Analysis of Free and Glycosylated Vitamin B6 in Wheat by High-Performance Liquid Chromatography[1]

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ABSTRACT

We developed an high-performance liquid chromatography (HPLC) method for analysis of vitamin B6 in wheat, based on modifications of existing reverse-phase ion-pairing procedures. The method is much simpler than multistep HPLC-based procedures required for exhaustive analysis of all B6 forms in foods, including minor components, or methods based on microbiological analysis. The method is suitable for analysis of major B6 forms in wheat, including pyridoxine glucoside. The method entails extraction of wheat B6 using metaphosphoric acid followed by quantitation of B6 in a single binary-gradient HPLC separation. Analysis of three randomly selected wheat cultivars with this method revealed significant variation in content of pyridoxine (2.2–3.3 nmol/g) and pyridoxine glucoside (1.8–9.4 nmol/g). The method should facilitate analysis of different wheat cultivars for vitamin B6 content.

Wheat is an important food source for both humans and animals in many cultures of the world (Bushuk 1986), and it is a significant source of vitamin B6, particularly in nonmeat diets (National Research Council 1989). More than 50% of vitamin B6 consumed by Americans comes from plant foods (Kant and Block 1990). Adequate intake of vitamin B6 is essential because of its key coenzymatic role in over 100 enzymes of protein, lipid, and carbohydrate metabolism (Leklem 1988). The recommended dietary intake for vitamin B6 is 2.0 mg/day and 1.6 mg/day for men and women, respectively (National Research Council 1989).

Marginal vitamin B6 nutritional status is prevalent in the United States; data show that 71 and 90% of American males and females, respectively, consume less than the recommended daily allowance (RDA) for vitamin B6 (Kant and Block 1990). Selhub et al (1993) recently reported that about half of the elderly subjects in the Framingham study consumed less than the RDA of B6, and that such intake is associated with significantly elevated plasma homocysteine, which in turn significantly increases risk for development of cardiovascular disease. In an accompanying editorial, Stampfer and Willet (1993) conclude that vitamin supplementation (for folate and B6) may be appropriate for many elderly Americans. Using serum metabolic markers, Joosten et al (1993) concluded that vitamin deficiencies, including vitamin B6, may be more prevalent in the elderly than plasma markers for those vitamins suggest.

Vitamin B6 includes a group of three 3-hydroxy-2-methylpyridine derivitives that exist in foods (and mammalian tissues) in phosphorylated and nonphosphorylated forms. These forms include pyridoxine (PN), pyridoxal (PL), and pyridoxamine (PM), as well as phosphorylated forms for PL and PM (PLP and PMP, respectively). These B6 vitamers all have high bioavailability, meaning they are well-absorbed and well-utilized in both humans and animals.

An additional glycosylated adduct of PN, 5′-*O*-(β-D-glucopyranosyl)pyridoxine, or pyridoxine glucoside (PNG), was first identified in rice bran (Yasumoto et al 1977) and occurs in many plant foods. PNG has potential nutritional significance due to its low bioavailability compared to nonglycosylated B6 vitamers (Gregory and Kirk 1981). Kabir et al (1983) found an inverse relationship between the PNG content of the diet and its bioavailability in humans. Reynolds (1988) found the percent PNG in foods was a strong predictor of bioavailability. Studies have shown that PNG bioavailability is low compared to PN in both rats (10–34%) (Ink et al 1986, Trumbo et al 1988) and humans (58%) (Gregory et al 1991b). Recent evidence suggests that PNG may inhibit transport of nonglycosylated B6 into liver cells (Zhang et al 1993) and may impair metabolism of nonglycosylated B6 in both rats (Gilbert and Gregory 1992) and humans (Gregory et al 1991a). The net effect of PNG low bioavailability may be to depress B6 nutritional status (Trumbo et al 1988).

Existing methods for analysis of B6 in wheat and food require time-consuming microbiological procedures (AACC 1983, AOAC 1990), multistep extraction or analysis procedures (Gregory and Sartain 1991), or sophisticated detection equipment (Guilarte et al 1981). The method of Gregory and Sartain (1991) gives complete information about vitamin B6 content but involves separate sample analyses for PN, PL, PM, PMP, and PNG; for PNG and PLP; for "B6X" content (polyglycosylated adducts); and for total B6. In addition, some of these procedures do not resolve PNG from other B6 forms (AACC 1983, AOAC 1990, Reitzer-Bergaentzle et al 1993) or have not been applied to analysis of cereal grains, including wheat (Bitsch and Moller 1989, Tadera and Naka 1991). Although there are many reports in the literature dealing with B6 content of wheat (reported as total B6 or as individual nonphosphorylated B6 vitamers, generally measured by microbiological assay), there is a paucity of reports dealing with PNG content in wheat.

The objectives of this study were: 1) to develop and validate a simple and reliable method for analysis of vitamin B6 forms, including PNG, in wheat based on modification of existing extraction and high-performance liquid chromatography (HPLC) methods; and 2) to demonstrate that the method permits detection of varying amounts of PN, PNG, and other B6 vitamers in different wheat cultivars.

MATERIALS AND METHODS

Reagents

4′-Deoxypyridoxine (dPN), PMP, PM, PL, PN, PLP, 4-pyridoxic acid (4-PA), sodium bisulfite and β-glucosidase (type 1) were obtained from Sigma Chemical (St. Louis, MO). Metaphosphoric acid, phosphoric acid, and potassium phosphate were obtained from J. T. Baker Chemical Company (Phillipsburg, NJ). Distilled, deionized water (ddH2O) was used in all procedures. 1-Octanesulfonic acid was obtained from Eastman-Kodak (Rochester, NY). Other chemicals were analytical grade.

Samples

Three cultivars of wheat were used: C1, Waldron, a hard red spring wheat from Fort Collins, CO (harvested in 1991); C2, a hard red winter wheat from Fort Collins, CO, (harvested in 1993); and C3, Spelt (harvested in Canada in 1993), obtained from Arrowhead Milling Co. The samples were stored frozen at −20°C

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in sealed 100-ml polycarbonate bottles until analysis. Before removing 10-g samples for analyses, bottle contents were mixed 1 min to assure homogeneity. Five replicates of each sample were analyzed to provide B6 vitamer concentration data.

HPLC Equipment and Methods

The HPLC system, mobile phases, and binary gradient program were modifications of previous methods (Sampson and O'Connor 1989). The HPLC system consisted of a Spectra Physics system (San Jose, CA) with an 8800 Ternary pump, 8875 Autosampler, 4400 Integrator, and CHROMNET/WINER software for system control and data acquisition; a Rheodyne model 7010 injection valve; an Ultremex C18 ion-pair analytical column (octadecylsilane; 4.6 mm × 15 cm; 3 μm particle size (Phenomenex, Torrance, CA) preceded by a Brownlee RP18 7-μm Newguard guard column (replaced after 100 samples); a postcolumn pump (Milton-Roy model 198-31) with a Li-Chroma Pulse Dampener (Chrom Tech, Apple Valley, MN); a McPherson FL-750 BX variable wavelength fluorescence detector (Acton, MA) equipped with a mercury-xenon lamp set to an excitation wavelength of 311 nm, a 24-μl capillary flowcell, a UT-1 excitation filter, a 360-nm emission long-pass filter, and an ACT-40 286 personal computer (an IBM clone; Advanced Computer Technology, Fort Collins, CO).

Buffered mobile phases used were: solvent A, 0.033*M* phosphoric acid and 0.008*M* 1-octanesulfonic acid, adjusted to pH 2.2 with 6*N* KOH; solvent B, 0.033*M* phosphoric acid and 10% (v/v) acetonitrile, adjusted to pH 2.2 with 6*N* KOH; postcolumn reagent, 1.0 mg/ml sodium bisulfite in 1.0*M* potassium phosphate buffer, adjusted to pH 7.5 with 6*N* KOH. Mobile phases were prepared fresh daily from 10X stock solutions that were prepared bi-weekly and stored at 4°C. Buffered solutions were filtered using an all-glass microfilter apparatus fitted with 0.2-μm Nylon-66 filter membranes (Chrom Tech). The postcolumn reagent was used to enhance B6 vitamer fluorescence (Coburn and Mahuren 1983) and was pumped in to the postcolumn eluate at 0.2 ml/min.

A binary gradient program was used to separate B6 vitamers. A linear gradient changed from 100% mobile phase A to 100% mobile phase B 10 min after injection; remained at 100% B for 15 min; then returned to 100% A in 4.5 min, followed by 5.5 min of re-equilibration in A before the next injection. Flow rate was 1.2 ml/min.

Sample Preparation

Wheat cultivars were extracted and analyzed under yellow fluorescent lighting (Gold F20T12/GO) to minimize photodegradation of B6 vitamers (Schaltenbrand et al 1987). Samples were held on ice during preparation. Samples (10 g) were ground in a micro-mill (Lab Apparatus Co., Cleveland, OH) and homogenized 3 min on speed setting 50 in 40 ml of ddH2O after addition of a known amount of the internal standard (dPN) using a Virtis Hi-Speed Homogenizer (model 45, Virtis Co., Gardiner, NY). Homogenates were deproteinized by addition of 5% (w/v) metaphosphoric acid, then centrifuged at 4°C for 15 min at 10,000 × *g*. Supernatants were transferred to clean tubes, clarified by centrifugation as above, filtered through 0.2-μm Nylon-66 filters (Rainin Instrument Co., Woburn, MA) and stored at −70°C. For each sample, duplicate aliquots of 20 μl were analyzed by HPLC.

Recovery Experiment

Five unspiked and five spiked samples of C1 were analyzed. Spikes of 9.5–89.9 nmol/g were added to the samples before homogenization. Recoveries were calculated as: Recovery = $(S - U) \times 100/Sp$, where S and U are analyzed vitamer concentrations in the spiked and unspiked samples, respectively, and Sp is the amount of added spike.

Glucosidase Experiment

We used a procedure similar to that described by Gregory and Sartain (1991). Aliquots of sample supernatants, prepared as described above, were adjusted to pH 5.0 using 6*N* NaOH. An aliquot (0.05 ml) of β-glucosidase solution (1.0 mg/ml of enzyme containing 20–40 U/mg) or distilled water was added to 2.0 ml of sample, followed by incubation at 37°C for 2.5 hr. The incubation was stopped by addition of solid meta-phosphoric acid to 5% (w/v). The samples were clarified by centrifugation for 10 min at 13,000 g using a microcentrifuge. The supernatants were drawn off and stored at −70°C until HPLC analysis. This experiment was done in duplicate.

In all experiments, B6 vitamers and PNG were quantified relative to peak areas of an internal standard, deoxypyridoxine, to correct for vitamer loss during sample preparation and for volumetric error during sample injection (Sampson and O'Connor 1989). PNG was quantified assuming that its molar fluorescence was equivalent to that of PN, as was shown to be the case by Gregory and Ink (1987). Identity of PNG in wheat samples was confirmed by demonstrating that the putative PNG peak was shifted stoichiometrically to PN following hydrolysis with almond β-glucosidase (Gregory and Ink 1987).

RESULTS AND DISCUSSION

Recovery Experiments

Recovery data for exogenous vitamer spikes are reported in Table I. Figure 1 shows a typical chromatogram for spiked wheat samples. Recoveries were near 100% for PN and PM (Table I). This is significant for PN, because of the body of literature

TABLE I
Recovery of B6 Vitamers from Wheat[a]

Vitamer[b]	Endogenous[c] (nmol/g)	Spike[d] (nmol/g)	Recovery[e]
PN	2.7	30	101.3 ± 1.7
PMP	0.8	10	79.3 ± 1.8
PLP	0.8	90	34.1 ± 0.7
PL	ND[f]	30	57.3 ± 1.8
PM	ND	19	96.2 ± 1.4

[a] Values are means ± standard error of the mean of five replicates.
[b] PN = pyridoxine; PL = pyridoxal; PM = pyridoxamine; PMP and PLP = phosphorylated form of PL and PM, respectively.
[c] Endogenous concentrations are from Table II for cultivar C1.
[d] Spike concentrations were varied to give full-scale peaks in sample chromatograms.
[e] Recoveries of PNG and 4-PA were not measured.
[f] Not detected.

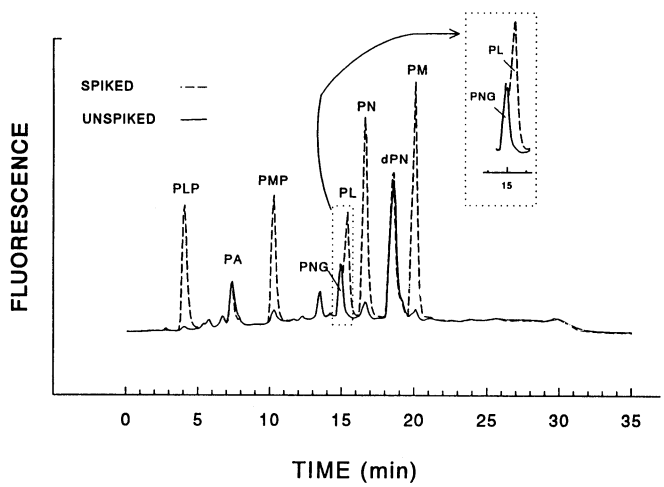

Fig. 1. Vitamin B6 recoveries. High-performance liquid chromatograms of a wheat sample with and without addition of exogenous B6 vitamer spikes. Insert shows that pyridoxal (PL) eluted 0.6 min after the putative pyridoxine glucoside (PNG) peak, with less than 15% of the PL area overlapping with PNG. PN = pyridoxine, PM = pyridoxamine, dPN = 4′-deoxypyridoxine, PLP and PMP = phosphorylated forms for PL and PM, respectively.

suggesting that vitamer predominates in plant foods (Toepfer and Lehmann 1961, Polansky et al 1964, Toepfer and Polansky 1970, Toepfer et al 1972, Michaela and Lorenz 1976, Vanderslice et al 1984). Lack of purified PNG precluded measuring PNG recovery for this method. We assume that the complete recovery of PN indicates similar high recovery of PNG, although we have no direct evidence bearing on this point.

We have not detected PM in wheats analyzed to-date, thus, high recovery for that vitamer is of little practical significance. Low recovery for PL appears of little practical concern in that we have not detected that vitamer in any wheat cultivars. Similarly, we have detected PMP at low concentrations in only one of three cultivars analyzed, which minimizes practical quantitative consequences of that vitamer's 79% recovery. The 34% recovery for PLP is more troubling because significant PLP is present in some plant foods (8, 15, 26, and 36% of total B6 in carrots, bananas, green beans, and broccoli, respectively) (Gregory and Ink 1987). Our results show that relatively little PLP is present in wheat, compared to PN and PNG, and no PLP was seen in two of three cultivars. Gregory and Sartain (1991), using an HPLC separation similar to the one in this study, reported that coeluting peaks made PLP concentrations appear artificially high in oat and rice bran as well as carrots. These authors reported that PLP in wheat bran could not be quantitated accurately due to coeluting peaks. Taken together, these observations lessen concern about the low PLP recovery value reported in Table I, at least for wheat samples that contain as little PLP as the cultivars we analyzed.

The insert in Figure 1 shows that the baseline did not resolve completely between PNG and PL. However, PL did elute consistently 0.60 min later than PNG, with less than 15% of the PL area overlapping with PNG (see also the insert in Fig. 2). Similar results for closely eluting PL and PNG have been reported by other investigators (Gregory and Ink 1987, Gregory and Sartain 1991). We have not detected PL in any wheat sample analyzed to-date, so that the incomplete resolution does not appear to be a practical problem.

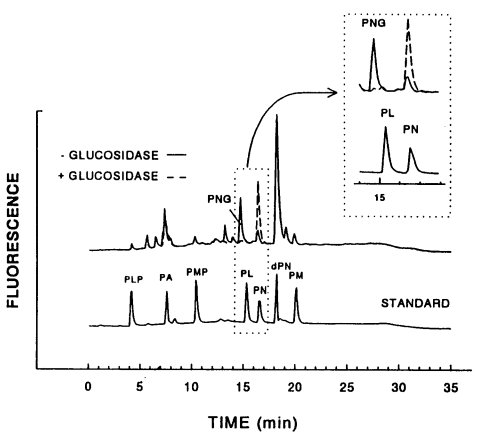

Fig. 2. Glucosidase incubation. High-performance liquid chromatograms of a wheat sample with and without addition of β-glucosidase. Insert shows that β-glucosidase incubation resulted in disappearance of the putative pyridoxine glucoside (PNG) peak, with a corresponding increase in the pyridoxine (PN) peak. PL = pyridoxal, PM = pyridoxamine, dPN = 4′-deoxypyridoxine, PLP and PMP = phosphorylated forms for PL and PM, respectively.

TABLE II
Effects of β-Glucosidase Incubation on Pyridoxine Glucoside (PNG) and Pyridoxine (PN) Content of Wheat[a]

	− Enzyme (nmol/g)	+ Enzyme (nmol/g)
PNG	7.62 ± 0.12	ND
PN	2.08 ± 0.08 a	10.40 ± 0.05 b
Total B6	9.70 ± 0.20 a	10.40 ± 0.05 a

[a]Values are mean ± standard error of the mean of two replicates. Means within rows not sharing common letters are significantly different at $P < 0.05$ (Student's t-test). ND = not detected.

Glucosidase Experiment

Because purified PNG standard is not available commercially, we confirmed the identity of our putative PNG peak in wheat samples by showing that treatment with β-glucosidase caused complete conversion of that peak to PN (Gregory and Ink 1987, Gregory and Sartain 1991). Incubation of extracted samples with β-glucosidase eliminated the peak seen at 15 min in untreated samples, with a concomitant increase in the PN peak (Fig. 2 and Table II). The magnitude of increase in the PN peak in β-glucosidase-treated samples (from 2.1 to 10.4 nmol/g) matched the size of the PNG peak in untreated samples (7.6 nmol/g), suggesting stoichiometric conversion of PNG to PN by β-glucosidase treatment. These results are consistent with identity of the 15-min peak in wheat samples as genuine PNG.

The peak at 7.5 min in Figures 1 and 2, where elution time corresponds to 4-pyridoxic acid (PA) was unexpected. We have seen this peak in only one of three wheat cultivars analyzed, and its identity as PA requires confirmation by mass spectrometry. We did not evaluate recovery of this peak with a PA standard. PA is the excretory form of B6 in mammalian tissues (Ink and Henderson 1984) and has not been reported, to our knowledge, in plant foods (Gregory and Sartain 1991). This form of B6 would not be bioavailable to subjects consuming it in foods, because humans lack pathways to convert the C-4′ carboxylic group of PA into PLP. It may be of nutritional interest in future work to confirm presence of PA in some wheat cultivars.

B6 Vitamer Content of Different Wheat Cultivars

Analysis of three different wheat cultivars using our method showed that PN and PNG are the predominant B6 forms (Table III and insert in Fig. 3), which is in agreement with data for wheat bran reported by Gregory and Sartain (1991). We observed small but detectable amounts of PMP, PLP, and PA in one cultivar. A striking point in the data of Table III is the variation in B6 content in the cultivars tested, both in total B6 (from 5.0 to 12.7 nmol/g, $P < 0.05$), as well as in amount of individual B6 forms (2.2 to 3.3 nmol/g for PN, $P < 0.05$), corresponding to 19–64% of total B6; and 1.8 to 9.4 nmol/g for PNG ($P < 0.05$), corresponding to 36–81% of total B6). Such variation, especially for PNG, in these randomly selected wheats suggests the possibility that future studies may identify wheat cultivars high in total B6 but low in PNG. The significantly lower total B6 for cultivar C3 in Table III (Spelt) may reflect genetic differences between it and the other two cultivars, which were both hard red bread wheats.

Total B6 concentration in these wheat cultivars (5–13 nmol/g of wheat) is substantially lower than the values of 19–23 nmol/g in most earlier reports (Polansky et al 1964; Toepfer and Polansky 1964, 1970; Toepfer et al 1972; Vanderslice et al 1984), although

TABLE III
B6 Vitamer Concentrations in Three Wheat Cultivars[a]

	Cultivars[c,d]		
Vitamer[b]	C1 (nmol/g)	C2 (nmol/g)	C3 (nmol/g)
PN	2.69 ± 0.15 a	2.16 ± 0.15 a	3.24 ± 0.22 b
PNG	7.14 ± 0.06 a	9.44 ± 0.59 b	1.79 ± 0.05 c
PMP	0.76 ± 0.15	ND	ND
PLP	0.77 ± 0.08	ND	ND
4-PA	1.31 ± 0.11	ND	ND
Total	12.67 ± 0.25 a	11.60 ± 0.25 a	5.03 ± 0.25 b

[a]Values are means ± standard error of the mean of five replicates.
[b]PN = pyridoxine; PNG = pyridoxine glucoside; PMP and PLP = phosphorylated form of pyridoxal (PL) and pyridoxamine (PM) (PL and PM were not detected in these cultivars); 4-PA = 4-pyridoxic.
[c]Identities of cultivars are given in the text.
[d]Means within row not sharing common letters are significantly different at $P < 0.05$ (Student's t-test). ND = not detected.

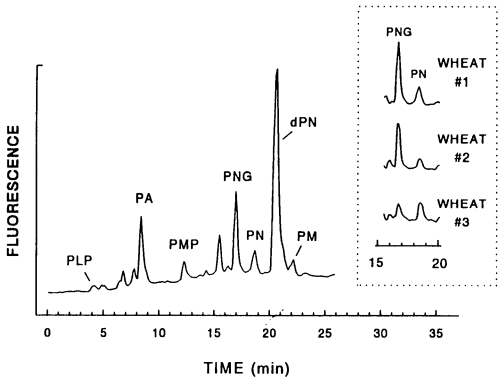

Fig. 3. Representative high-performance liquid chromatograms. Insert shows the significant variation in pyridoxine glucoside (PNG) and pyridoxine (PN) peak areas observed in three different wheat cultivars. 1–3 in the insert correspond to cultivars C1–C3, respectively, in Table III. PL = pyridoxal, PM = pyridoxamine, dPN = 4′-deoxypyridoxine, PLP and PMP = phosphorylated forms for PL and PM, respectively.

our values are higher than the 2–4 nmol/g range in two reports (Michaela and Lorenz 1976, Keagy et al 1980). Most literature reports are based on microbiological assay following vigorous acid hydrolysis. Such procedures will give artificially high PN concentration due to acid hydrolysis of PNG to PN (Ink et al 1986, Gregory 1988), but would not account for high total B6. We cannot exclude the possibility that our method involves incomplete recovery of PNG, or that recovery of endogenous wheat PN is lower than the 101±2% recovery of exogenous PN spikes that we observed. The latter possibility can only be evaluated using intrinsic labeling of endogenous B6 forms in wheat (Gregory 1988), which was beyond the scope of the present study.

We are unaware of other reports dealing with B6 analysis of whole wheat by HPLC, or with direct comparisons of results obtained with different B6 methods for wheat (e.g., HPLC vs. microbiological assay vs. radiometric-microbiological assay). Guilarte (1991a,b) has recently discussed advantages of his radiometric-microbiological method for analysis of B6 compared to that of the traditional microbiological method (AOAC 1990). This method gives B6 concentrations comparable to those obtained by HPLC for mammalian samples (Coburn et al 1984). A future study comparing data obtained by HPLC vs. traditional microbiological assay vs. radiometric-microbiological assay would be useful in clarifying reported variation in B6 content of wheat.

The differences between our wheat B6 values and those in the literature, as well as the differences we observed in PN, PNG, and total B6 concentration in the three cultivars we analyzed, raise other questions about sources of variation of B6 in wheat. It may be of interest in future work to systematically evaluate how factors such as growing regimen, season of the year, and storage technique, as well as cultivar, extraction procedure, and analytical method affect measured values of B6 in wheat.

We suggest that our method's main utility is as a quick screen that will permit between-cultivar comparison of relative amounts of PN and PNG in wheat. In particular, the method should facilitate identification of wheat cultivars high in total B6 but low in PNG, if such cultivars exist.

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