Development of Simultaneous Enzymatic Assay Method for All Six Individual Vitamin B₆ Forms and Pyridoxine- β -Glucoside

Toshiharu YAGI, Ryutaro MURAYAMA, Huong Thi Viet DO, Youhei IDE,
Andrew Njagi MUGO and Yu YOSHIKANE

Faculty of Agriculture and Agricultural Science Program, Graduate School of Integrated Arts and Sciences,
Kochi University, Monobe-Otsu 200, Nankoku, Kochi 783–8502, Japan

(Received January 6, 2010)

Summary A method for determining all of the six natural vitamin B₆ compounds and pyridoxine- β -glucoside in urine from humans consuming their usual diet was developed. These compounds were specifically converted with 5 enzymes into a high fluorescent 4-pyridoxolactone which was supersensitively determined by an isocratic HPLC. All of the compounds in urine from humans consuming their usual diets were for the first time determined together. The preparation procedure for urine samples was easy without HCl-hydrolysis, and the enzyme reactions took only 2 or 3 h. It required only 5 μ L of the urine sample for analysis of one of the compounds. Urine samples from five young Japanese males consuming their usual diet contained pyridoxal, pyridoxamine, and pyridoxine- β -glucoside but not pyridoxine or phosphoester forms. The contents of 4-pyridoxic acid and pyridoxal correlate well with a correlation coefficient of 0.98. On the other hand, the content of pyridoxamine did not correlate with that of 4-pyridoxic acid.

Key Words vitamin B₆, human urine, pyridoxal, pyridoxamine, pyridoxine

There are six natural forms of vitamin B₆: pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), pyridoxine 5'-phosphate (PNP), pyridoxal 5'-phosphate (PLP), and pyridoxamine 5'-phosphate (PMP). The vitamin B₆ compounds have similar nutritional value because they are inter-converted (1). Generally, it has been assumed that PN, PM and PL are adsorbed through the intestinal mucosa, and then phosphorylated and converted into PLP in the liver. Some PLP exits the liver and travels in the blood on albumin, turning over slowly. PL is the form most actively transported to other cells from the liver. The cells adsorb PL and then phosphorylate it to make PLP, the coenzyme form of vitamin B₆, which plays essential roles in many enzymes involved in amino acid metabolism. The main excretory form of vitamin B₆ is 4-pyridoxic acid (4-PA), lost in urine. In addition to the six vitamin B₆ compounds, there is also a storage form of vitamin B₆, pyridoxine- β -glucoside (PNG), which is also adsorbed through the intestinal mucosa and metabolized like PN although its bioavailability is lower than that of PN (1).

Although there are many studies describing the content of the individual vitamin B₆ compounds in urine

from humans consuming their usual diet, the figures are contradictory among them. The 24-h urine from five American young males contained, on average, 491, 226, 47 nmol of PL, PM, and PN, respectively, when the vitamin B₆ compounds were assayed using *Saccharomyces cerevisiae* after the urine was autoclaved in 0.055 N HCl for 3 h, and then applied to an ion-exchange column chromatography to separate them (2). The 24-h urine contained 5,500 nmol of 4-PA. On the other hand, a recent comparative study, in which the vitamin B₆ compounds were assayed by an HPLC, reported that only 4-PA was detectable in the 24-h urine from ten young American males (3). Actually, it has been difficult to elucidate reliable contents of the six natural vitamin B₆ compounds in urine of humans consuming their usual diet with the assay methods so far developed, including microbioassay and HPLCs.

To determine vitamin B₆ in urine, both the specificity and sensitivity of the assay method should be improved. One promising way to increase the specificity is to harness the functions of enzymes. Recently, we have developed an enzyme-HPLC method, in which vitamin B₆ compounds are specifically converted into a highly fluorescent vitamin B₆-derivative, 4-pyridoxolactone (4-PLA), and then 4-PLA is measured in pmole amounts by HPLC (4). The enzyme-HPLC method suffered the disadvantage of using HCl-hydrolysis under high pressure for hydrolyzing the phosphate forms to free ones. Although HCl-hydrolysis procedure is an official (AOAC) method for analysis of vitamin B₆ in foods, it could cause degradation of some of the vitamin B₆ compounds by reactions with various food or urine components.

E-mail: yagito@kochi-u.ac.jp

Abbreviations: AEH, all enzymatic HPLC; ALP, *Escherichia coli* alkaline phosphatase; 4-PA, 4-pyridoxic acid; PL, pyridoxal; 4-PLA, 4-pyridoxolactone; PLDH, PL 4-dehydrogenase; PLP, pyridoxal 5'-phosphate; PM, pyridoxamine; PMP, pyridoxamine 5'-phosphate; PN, pyridoxine; PNG, pyridoxine- β -glucoside; PNOX, pyridoxine 4-oxidase; PNP, pyridoxine 5'-phosphate; PPAT, PM-pyruvate aminotransferase; TCA, trichloroacetic acid; YM, young male subject.

Here, we have reformed the enzyme-HPLC method by application of alkaline phosphatase and β -glucosidase, and developed a new method for assay of vitamin B₆ in human urine. The new (all enzymatic HPLC, AEH) method was able to measure all of the vitamin B₆ compounds and PNG together.

MATERIALS AND METHODS

Materials. Recombinant PL 4-dehydrogenase (PLDH) (5), PN 4-oxidase (PNOX) (6), and PM-pyruvate aminotransferase (PPAT) (7) were prepared as previously reported. *Escherichia coli* alkaline phosphatase (ALP) with a His-tag at a C-terminus was over-expressed and purified according to the general protocols. PNP was prepared from PLP by reduction with sodium borohydride. PNG was prepared from PN and cellobiose based on a reverse reaction of β -glucosidase (8). PN and PLP were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and PM and PL from Sigma Chemical Co. (St. Louis, MO). PMP was a gift from Daiichi Fine Chemicals Co. (Takaoka, Japan).

Urine sample. Early morning urine samples were collected from five young healthy men (aged between 21 and 22). The body mass index was 23.9 ± 5.9 (means $\pm$ SD) kg/m², and their height was 170.2 ± 2.6 cm. They were consuming their usual diet without a vitamin B₆ supplement. They gave written informed consent.

Sample preparation. To 5 mL of the urine was added 0.1 mL of 50% (w/v) trichloroacetic acid (TCA). The acidic urine was incubated at 100°C for 5 min. After cooling on ice, the mixture was centrifuged at 8,000 rpm for 10 min at 4°C. The supernatant (5 μ L) was then used for analysis.

Reaction conditions for the assay. The compositions of the reaction mixtures and the reaction steps are shown in Scheme 1. To determine PL, the urine sample (5 μ L) was added to a reaction mixture (375 μ L) consisting of 25 mM Tris-HCl (pH 9.0), 1 mU of PLDH and 0.5 mM NAD⁺. The reaction mixture was then incubated at 30°C for 1 h, and then 20 μ L of 0.55 M HCl was added to stop the enzymatic reaction. An aliquot (100 μ L) of the reaction mixture was applied to a HPLC column after the filtration with a Dismic 13 syringe filter (pore size of 0.2 μ m, Advantech, Tokyo, Japan). To make a control (no-reaction) reaction mixture, HCl was added before the addition of urine. PM and PN were determined in the same way as the PL but using additional enzymes and substrate or cofactor (Scheme 1). For the determination of PLP, a urine sample (5 μ L) was added to a reaction mixture (377 μ L) consisting of 25 mM Tris-HCl (pH 9.0), 0.5 mM MgCl₂ and 10 mU ALP. The reaction mixture was incubated at 30°C for 2 h and then 1 mU (final concentration) PLDH and 0.5 mM (final concentration) NAD⁺ were added to the reaction mixture and incubated further for 1 h at 30°C. The enzyme reaction was then stopped by the addition of 20 μ L 0.55 M HCl. Then, 100 μ L of the reaction mixture was applied as described above. PMP and PNP were determined in the same way as the PLP but using the

additional enzymes and the substrate or the cofactor (Scheme 1). For the determination of PNG, a urine sample (5 μ L) was added to a reaction mixture (200 μ L) consisting of 50 mM sodium acetate (pH 5.0), 100 mM NaH₂PO₄ and 50 mU β -glucosidase (NaH₂PO₄ was added to inhibit phosphatase activity in the almond β -glucosidase preparation). The reaction mixture was incubated at 30°C for 2 h. Then, 0.5 M NaOH (80 μ L) and 0.25 M Tris-HCl (pH 9.0, 16 μ L) were added to the reaction mixture to change its pH, and then to this reaction mixture 1 mU PLDH, 0.5 mM NAD⁺, 1 mU PNOX and 2.5 μ M FAD were added. Finally after an incubation at 30°C for 1 h, 20 μ L 2.0 M HCl was added.

Calculation. Because urine may contain all forms of vitamin B₆, the pyridoxal content was first determined directly from the peak height or area of 4-PLA on the elution profile of the HPLC of reaction 1. PM, PN, and PLP were determined by subtracting the peak height or area found in the elution profile of reaction 1 from that of reactions 2, 3, and 4, respectively. PMP was determined by subtracting the peak height or area found in the elution profile of reaction 5 from that of reaction 4, followed by an additional subtraction of the peak height or area corresponding to that of PM. PNP was determined by subtracting the peak height or area found in the elution profile of reaction 6 from that of reaction 4, followed by an additional subtraction of the peak height or area corresponding to that of PN. PNG was determined by subtracting the peak height or area found in the elution profile of reaction 7 from that corresponding to PN.

HPLC system. 4-PLA was measured by a reversed-phase isocratic HPLC method. It was separated with a Jasco HPLC system (JASCO, Tokyo, Japan) equipped with an AS-2055 autosampler, a PU-2080 pump, and an FP-920 fluorescence detector. Optimum separation was obtained on a Cosmosil 5C18MS-II column (250 $\times$ 4.6 mm; Nacalai Tesque), with a mobile phase consisting of 20 mM potassium phosphate buffer (pH 7.0) and 10% (v/v) methanol. The flow rate and sample volume were 0.5 mL/min and 100 μ L, respectively. The fluorescence intensity of the eluted 4-pyridoxolactone was monitored at 430 nm (excitation at 360 nm).

Enzyme, 4-PA, and creatinine assays. PLDH (5), PNOX (6), PPAT (7), ALP (9), and β -glucosidase (10) were assayed as described previously. The activities of the enzymes were assayed every time just before usage for enzymatic conversion of vitamin B₆ compounds into 4-PLA. 4-PA was determined by a HPLC method (11). Creatinine was determined by a colorimetric peroxidase-coupled procedure (12).

RESULTS

Determination of vitamin B₆ compounds in urine

Urine samples from five young males were analyzed by the AEH method. The chromatograms of the analysis of PL, PM, and PN in YM-3 (the number 3 subject of the young males) urine are shown as an example in Fig. 1. 4-PLA was eluted at about 11 min as shown with an arrow, together with unknown compounds in the

Scheme 1

urine. The elution time was changed from 11 to 14 min depending on a lot number of the HPLC column, although the over-all elution profile was not changed. Although the control reaction mixture showed a low peak of 4-PLA (PL, No-R in Fig. 1A), which was probably produced from 4-PA during the urine sample preparation under the acidic conditions, its height and area increased when the urine was subjected to the enzymatic conversion with reaction 1 in Scheme 1 for converting PL in the urine into 4-PLA (PL, R). The amount of PL present corresponded to fluorescence intensity (PL, R) minus (PL, No-R). Thus, it was possible to determine PL content in the urine using the PL standard curve. The urine sample, to which 2 pmol of PL had been added, showed a higher peak (PL+Sd, R) because the corresponding amount of 4-PLA was additionally produced by enzymatic conversion. In contrast, the urine sample containing 2 pmol of PL without the enzymatic conversion did not show this increase: its peak height was the same as that of the control reaction mixture (PL+Sd, No-R). Thus, PL in the urine was satisfactorily determined. PM in the urine was determined by the reaction 2 in Scheme 1. Elution patterns of the two control reaction mixtures, and reaction mixture with or

without 2 pmol of PM are shown in Fig. 1B. Two controls (PM, No-R and PM+Sd, No-R) showed the same peak height as those of (PL, No-R and PL+Sd, No-R), respectively. In contrast, the peak height of the reaction mixture (PM, R) was higher than that of the reaction mixture (PL, R) because the former peak was a total of PL and PM. Thus, subtraction of the peak height or area of the reaction mixture (PL, R) from that of the reaction mixture (PM, R) gave a 4-PLA amount corresponding to PM. Standard PM (2 pmol) was quantitatively converted into 4-PLA and determined (PM+Sd, R). PN in the urine was determined by reaction 3 in Scheme 1. Elution patterns are shown in Fig. 1C. Like PL and PM, PN was also quantitatively converted into 4-PLA and determined (PN+Sd, R). The peak height of the reaction mixture (PN, R) was the same as that of the reaction mixture (PL, R), showing that PN was not contained in the urine.

Figure 2 shows that PLP (PLP+Sd, R), PMP (PMP+Sd, R), PNP (PNP+Sd, R), and PNG (PNG+Sd, R) added in the urine are also quantitatively converted into 4-PLA and determined. In this experiment, a different lot number of the HPLC column was used, and 4-PLA was eluted at around 13.7 min.

Fig. 1. HPLC chromatograms for the analyses of free forms of vitamin B₆. (A) PL in urine from YM-3 was determined. The urine sample (5 μ L) was subjected to enzymatic conversion (reaction 1 in Scheme 1) and applied to HPLC (PL, R). The urine sample, to which 2 pmol of PL had been added, was subjected to enzymatic conversion (PL+Sd, R). Their controls were also done (PL, No-R, and PL+Sd, No-R). (B) PM in urine from YM-3 was determined. The urine sample (5 μ L) was subjected to enzymatic conversion (reaction 2 in Scheme 1) and applied to HPLC (PM, R). The urine sample, to which 2 pmol of PM had been added, was subjected to enzymatic conversion (PM+Sd, R). Their controls were also done (PM, No-R, and PM+Sd, No-R). (C) PN in urine from YM-3 was determined. The urine sample (5 μ L) was subjected to enzymatic conversion (reaction 3 in Scheme 1) and applied to HPLC (PN, R). Two pmol of PN was added to the urine sample, and then subjected to enzymatic conversion (PN+Sd, R). Their controls were also done (PN, No-R, and PN+Sd, No-R). Arrows show PLA peaks.

Fig. 2. HPLC chromatograms for the analyses of phosphoester forms of vitamin B₆ and PNG. Two pmol of PLP, PMP, PNP or PNG was added to the YM-3 urine sample, and then they were subjected to enzyme conversion as shown reactions 4, 5, and 6 in Scheme 1, respectively. Their elution patterns are shown as PLP+Sd, PMP+Sd, PNP+Sd, and PNG+Sd, respectively.

Table 1. Contents of vitamin B₆ compounds and 4-pyridoxic acid in the urine of five young males.

Young male	PL (nmol/mL)	PM (nmol/mL)	PNG (nmol/mL)	4-PA (nmol/mL)
YM-1	0.31±0.02	0.10±0.00	0.42±0.08	1.08±0.03
YM-2	0.73±0.01	0.06±0.00	ND	2.98±0.02
YM-3	0.42±0.01	0.10±0.00	0.2±0.01	1.02±0.37
YM-4	0.22±0.00	ND	0.31±0.10	0.44±0.00
YM-5	0.21±0.00	0.09±0.00	0.23±0.04	0.48±0.00

PN, PLP, PMP, or PNP was not detected. ND means not detected.

The results clearly showed that the AEH method could be applied for determination of contents of individual vitamin B₆ compounds and PNG in urine of human consuming a normal diet. The elution pattern of the control reaction mixture showed a characteristic elution profile for different individuals, showing that their urines contain different amount of unknown compounds (data not shown). However, no compound interfered with the elution pattern of 4-PLA. In some cases, an unknown compound adsorbed firmly on the HPLC column and was eluted as a broad peak on the chart of the next analysis, because the HPLC was an isocratic system. Such compounds, however, showed no effect on the elution pattern of 4-PLA. A 5 μ L urine sample gave the best result, especially when the phosphate forms were analyzed: some unknown compounds in the urine samples strongly inhibited the alkaline phosphatase reaction when higher amounts were used. *Contents of vitamin B₆ compounds, PNG and 4-PA in five young males.*

The urine samples contained PL, PM and PNG but not PN or the phosphoester forms (Table 1). The PL, PM, and PNG contents were within a narrow range (0.21–0.73, 0.00–0.10, and 0.00–0.42 nmol/mL, respectively). The average recoveries of the internal standard were 89% (86–93%), 78.4% (76–81%), and 85% (67–96%) for PL, PM, and PNG, respectively. The PL content was 5.4 fold higher than the PM content on

average. The PNG contents were similar to that of PL. The 4-PA contents were 3.2 fold higher than the PL contents on average. The correlation coefficient between PL and 4-PA contents was 0.98, showing a strong correlation between the values. In contrast, no correlation (0.07 of correlation coefficient) was found between PM and 4-PA contents. The correlation (0.09) between PL and PM contents was not significant. The correlation coefficients between the PNG content and the 4-PA, PL, or PM content were –0.76, –0.81, and 0.06, respectively. The negative correlation between PNG and 4-PA contents has been reported (13). The results showed that 4-PA content in urine, which is generally used to estimate B₆ nutritional state in a body, reflected only PL content, and conform to the fact that PL is an intimate precursor of 4-PA. On the other hand, a direct conversion of PM to 4-PA has not been demonstrated so far, and thus its concentration may not correlate with that of 4-PA.

The contents were corrected with the creatinine contents, and the values (μ mol of PL, PM, PNG or 4-PA/g of creatinine) are shown in Table 2. The correlation coefficients between the corrected PL and 4-PA contents, the corrected PM and 4-PA contents, and the corrected PM and PL contents were 0.82, –0.32, and –0.51, respectively. Those between the corrected PNG and PL contents, the corrected PNG and PM contents, and the corrected PNG and 4-PA contents were –0.23, 0.54, and

Table 2. Content of creatinine, and μmol of PL, PM, PNG and 4-PA per gram of creatinine.

Young male	Creatinine (mg/dL)	PL ($\mu\text{mol}/\text{Cr}$, g)	PM ($\mu\text{mol}/\text{Cr}$, g)	PNG ($\mu\text{mol}/\text{Cr}$, g)	4-PA ($\mu\text{mol}/\text{Cr}$, g)
YM-1	225.4	0.138	0.044	0.186	0.479
YM-2	217.8	0.335	0.028	0.000	1.368
YM-3	163.5	0.257	0.061	0.122	0.624
YM-4	81.8	0.269	ND	0.379	0.538
YM-5	152.2	0.138	0.059	0.151	0.315

0.63, respectively. Thus, the higher correlation coefficient between PL and 4-PA contents was obtained when the original uncorrected PL and 4-PA contents rather than the corrected ones were compared.

DISCUSSION

Here we have developed an analytical method (the AEH method) of vitamin B₆ and PNG for urine of humans consuming their usual diet and for the first time determined six vitamin B₆ compounds and PNG together in individual human urine. The preparation procedure for the urine sample is easy without HCl-hydrolysis, and the enzyme reactions take only 2 or 3 h. It requires only 5 μL of the urine sample for the analysis of one of the compounds.

The average ratio of vitamin B₆ (PL+PM) and 4-PA contents in the urine was 0.37 (2.24/6) in this study. The results coincide with the general consensus that the major excretory metabolite of vitamin B₆ in humans is urinary 4-PA. Urinary 4-PA excretion is a direct measure of vitamin B₆ status and is considered to be a short-term indicator of a recent vitamin B₆ intake (14). Studies have also shown that with a conventional food-based diet, urinary 4-PA accounts for <40–60% of the vitamin B₆ intake (15). Here, we have found that PL content correlated well with 4-PA contents. Thus, vitamin B₆ status of humans may be estimated directly from PL content in urine by the AEM method. Further studies are required to examine which value (PL or 4-PA content) has the advantage for estimation of vitamin B₆ status in humans under various health conditions.

The urine samples contained various amounts (0–0.06 nmol/mL) of PM. Recently, many studies (16, 17) have shown that PM is able to prevent arterial hardening in diabetes and progression of diabetic complications. If PM content in urine reflects its concentration in a body, the content may be used as a biomarker for estimation of susceptibility to the diabetic complications. It has been reported that vitamin B₆ in plasma is mainly PLP (60%), PN (15%) and PL (14%) (1). Thus, the present results may not reflect the vitamin B₆ content in the plasma, suggesting that the PM content in urine does not necessarily reflect the PM content in the body. A systematic study on the validity of PM content in urine as a biomarker for the diabetic complications is therefore required.

The phosphoester forms of vitamin B₆ were not detected in the urine used here. A highly sensitive assay with apoaspartate aminotransferase showed that a

human urine sample contained no PLP or PMP (18). Together with the present results, it may be concluded that the phosphate forms of vitamin B₆ including PNP are not excreted into normal human urine. Because they are not permeable to cell membranes (1), the phosphate forms of vitamin B₆ may not be filtered out of the blood by the glomerular filtration. A systematic screening of human urine could find some malady in which the phosphate forms are excreted into urine.

PNG was found in the urine samples and its contents were similar to those of PL. It has been reported that the urine from ten healthy American women contained PNG, and its contents reflected the consumed PNG amounts (13). The content in 24 h-urine samples from the above study under a low and high PNG diet was 0.075 and 0.216 ($\mu\text{mol}/\text{d}$), respectively. Therefore, the difference among the values of five Japanese young men may reflect the composition of the food they had ingested. Because PNG comprises 5–80% of the total vitamin B₆ content of various fruits and vegetables, the man whose urine contained the higher PNG could have eaten a larger amount of vegetable foods.

REFERENCES

- 1) Spinneker A, Sola R, Lemmen V, Castillo MJ, Pietrzik K, González-Gross M. 2007. Vitamin B₆ status, deficiency and its consequences—An overview. *Nutr Hosp* **22**: 7–24.
- 2) Kelsay J, Baysal A, Linkswiler H. 1968. Effect of vitamin B₆ depletion on the pyridoxal, pyridoxamine and pyridoxine content of the blood and urine of men. *J Nutr* **94**: 490–494.
- 3) Zempleni J, Kübler W. 1995. Metabolism of vitamin B₆ by human kidney. *Nutr Res* **15**: 187–192.
- 4) Nishimura S, Nagano S, Crai CA, Yokochi N, Yoshikane Y, Ge F, Yagi T. 2008. Determination of individual vitamin B₆ compounds based on enzymatic conversion to 4-pyridoxolactone. *J Nutr Sci Vitaminol* **54**: 18–24.
- 5) Yokochi N, Nishimura S, Yoshikane Y, Ohnishi K, Yagi T. 2006. Identification of a new tetrameric pyridoxal 4-dehydrogenase as the second enzyme in the degradation pathway for pyridoxine in a nitrogen-fixing symbiotic bacterium, *Mesorhizobium loti*. *Arch Biochem Biophys* **452**: 1–8.
- 6) Yuan B, Yoshikane Y, Yokochi N, Ohnishi K, Yagi T. 2004. The nitrogen-fixing symbiotic bacterium *Mesorhizobium loti* has and expresses the gene encoding pyridoxine 4-oxidase involved in the degradation of vitamin B₆. *FEMS Microbiol Lett* **234**: 225–230.
- 7) Yoshikane Y, Yokochi N, Ohnishi K, Hayashi H, Yagi T. 2006. Molecular cloning, expression and characteriza-

- tion of pyridoxamine-pyruvate aminotransferase. *Biochem J* **396**: 499–507.
- 8) Iwami K, Yasumoto K. 1986. Synthesis of pyridoxine- β -glucoside by rice bran β -glucosidase and its in situ absorption in rat small intestine. *Nutr Res* **6**: 407–414.
- 9) Xu X, Kantrowitz ER. 1993. Binding of magnesium in a mutant *Escherichia coli* alkaline phosphatase changes the rate-determining step in the reaction mechanism. *Biochemistry* **32**: 10683–10691.
- 10) Bauer MW, Bylina EJ, Swanson RV, Kelly RM. 1996. Comparison of a β -glucosidase and a β -mannosidase from the hyperthermophilic archaeon *Pyrococcus furiosus*. Purification, characterization, gene cloning, and sequence analysis. *J Biol Chem* **271**: 23749–23755.
- 11) Schuster K, Bailey LB, Cerda JJ, Gregory III JF. 1984. Urinary 4-pyridoxic acid excretion in 24-hour versus random urine samples as a measurement of vitamin B₆ status in humans. *Am J Clin Nutr* **39**: 466–470.
- 12) Artiss JD, McEnroe RJ, Zak B. 1984. Bilirubin interference in a peroxidase-coupled procedure for creatinine eliminated by bilirubin oxidase. *Clin Chem* **30**: 1389–1392.
- 13) Hansen CM, Leklem JE, Miller LT. 1996. Vitamin B-6 status indicators decrease in women consuming a diet high in pyridoxine glucoside. *J Nutr* **126**: 2512–2518.
- 14) Leklem JE. 1990. Vitamin B-6. A status report. *J Nutr* **120**: 1503–1507.
- 15) Huang Y-C, Chen W, Evans MA, Mitchell ME, Shultz TD. 1998. Vitamin B-6 requirement and status assessment of young women fed a high-protein diet with various levels of vitamin B-6. *Am J Clin Nutr* **67**: 208–220.
- 16) Chetyrkin SV, Mathis ME, Ham A-JL, Hachey DL, Hudson BG, Voziyan PA. 2008. Propagation of protein glycation damage involves modification of tryptophan residues via reactive oxygen species: inhibition by pyridoxamine. *Free Radic Biol Med* **44**: 1276–1285.
- 17) Chang K-C, Liang J-T, Tsai P-S, Wu M-S, Hsu K-L. 2009. Prevention of arterial stiffening by pyridoxamine in diabetes is associated with inhibition of the pathogenic glycation on aortic collagen. *Brit J Pharm* **157**: 1419–1426.
- 18) Yang BI, Sawhney AK, Pitchlyn RC, Peer PM. 1981. Simple assay for femtomoles of pyridoxal and pyridoxamine phosphates. In: *Methods in Vitamin B-6 Nutrition* (Leklem JE, Reynolds RD, eds), p 79–97. Plenum Press, New York.