J Nutr Sci Vitaminol, **54**, 18–24, 2008

Determination of Individual Vitamin B_6 Compounds Based on Enzymatic Conversion to 4-Pyridoxolactone

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(Received July 23, 2007)

Summary A determination method for individual natural vitamin B_6 compounds was developed. The vitamin B_6 compounds were specifically converted into 4-pyridoxolactone (PAL), a highly fluorescent compound, through a combination of enzymatic reactions and HCl-hydrolysis. PAL was then determined by HPLC. Pyridoxal was completely oxidized to PAL with pyridoxal 4-dehydrogenase (PLDH). Pyridoxine and pyridoxamine were totally converted into PAL through a coupling reaction involving pyridoxine 4-oxidase and PLDH, and one involving pyridoxamine-pyruvate aminotransferase and PLDH, respectively. The 5′-phosphate forms and pyridoxine-β-glucoside were hydrolyzed with HCl, and then determined as their free forms. Pyridoxine 5′-phosphate and pyridoxine-β-glucoside were not separately determined here. Three food samples were analyzed by this method.

Key Words vitamin B_6, 4-pyridoxolactone, pyridoxal 4-dehydrogenase, pyridoxine 4-oxidase, pyridoxamine-pyruvate aminotransferase

There are six natural forms of vitamin B_6: pyridoxine, pyridoxal, pyridoxamine, pyridoxine 5′-phosphate, pyridoxal 5′-phosphate, and pyridoxamine 5′-phosphate (Fig. 1). In addition, plants contain pyridoxine-β-glucoside, a storage form of vitamin B_6; it is not counted as vitamin B_6 because its nutritional availability as a form of vitamin B_6 in the human body is controversial (*1*). The above six vitamin B_6 compounds have identical nutritional functions. On the other hand, recent studies have shown that individual vitamin B_6 compounds exhibit specific biochemical functions; pyridoxamine, in particular, can detoxify active carbonyl products and is undergoing a phase II clinical trial for the treatment of complications of diabetes (*2*). Vitamin B_6 compounds are strong quenchers of singlet oxygen (*3*), and pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate, coenzyme forms of vitamin B_6, show stronger protection of yeast cells from oxidative death than vitamin C, a representative antioxidative vitamin (*4*). Thus, the development of a method for the determination of individual vitamin B_6 compounds plus pyridoxine-β-glucoside in foods is required to estimate their functionality.

Although many methods have been developed for the determination of individual vitamin B_6 compounds, no method can be satisfactorily applied to the analysis of food samples. Thus, a microbioassay involving a budding yeast, *Saccharomyces cerevisiae* (former name, *S. uvarum*), which was developed about 40 y ago (*5*), has been used to determine the total content of vitamin B_6 in food samples; in the figure, the content of pyridoxine-β-glucoside is included because food samples are prehydrolyzed with HCl for the microbioassay, and pyridoxine-β-glucoside in the samples is converted into pyridoxine. The best method so far developed for individual determination may be cation-exchange HPLC with a fluorescence detection system (*6*), which is applicable to biological samples, such as serum, brain, liver, milk and so on, from experimental animals. The second method applicable to samples containing specifically high amounts of vitamin B_6 compounds may be reversed-phase isocratic HPLC with a fluorescence detection system (*7*). However, these methods and other ones recently reported (*8–11*), which are based on HPLC separations, suffer the disadvantage of difficulties for applying to analyses of foods, especially plants and beans, because they contain only low amounts of individual vitamin B_6 compounds together with much higher amounts of fluorescent components which are eluted as interfering peaks on HPLC.

To determine individual vitamin B_6 compounds in such foods, both the specificity and sensitivity of the methods should be improved. One promising way to increase the specificity is to harness the functions of enzymes. Recently, we cloned and expressed several enzymes involved in the degradation pathway for pyridoxine (*12–14*). Three of them, when they are used alone or in combination, can specifically convert the free (unphosphorylated) forms of vitamin B_6 into a highly fluorescent vitamin B_6-derivative, 4-pyridoxolactone (*15*), which can be measured in pmol amounts by HPLC.

Here, we have developed a new method (designated as the Enzyme-HPLC method) for the determination of individual vitamin B_6 compounds. We previously developed an enzymatic fluorometric assay for pyridoxal

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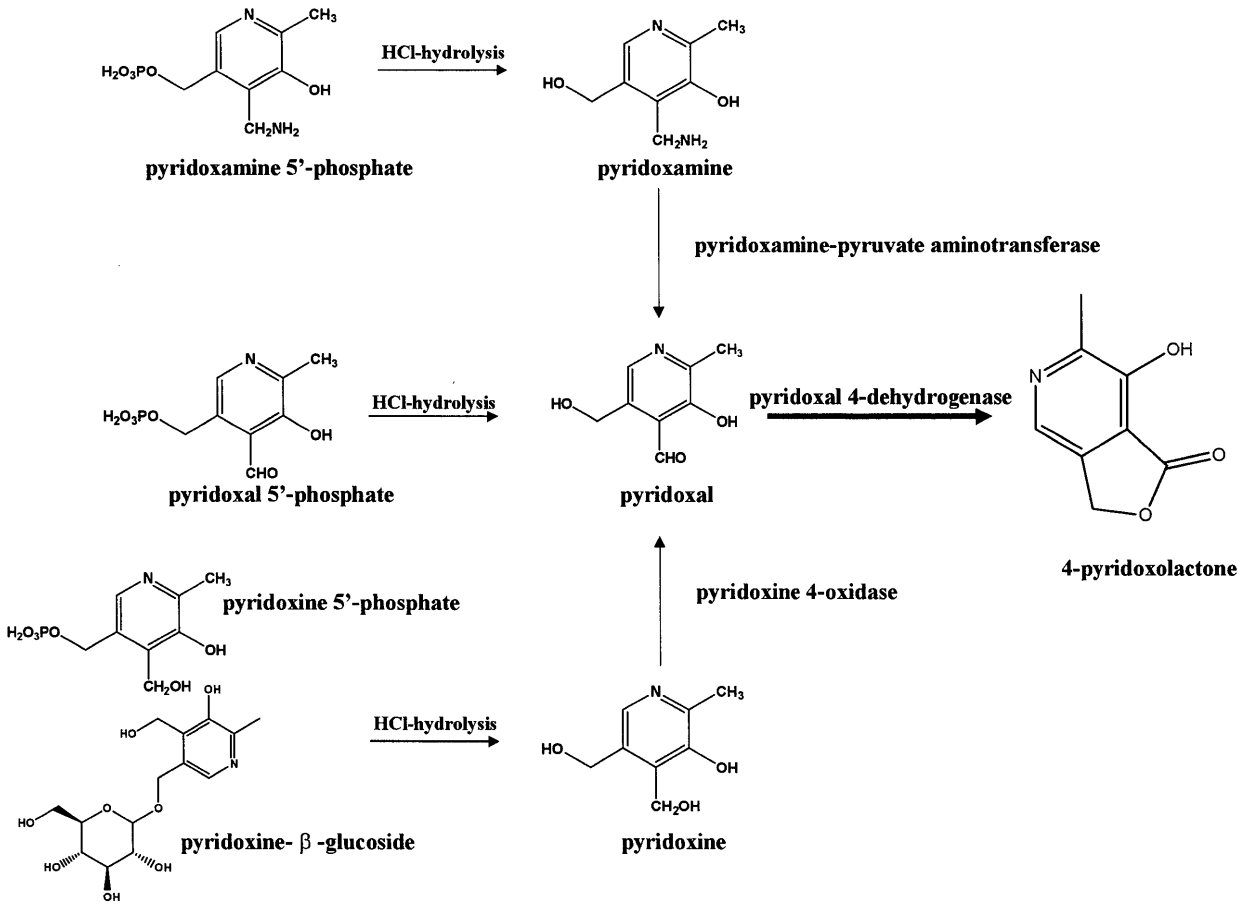

Fig. 1. Strategy for the enzyme-HPLC assay for natural vitamin B_6 compounds and pyridoxine-β-glucoside. All vitamin B_6 compounds were enzymatically converted into 4-pyridoxolactone, a highly fluorescent compound, which was then determined by HPLC.

with high specificity and sensitivity (*16*), and the new method is an advanced version for individually determining natural vitamin B_6 compounds.

MATERIALS AND METHODS

Strategy. The strategy for the Enzyme-HPLC method for the determination of individual vitamin B_6 is shown in Fig. 1. Pyridoxine, pyridoxamine, and pyridoxal are specifically converted into 4-pyridoxolactone by three enzymes involved in the degradation pathway for vitamin B_6, and then 4-pyridoxolactone is determined by reversed-phase isocratic HPLC. Pyridoxal is converted into 4-pyridoxolactone by pyridoxal 4-dehydrogenase, which catalyzes the irreversible oxidation of pyridoxine to 4-pyridoxolactone (*14*). Pyridoxine is converted into 4-pyridoxolactone through a coupling reaction involving pyridoxine 4-oxidase (*12*) and pyridoxal 4-dehydrogenase: both reactions are irreversible. Pyridoxamine is converted into 4-pyridoxolactone through a coupling reaction involving pyridoxamine-pyruvate aminotransferase (*13*) and pyridoxal 4-dehydrogenase. Although the transamination reaction is reversible, the coupled reaction becomes irreversible because pyridoxal 4-dehyrogenase catalyzes no reverse reaction. Thus, pyridoxamine would be totally converted into 4-pyridoxolactone.

Three phosphate forms and pyridoxine-β-glucoside were hydrolyzed at 121°C with HCl to convert them into the corresponding free forms according to the AOAC official method for the determination of vitamin B_6 in foods samples (AOAC Official Method 961.15). Then, they are converted into 4-pyridoxolactone as described above.

Materials. Recombinant pyridoxal 4-dehydrogenase (*14*), pyridoxine 4-oxidase (*12*), and pyridoxamine-pyruvate aminotransferase (*13*) were prepared as previously reported. Pyridoxine 5′-phosphate was prepared from pyridoxal 5′-phosphate through reduction with sodium borohydride. Pyridoxine and pyridoxal 5′-phosphate were purchased from Nacalai Tesque, Inc. (Kyoto, Japan), and pyridoxamine and pyridoxal from Sigma Chemical Co. (St. Louis, MO). Pyridoxamine 5′-phosphate was a gift from Daiichi Fine Chemicals Co. (Takaoka, Japan). Pyridoxine-β-glucoside was a gift from Dr. H. Tsuge (Chubu University, Aichi, Japan) and Dr. K. Tadera (Kagoshima University, Kagoshima, Japan).

Sample preparation. All food samples were dried according to the AOAC official method, and then ground with a Mini Blender (Osaka Chemicals, Osaka, Japan). For the determination of pyridoxine, pyridoxal and pyridoxamine, the dried powder (0.05 g) of each vegetable, legume, and grain was suspended in 10 mL of cold 0.88 M HCl, and then kept for 5 h on ice. For

determination of their 5′-phosphate forms and pyridoxine-β-glucoside, the suspensions were autoclaved at 121°C for 5 h. The cold extracts and autoclaved solutions were neutralized with 8.8 M NaOH, and then the neutralized solutions were passed through a glass filter, GF/A (Whatman, Maidstone, England). Generally, 50 μL of a filtrate was used for the next enzymatic reaction. For other vegetable foods such as pepper and garlic, 0.44 M HCl was used. Meat was treated with 0.055 M HCl.

Internal standards (almost the same amount as that found in the food samples for each vitamin B_6 compound) were added to the ground food samples just before making the suspension.

Analytical method

HPLC system: 4-Pyridoxolactone was measured by a reversed-phase isocratic HPLC method. It was separated with a Jasco HPLC system (JASCO, Tokyo, Japan) equipped with a PU-2080 pump, an AS-2055 autosampler, and an FP-920 fluorescence detector. Optimum separation was obtained on a Cosmosil 5C18MS-II column (250×4.6 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 μL, respectively. The fluorescence intensity of the eluted 4-pyridoxolactone was monitored at 430 nm (excitation at 360 nm).

For regression analyses, reversed-phase isocratic HPLC with a fluorescence detection system for measurement of the standard natural vitamin B_6 compounds was performed as described previously (*17*). This method is designated as classic HPLC in Table 1.

Enzyme assays: Pyridoxal 4-dehydrogenase (*14*), pyridoxine 4-oxidase (*12*), and pyridoxamine-pyruvate aminotransferase (*13*) were assayed as described previously. The activities of the enzymes were assayed every time just before usage for enzymatic conversion of vitamin B_6 compounds into 4-pyridoxolactone.

Table 1. Slopes and R^2 of the linear regression lines between values determined with the Enz-HPLC method and classic HPLC.

Analyzed vitamin B_6 compound	Slope	R^2
Pyridoxal	1.126	0.99
Pyridoxamine	0.949	0.99
Pyridoxine	1.114	0.99
Pyridoxal 5′-phosphate		
0.055 M HCl	0.994	0.98
0.44 M HCl	0.939	0.99
0.88 M HCl	0.950	0.99
Pyridoxamine 5′-phosphate		
0.055 M HCl	1.056	0.99
0.44 M HCl	0.993	0.99
0.88 M HCl	0.949	0.99
Pyridoxine 5′-phosphate		
0.055 M HCl	1.052	0.99
0.44 M HCl	0.969	0.99
0.88 M HCl	0.990	0.99

Reaction conditions for the enzymatic conversion: Pyridoxal was converted into 4-pyridoxolactone at 30°C for 1 h in a 600 μL capped Eppendolf tube containing 180 μL of a reaction mixture consisting of 20 mM sodium phosphate buffer (pH 8.0), 1 mM NAD, 1 mU pyridoxal 4-dehydrogenase, and 0.5–200 pmol of pyridoxal. The enzyme reaction was stopped by the addition of 20 μL of 0.44 M HCl. The reaction mixture was filtered through a Dismic-13 cp (pore size of 0.2 μm, Advantec, Tokyo, Japan), and then 100 μL of the filtrate was injected into the HPLC column. To convert pyridoxamine and pyridoxine into 4-pyridoxolctone, 4 mM pyruvate and 1 mU pyridoxamine-pyruvate aminotransferase, and 5 μM FAD and pyridoxine 4-oxidase, respectively, were added to the reaction mixture.

Calculation of contents: Because food samples contain all forms of vitamin B_6, the pyridoxal content in them was determined with the cold extract solution at first. The pyridoxal 5′-phosphate content was then calculated by subtracting the pyridoxal content from the value obtained with the autoclaved solution. The pyridoxine content was calculated by subtracting the pyridoxal content from the value obtained with the cold extract through the coupling reaction involving pyridoxine oxidase and 4-pyridoxal dehydrogenase. The contents of pyridoxine 5′-phosphate and pyridoxine β-glucoside were calculated by subtracting the contents of pyridoxal, pyridoxal 5′-phosphate, and pyridoxine from the value obtained with the autoclaved sample through the coupling reaction involving the two enzymes. The pyridoxamine content was calculated by subtracting the pyridoxal content from the value obtained with the cold extract through the coupling reaction involving pyridoxamine-pyruvate aminotransferase and pyridoxal 4-dehydrogenase. The pyridoxamine 5′-phosphate content was calculated by subtracting the contents of pyridoxal, pyridoxal 5′-phosphate, and pyridoxamine from the value obtained with the autoclaved sample through the coupling reaction involving the two enzymes.

RESULTS

Determination of 4–pyridoxolactone by HPLC, and conversion of pyridoxal, pyridoxine, and pyridoxamine through the enzyme reactions

Standard 4-pyridoxolactone was eluted as a sharp single peak at around 11 min (Fig. 2A). The lower and upper detection limits were 0.1 pmol and 100 pmol, respectively. Pyridoxal, pyridoxine, and pyridoxamine, which had been changed to 4-pyridoxolactone by the enzymatic reactions, were eluted at the same elution time as that of the standard 4-pyridoxolactone as a single peak with the same peak height and area as those of the standard one. The elution pattern of pyridoxamine after conversion into 4-pyridoxolactone with pyridoxamine-pyruvate aminotransferase and pyridoxal 4-dehydrogenase is shown in Fig. 2B. Their 5′-phosphate forms and pyridoxine-β-glucoside, which had been changed to 4-pyridoxolactone by the HCl-hydrolysis

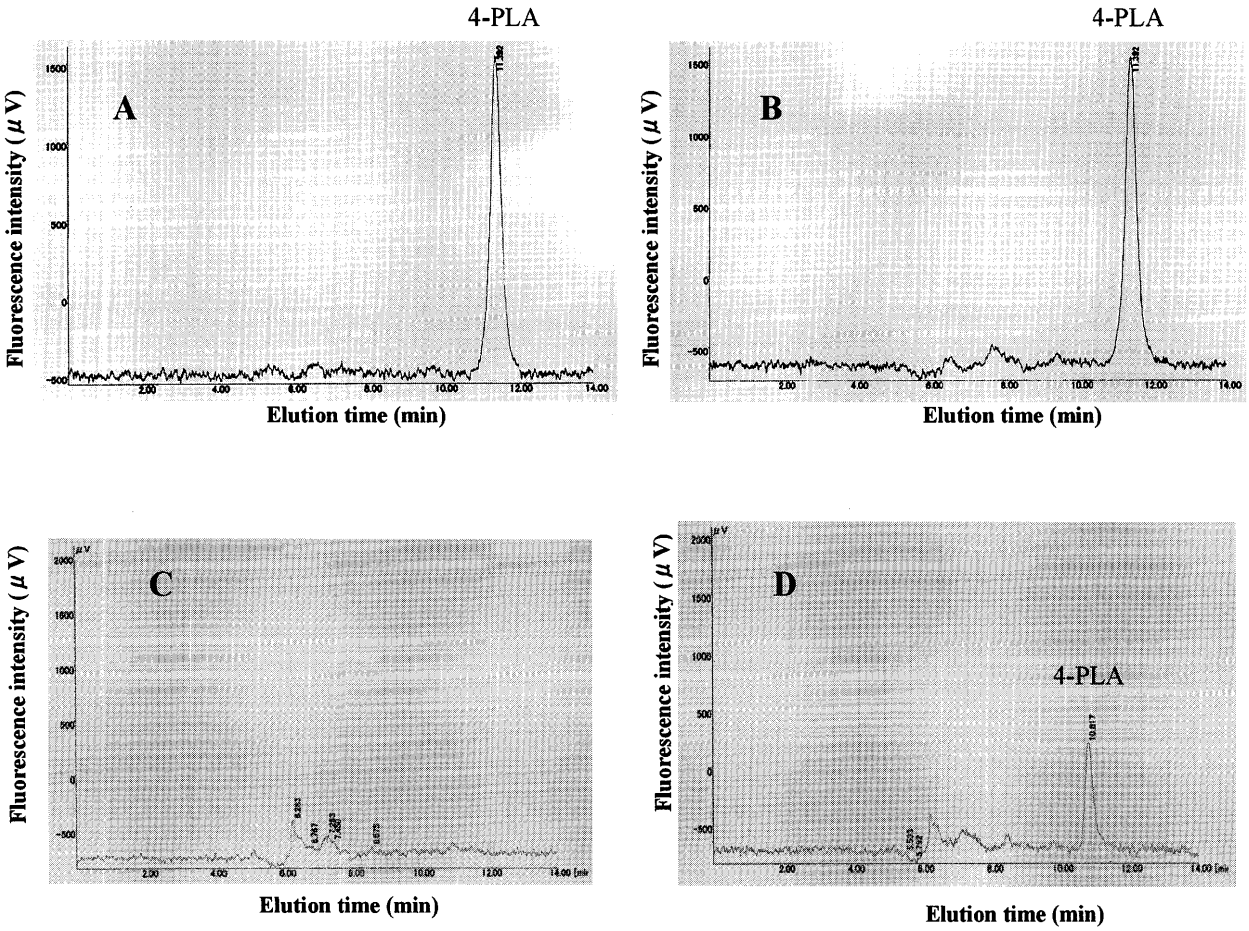

Fig. 2. HPLC chromatograms. (A) Standard 4-pyridoxolactone (4-PLA), 1 pmol. (B) Standard pyridoxamine (1 pmol) was enzymatically converted into 4-pyridoxolactone. (C) The cold extract derived from chicken white meat was subjected to enzymatic conversion with pyridoxamine-pyruvate aminotransferase and pyridoxal 4-dehydrogenase, and the reaction was stopped at 0 min. (D) The cold extract derived from chicken white meat was subjected to enzymatic conversion with pyridoxamine-pyruvate aminotransferase and pyridoxal 4-dehydrogenase, and the reaction was stopped at 60 min.

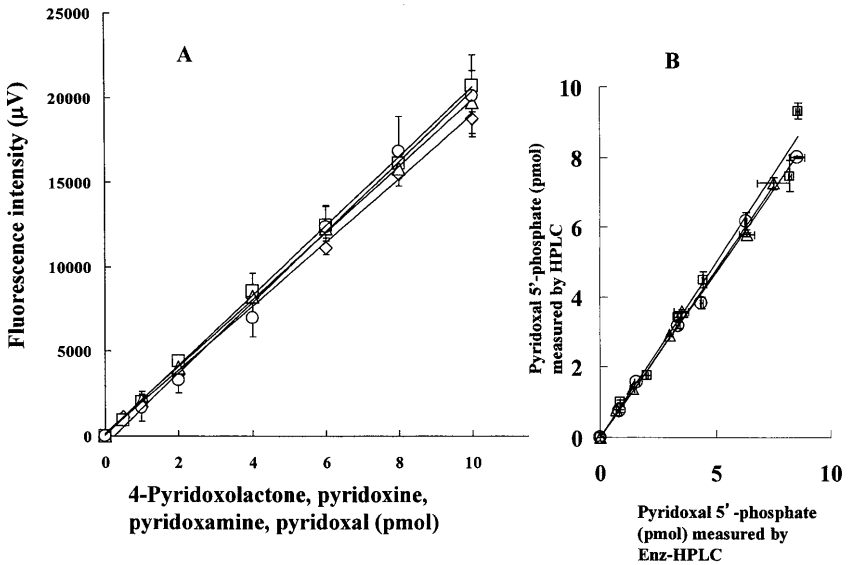

Fig. 3. Calibration curves for 4-pyridoxolactone, pyridoxal, pyridoxine and pyridoxamine, and correlation curves. (A) Calibration curves for: ◇, 4-pyridoxolactone; □, pyridoxal; ○, pyridoxine; △, pyridoxamine. (B) Correlation plots and linear regression lines of pyridoxal 5′-phosphate values determined with the HPLC and Enz-HPLC methods. Pyridoxal 5′-phosphate was hydrolyzed with 0.055 M (□), 0.44 M (○), and 0.88 M (△) HCl. Each point represents the mean and SD of duplicate experiments.

Table 2. Contents and recoveries of vitamin B_6 compounds in three food samples.

Materials	Contents (μg/100 g) and Yields (%)					
	PL[a]	PLP[a]	PM[a]	PMP[a]	PN[a]	PNP(G)[a]
Chicken white meat						
Contents	93.4±4.0	879.7±45.4	98.4±10.8	1,219.7±111.4	ND[b]	ND[b]
Yields[d]	84.9±2.1	58.1±3.5	70.3±15.4	90.4±5.1	—	—
Pepper						
Contents	89.1±3.8	138.1±0.5	66.7±14.7	242.5±31.4	910±2.0	5,468±134.3
Yields[d]	77.2±26.2	ND[b]	94.5±38.3	NE[c]	55.5±5.3	41.9±10.9
Garlic						
Contents	69.9±1.3	64.7±38	152.6±19.8	ND[b]	136.5±11.3	2,243.6±88.1
Yields[d]	78.9±7.2	NE[c]	NE[c]	—	128.8±5.3	42.1±0.9

[a] PL, PLP, PM, PMP, PN, and PNP(G) mean pyridoxal, pyridoxal 5′-phosphate, pyridoxamine, pyridoxamine 5′-phosphate, pyridoxine, and pyridoxine 5′-phosphate(-β-glucoside), respectively.
[b] ND and [c] NE represent not detectable and not evaluated, respectively.
[d] Yields were determined by the internal standard method. For example, 100 μg of PL was added to the dried powder (100 g) of chicken white meat. Then, the sample with or without standard PL was suspended, and the amount of PL was determined as described under "Materials and Methods." The amount of PL in the sample with or without standard PL was 164.2 (μg/100 g) or 79.3 (μg/100 g), respectively. Thus, yield in this case was calculated as follows: (164.2−79.3)/100×100=84.9 (%). For determination of yield of PMP, 1,300 μg of PMP was added to the dried powder (100 g). Likewise, a similar amount of the standard vitamin B_6 compounds as that determined by this method was added as the internal standard.

and the enzyme reactions, were also eluted at the same elution time as that of the standard 4-pyridoxolactone as a single peak with the same peak height and area as those of the standard one. The calibration curves (Fig. 3A) showed that pyridoxine, pyridoxal, and pyridoxamine were determined with the same sensitivity as 4-pyridoxolactone, showing that they were completely converted into 4-pyridoxolactone through the enzyme reactions. The three phosphate forms and the glucoside, after hydrolyses with HCl, also gave the same calibration curves (data not shown). Thus, all vitamin B_6 compounds could be determined with the same sensitivity by the Enzyme-HPLC method.

Correlation plots and regression lines

The concentrations of the vitamin B_6 compounds and pyridoxine-β-glucoside were measured by both the HPLC method and the Enzyme-HPLC method. The correlation plots and linear regression lines are shown in Fig. 3B, pyridoxal 5′-phosphate being determined by both methods. Three lines were obtained because it was hydrolyzed with three different HCl concentrations. The slopes and R^2 values are shown in Table 1. The same plots and lines were obtained when pyridoxine 5′-phosphate and pyridoxamine 5′-phosphate were determined. The slopes and R^2 values are also shown in Table 1. For pyridoxal, pyridoxine, and pyridoxamine, a single correlation plot and linear regression line was obtained for each, because they did not need HCl-hydrolysis. They also exhibited good correlation coefficients, as shown in Table 1. Because we only had a limited amount of pyridoxine-β-glucoside, only 2 and 10 pmol were examined with the two methods, the results correlated well at these concentrations (data not shown).

Contents in several samples

The contents and yields of individual vitamin B_6 compounds in three food samples were determined by the Enzyme-HPLC method, as shown in Table 2. As a representative example, the elution pattern of 4-pyridoxolactone corresponding to the amount of pyridoxal plus pyridoxamine in chicken white meat is shown in Fig. 2D. Figure 2C shows the elution pattern of a control sample, in which these vitamin B_6 compounds were not converted into 4-pyridoxolactone because the enzyme reaction was stopped at 0 min. The fluorescence signal of the control at around 11 min was very low and thus did not prevent accurate determination of 4-pyridoxolactone derived from the food sample.

Chicken white meat contained neither pyridoxine nor pyridoxine 5′-phosphate. It contained high amounts of pyridoxamine 5′-phosphate and pyridoxal 5′-phosphate. The recoveries determined with internal standards were fairly good. The sum of the vitamin B_6 compounds was 0.63 mg/100 g of dried edible part. This value was similar to the 0.6 mg/100 g of dried edible part shown in the Standard Tables of Food Composition in Japan, 5th Revised and Enlarged Edition (STFCJ), for which the total contents were determined by means of a microbiological assay. Red pepper (Japanese tougarashi) contained a large amount of pyridoxine 5′-phosphate or pyridoxine-β-glucoside (probably the latter as described later), and a high amount of pyridoxine. Garlic also contained a large amount of pyridoxine 5′-phosphate or pyridoxine-β-glucoside (also probably the latter as described later). Both vegetable foods contained low amounts of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate. The recoveries of pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, and pyridoxamine for vegetable foods could not be determined precisely for an unknown reason.

DISCUSSION

All natural vitamin B_6 compounds were individually determined by specifically converting them into 4-pyridoxolactone with a combination of enzyme reactions and HCl-hydrolysis. We have found that the official AOAC conditions for HCl hydrolysis, 0.44 M–2 h and 0.88 M–3 h, were not enough for the hydrolysis of pyridoxamine 5′-phosphate or pyridoxine 5′-phosphate, and so the period of hydrolysis should be extended up to 5 h. Several studies have indicated that the official AOAC conditions are not enough for the hydrolysis of the phosphate forms of vitamin B_6 in vegetable foods (*18*). On the other hand, such a long period of hydrolysis with HCl may result in loss of vitamin B_6 compounds through some reactions between them and other components, such as amino acids, in foods. Indeed, as shown in Table 2, the yield of pyridoxal 5′-phosphate and pyridoxamine 5′-phosphate in red pepper could not be determined probably because they reacted with other components in it. Thus, although here we have used the HCl-hydrolysis according to the general AOAC method currently applied for food analyses, rapid and mild conditions for the hydrolysis, which will not cause side reactions, are required. We are currently developing such an enzymatic hydrolysis procedure.

Chicken white meat contained high amounts of pyridoxamine 5′-phosphate and pyridoxal 5′-phosphate but no pyridoxine or pyridoxine-β-glucoside (or pyridoxine 5′-phophate). In contrast, two vegetable foods, red pepper and garlic contained very high amounts of pyridoxine-β-glucoside or pyridoxine 5′-phosphate; probably the former compound because it is a major storage form of vitamin B_6 in plant-derived foods (*19*). We are developing an enzymatic hydrolysis method in which pyridoxine-β-glucoside is specifically hydrolyzed by a β-glucosidase from a microorganism, which is specific for the substrate as a porcine enzyme (*20*).

The Enz-HPLC method has an advantage over the classic HPLC methods in its high specificity. So, even samples containing many and high amounts of contaminants, which are co-eluted at the same elution times as those of vitamin B_6 compounds and interfere with their determination, can be analyzed. On the other hand, the former method needs three enzymatic reaction steps, and takes a longer time to get results, compared to the latter one. Some way to make the enzymatic reaction step easy should be developed to spread this method for a general individual determination of vitamin B_6 compounds.

Acknowledgments

We would like to express our gratitude to Dr. Haruto Tsuge, Chubu University, and Dr. Kenjiro Tadera, Kagoshima University for their kind offering of pyridoxine-β-glucoside.

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