Improved Chromatographic Determination of Free and Glycosylated Forms of Vitamin B_6 in Foods[†,‡]

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Glycosylated forms of vitamin B_6, including 5′-*O*-(β-D-glucopyranosyl)pyridoxine (PN-glucoside) and several derivatives, often comprise a major fraction of the total vitamin in plant-derived foods. An ion-pair reverse-phase HPLC procedure has been previously reported that is suitable for the measurement of PN-glucoside and all free (nonglycosylated) forms of the vitamin. The present research was conducted to evaluate the efficacy of procedures for selective enzymatic and acid-catalyzed hydrolysis of glycosylated forms of the vitamin. Application of these procedures permits a determination of total vitamin B_6 as well as the individual free and glycosylated fractions. Glycosylated forms of pyridoxine were found to comprise 0, 57, 67, 77, and 82% of the total vitamin B_6 in oat bran, wheat bran, soy flour, carrots, and rice bran, respectively.

INTRODUCTION

Although evidence of uncharacterized "bound" forms of vitamin B_6 in plant-derived foods has long been apparent (Siegel et al., 1943; Rabinowitz and Snell, 1947; Yasumoto et al., 1976; Nelson et al., 1977), 5′-*O*-(β-D-glucopyanosyl)-pyridoxine (PN-glucoside) from rice bran was the first glycosylated form of the vitamin to be identified (Yasumoto et al., 1977). Additional derivatives having acyl groups (malonyl or 3-hydroxy-3-methylglutaryl) esterified to the C-6 position of the glucopyranosyl moiety of PN-glucoside (Tadera et al., 1983, 1985) have been reported to exist as a low percentage of the total vitamin B_6 in pea seedlings germinated in the presence of added pyridoxine (PN). Whether these PN-glucoside esters occur naturally in significant quantities is unclear. Three other pyridoxine glucosides have been isolated and identified from rice bran, including 4′-*O*-(β-D-glucosyl)-5′-*O*-(β-D-glucosyl)pyridoxine, 5′-*O*-(β-D-cellobiosyl)pyridoxine, and a partially characterized 5′-*O*-(β-D-glucotriosyl)pyridoxine. The significance of these B_6 oligosaccharides in common foods has not been determined.

The nutritional significance of glycosylated vitamin B_6 is related to its incomplete bioavailability. Kabir et al. (1983a) first observed an inverse correlation between the net bioavailability of vitamin B_6 for human subjects and the proportion of glycosylated vitamin B_6 in several tested foods, although subsequent studies with additional foods demonstrated considerable variability in this relationship (Bills et al., 1987). Studies of the bioavailability for human subjects of purified deuterium-labeled forms of PN-glucoside and PN indicated a bioavailability of 58 ± 10% (mean and SEM) of orally administered PN-glucoside relative to that of PN (Gregory et al., 1991). In contrast, the bioavailability of PN-glucoside in the rat is approximately 20–30% relative to PN (Ink et al., 1986; Trumbo et al., 1988a,b). Because the proportion of glycosylated vitamin B_6 is a determinant of the net bioavailability of dietary B_6, the ability of an assay to differentiate between total and glycosylated vitamin B_6 would thus be of value in the nutritional assessment of diets.

Assays of total vitamin B_6 have traditionally been based on the acid-catalyzed hydrolysis of vitamin B_6 5′-phosphates and bound (i.e., glycosylated) derivatives by autoclaving in HCl (e.g., 0.44 M HCl, 2 h, 121 °C; AOAC, 1970; Gregory, 1988). Aside from early studies of the assay of vitamin B_6 from several plant-derived foods (Rabinowitz and Snell, 1947), little information is available concerning the adequacy of this treatment for the hydrolysis of PN from PN-glucoside and other glycosylated derivatives. Kabir et al. (1983b) reported a differential microbiological assay for the measurement of total and glycosylated vitamin B_6 in foods using *Saccharomyces uvarum*. This procedure was based on the use of sample pretreatments by acid hydrolysis in 0.44 M HCl (2 h, 121 °C) for the determination of total vitamin B_6, while glycosylated vitamin B_6 was determined by assay of a buffer extract of the sample conducted before and after treatment with a β-glucosidase. The procedure of Kabir et al. (1983a) was modified by Tadera et al. (1986). These authors reported the existence of a type of glycosylated vitamin B_6 in certain plant-derived foods that could be released by β-glucosidase treatment only after prior incubation in strong base (0.7 M KOH) for 3 h. Although this form of glycosylated vitamin B_6, termed "B6X", was not identified, Tadera et al. (1986) proposed that it may be composed of PN-glucoside esterified to an organic acid which is released by saponification. The B6X fraction was present in podded peas (21% of total B_6), defatted soybeans (27% of total), rice bran (38% of total), and wheat bran (19% of total) but was not detected in cauliflower, spinach, pumpkin, and broad beans (Tadera, 1986). The quantitative significance of B6X in other foods has not been determined.

As an extension of methodology developed for the measurement of vitamin B_6 in foods and other biological materials (Gregory and Feldstein, 1985), Gregory and Ink (1987) reported that PN-glucoside could be measured directly by HPLC analysis of food extracts or indirectly as free pyridoxine following treatment with β-glucosidase. Of the plant-derived foods analyzed, including broccoli, bananas, green beans, carrots, orange juice, and alfalfa sprouts, the content of glycosylated vitamin B_6 whether measured directly as PN-glucoside or indirectly as free pyridoxine released by enzymatic treatment was approximately equivalent. These results indicated that PN-glucoside accounted for most or all of the glycosylated vitamin

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B_6 in these samples (5–75% of total vitamin B_6), although the absence of a saponification during preparation of the extracts precluded assessment of the B6X fraction. There was no evidence of significant glycosylation of pyridoxal (PL) or pyridoxamine (PM).

This study was intended to extend the application of our previous HPLC method for the measurement of free and glycosylated vitamin B_6 in plant-derived foods. Specifically, selected foods were analyzed by measurement of PN-glucoside and nonglycosylated forms of vitamin B_6 before and after enzymatic and acid-catalyzed hydrolysis techniques. The objectives of this study were to determine sample preparation and analytical techniques appropriate for the determination of all forms of vitamin B_6 in selected food samples.

MATERIALS AND METHODS

Vitamin B_6 Compounds, Reagents, and Food Samples. Pyridoxine (PN), pyridoxal (PL), pyridoxamine (PM), and 4′-deoxypyridoxine (4dPN) were purchased as hydrochloride salts from Sigma Chemical Co. (St. Louis, MO). Pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) were also purchased from Sigma. These compounds were used in the preparation of standards without further purification. PN-glucoside was prepared by biological synthesis that involved the germination of alfalfa seeds in the presence of added pyridoxine (Ink et al., 1986). PN-glucoside was extracted and purified by cation-exchange and gel filtration chromatography (Trumbo et al., 1988a,b).

β-Glucosidase from almonds was obtained from Sigma (Catalog No. G4511, EC 3.2.1.21, label claim 39 units/mg solids, where 1 unit liberates 1 μmol of glucose/min from salicin at pH 5.0 at 37 °C). Acid phosphatase from potatoes was also obtained from Sigma (Catalog No. P1146, EC 3.1.3.2, 3–10 units/mg solids, where 1 unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate/min at pH 4.8 at 37 °C).

2-Propanol (HPLC grade) and 1-octanesulfonic acid (Certified ACS, as sodium salt) were obtained from Fisher Scientific Co. (Pittsburgh, PA). All other reagents were of analytical grade.

Food samples, obtained at a local grocery store, included fresh carrots, rice bran, oat bran, wheat bran, and whole soybean flour. For the purposes of this study, only a single commercial sample of each material was obtained. Each extraction and analysis was performed in triplicate.

Extraction and Sample Preparation Procedures. The extraction procedure involving sulfosalicylic acid was based on that employed previously (Gregory and Feldstein, 1985; Gregory and Ink, 1987), which was based on the method originally described by Vanderslice et al. (1980). For a typical extraction of dry food samples, 2 g was weighed and transferred to a 50-mL polypropylene centrifuge tube, mixed with 18 mL of 5% (w/v) sulfosalicylic acid (SSA) and 2 mL of a solution of 4dPN (typically 600 nmol/mL) as an internal standard, and then homogenized with a polytron device (Model PTA 10S probe; Brinkmann Instruments Co., Westbury, NY) for 30 s at full power. Methylene chloride (10 mL) was added and the mixture homogenized for an additional 20 s, followed by centrifugation at 9000*g* for 15 min at 2°C. Portions of the supernatants were retained for anion-exchange purification, selective hydrolysis of glycosylated forms, and HPLC analysis. The ratio of sample to 5% SSA was increased as needed (e.g., 3 g of chopped carrot and 9.5 mL of SSA).

Anion-exchange chromatography was performed to remove SSA and provide partial purification of the extracts by a modification of the method of Vanderslice et al. (1980). Disposable polypropylene columns (ca. 8 mm i.d., Model QS-Q, Isolab Inc., Akron, OH) were slurry-packed with 3 mL of Bio-Rad AG 2-x8 (200–400 mesh, Cl^- form; Bio-Rad Laboratories, Richmond, CA) and equilibrated with 0.1 M HCl. A 1-mL portion of each extract was applied to an AG 2-x8 column and the corresponding 1-mL effluent discarded. All forms of vitamin B_6 and the internal standard were then eluted with 5 mL of 0.1 M HCl. Preliminary studies indicated complete recovery from these columns.

Portions of the purified extracts were subjected to several hydrolytic treatments essentially as described by Tadera et al. (1986), as follows: (a) A 1-mL aliquot of the purified extract was retained and analyzed by HPLC with no further treatment. (b) Another portion (2 mL) of the purified extract was adjusted to pH 5.0 with KOH and incubated with 0.2 mL of 1 mg/mL aqueous solution of β-glucosidase at 37 °C for 2.5 h. [These conditions were previously shown to yield complete hydrolysis of PN-glucoside (Gregory and Ink, 1987).] (c) A third portion (2 mL) was combined with 0.265 mL of 6 M KOH and incubated in the dark for 3 h at ambient temperature. A portion of this was neutralized with HCl and retained for HPLC analysis. (d) Another portion of the KOH-treated extract above was adjusted to pH 5.0 and incubated with β-glucosidase as described previously. Each extract was analyzed by HPLC as described below. Prior to HPLC analysis, the enzyme-treated samples were subjected to centrifugal ultrafiltration through YMT membranes (Micropartition System, Amicon, Danvers, MA) which allowed vitamin B_6 compounds to pass freely but retained protein. In selected samples, treatment b was modified to include simultaneous treatment with acid phosphatase and β-glucosidase as described below. The inclusion of acid phosphatase was useful in the measurement of PLP as PL in plant samples that contained interfering compounds that eluted near PLP.

For comparison, selected samples were also subjected to acid-catalyzed hydrolysis (AOAC, 1970). Three grams of chopped carrot was mixed with 0.5 mL of 600 μM 4dPN and 9.5 mL of 0.59 M HCl and homogenized by using a polytron (final acid concentration approximately 0.44 M). After heating in an autoclave at 121 °C for 2 h, the mixtures were cooled to ambient temperature and extracted with methylene chloride as described previously. Two 2-mL portions of the aqueous layer were removed, combined with an equal volume of 0.2 M trisodium citrate, and adjusted to pH 5.0 with KOH. One portion was mixed with 0.1 mL of acid phosphatase (2 mg/mL in 0.8 M potassium acetate, pH 5.0) and 0.2 mL of β-glucosidase (1 mg/mL aqueous solution), incubated at 37 °C for 3 h to dephosphorylate any remaining PLP or PMP and to hydrolyze any residual glycosylated vitamin B_6, and then subjected to ultrafiltration. Each solution was then analyzed by HPLC.

Analytical Procedures. HPLC analyses were performed according to a modification of our previously described procedures (Gregory and Feldstein, 1985; Gregory and Ink, 1987). The HPLC system consisted of a gradient liquid chromatograph (Rainin Instruments, Woburn, MA) equipped with two rabbit HP pumps, a Model 7125 injection valve (Rheodyne, Cotati, CA), and a gradient controller. A Model FD300 fluorescence detector equipped with a 20-μL flow cell (Spectrovision, Chelmsford, MA) was employed, operated at an excitation wavelength of 295 nm and an emission wavelength of 405 nm. Chromatographic peaks were quantified relative to the area of the internal standard by using a Model 3396A or 3388A integrator (Hewlett-Packard Co., Avondale, PA).

A Beckman Ultrasphere ion-pair (IP) column (4.6 mm i.d. × 25 cm; octadecylsilyl, 5-μm particle size; Beckman Instruments, Inc., San Ramon, CA) was used for all analyses. The mobile phases consisted of (a) 0.033 M phosphoric acid and 8 mM octanesulfonic acid, pH 2.2, and (b) 0.033 M phosphoric acid and 17% (v/v) 2-propanol. The separation was performed at ambient temperature at a flow rate of 1.0 mL/min, with a slightly concave gradient from 17% solvent b to 31% solvent b in 11 min, followed by a linear increase to 100% solvent b in 4 min and a 15-min hold at 100% solvent b. The system was re-equilibrated with 17% solvent b for 20 min prior to the next injection. All injections (100 μL) were made by using a filled-loop technique. A blank gradient was run before the injection of standards and samples. Methanol was the storage solvent for the HPLC system. Water used in the preparation of the mobile phases was purified by using a Milli-Q system (Millipore Corp., Bedford, MA).

RESULTS AND DISCUSSION

The HPLC separation reported here is a modification of that reported previously with respect to the binary rather than ternary gradient employed and the different procedures for sample preparation. Typical separations were shown in Figure 1. In contrast to the column employed previously (Perkin-Elmer 3 × 3 C18), the Ultrasphere IP

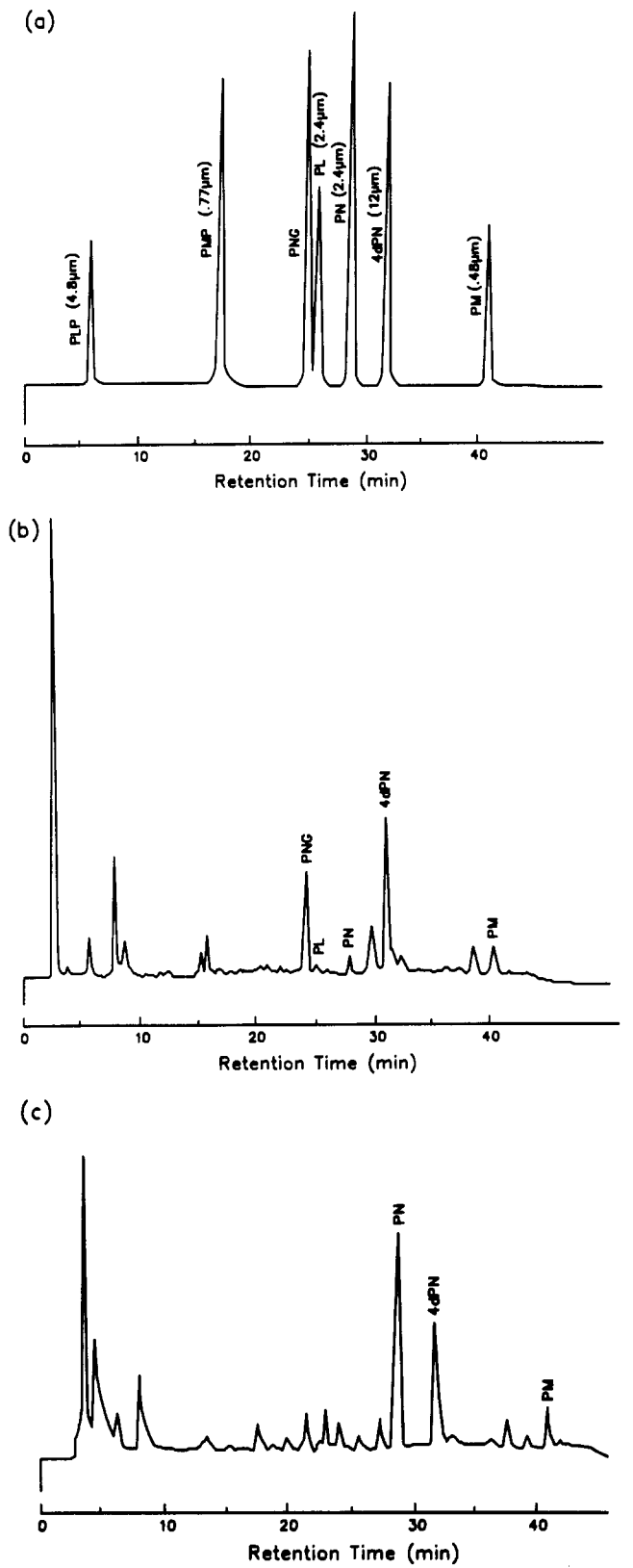

Figure 1. Typical chromatograms from (a) standard mixture of vitamin B_6 compounds, (b) SSA extract of rice bran following AG 2-x8 column, and (c) SSA extract of rice bran following AG 2-x8 column, then sequential treatment with KOH and combined acid phosphatase and β-glucosidase.

column used in this study was chosen because of its more effective separation of PLP, the first vitamin B_6 component eluted, from the solvent front. When analyzed by using the form of Perkin-Elmer 3 × 3 column currently marketed, PLP is retained very weakly, which makes its measurement difficult. There have been reported similar separations using other commercially available columns (Hollins and Henderson, 1986; Sampson and O'Connor, 1989; Bitsch and Muller, 1989).

The extraction with SSA, as developed by Vanderslice et al. (1980), has been shown to be effective in extracting various forms of vitamin B_6 from foods of plant and animal origin (Vanderslice et al., 1980; Gregory and Feldstein, 1985; Gregory and Ink, 1987). This procedure also prevents enzymatic interconversion of B_6 compounds following homogenization of samples. The vitamin B_6 analogue 4′-deoxypyridoxine (4dPN) is effective as an internal standard because of its stability under conditions of this analysis and its separation from other sample components in the HPLC analysis (Gregory and Feldstein, 1985).

Enzymatic Hydrolysis Procedures. In this study, samples were prepared for HPLC analysis by several methods including the following: (1) extraction using SSA followed by direct analysis (Vanderslice et al., 1980); (2) measurement of PN-glucoside and other forms of vitamin B_6 before and after treatment of SSA extracts with β-glucosidase (Gregory and Ink, 1987); (3) analysis after treatment of SSA extracts with KOH and β-glucosidase (Tadera et al., 1986); and (4) extraction by incubation in 0.44 M HCl for 2 h at 121 °C (AOAC, 1970) followed by HPLC analysis. Acid phosphatase treatment for the selective dephosphorylation of PLP and PMP in food extracts was also employed to aid in identification of these peaks (Gregory and Kirk, 1978). The high resolution and efficiency of the chromatographic separation is illustrated by the chromatogram in Figure 1a. Standards were ordinarily prepared without pyridoxine 5′-phosphate because it is a negligible constituent of the total vitamin B_6 in foods.

In initial studies (Table I), selected foods (soy flour, wheat bran, rice bran, and oat bran) were subjected to extraction with SSA, followed by analysis of purified extracts with and without the following: (1) treatment with β-glucosidase (for oat bran with and without concurrent treatment with acid phosphatase), (2) saponification with 0.7 M KOH for 3 h, and (3) sequential treatment with 0.7 M KOH and then β-glucosidase. In analysis of each of these foods, a cluster of three or four peaks eluted in the vicinity of PLP, which rendered the accurate measurement of PLP difficult or impossible (Figure 1). Other peaks could be observed in varying number and magnitude, which varied with the sample origin. These replicate analyses were less reproducible than observed previously (Gregory and Feldstein, 1985: Gregory and Ink, 1987). This may be due to variability in the effectiveness of hydration in extraction of dry samples and/or to the analyte concentrations which were low relative to the sensitivity of this detector (Table I).

Comparisons of the results of HPLC analyses following the various hydrolytic pretreatments provided evidence of the nature of the form(s) of glycosylated vitamin B_6 present in each sample (Table I). In soybean flour, the total vitamin B_6 content (i.e., the sum of PN-glucoside and PN) was approximately constant regardless of the hydrolytic method employed. Direct measurement of PN-glucoside yielded approximately the same value as the incremental increase in PN following treatment of the extracts with β-glucosidase, which indicated that PN-glucoside was the only enzymatically labile form of β-linked glycosylated vitamin B_6. Similarly, β-glucosidase treatment after KOH treatment did not increase the total vitamin B_6 over that detected in the analysis of untreated extracts, which indicated the absence of saponifiable esterified forms of glycosylated vitamin B_6 (i.e., B6X). The

Table I. Concentration of Vitamin B_6 Compounds in Selected Foods Determined by HPLC Analysis following Extraction with SSA and Selective Hydrolysis

		concn,[a,b] nmol/g						
sample	pretreatment	PLP	PMP	PN-glycoside	PL	PN	PM	total
soy flour	none	0	0	19.9 ± 2.7	0	10.4 ± 3.7	0	30.3 ± 1.2
	β-glucosidase	0	0	0	0	25.0 ± 1.0	0	25.0 ± 1.0
	KOH	0	0	21.4 ± 3.9	0	7.1 ± 3.7	0	28.6 ± 4.6
	KOH + β-glucosidase	0	0	0	0	30.1 ± 6.4	0	30.1 ± 6.4
oat bran	none	*	0	0	1.52 ± 0.33	2.01 ± 0.53	3.12 ± 0.66	6.77* ± 1.6
	β-glucosidase	*	0	0	1.59 ± 0.64	2.03 ± 0.12	3.61 ± 0.31	7.37 ± 0.52
	β-glucosidase + acid phosphatase	0	0	0	4.46 ± 0.65	2.40 ± 0.78	3.10 ± 0.28	9.90 ± 1.74
	KOH	*	0	0	1.42 ± 0.42	2.16 ± 0.33	3.45 ± 0.33	7.03 ± 0.74
	KOH + β-glucosidase	*	0	0	3.41 ± 0.89	2.31 ± 0.85	3.27 ± 0.20	8.46 ± 2.04
wheat bran	none	*	0	19.1 ± 4.8	0	24.0 ± 5.2	0	46.9* ± 11.2
	β-glucosidase	*	0	0	0	50.1 ± 7.9	0	50.1* ± 7.9
	KOH	*	0	20.5 ± 4.3	0	29.3 ± 7.8	0	49.8 ± 5.2
	KOH + β-glucosidase	*	0	0	0	56.4 ± 3.0	0	56.4 ± 3.0
rice bran	none	*	0	28.8 ± 4.2	0	7.9 ± 2.9	0	36.7* ± 5.0
	β-glucosidase	*	0	0	0	62.5 ± 10.9	0	62.5* ± 10.9
	KOH	*	0	31.4 ± 3.7	0	6.9 ± 4.0	0	38.4 ± 5.0
	KOH + β-glucosidase	*	0	0	0	103 ± 19	0	103 ± 19

[a] Values are means and SD, $n = 3$ independent analyses ($n = 6$ for rice bran). A zero (0) indicates a concentration at or below the limits of quantitation of approximately 0.3 nmol/g for each peak. The oat bran extract was prepared by using the β-glucosidase pretreatment alone and in combination with acid phosphatase. [b] Asterisk (*) indicates that interference precluded accurate measurement of PLP. The calculated values for total vitamin B_6 in these samples did not include apparent PLP concentration.

Table II. Concentration of Vitamin B_6 Compounds in Rice Bran and Carrots Determined by Using Extraction with SSA, Selective Hydrolysis, and HPLC Analysis[a]

		concn,[b,c] nmol/g						
sample	pretreatment	PLP	PMP	PN-glucoside	PL	PN	PM	total
rice bran	none	*	0	45.1 ± 3.4	4.9 ± 2.0	6.1 ± 1.2	5.7 ± 1.2	63.6* ± 6.8
	β-glucosidase + phosphatase	0	0	0	7.3 ± 0.5	54.2 ± 16.3	9.9 ± 1.1	71.5 ± 16.4
	KOH + β-glucosidase + phosphatase	0	0	0	15.2 ± 1.4	99.9 ± 15.6	10.8 ± 2.5	126 ± 18
carrots	none	*	0.7 ± 0.3	4.4 ± 0.7	0.2 ± 0.2	0.5 ± 0.2	0.2 ± 0.05	5.9* ± 1.2
	β-glucosidase + phosphatase	0	0	0	1.4 ± 0.3	4.0 ± 1.7	0.6 ± 0.1	6.0 ± 1.9
	KOH + β-glucosidase phosphatase	0	0	0	1.4 ± 0.2	5.0 ± 0.6	0.5 ± 0.1	6.8 ± 0.7

[a] The enzymatic treatments involved the use of *both* acid phosphatase and β-glucosidase. [b] Values are means and SD, $n = 3$ independent analyses. A zero (0) indicates a concentration at or below the limits of quantitation of approximately 0.3 nmol/g for each peak. [c] Asterisk (*) indicates interference which precluded accurate measurement of PLP. Total vitamin B_6 in these samples did not include apparent PLP concentration.

composition of oat bran was unusual with respect to its absence of detectable PN-glucoside.

Although PN-glucoside was the major form of glycosylated vitamin B_6 in wheat bran and rice bran, each exhibited evidence of more complex glycoconjugates of the vitamin. Following β-glucosidase treatment, there was a small increase in measured vitamin B_6 relative to that measured with no hydrolysis. This is evidence of enzymatically labile β-glucosides of pyridoxine other than PN-glucoside, possibly various pyridoxine oligosaccharides reported by Tadera et al. (1988). Treatment with KOH followed by β-glucosidase yielded a significant increase in total vitamin B_6 in each case, which is evidence of esterified glycoconjugates of pyridoxine requiring saponification to render them susceptible to hydrolysis by this enzyme.

To alleviate the problems encountered in the measurement of PLP by direct analysis, the use of an enzymatic step to hydrolyze PLP to PL was incorporated (Tables I and II). Acid phosphatase was used diagnostically in the analysis of oat bran to examine the measurement of PLP directly (i.e., before hydrolysis) or as PL following enzymatic hydrolysis (Table I). Direct measurement of PLP prior to any hydrolytic pretreatment yielded an apparent concentration of 46.3 nmol/g in oat bran (data not shown). In contrast, the increase in PL following treatment with acid phosphatase was 2.86 nmol/g, which was indicative of the actual PLP concentration. This approach to the measurement of PLP was extended in studies shown in Table II, in which all SSA extractions and hydrolytic treatments were conducted as described above, with the exception that the enzymatic treatment of the extract consisted of a combination of β-glucosidase and acid phosphatase rather than β-glucosidase alone. This procedure was applied to rice bran, which exhibited interference in the previous experiment (Table I and Figure 1), and raw carrots. The efficacy of the acid phosphatase was verified by observing the ability of this treatment to convert fully PLP standards (alone or added to carrot extract) to PL (data not shown). The results of Table II indicate that free PL and PLP represented a total of 7.3 ± 0.5 nmol/g of rice bran when determined following enzymatic treatment, which is in marked contrast to the apparent PLP content of 49.6 nmol/g determined by direct analysis of the SSA extract. Apparent interference in the PLP peak was also observed in the raw carrots. Direct analysis of SSA extracts of carrots yielded an apparent PLP content of 9.3 nmol/g, in great excess of the total determined as PL + PLP (1.4 nmol/g) following enzymatic hydrolysis. In addition, residual peaks near the retention time of PLP were observed following acid phosphatase treatment conducted under conditions that gave full hydrolysis of PLP. It thus appears that the incorporation of a phosphatase treatment in routine analysis would be useful to alleviate the problem of interference in the measurement

Table III. Concentration of Vitamin B_6 Compounds in Rice Bran and Carrots Determined by Using Extraction with 0.44 M HCl (121 °C, 2 h), Selective Hydrolysis, and HPLC Analysis[a]

		concn,[b] nmol/g						
sample	pretreatment	PLP	PMP	PN-glucoside	PL	PN	PM	total
rice bran	none	0	0	32.0 ± 5.2	14.0 ± 1.4	83.3 ± 12.7	0	129 ± 18
	β-glucosidase	0	0	0	12.9	110	0	125
carrots	none	0	0	0	4.9 ± 0.7	5.3 ± 0.5	1.7 ± 0.5	11.0 ± 1.4
	β-glucosidase	0	0	0	3.8 ± 1.8	4.8 ± 0.7	2.0 ± 0.05	10.3 ± 2.5

[a] The enzymatic (β-glucosidase) treatments were conducted after autoclaving and pH adjustment. [b] Values are means and SD, $n = 3$ independent analyses ($n = 1$ for rice bran extract treated with β-glucosidase). A zero (0) indicates a concentration at or below the limits of quantitation of approximately 0.3 nmol/g for each peak.

of PLP in certain plant-derived foods when the SSA extraction procedure is used.

Coburn and Mahuren (1983) developed a postcolumn derivatization method for enhancing the sensitivity of detection for PLP by using a phosphate-buffered bisulfite solution to form the hydroxylsulfonate adduct of PLP. We employed this procedure in our previous HPLC methods (Gregory and Feldstein, 1985; Gregory and Ink, 1987). In the present study, the postcolumn derivatization provided no increase in selectively of detection; i.e., interferences were observed with or without postcolumn derivatization. Thus, in the interest of simplicity, postcolumn, derivatization was not routinely employed in this study.

Acid-Catalyzed Hydrolysis. The effects of autoclaving samples for 2 h in 0.44 M HCl were investigated to determine the suitability of this procedure in the measurement of total vitamin B_6 in plant-derived food samples. The major consideration was the extent of hydrolysis of glycosylated and phosphorylated forms of the vitamin. Under the conditions used in this study (AOAC, 1970), PMP and PLP underwent complete hydrolysis to PM and PL, respectively. Purified PN-glucoside, when heated at 121 °C for 2 h in 0.44 M HCl, also was completely converted to PN (Gregory and Ink, 1987). Aside from the hydrolysis of glycosylated and phosphorylated forms of the vitamin, little or no degradation occurs during extraction under these conditions. Carrots were selected for the present study because of their comparatively high concentration of PN-glucoside (ca. 60–70% of the total vitamin B_6), whereas rice bran was chosen in view of its high content of various glycosylated forms of vitamin B_6 (Table I).

In the analysis of carrots following HCl treatment (Table III), no residual PN-glucoside, PLP, or PMP was observed, which indicated their complete hydrolysis. The total content of vitamin B_6 was greater in this analysis (11.0 ± 1.4 nmol/g) than obtained when SSA was used as the extract (5.9 ± 1.2 nmol/g; Table II). It is unclear whether this difference is due to natural variation in composition of the carrots or a difference in the effectiveness of the extraction procedures. In contrast to the absence of PN-glucoside in the autoclaved carrots, rice bran samples exhibited significant residual PN-glucoside after the 2-h autoclave treatment. The identity of the apparent PN-glucoside peak was confirmed by hydrolysis with β-glucosidase, which fully converted it to PN. These results suggest that various forms of glycosylated vitamin B_6 are slowly hydrolyzed to yield PN-glucoside and ultimately PN when autoclaved in 0.44 M HCl. Thus, foods containing a high proportion of glycosylated vitamin B_6 in forms other than PN-glucoside may exhibit incomplete hydrolysis during the use of these standard extraction conditions.

The use of acid-catalyzed hydrolysis in preparation of food samples for HPLC analysis has been also employed by Bognar (1985), who incubated samples at 120 °C in 0.2 N sulfuric acid for 30 min. While this method yielded complete hydrolysis of phosphate esters of PLP and PMP, the results of the present study indicate that the comparatively mild treatment used by Bognar would only partially hydrolyze PN-glucoside and other glycosylated forms of vitamin B_6. Thus, the procedure of Bognar would underestimate total vitamin B_6 in most plant-derived foods. Toukairin-Oda et al. (1989) reported an HPLC method for measurement of vitamin B_6 in foods following extraction with 1 M perchloric acid. No attempt was made to hydrolyze glycosylated forms of the vitamin, which accounted for the fact that HPLC-derived values were lower than data obtained when acid hydrolysis and microbiological assay were used.

Table IV. Comparison of Total Vitamin B_6 As Determined in This Study and As Published Previously

	percentage of total vitamin B_6[a]			total vitamin B_6,[b] nmol/g		
sample	glycosylated (all forms)	PN-glucoside	B6X	this study	USDA	other publications
soy flour	67	67	0	30	27.3	
oat bran	0	0	0	14	9.76	
wheat bran	45	34	11	56	76.9	62[c]
rice bran	82	36	43	125	241	285[c]
carrots, raw	77	65	12	6.8–13.8	8.70	26 (70)[d] 10 (51)[e]

[a] Glycosylated vitamin B_6 includes PN-glucoside determined by direct measurement, B6X (i.e., PN released after treatment with KOH and β-glucosidase), and additional glycosylated forms that release PN with β-glycosidase but not requiring KOH treatment. [b] USDA refers to references in Agricultural Handbook 8 series (soy flour, Handbook 8-16, 1986; oat bran, wheat bran, and rice bran, Handbook 8-20, 1989; carrots, Handbook 8-11, 1984). The numbers in parentheses are percentages of glycosylated vitamin B_6 reported. [c] From Tadera et al. (1986). [d] From Gregory and Ink (1987). [e] From Kabir et al. (1983).

Summary of Data and Comparison with Previous Studies. With the exception of limited data reported by Tadera et al. (1986), little quantitative information has been previously available concerning the distribution and identity of the various glycosylated forms of vitamin B_6 in foods. The study of Kabir et al. (1983b) involved the measurement of "glycosylated vitamin B_6" calculated differentially by microbiological assay with or without pretreatment with β-glucosidase. From the results of the present study, we have calculated the proportions of various classes of glycosylated vitamin B_6 in the several foods examined (Table IV). Tadera et al. (1986) reported significant amounts of the B6X fraction (i.e., that released by β-glucosidase treatment following saponification in KOH) in rice bran, wheat bran, and soy flour. This was in general agreement with the results found for wheat bran and rice bran, which contained measurable quantities of such a B6X fraction, although little or no B6X was found in soy flour. In contrast to the other brans examined, no forms of glycosylated vitamin B_6 were detected in oat bran.

A small amount of B6X may have been present in carrots, although the analytical and nutritional significance of this observation is questionable in view of the variability of the data. Generally good agreement was observed between the results of this study and data reported by the USDA that were obtained by using the AOAC method of acid-catalyzed hydrolysis and microbiological assay. The results of this study indicate that total vitamin B_6, as well as individual fractions of free and glycosylated forms of the vitamin, can be effectively measured by reverse-phase HPLC with fluorometric detection. Because of the absence of readily obtainable standards for certain glycosylated forms (i.e., pyridoxine oligosaccharides and pyridoxine glucoside esters) and in view of the problem of interference in the measurement of PLP, the analytical approach taken is by necessity one of multiple pretreatments followed by HPLC analysis.

Summary and Conclusion. The most definitive qualitative and quantitative information concerning vitamin B_6 in foods has been derived from a multistep analytical procedure that involves SSA extraction followed by HPLC analysis of (1) untreated extracts (which provides data regarding PN-glucoside, PMP, PL, PN, and PM); (2) extracts treated simultaneously with acid phosphatase and β-glucosidase (which provides differential information regarding the content of PLP, PMP, PN-glucoside, and PN oligosaccharides); and (3) extracts treated with KOH followed by the combined enzymes (which yields differential information concerning the esterified PN-glucosides, i.e., the B6X fraction).

Alternatively, acid-catalyzed hydrolysis (2 h, 0.44 M HCl) may be used to provide a measurement of total vitamin B_6 (measured as PL, PN, and PM). Care must be taken when this approach is used in view of the incomplete hydrolysis of glycosylated forms of B_6 in rice bran observed in this study. Such incomplete hydrolysis would cause underestimation of total vitamin B_6 in such samples whether HPLC or traditional microbiological analyses are used. The effect of β-glucosidase treatment following acid hydrolysis as used in this study should be evaluated, at least diagnostically, when routine assays of a particular sample are established to determine whether incomplete hydrolysis of glycosylated forms of the vitamin may have occurred.

In summary, the accuracy of determinations of vitamin B_6 depend heavily on the effectiveness of the preparative methods employed. The methods reported here provide a valid approach to the measurement of free and glycosylated forms of the vitamin. This will be useful in food analysis to provide information concerning total vitamin B_6 as well as the glycosylated species which exhibit incomplete bioavailability.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; PN, pyridoxine; PL, pyridoxal; PM, pyridoxamine; 4dPN, 4′-deoxypyridoxine; PLP, pyridoxal 5′-phosphate; PMP, pyridoxamine 5′-phosphate; PN-glucoside, 5′-*O*-(β-D-glucopyranosyl)pyridoxine.

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Registry No. PN, 65-23-6; PL, 66-72-8; PM, 85-87-0; PLP, 54-47-7; PMP, 529-96-4; PN-glucoside, 63245-12-5; vitamin B_6, 8059-24-3.