Determination of Vitamin B-6 in Foods and Other Biological Materials by Paired-Ion High-Performance Liquid Chromatography

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A paired-ion reverse-phase high-performance liquid chromatographic (HPLC) method was developed for the determination of the individual B-6 vitamers in foods and other biological materials. Samples were extracted with sulfosalicyclic acid and extracts purified by preparative anion exchange chromatography. The analytical separation of the B-6 vitamers and the internal standard 4′-deoxypyridoxine was achieved by gradient elution with an octadecylsilyl column within 23 min. The use of a post-column bisulfite reagent permitted sensitive fluorometric detection of all B-6 vitamers. High recovery and precision were obtained. The results compared favorably with previously reported data for a variety of samples. This procedure provides a convenient alternative to previous methods for the determination of vitamin B-6.

INTRODUCTION

The determination of vitamin B-6 in foods and other biological materials is complicated by the existence of six structurally related forms of the vitamin in complex organic matrices. Because of the lengthy nature and often limited precision of microbiological assay methods for vitamin B-6, HPLC has been adapted to several aspects of vitamin B-6 analysis. In addition to having greater precision, HPLC methods permit a direct determination of the concentration of the individual B-6 vitamers that is difficult to achieve by using microbiological methods.

Initial approaches to the determination of vitamin B-6 with HPLC have been discussed in a previous review (Gregory and Kirk, 1981). Reverse-phase HPLC, with no ion-pairing agent in the mobile phase, was successfully employed for the fluorometric determination of the vitamin B-6 metabolite 4-pyridoxic acid (4PA) in urine (Gregory and Kirk, 1979) and pyridoxine (PN) in fortified breakfast cereals (Gregory, 1980a). This method, with the addition of pre-column or post-column derivatization with semicarbazide, also permitted the determination of pyridoxal (PL) and pyridoxal phosphate (PLP) in animal tissues and blood plasma (Gregory, 1980b; Schrijver et al., 1981; Gregory and Litherland, unpublished results). Attempts to extend reverse-phase HPLC to the determination of all naturally occurring forms of the vitamin were only partially successful because of often marginal resolution, lengthy derivatization, and variable precision (Gregory et al., 1981).

As an alternative to reverse-phase methods, Vanderslice et al. (1979, 1980, 1981abcd; Vanderslice and Maire, 1980) devized an anion exchange HPLC procedure for the separation and quantitation of the six B-6 vitamers and an internal standard in various foods and biological materials. This method involved programmed switching between two anion exchange columns, in situ derivatization of PL and PLP with semicarbazide, and automated changes in the excitation and emission wavelengths of the fluorometric detector. An unresolved difficulty with this method is the current lack of commercial availability of a suitable form of the anion exchange resin in the analytical system. The Vanderslice group also developed rapid and effective sample extraction and purification methods which were compatible with the HPLC procedure (Vanderslice et al., 1980).

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Coburn and Mahuren (1983) reported a cation exchange HPLC method which employed a ternary solvent program and post-column derivatization of PL and PLP with sodium bisulfite. This method yielded effective separation and sensitive fluorometric detection of B-6 vitamers and an internal standard in extracts of biological materials. The lack of purification of sample extracts did not pose a problem for most animal tissues and biological fluids analyzed, although interference in the determination of PLP was encountered with certain plasma samples. No attempts were made to apply this method to the analysis of other types of biological materials or food samples. The ternary gradient may present difficulties in some laboratories, and the comparatively short lifetime of the silica-based column may be a limitation.

In an alternate approach to ion exchange or conventional reverse-phase HPLC, Tryfiates and Sattsangi (1982) developed an ion-pair reverse-phase HPLC system capable of separating the major vitamin B-6 compounds. This method employed a simple step gradient to achieve the separation. The elution was monitored with an ultraviolet absorption detector, which lacked the sensitivity and specificity needed for the quantitative of B-6 vitamers in biological materials.

The procedure reported here was developed to provide a relatively simple method for the direct determination of the B-6 vitamers in a variety of foods and other biological materials. Various aspects of the methods of Vanderslice et al. (1980), Coburn and Mahuren (1983), and Tryfiates and Sattsangi (1982) have been incorporated and optimized in order to yield effective sample extraction and extract preparation, and rapid, efficient HPLC separation with common commercially available equipment and sensitive fluorometric detection.

MATERIALS AND METHODS

Reagents. Pyridoxal, pyridoxamine (PM), pyridoxine (PN), and 4′-deoxypyridoxine (4-dPN) were obtained as their hydrochloride forms from Sigma Chemical Co. (St. Louis, MO). Pyridoxal phosphate, 4-pyridoxic acid, and pyridoxamine phosphate also were obtained from Sigma. Purified water was prepared by using a Milli-Q system (Continental Water Systems Corp., El Paso, TX). 2-Propanol (HPLC grade) and sulfosalicylic acid (Certified A.C.S.) were obtained from Fisher Scientific Co. (Pittsburgh, PA). 1-Heptanesulfonic acid and 1-octanesulfonic acid (sodium salt forms) were purchased from Eastman Kodak Co. (Rochester, NY). All other chemicals were of analytical grade.

Center cut pork loin chops, frozen broccoli spears, and whole pasteurized homogenized cow's milk were purchased

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locally. Samples of human plasma and milk were obtained from healthy donors consuming balanced diets. The plasma donor was taking a multivitamin supplement which provided 2 mg of PN–HCl daily. Rat liver and muscle samples were excised immediately after decapitating male rats (500–600 g, Sprague–Drawley Crl:CD(SD)BR, Charles River Breeding Laboratories, Wilmington, MA) which had been fed a commercial ration (Purina Rodent Chow No. 5001, Ralston Purina Co., St. Louis, MO).

Sample Extraction and Purification. A sample purification system was assembled as described by Vanderslice et al. (1980), which included a peristaltic pump (Gilson Medical Electronics, Middleton, WI), a glass column (9 mm id × 250 mm, Model 252-06, Altex Scientific, Berkeley, CA) equipped with an adjustable plunger (Altex Model 252-11), a Tefzel injection valve with 1.1-mL sample loop, and a Tefzel 4-way slider valve (Altex Model 201-52) to reverse the flow during column regeneration. The column was packed with Bio-Rad AG2-X8 anion exchange resin (200–400 mesh, chloride form, Bio-Rad Laboratories, Richmond, CA). The elution of the B-6 vitamers was monitored by using a fluorometric detector (Fluoro-Monitor, American Instrument Co., Silver Spring, MD) equipped with a General Electric Germicidal lamp (Model C4T41), a 70-μL flow cell, a 295-nm interference excitation filter (American Instrument Co.), and a 405-nm narrow pass emission filter (Dell Optics Co., Inc., North Bergen, NJ). The mobile phase 0.1 M HCl, was pumped at a flow rate of 1.0 mL/min.

Extractions were performed essentially as described by Vanderslice et al. (1980, 1981b, 1983). Plant and animal tissues (2 g) were weighed and transferred into 50-mL polypropylene centrifuge tubes (Fisher Scientific Co., Pittsburgh, PA), mixed with 9 mL of 5% (w/v) sulfosalicylic acid (SSA) and 1 mL of a solution of 4-dPN (typically 120 nmol/mL in 5% SSA), and then homogenized with a Polytron homogenizer with a Model PTA 10S generator (Brinkmann Instruments Co., Westbury, NY) for 45 s at power setting 7. Following the addition of 10 mL of methylene chloride, the mixture was blended for another 20 s at power setting 5. After homogenization, the tubes were tightly sealed and centrifuged at 9000*g* for 15 min at 2 °C. For the preparation of samples in the determination of the recovery, 1 mL of a mixed solution of the B-6 vitamers in 5% SSA was added along with the 8 mL of 5% SSA and 1 mL of the internal standard solution prior to blending.

Milk and plasma samples (3 mL) were blended in 20-mL centrifuge tubes with 1 mL of 20% (w/v) SSA and 25 μL of a 120 nmol/mL solution of 4-dPN by using the Brinkmann Polytron with a Model PTA 7 generator for 45 s at power setting 7. After the addition of 4 mL of methylene chloride, the mixture was blended for 20 s at a power setting of 5. The mixtures were centrifuged as described above.

Following centrifugation, an aliquot of the aqueous layer of each sample was removed and injected, by using a filled loop technique, into the preparative chromatographic system. The biologically active B-6 vitamers and 4-dPN were manually collected as they eluted as a single fluorescing peak near the void volume with a retention time of approximately 4 min. Approximately 30 injections could be made before the preparative column required regeneration with a solution of 0.1 M HCl, 0.7 M NaCl, and 2.0% (w/v) ferric chloride, as described by Vanderslice et al. (1980).

Analytical Procedures. The analytical system consisted of two Model 110A solvent metering pumps, a Model 410 gradient controller, and a Model 905-40 injection valve with a 50-μL sample loop, all obtained from Altex Scientific (Berkeley, CA). A 100-mL sample loop was employed to increase sensitivity during the analysis of plasma. A Rheodyne (Cotati, CA) Model 5302 3-way slider valve, actuated by a Rheodyne Model 7163 solenoid valve, was installed at the solvent inlet of pump B to permit stepwise switching between solvent A2 and solvent B (described below). Valve switching was controlled by the integrator (Model 3388A, Hewlett-Packard, Avondale, PA). The analytical column was a Perkin-Elmer 3 × 3 (3 μm octadecylsilica, 4.6 mm id × 3 cm; Perkin-Elmer, Norwalk, CT). The reagent used in post-column derivatization (1 mg/mL of sodium bisulfite in 1.0 M sodium phosphate buffer, pH 7.5; Coburn and Mahuren, 1983) was metered at 0.16 mL/min into the column eluent stream by way of a Teflon T-junction (0.8 mm id, Altex Model 200-22) using a peristaltic pump (Model III, Technicon Corp., Tarrytown, NY). All tubing in the post-column system was 0.8 mm id Teflon (Rainin Instruments, Woburn, MA). The bisulfite reagent was made fresh daily and was filtered through a 0.45-μm pore size membrane prior to use. The combined reagent and eluate stream was conducted to the fluorometer by a 100-cm delay tube (0.8 mm Teflon). Fluorescence was monitored by using a Model LS-5 spectrophotofluorometer (Perkin-Elmer, Norwalk, CT) equipped with a 20-μL flow cell. An excitation wavelength of 330 nm (15-nm bandpass), an emission wavelength of 400 nm (20-nm bandpass), and a response factor of 4 were employed. A schematic diagram of the analytical system is shown in Figure 1.

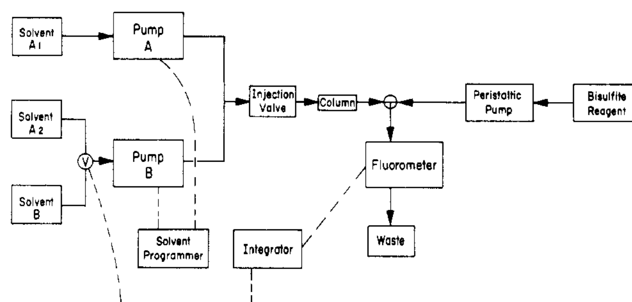

Figure 1. Schematic diagram of the analytical HPLC system. V = electrically controlled solvent switching valve; (—) liquid flow pathways; (--) electrical connections.

The following mobile phases were employed in the HPLC separation: solvent A1 = 0.033 M potassium phosphate and 8 mM octanesulfonic acid (pH 2.2); solvent A2 = 0.033 M potassium phosphate, 8 mM octanesulfonic acid, and 2.5% (v/v) 2-propanol (pH 2.2); solvent B = 0.033 M potassium phosphate and 6.5% 2-propanol (pH 2.2). The separation was performed at ambient temperature by using a linear gradient (1.8 mL/min total flow rate) from 100% solvent A1 to 100% solvent A2 in 12 min, followed by a programmed switch to 100% solvent B 15 min after injection. After the elution of PM, approximately 10 min was required for reequilibration prior to the next injection. An initial blank gradient was run prior to the injection of standard and samples. Methanol was the solvent used for column storage. Quantitation of B-6 vitamers was performed relative to the height of the internal standard peak, which corrected for possible variation in the volumn injected and in the dilution encountered in the preparative system.

RESULTS AND DISCUSSION

The HPLC method described here yielded complete resolution of the B-6 vitamers with high chromatographic

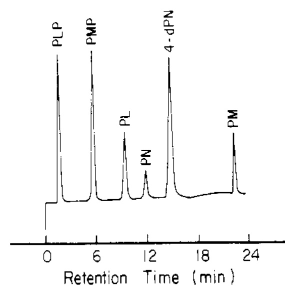

Figure 2. Chromatogram of B-6 vitamers and 4′-deoxypyridoxine obtained by using the ternary gradient elution HPLC procedure.

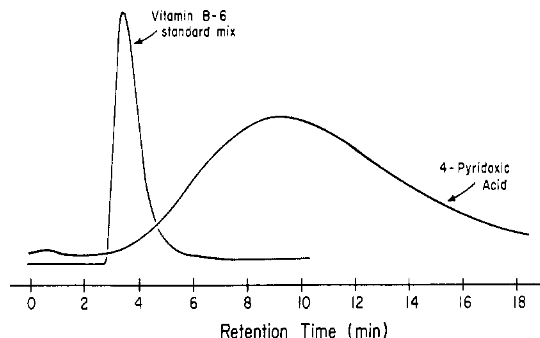

Figure 3. Preparative anion exchange chromatography of mixed B-6 vitamers and 4-pyridoxic acid (4PA). Note the stronger retention of 4PA.

efficiency (Figure 2). The ternary elution system described was employed by using HPLC equipment designed for running binary gradients simply by adding the externally controlled switching valve at the inlet of pump B. 4-Deoxypyridioxine was found to be well suited for use as an internal standard because of its stability, similar behavior to that of the B-6 vitamers in the preparative chromatographic system, and its separation from the B-6 vitamers during HPLC analysis of purified sample extracts. Post-column addition of a strongly buffered solution of sodium bisulfite permitted fluorometric quantitation of PLP and PL as their more highly fluorescent hydoxysulfonate derivatives and permitted the determination of the native fluorescence of PMP, PM, PN, and 4-dPN at the same excitation and emission wavelengths (Coburn and Mahuren, 1983). Pyridoxine phosphate was not evaluated in this study because of its minor significance as a naturally occurring form of vitamin B-6 (Vanderslice et al., 1980, 1981b, 1983; Coburn and Mahuren, 1983).

The vitamin B-6 metabolite 4-pyridoxic acid (4PA) eluted between PLP and PMP in the HPLC system and could be detected fluorometrically. This method was not capable of providing accurate quantitation of 4PA, however, because of the observed affinity of this compound for the AG2-X8 resin of the preparative system (Figure 3). Direct HPLC analysis of extracts of biological materials prepared by using trichloroacetic acid or perchloric acid for extraction may permit quantitation of this biologically inactive metabolite when necessary.

In preliminary studies, analyses were performed by monitoring the native fluorescence of the B-6 vitamers in the acidic mobile phase (excitation wavelength 295 nm, emission wavelength 405 nm). This provided sufficient sensitivity and specificity for the quantitation of vitamin B-6 in samples having relatively high levels of the B-6 vitamers (e.g., rat liver and muscle), although the greater sensitivity achieved when using the post-column reagent was found to be an advantage in all analyses. During the preliminary studies, HPLC separations were performed by using a stepwise binary gradient. For this separation,

Table I. Concentration of the Principal B-6 Vitamers in Rat Muscle[a]

vitamer	this study[b]	Thiele and Brin (1966)[c]	Lumeng and Li (1980)[d]	Vanderslice et al. (1981a)[e]
PLP	36.0 ± 5.4	18.2	21.8	32.3
PMP	5.09 ± 1.09	7.2	4.6	7.0

[a] Data from this study were determined using the binary step gradient method of HPLC separation and monitoring of the native fluorescence of the B-6 vitamers. All values in nmol/g. [b] Mean and SD: $n = 2$. [c] Microbiological determination after acid hydrolysis and ion exchange chromatographic separation, $n = 5$. [d] Fluorometric determination after ion exchange chromatographic separation, $n = 6$. [e] Anion exchange HPLC, $n = 1$.

Table II. Precision of Preparative and Analytical HPLC Systems Determined by Four Repeated Analyses of a Mixed B-6 Vitamer Solution

vitamer	concn, nmol/mL actual	concn, nmol/mL obsd	obsd/actual, %
PLP	10	10.28 ± 0.24	102.8 ± 2.4
PMP	4	4.05 ± 0.07	101.2 ± 1.8
PL	5	5.01 ± 0.04	100.2 ± 0.7
PN	5	5.02 ± 0.03	100.4 ± 0.6
PM	2	2.00 ± 0.03	99.8 ± 0.1

[a] Mean and SD of 4 analyses with the first sample as the standard.

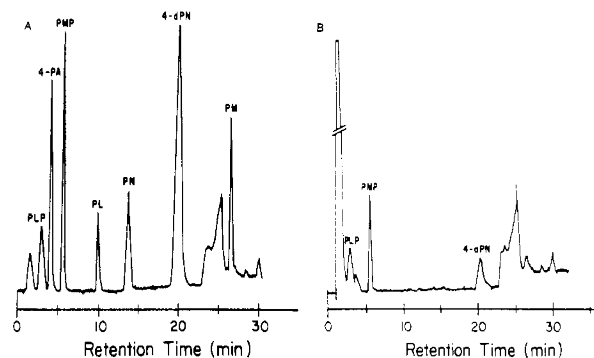

Figure 4. Chromatograms of (A) vitamin B-6 standard and (B) rat muscle extract with the binary stepwise gradient HPLC procedure.

solvent A was comprised of 0.033 M potassium phosphate, pH 2.2, with 4 mM each of heptanesulfonic acid and octanesulfonic acid and 1.5% (v/v) 2-propanol, and solvent B was 0.033 M potassium phosphate with 5% (v/v) 2-propanol. The separation was isocratic with solvent A until the elution of 4-dPN (approximately 20 min) at which time isocratic elution with solvent B was initiated. An exponential flow rate program from 0.5 to 2.0 mL/min was employed to improve the separation. Although this HPLC procedure is somewhat simpler than the ternary gradient elution method, the problem of obtaining adequate separation of PLP from other early eluting components, while preventing broadening of later peaks, could not be solved fully. This method was found to permit accurate quantification of the principal B-6 vitamers in rat muscle (Figure 4, Table I), liver, and certain other samples, but the resolution in the region of PLP was inadequate for the analysis of many plant-derived samples. For this reason the ternary gradient system was found to be the method of choice.

The overall precision of the preparative and ternary-gradient analytical systems was evaluated by quadruplicate injection into the preparative system of aliquots of a standard solution of B-6 vitamers (in SSA), followed by HPLC analysis (Table II). At the micromolar concen-

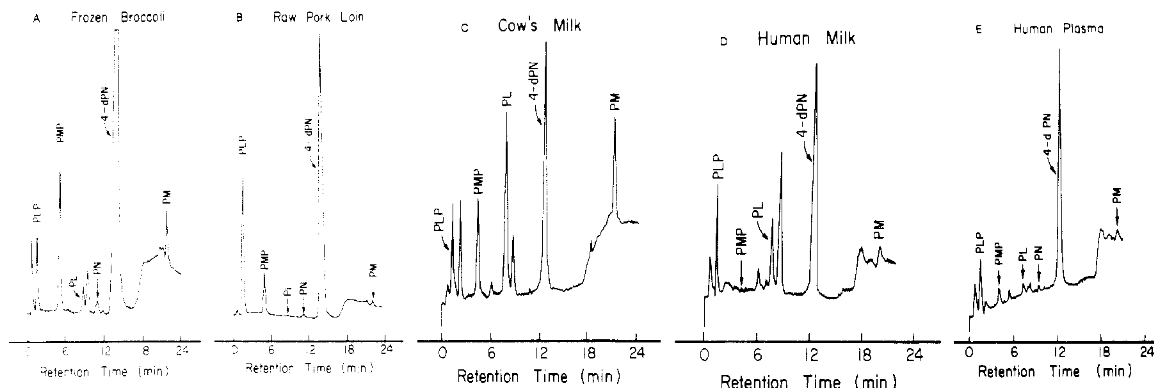

Figure 5. Chromatograms of extracts from foods and other biological materials with the ternary gradient HPLC procedure: A, frozen broccoli; B, raw pork loin; C, pasteurized homogenized cow's milk; D, human milk; E, human plasma.

Table III. Percentage Recovery of B-6 Vitamers Added to Broccoli and Pork Loin Samples Prior to Homogenization

vitamer	amount added, nmol/g	recovery,[a] % broccoli	recovery,[a] % pork loin
PLP	5	98.7 ± 2.0	78.7 ± 9.1
PMP	2	103.2 ± 2.1	96.5 ± 3.8
PL	2.5	98.9 ± 1.5	86.7 ± 0.9
PN	2.5	99.5 ± 2.5	93.3 ± 3.8
PL	1	101.7 ± 3.8	98.1 ± 1.2

[a] Mean and SD. Values represent triplicate extractions and analyses.

Table IV. Concentration of Vitamin B-6 in Raw Pork Loin[a]

vitamer	this study[a]	Vanderslice et al. (1981a)	Polansky and Toepfer (1969)[b]
PLP	28.39 ± 2.12	31.2	
PMP	2.64 ± 0.29	4.3	
PL	0.19 ± 0.02	ND	16.4
PN	1.56 ± 0.14	1.2	3.4
PM	0.38 ± 0.07	ND	5.5
total	31.1 ± 2.43	37.1	24.9

[a] Mean and SD, n = 3. ND, not detected. Values given in nmol/g. [b] Determined microbiologically after acid hydrolysis and chromatographic separation.

trations employed, all vitamers exhibited relative standard deviations of less than 2%.

The effectiveness of extraction was examined by measurement of the recovery of B-6 vitamers added prior to the homogenization of pork loin and broccoli samples (Table III). Recovery values for the B-6 vitamers added to broccoli samples ranged from 98.7 to 103.2% and exhibited relative standard deviations of no greater than 3.71%. Mean recovery values were lower for PLP and PL in pork loin (78.7 and 86.7%, respectively), which presumably reflects limited entrapment or binding of these vitamers by muscle proteins even in the presence of 5% SSA. Recovery studies were not performed on the milk and plasma samples in view of the high values obtained by Vanderslice et al. (1981, 1983) on the basis of similar extraction procedures. Overall, the use of SSA as an extractant yielded high precision and generally high recovery of B-6 vitamers, which is consistent with the results of Vanderslice (1980, 1981ab, 1983).

Typical HPLC chromatograms obtained by using the ternary-gradient HPLC system are shown in Figure 5. The preparative system provided effective purification of the sample extracts in addition to removal of SSA. Few peaks other than those of known B-6 vitamers were encountered during the analysis of foods and other biological samples (Figure 5). The observed concentrations of B-6

Table V. Concentration of Vitamin B-6 in Broccoli[a]

vitamer	this study[a]	Polansky and Toepfer (1969)[b]
PLP	1.69 ± 1.04	
PMP	2.17 ± 0.29	
PL	0.67 ± 0.30	7.93
PN	1.02 ± 0.22	3.55
PM	0.67 ± 0.19	0.77
total	6.22 ± 2.01	12.2

[a] Mean and SD, n = 3; commercially frozen broccoli. Values given in nmol/g. [b] Determined microbiologically after acid hydrolysis and chromatographic separation; fresh broccoli.

Table VI. Concentration of Vitamin B-6 in Pasteurized Homogenized Cow's Milk[a]

vitamer	this study[a]	Coburn and Mahuren (1983)	Polansky and Toepfer (1969)[b]
PLP	0.340 ● 0.013	0.613	
PMP	0.185 ± 0.048	0.231	
PL	0.794 ± 0.132	1.070	0.15
PN	ND	ND	0.06
PM	0.183 ± 0.011	0.175	0.41
total	1.50 ± 0.13	2.09	2.0

[a] Mean and SD, n = 3. ND, not detected. Values given in nmol/g. [b] Determined microbiologically after acid hydrolysis and chromatographic separation.

Table VII. Concentration of Vitamin B-6 in Human Milk[a]

vitamer	this study[a]	Coburn and Mahuren (1983)[b]	Vanderslice et al. (1983)[c]
PLP	0.148 ± 0.063	0.111	0.07
PMP	0.009 ± 0.001	0.017	ND
PL	0.349 ± 0.010	0.723	0.87
PN	ND	0.032	ND
PM	0.033 ± 0.007	0.005	ND
total	0.538 ± 0.066	0.888	0.94

[a] Mean and SD for single sample with triplicate extractions and analyses. ND, not detected. Values given in nmol/mL. [b] Mean of two samples. [c] Mean of values for four samples obtained over a 24-h period from a single individual consuming an unsupplemented diet.

vitamers are presented in Tables IV–VIII. The precision of the results was generally related to the concentration of the B-6 vitamers in the samples. Close correlations were observed between the results of this study and previously published data for most of the materials examined (Tables IV–VIII). The sensitivity of this method was marginal for the determination of B-6 vitamers in human plasma (Figure 5E), although acceptable signal/noise ratios were observed for other samples. The use of a signal processor (Vanderslice et al., 1981c) or the direct injection of samples

Table VIII. Concentration of Vitamin B-6 in Human Plasma[a]

vitamer	this study[a]	Coburn and Mahuren (1983)[b]	Vanderslice et al. (1981b)[c]	Lumeng et al. (1980)[d]
PLP	167	57	74	60
PMP	12	8	31	2
PL	19	23	ND	15
PN	37	19	180	30
PM	16	2	6	8
total	251	109	291	115

[a] Mean of duplicate analyses of a single sample. The plasma donor consumed a supplement of 2 mg of PN-HCl per day. The data from the other studies shown are for unsupplemented individuals. ND, not detected. Values given in pmol/mL. [b] Mean of 38 samples. [c] Mean of 2 samples. [d] Mean of 6 samples.

after deproteination with trichloroacetic acid (Coburn and Mahuren, 1983), omitting the cleanup step, would improve the applicability of this system to the routine analysis of plasma samples. The observation that PN was present in plasma at a concentration substantially lower than that of PLP corroborates the results of Coburn and Mahuren (1983) and Lumeng et al. (1980) and is in contrast to the report by Vanderslice et al. (1981b) that PN is a major form of vitamin B-6 in plasma. This suggests that interferences may exist in the region of PN in their anion exchange analysis of plasma extracts.

The existence of B-6 vitamers as 5′-O-β-D-glucosides and related conjugated forms (Yasumoto et al., 1977; Tadera et al., 1983) as varying proportions of the total vitamin B-6 of many plant tissues has been demonstrated (Kabir et al., 1983a). Although these conjugated forms have been reported to be biologically active and available for absorption in the rat (Tsuji et al., 1977), the glucosides appear to be largely unavailable in the human (Kabir et al., 1983b). Conventional microbiological assay methods for vitamin B-6 employ autoclaving in the presence of HCl for extraction, which also serves to hydrolyze phosphate esters and glycoside conjugates. Thus, analysis using conventional microbiological methods based on acid hydrolysis would tend to overestimate the concentration of biologically available B-6 in foods containing significant quantities of the β-glucoside conjugates. Analytical methods such as that reported here would not be susceptible to this problem. Recent studies with aqueous extraction and microbiological analysis before and after enzymatic treatment of food extracts have shown widely varying proportions of these conjugates in plant-derived foods (Kabir et al., 1983a). We have recently found that, with minor modifications, the HPLC method reported here would be capable of providing data concerning the concentration of the individual B-6 vitamers and their conjugated forms. This work will be the topic of a separate publication.

In conclusion, the method described permits the determination of vitamin B-6 in various types of foods and other biological materials. The effective preparative procedure, high resolution, and sensitivity all contribute to the validity of the method.

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Registry No. PLP, 54-47-7; PMP, 529-96-4; PL, 66-72-8; PN, 65-23-6; PM, 85-87-0.

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