magnesium deficiency is known to be associated with impaired PTH secretion.[20] Thus the question has now come to our attention whether skeletal impairment in hypovitaminosis D can be explained by secondary hyperparathyroidism alone. A recent paper from Finland also reported that serum PTH level was within the reference range despite hypovitaminosis D in 74.8% of the bedridden geriatric patients.[21] Patients in the lowest quartile of serum PTH level were associated with the history of hip fracture (odds ratio 2.9). Thus it is obvious that hypovitaminosis D is associated with increased risk of hip fracture, although further studies are required to determine whether it is mediated by secondary hyperparathyroidism or due to hypovitaminosis per se.

Compared to vitamin D, far smaller number of papers has been published on the relationship of vitamin K with hip fracture. Epidemiological studied have shown that higher intake of vitamin K is associated with lower risk of hip fracture.[11,23] Among the two vitamin K analogs studied here, PK seems to best represent the vitamin K status of these subjects. Kaneki *et al.* reported that there is a large geographic difference in serum MK-7 concentration in Japan, which could be accounted for by the frequency of consuming natto, which contains extraordinary amount of MK-7.[24] Blood concentrations of PK and MK-7 were consistently lower in fractured patients than control subjects in both genders.

Kawana *et al.* reported that there was no significant alteration in the circulating concentrations of PK and MK-7 in hip fractured patients.[25] In their paper, these concentrations were below the detection limit in the substantial number of subjects. Blood vitamin K levels were reported to be below the detection limit in other papers also.[24,26] In our data using newly developed LC-APCI-MS/MS method for the determination of circulating vitamin K levels, serum concentrations of PK and MK-7 were detectable in almost all subjects.[14] Thus, previous reports using less sensitive assay methods should be interpreted with caution.

In fractured subjects, serum albumin concentration was significantly lower in both genders, and hemoglobin level and serum cholesterol concentration was significantly lower in the females. Thus patients with hip fracture are malnourished. Then it was considered mandatory to analyze the relationship between the overall malnutrition and decreased levels of circulating these vitamins. We have studied it with two analytical procedures; logistic regression analysis and principal component analysis (PCA). Logistic regression analysis revealed that serum concentrations of 25OH-D, PK and albumin were significant contributing factors for fracture risk, and suggested that circulating 25OH-D and PK levels contributed to the increased risk of fracture independent of general malnutrition.

Finally PCA was done. Three components were obtained, representing overall nutritional status, vitamin D status, and vitamin K status, respectively. Since these components are, by their definition, independent of each other, these results strongly suggest that hypovitaminosis D and K in patients with hip fracture is not merely a manifestation of general malnutrition. At present, the reason for the association of MK-7 with the first component, representing the overall nutritional status is not known. We have also recently reported that institutionalized elderly subjects had high prevalence of hypovitaminosis D and K, which is independent of general malnutrition by PCA.[15]

One of the limitations of the current work is that it is a case control study, but not a prospective one. Since the association of hip fracture with the insufficiency of two bone-active vitamins; vitamin D and vitamin K has been scarce, we have done this study as the initial step.

Another limitation is that the nursing home residents adjacent to the hospital were the control subjects. It is unclear whether the control subjects represent the average Japanese elderly population or not. However, it is quite unlikely the nursing home residents have nutritional status far better than the average Japanese elderly. Rather, they are likely to be equal to or worse than the average. Thus, we believe that our finding that the blood levels of these vitamins in fractured patients were even lower than that in nursing home residents has clinical implications.

In summary, patients with hip fracture had lower serum concentration of vitamin K in both genders, and lower serum concentration of vitamin D in female subjects. Since blood samples were obtained within 24 hours after fracture, these data is likely to represent the patients' status before fracture. Lower serum albumin concentration in fractured patients suggests that these subjects are also generally malnourished. Insufficiency of these vitamins as well as the overall malnutrition is likely to predispose elderly people to hip fracture, and intervention study to correct these abnormalities is needed.

**ACKNOWLEDGMENTS**

This study was supported by Health and Labor Science Research Grant from the Ministry of Health, Labor and Welfare, Japan, and Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (JSPS).

**AUTHOR DISCLOSURES**

None of the authors have any conflicts of interest.

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