Determination of Vitamin B-6 in Animal Tissues by Reverse-Phase High-Performance Liquid Chromatography

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A reverse-phase high-performance liquid chromatographic (LC) method is described for the fluorometric determination of pyridoxal (PL), pyridoxamine (PM), and their 5′-phosphates (PLP and PMP) in a variety of animal tissues and milk. Samples are extracted with perchloric acid. Aliquants of neutralized extracts are treated with glyoxylic acid to deaminate PM and PMP, followed by derivatization with semicarbazide. LC analysis of the extracts prepared with and without glyoxylate treatment permits the individual quantitation of the B-6 vitamers. Fluorescence spectral analysis of collected LC fractions and correlation with microbiological assay results support the accuracy of the procedure.

The determination of vitamin B-6 in foods and other biological material is a difficult analytical problem because of the occurrence of six nutritionally active vitamers in complex matrices. Current methods for the determination of vitamin B-6 are based on cumbersome microbiological growth techniques (Toepfer and Lehmann, 1961; Toepfer and Polansky, 1970; Haskell and Snell, 1970; Barton-Wright, 1971) or laborious chemical methods (Fujita et al., 1955a–c; Hennessy et al., 1960; Kraut and Imhoff, 1967; Contractor and Shane, 1968; Loo and Badger, 1969; Columbini and McCoy, 1970a; Takanashi et al., 1970; Masukawa et al., 1971; Fiedlerova and Davidek, 1974; Chin, 1975; Gregory and Kirk, 1977; Spector, 1978; Chauhan and Dakshinamurti, 1979) of uncertain specificity. The need exists for a rapid procedure which would provide accurate, precise data for the B-6 vitamers. Such a method would facilitate nutritional and metabolic studies and aid in the assessment of the nutritional quality of foods.

High-performance liquid chromatography (LC) has recently been applied to the analysis of B-6 vitamers in certain foods and biological materials. Many of the reported LC methods for vitamin B-6 compounds have lacked the chromatographic efficiency, detection specificity, or sensitivity necessary for the analysis of complex materials (Williams and Cole, 1975; Wong, 1978; Yoshida et al., 1978; Williams, 1979; Lim et al., 1980). Yasumoto et al. (1975) described a procedure which was suitable for the determination of the free base (nonphosphorylated) B-6 vitamers in acid hydrolysates of biological materials using cation-exchange LC and postcolumn chromogenic labeling. Application of this method has been limited because of the complex instrumentation and lengthy separation time (~2 h/sample). Thompson (1978) reported a similar cation-exchange LC method with fluorometric detection which was successfully applied to the determination of vitamin B-6 in milk and fortified infant formulas. Vanderslice et al. (1979) recently devised an anion-exchange fluorometric LC method which is capable of separating and detecting the six B-6 vitamers. This anion-exchange method has been successfully applied to the determination of vitamin B-6 in a variety of foods (Vanderslice et al., 1980). Slow elution (~70 min) and the need for monitoring with two excitation wavelengths may be disadvantages to some analysts.

Reverse-phase LC based on the solvophobic technique described by Horvath et al. (1976, 1977) has been shown to be well suited for the rapid, high efficiency separation of vitamin B-6 compounds. This LC technique was first employed for the fluorometric determination of the B-6 vitamers in low-moisture cereal food systems (Gregory and Kirk, 1978). The reverse-phase method has been slightly modified for the rapid determination of vitamin B-6 in breakfast cereals (Gregory, 1980a) and 4-pyridoxic acid, the major urinary vitamin B-6 metabolite (Gregory and Kirk, 1979).

Recently, this reverse-phase LC technique was adapted to permit the determination of pyridoxal 5′-phosphate (PLP) in animal tissues using fluorometric detection of the PLP–semicarbazone derivative (Gregory, 1980b). The derivatization was employed because the semicarbazone derivative exhibits greater retention and fluorescence than unmodified PLP.

The procedure reported here is a modification of the reverse-phase semicarbazone technique for the determination of PLP. It permits the determination of the principal B-6 vitamers in animal tissues and selected foods of animal origin in which the vitamin B-6 activity is almost entirely due to pyridoxal (PL), pyridoxamine (PM), and their 5′-phosphates (PLP and PMP). This method is based on the conversion of PMP and PM to PLP and PL, respectively, by deamination with glyoxylate prior to derivatization. The applicability of the method, along with its precision and accuracy, were examined. A preliminary description of this procedure has been previously reported (Gregory and Kirk, 1981).

MATERIALS AND METHODS

Materials and Equipment. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, pyridoxal hydrochloride, pyridoxamine 5′-phosphate, pyridoxal 5′-phosphate, glyoxylic acid (Na salt), potato acid phosphatase (2 units/mg of solid), and semicarbazide hydrochloride were purchased from Sigma Chemical Co. All other chemicals employed were reagent grade, except acetonitrile which was Nanograde (Mallinckrodt, Inc.).

Rats (Sprague–Dawley, ~250 g, male; Charles River Breeding Laboratories) were fed ad libitum a commercial rodent chow diet or the same diet which had been powdered and fortified with pyridoxine (PN) hydrochloride to 25 μg of added PN/g of diet. Rat tissues were rapidly excised from the animals after decapitation, frozen, and stored at −20 °C until analyzed. Ground beef, frozen calf liver, homogenized whole milk, and canned evaporated milk were obtained locally. For cooking trials, ground beef was fried, and calf liver was cooked in a microwave oven.

Analyses were performed by using an Altex Model 312 liquid chromatograph. Fluorescence detection was performed with an American Instrument Co. fluoro-Monitor equipped with a 70-μL flow cell, Corning 7-51 excitation filter (365-nm transmission maximum), Wratten 8 emission filter (>400-nm transmission), and a General Electric lamp, Model F4T4-BL. The LC column was a 4.6 mm i.d. × 25

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cm Ultrasphere IP (5-μm particle size octadecylsilica with trimethylsilyl capping of residual silanol groups) obtained from Altex Scientific. The LC mobile phase consisted of a 0.033 M potassium phosphate buffer, pH 2.2, containing 2.5% (v/v) acetonitrile. Water for the mobile phase was prepared by passing deionized distilled water through a column of Porapak Q (Waters Associates).

Fluorescence excitation and emission spectra (uncorrected) of selected manually collected LC peaks were recorded by using a Perkin-Elmer Model 204-S spectrophotofluorometer (xenon lamp, slits providing a 10-nm band-pass).

Extraction and Derivatization. The extraction, derivatization, and LC analytical methods were based on previously described procedures (Gregory, 1980b). Soft tissue samples (~5 g) were homogenized with 2 volumes of 0.1 M KH_2PO_4, pH 7.0, by using a Brinkmann Polytron (2 min at speed setting 7; Model PT 10/35 with PT 10-ST probe generator). Cooked liver was finely chopped and homogenized in 2 volumes of the pH 7.0 phosphate buffer for 2 min with the Polytron. Fibrous tissue and ground beef (raw and cooked) were homogenized in 4 volumes of the pH 7.0 phosphate buffer for 2 min with the Polytron.

Four-milliliter portions of the homogenate or milk sample were transferred to 16 × 100 mm screw-cap test tubes. To tubes designated "recovery", 2 mL of a freshly prepared aqueous mixture of either PM and PMP or PL and PLP was added and mixed well (standard solutions were 10 μg of free base/mL with respect to each vitamer). Protein was precipitated by adding 4 mL of 3 N perchloric acid, mixing, and centrifuging for 15 min at 1500*g* in a clinical centrifuge. The supernatant was removed and the pellet was resuspended in 4 mL of 1 N perchloric acid, followed by recentrifugation. The second supernatant was combined with the first and adjusted to pH 6 to 7 with 3 N KOH. The neutralized extracts were diluted to a constant volume of 20 mL with distilled water. After the $KClO_4$ precipitate was allowed to settle for 15–30 min, aliquants of the supernatant were taken for derivatization.

Duplicate portions (1.5 mL) of each extract were transferred to 16 × 100 mm screw-cap test tubes and diluted with 0.4 mL of 1.0 M KH_2PO_4, pH 7.5. Water (0.1 mL) or 0.1 mL of aqueous 0.5 M sodium glyoxylate was added, followed by mixing, capping tightly, and incubating in a boiling water bath for 15 min. The purpose of the glyoxylate treatment was to convert PM and PMP to PL and PLP, respectively. The tubes were cooled under running tap water to ambient temperature. Semicarbazide (0.5 mL of a 0.2 M aqueous solution) was added to each tube, followed by a 5-min incubation in a boiling water bath. The derivatized solutions were filtered through a 0.45 μm pore size membrane prior to LC analysis.

Standards were prepared from fresh aqueous solutions of PM and PMP or PL and PLP (typically 1.0 μg of free base/mL for each vitamer) in a manner identical with that described for sample extracts. The standard solutions yielded a final concentration of 0.6 μg of free base/mL for each vitamer. Standards and samples were analyzed on the same day that they were derivatized.

Liquid Chromatographic Analysis. The components of the mobile phase, acetonitrile and 0.033 M potassium phosphate (pH 2.2), were separately filtered under vacuum through a 0.45 μm pore size membrane and degassed by sonication under vacuum. The acetonitrile was added to the phosphate buffer to provide a 2.5% (v/v) final concentration of acetonitrile. Chromatographic analysis of samples and standards was ordinarily performed at a flow rate of 2.0 mL/min at ambient temperature, which provided an inlet pressure of ~3000 psi. Samples and standards were injected by using a filled loop technique with a 50- or 100-μL injection volume.

After several hours of LC analysis of certain tissue samples (e.g., liver), broad, slow eluting peaks and base-line drift were normally encountered. These contaminants were washed from the column by pumping the mobile-phase buffer containing 20% (v/v) acetonitrile for 20–30 min prior to reequilibration and continuation of sample analysis. Prior to shut down at the end of the analysis period, the system was sequentially flushed with water and acetonitrile.

Quantitation was based on the measurement of peak heights for the PL- and PLP-semicarbazone peaks prepared in the presence and absence of glyoxylate. Quantitation of the individual vitamers was performed by using the following calculation method. Letting F = peak height (relative fluorescence), C = calibration factor (response to standard; relative fluorescence per microgram per milliliter), D = dilution factor (milliliters of injection solution per gram of sample), and R = recovery of each added vitamer, then

$$F_{\text{total B-6 phosphates}} = F_{\text{PMP}} + F_{\text{PLP}} \quad (1)$$

$$F_{\text{total free bases}} = F_{\text{PM}} + F_{\text{PL}}$$

where $F_{\text{total B-6 phosphates}}$ and $F_{\text{total free bases}}$ refer to the peak heights observed with glyoxylate treatment.

$$(\mu\text{g of vitamer/g})_X = F_X(1/C)_X(1/D)(1/R)_X \quad (2)$$

$$\mu\text{g of PMP/g} = (F_{\text{total B-6 phosphates}} - F_{\text{PLP}})(1/C)_{\text{PMP}}(1/D)(1/R)_{\text{PMP}}$$

$$\mu\text{g of PLP/g} = F_{\text{PLP}}(1/C)_{\text{PLP}}(1/D)(1/R)_{\text{PLP}}$$

$$\mu\text{g of PM/g} = (F_{\text{total free bases}} - F_{\text{PL}})(1/C)_{\text{PM}}(1/D)(1/R)_{\text{PM}}$$

$$\mu\text{g of PL/g} = F_{\text{PL}}(1/C)_{\text{PL}}(1/D)(1/R)_{\text{PL}}$$

All vitamin B-6 concentrations were expressed as micrograms of free base per gram of sample, permitting the addition of values for phosphorylated and nonphosphorylated vitamers.

As a test of the applicability and reproducibility of the procedure, samples of selected animal tissues and milk products were each analyzed in triplicate as described above. Triplicate recovery determinations were performed for each B-6 vitamer in these samples.

Microbiological Assay Procedure. Selected samples were analyzed microbiologically for the B-6 vitamers for comparison with the LC assay results. Five-milliliter portions of the neutralized perchlorate extracts were mixed with 1 mL of a 1 unit/mL potato acid phosphatase solution prepared in 0.2 M potassium acetate, pH 4.5. The mixtures were incubated at 37 °C for 1 h, which had been previously shown to provide quantitative hydrolysis of the phosphorylated B-6 vitamers (Takanashi et al., 1970; Gregory and Kirk, 1978). The enzyme reactions were terminated, and the protein was precipitated by incubation for 5 min in a boiling water bath, followed by centrifugation in a clinical centrifuge.

The B-6 vitamers in the extracts were fractionated by reverse-phase LC by using the method previously described (Gregory and Kirk, 1978; Gregory, 1980a). The separations were performed by using a 0.033 M potassium phosphate mobile phase, pH 2.2, a Partisil 10 ODS-3 column (Whatman, Inc.; 4.6 mm × 25 cm), and a flow rate of 2.0 mL/min. Preliminary injections (100 μL) of a 1.0 μg/mL mixture of PM, PL, and PN were used to determine the retention times and peak widths of these B-6 vitamers (T_R

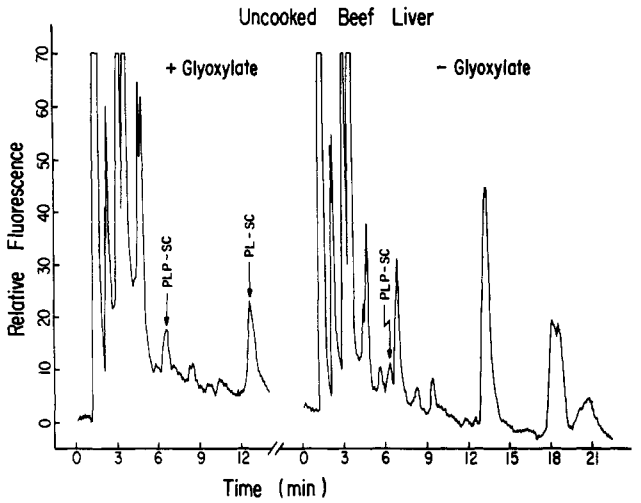

Figure 1. Chromatograms from the LC determination of vitamin B-6 in uncooked beef liver.

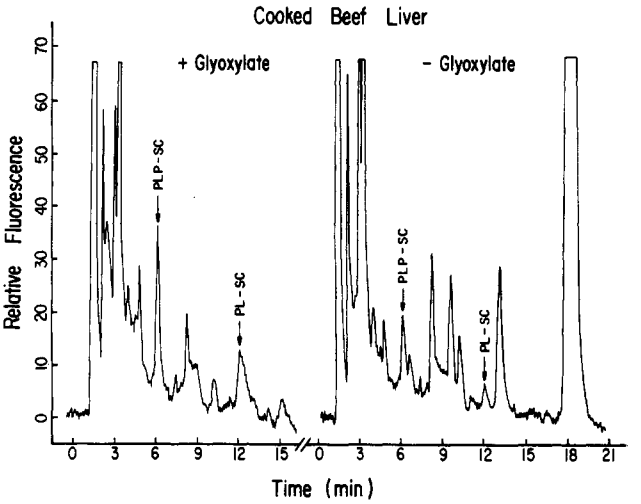

Figure 2. Chromatograms from the LC determination of vitamin B-6 in cooked beef liver.

= 2.2, 5.6, and 7.7 min, respectively). Base-line resolution was obtained. Several 100-μL portions of the extracts were chromatographed, and the fractions corresponding to the B-6 vitamers manually collected.

The microbiological assay procedure was that of Haskell and Snell (1970) with *Saccharomyces uvarum* as the test organism and Pyridoxine Y Medium (Difco Laboratories) as the growth medium. The vitamin B-6 concentration of each LC fraction was determined relative to a PN standard curve which was run over the range of 0–8 ng of PN/assay tube (5-mL total volume). One-milliliter portions of each fraction were analyzed in triplicate. The tubes were incubated with shaking in a 30 °C water bath for 18 h. Turbidity was determined by measuring the absorbance at 650 nm with a Gilford Model 250 spectrophotometer.

Because of the photolability of the B-6 vitamers, all LC and microbiological assays were performed in the absence of white fluorescent or incandescent light. When necessary, lighting was provided with gold fluorescent lamps (General Electric Model F40G0).

RESULTS AND DISCUSSION

The reverse-phase LC method described here is a modification and extension of the procedure developed for the determination of PLP in animal tissues (Gregory, 1980b). The principal modification was the incorporation of a glyoxylate treatment to permit the determination of PM and PMP in addition to PL and PLP. Another modification was the use of a 5 μm particle size reverse-phase column to provide greater chromatographic efficiency.

This assay procedure was applied to a variety of animal tissues and products in order to assess the suitability of the chromatographic separation, recovery, accuracy, and precision of the method. Typical chromatograms for the analysis of calf liver (raw and cooked), cooked ground beef, and milk (homogenized whole and evaporated) are presented in Figures 1–4. The differences in height of PL- and PLP–semicarbazone peaks in the presence and absence of glyoxylate were the basis of the quantitation of the individual B-6 vitamers. These chromatograms illustrate the typical resolution and efficiency obtained under these conditions. Partially incomplete resolution of PLP-semicarbazone from other components was noted with certain samples. In all analyses, resolution was sufficient to permit accurate quantitation. The chromatograms obtained for muscle and homogenized milk (Figures 3 and 4) and brain tissue (Gregory and Kirk, 1981) were remarkably free of extraneous fluorescing components.

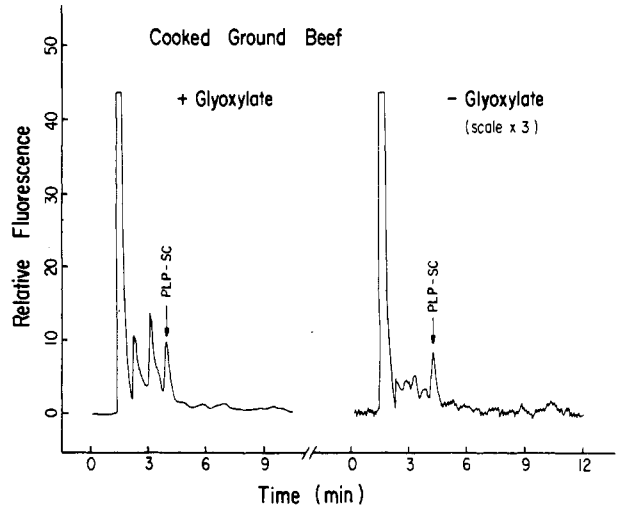

Figure 3. Chromatograms from the LC determination of vitamin B-6 in cooked ground beef. The detector sensitivity for the right chromatogram (no glyoxylate) was 3 times greater than that employed for the left chromatogram.

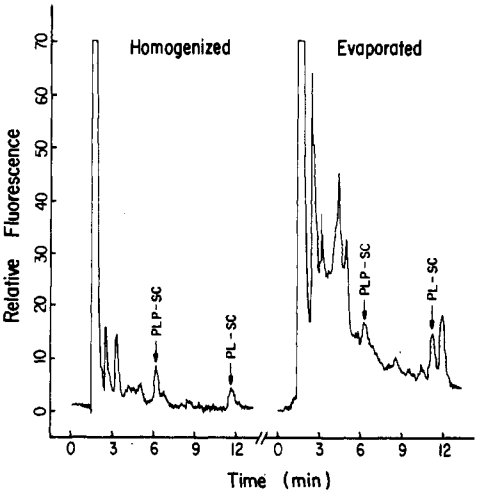

Figure 4. Chromatograms from the LC determination of vitamin B-6 in homogenized whole milk and canned evaporated milk. Note that the chromatograms from only the *glyoxylate-treated* extracts are shown.

As previously reported (Gregory, 1980b), the fluorescence response was linear over a range of at least 5–1500 ng of PLP/injection and was independent of injection volume over 10–100 μL. Similar linearity of fluorescence

Table I. B-6 Vitamer Content of Selected Foods and Tissues of Animal Origin[a,b]

sample	units	PL	PLP	PM	PMP	total
calf liver[c]						
raw	μg/g	ND	0.48 ± 0.02	1.15 ± 0.08	0.33 ± 0.11	1.96 ± 0.14
	nmol/g	ND	2.86 ± 0.12	6.85 ± 0.48	1.96 ± 0.65	11.7 ± 0.8
cooked	μg/g	1.40 ± 0.28	2.80 ± 0.17	2.30 ± 0.31	5.05 ± 0.08	11.6 ± 0.5
	nmol/g	8.33 ± 1.67	16.7 ± 1.0	13.7 ± 1.8	30.1 ± 0.5	68.8 ± 2.7
rat brain[d]	μg/g	ND	0.60 ± 0.06	ND	1.33 ± 0.13	1.93 ± 0.14
	nmol/g	ND	3.57 ± 0.36	ND	7.91 ± 0.77	11.5 ± 0.8
ground beef						
raw	μg/g	ND	0.66 ± 0.19	ND	1.19 ± 0.29	1.85 ± 0.35
	nmol/g	ND	3.93 ± 1.13	ND	7.08 ± 1.73	11.0 ± 2.1
cooked	μg/g	ND	2.48 ± 0.49	ND	1.40 ± 0.23	3.88 ± 0.54
	nmol/g	ND	14.8 ± 2.9	ND	8.33 ± 1.37	23.1 ± 3.2
whole milk	μg/g	0.30 ± 0.01	0.11 ± 0.00	0.04 ± 0.02	0.04 ± 0.01	0.49 ± 0.03
	nmol/g	1.79 ± 0.06	0.66 ± 0.02	0.24 ± 0.12	0.24 ± 0.06	2.93 ± 0.15

[a] Mean ± standard deviation, triplicate analyses. Data expressed as micrograms of free base (nonphosphorylated) vitamer per gram and nanomoles per gram. [b] ND = not detected (0.1–0.2 μg/g for animal tissues). [c] Raw and cooked data not for same liver sample. [d] Rat fed a commercial laboratory rodent chow diet.

has been observed for PL, PM, and PMP in this analysis. A detection limit of ~1–2 ng for each vitamer (signal/noise = 2) was routinely observed with the present detection system. Under these conditions, the limit of detection was approximately 0.2 μg/g for each vitamer in animal tissue samples and 0.05 μg/mL for milk samples. This sensitivity was sufficient for the analysis of all tissues examined. Although milk can be successfully analyzed, the quantitation of certain samples was difficult because the low vitamin B-6 content of milk and the resulting low signal/noise ratios.

Previous examination of the fluorescence spectra of manually collected PLP–semicarbazone LC fractions from standards and tissues strongly supported the specificity of the assay procedure (Gregory, 1980b). In the present study, the PL– and PLP–semicarbazone fractions from standards and milk extracts (homogenized whole and evaporated) were collected and their fluorescence spectra examined. The excitation and emission spectra of the semicarbazone fractions from each sample extract were nearly identical with those of the respective standards. Typical spectra are presented in Figure 5. These results further support the qualitative validity of the assay procedure.

The glyoxylate reaction was found to provide quantitative deamination of PM and PMP under the conditions employed. Initial studies were conducted to compare the suitability of several previously reported methods which utilized either Al^{3+} (Toepfer et al., 1961; Contractor and Shane, 1968), Cu^{2+} (Suelter et al., 1976), or no metal catalyst (Gregory and Kirk, 1977). The procedures utilizing Al^{3+} or no catalyst provided complete deamination, while the reaction in the presence of Cu^{2+} provided only 50–70% yield under these conditions. The procedure based on the use of no added metal catalyst was selected because the PL– and PLP–semicarbazone derivatives were subsequently found to be more stable in the absence of Al^{3+} ions. Even in the absence of metal ions, typical losses of 20–30% for the semicarbazone derivatives have been encountered when derivatized samples were stored at 2 °C for 2–3 days prior to LC analysis. These data indicate the need for quantitation of semicarbazone derivatives within 1 day of sample preparation. Storage of derivatized samples at ambient temperature for at least 6 h did not cause significant losses of the semicarbazone derivatives.

Preliminary studies were conducted to examine the applicability of treating the extracts with sodium borohydride prior to glyoxylate treatment, as described by Suelter et al. (1976). Borohydride treatment results in the reduction of PL and PLP to PN and PNP, which cannot

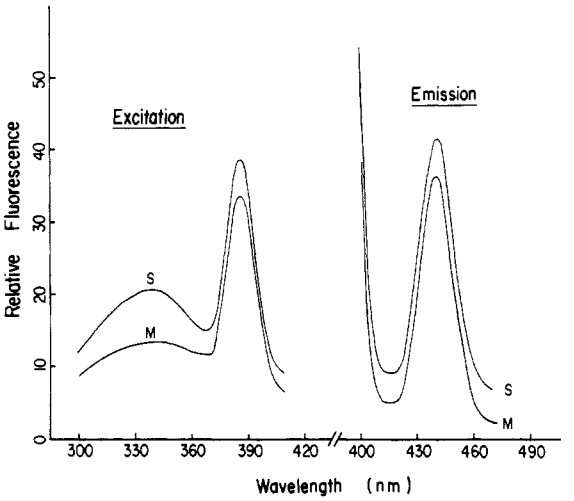

Figure 5. Fluoroescence spectra (uncorrected) of manually collected pyridoxal–semicarbazone fractions from standards (S; ~30 ng as PL) and homogenized whole milk extract (M). Excitation spectrum: emission wavelength = 446 nm. Emission spectrum: excitation wavelength = 380 nm. Spectra offset 5 units vertically for clarity.

be detected with this assay procedure, consequently permitting direct quantitation of PM and PMP. Under the conditions employed, concentrations of sodium borohydride up to 5.0 mM (twice that recommended) were insufficient to completely reduce PL and PLP in sample extracts or standard solutions. Because the borohydride treatment provided no advantage in analysis time or specificity, it was subsequently omitted.

The results of the LC analysis of selected animal tissue and milk samples (Table I) illustrate the high precision of the method. The data for rat brain and uncooked ground beef, showing 55–70% PMP and no detectable PM or PL, were typical of the B-6 vitamer distribution of fresh animal tissue. High concentrations of PM in the calf liver sample were probably the result of enzymatic dephosphorylation during commerical handling or storage prior to its purchase. These data for whole milk are in contrast to the values reported by Masukawa et al. (1971) which indicated that PLP, PM, and PMP did not occur naturally in milk. The presence of PMP in milk has been previously reported (Gregory and Mabbitt, 1961; Vanderslice et al., 1980).

Results obtained by using the reverse-phase LC procedure were found to be in generally close agreement with previously published data (Table II). The vitamin B-6 content of the calf liver sample was lower than that or-

Table II. Comparison of Reverse-Phase LC Results with Previously Published Data for B-6 Vitamers in Selected Biological Materials

		nmol/g wet weight					
		reverse-phase LC	previously published				
sample	vitamers						
calf liver, raw	PL + PLP	2.9	5.1				
	PM + PMP	8.8	24.1				
	PN + PNP		3.1				
	total	11.7	32.3[a]				
rat liver, B-6 sufficient	PL + PLP	18.5	30.8	21.8	16.4	14.8	
	PM + PMP	23.2	32.0	28.3	41.7	41.1	
	PN + PNP			1.4	1.6	3.9	
	total	41.7	62.8[b]	51.5[c]	59.7[d]	59.8[e]	
rat brain, B-6 sufficient	PL + PLP	3.6	5.7	7.6	4.6	4.6	6.1
	PM + PMP	7.9	9.8	11.7	8.8	9.3	5.8
	PN + PNP			1.4	0.6	0.7	0.1
	total	11.5	15.5[b]	15.5[b]	14.0[d]	14.6[e]	12.0[f]
hamburger, raw	PL + PLP	3.9	8.7	8.6	4.2		
	PM + PMP	7.1	8.6	6.0	3.0		
	PN + PNP		1.8	0.6	1.2		
	total	11.0	19.1[g]	15.2[h]	8.4[i]		
whole milk, homogenized	PL + PLP	2.5	1.6	0.5			
	PM + PMP	0.5	0.4	0.5			
	PN + PNP		0.06	0.0			
	total	3.0	2.1[j]	1.0[i]			

[a] Toepfer and Lehmann (1961). [b] Lumeng and Li (1980). [c] Thiele and Brin (1966). Rats fed ad libitum 4 μg of PN/g of diet. [d] Thiele and Brin (1968). Rats fed ad libitum 4 μg of PN/g of diet. [e] Brin and Thiele (1967). Rats fed ad libitum 2 μg of PN/g of diet. [f] Loo and Badger (1969). [g] Polansky and Toepfer (1969). Value is for ground chuck. [h] Vanderslice et al. (1980). Value is for a lean sample. [i] Fujita et al. (1955b). [j] Polansky and Toepfer (1969).

Table III. Recovery of B-6 Vitamers Added to Sample Homogenates prior to Extraction and Liquid Chromatographic Analysis[a,b]

	% recovery			
sample	PL	PLP	PM	PMP
calf liver				
raw	111.7 ± 7.2	82.0 ± 5.0	110.3 ± 9.1	94.0 ± 1.4
cooked		72.6 ± 14.2	82.7 ± 9.8	59.4 ± 7.3
rat brain	107.0 ± 1.0	94.3 ± 1.3	101.0 ± 2.7	90.7 ± 1.5
ground beef				
raw	90.3 ± 6.7	84.5 ± 9.7	97.8 ± 8.7	95.0 ± 11.3
cooked	117.7 ± 2.5	79.3 ± 0.6	97.0 ± 2.0	98.7 ± 1.2
milk				
whole	88.7 ± 2.9	97.0 ± 4.0	130.6 ± 3.2	113.2 ± 1.5
evaporated	115.3 ± 7.5	67.3 ± 0.6	96.0 ± 6.9	88.3 ± 9.1
mean recovery	105.1	82.4	102.2	91.3
mean C.V., %[c]	4.4	6.3	6.2	5.7

[a] Mean ± standard deviation, triplicate analyses. [b] Recovery refers to the recovery of vitamers which were added to sample homogenates prior to extraction and analysis. Recovery of PMP and PM was determined separately from PLP and PL. [c] C.V. = coefficient of variability (mean relative standard deviation).

dinarily observed for liver, possibly because of B-6 vitamer degradation during commercial frozen storage. LC results for vitamin B-6 in rat brain and liver were slightly lower than published values for animals of similar dietary vitamin B-6 intake. Differences between observed B-6 vitamer concentrations and published data may be due in part to the specificity of microbiological assay procedures and the marked effect of age and vitamin B-6 nutriture on the vitamer content of animal tissues and milk.

The recovery data for B-6 vitamers added to sample homogenates (or liquid milk samples) prior to extraction are shown in Table III. The high precision of the method is confirmed by the mean coefficients of variability which ranged from 4.4 to 6.2% for the recovery of the B-6 vitamers. Recovery values for each vitamer were highly dependent upon the nature of the sample. The variably higher recovery for PL and PM indicates the occasional occurrence of partial dephosphorylation of PLP and PMP under these assay conditions. These data indicate the necessity of running several recovery samples for each type of material analyzed. The recovery is sufficiently consistent among samples of similar composition that determination of the recovery for each sample is not required. Previous studies by Johansson et al. (1968) using radiolabeled vitamin B-6 have shown that the extraction efficiency of the perchloric acid method is at least 85% for all B-6 vitamers in animal tissue. The high extraction efficiency and the observed precision (Table III) justify the use of recovery values in the calculation of B-6 vitamer concentrations.

Limited investigations were conducted to compare the results of the LC method with those of the *S. uvarum* growth procedure. Because *S. uvarum* does not respond to the phosphorylated B-6 vitamers, the perchloric acid extracts were treated with acid phosphatase prior to LC fractionation. Fair correlation was observed between the results of the semicarbazone LC and microbiological procedures for the individual B-6 vitamers and total vitamin

Table IV. Comparison of Results of Microbiological (*S. uvarum*) and Liquid Chromatographic Analyses for Vitamin B-6 in Rat Liver and Homogenized Whole Milk[a]

	liquid chromatography		*S. uvarum*[b]	
sample	vitamer	concn, nmol/g	vitamer	concn, nmol/g
rat liver[c]	PL	ND	PL + PLP	14.3 ± 1.8
	PLP	18.5 ± 0.6	PM + PMP	28.6 ± 3.6
	PM	ND	PN + PNP	1.8 ± 0.6
	PMP	23.2 ± 0.6		
	total[d]	41.7 ± 0.6	total	44.6 ± 4.2
whole milk	PL	2.02 ± 0.36	PL + PLP	3.04 ± 1.07
	PLP	2.02 ± 0.24	PM + PMP	1.37 ± 0.12
	PM	1.25 ± 0.48	PN + PNP	ND
	PMP	0.48 ± 0.24		
	total[d]	5.77 ± 0.66	total	4.41 ± 1.07

[a] Mean ± standard deviation, triplicate analyses. ND = not detected. [b] Microbiological assay of phosphatase-treated perchlorate extracts of samples following fractionation by reverse-phase liquid chromatography. Values determined with pyridoxine hydrochloride as a standard. [c] Rats fed a commercial laboratory rodent chow diet which contained experimentally added PN-HCl at 25 μg of PN/g. [d] Values for total vitamin B-6 not significantly different ($p > 0.2$) as determined by a two-tailed t test.

B-6 in these samples (Table IV). Within the limited precision of the *S. uvarum* procedure, these data support the accuracy of the LC assay.

Another objective of the microbiological assay of the fractionated extracts was to determine the significance of PN and PNP as sources of vitamin B-6 activity in animal tissue and milk. Because the semicarbazone procedure, as described here, does not detect PN and PNP, the content of these vitamers is crucial to the accuracy of the method. Preparative reverse-phase LC was used in order to assure complete resolution of the B-6 vitamers. For rat liver, PN and PNP were found to account for 4% of the total vitamin B-6, while these vitamers were not detectable in the whole milk sample. In this study a diet containing ~20 times the nutritional requirement for the rat was fed ad libitum for 4 days. Analysis showed that liver PN and PNP levels were minimal. These data suggest that the in vivo enzymatic phosphorylation and oxidation of dietary PN are sufficiently rapid that tissue levels of PN and PNP remain very low. The results of several radiosotopic PN metabolism studies are in support of this conclusion (Johansson et al., 1968, 1974; Columbini and McCoy, 1970b). Extensive data which are based on ion-exchange fractionation and microbiological assay [e.g., Polansky and Toepfer (1969)] also indicate that ~95% of the vitamin B-6 in milk is comprised of the aldehyde and amine forms of the vitamin. Thus, the measurement of PM, PL, and their 5′-phosphates by the reverse-phase semicarbazone procedure provides an accurate assessment of the vitamin B-6 content of animal tissues and products.

Recently Vanderslice and co-workers (Vanderslice and Maire, 1980; Vanderslice et al., 1980, 1981) reported an anion-exchange LC procedure which is capable of fluorometrically quantifying the individual B-6 vitamers in animal tissues and blood plasma. The extraction and cleanup procedure of the anion-exchange method are rapid and suitable for partial automation; however, the lengthy analytical separation (~70 min/sample) is rate limiting. The reported precision of the anion-exchange method is slightly greater than that of the reverse-phase procedure. The fact that it employs a direct analysis for all vitamers, as opposed to the differential calculation of PM and PMP of the reverse-phase procedure, contributes to the greater precision. The use of an internal standard in the anion-exchange method is also advantageous. Several pyridine derivatives have been examined for use as internal standards in the reverse-phase procedure, although none has been found to be fully satisfactory. The reverse-phase semicarbazone procedure provides a favorable alternative to the anion-exchange method because of its greater rapidity for multiple analysis, less complex instrumentation, and comparative simplicity.

The reverse-phase semicarbazone method has been found to be compatible with pretreatment of sample extracts with manganese dioxide to oxidize PN and PNP to PL and PLP, respectively. This would permit the analysis of these vitamers using the semicarbazone derivatization procedure. Further studies are in progress to determine the effectiveness of determining PN and PNP by semicarbazone derivatization or direct fluorometric assay (Gregory, 1980a) for the determination of naturally occurring vitamin B-6 in foods such as cereal grains, fruits, and vegetables.

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Rapid High-Performance Liquid Chromatographic Determination of Ascorbic Acid and Combined Ascorbic Acid–Dehydroascorbic Acid in Beverages

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A simple HPLC method is described for the rapid estimation of ascorbic acid and dehydroascorbic acid, the physiologically active forms of vitamin C. Isocratic separation of ascorbic acid was accomplished in 6 min on a μBondapak NH_2 column using a 50:50 (v/v) methanol–0.25% KH_2PO_4 buffer (pH 3.5) solvent. Total ascorbic acid is determined by first reducing the dehydroascorbic acid to ascorbic acid by treatment with DL-homocysteine. This reaction is complete in 15 min and total ascorbic acid analysis is performed immediately. This HPLC procedure has been applied to the analysis of ascorbic acid in selected beverages.

Three classic methods have been developed for the estimation of both ascorbic acid (AA) and dehydroascorbic acid (DAA). Roe et al. (1948) determined DAA by condensation with 2,4-dinitrophenylhydrazine and total ascorbic acid (TAA) by oxidation of AA to DAA and subsequent osazone formation. The procedure of Tillmans et al. (1932) was based on the titration of AA with 2,6-dichloroindophenol. Reduction of DAA followed by titration permitted the estimation of TAA. Finally, DAA was treated with *o*-phenylenediamine which yielded an easily detected fluophor (Deutsch and Weeks, 1965). TAA could be determined after oxidation of AA. Each of these methods has been adapted for semiautomated continuous flow analysis of ascorbic acid in pharmaceutical and food products by Pelletier and Brassard (1975), Garry and Owen (1968), and Kirk and Ting (1975). However, these procedures are nonspecific for ascorbic acid, require the preparation and analysis of blanks, and are time consuming.

Several HPLC methods have been developed for the analysis of AA. These assays utilized various column packings and solvent compositions, and detection is based on the UV absorbance or the electrochemical properties of AA (Sood et al., 1976; Pachla and Kissinger, 1976). No HPLC method has been reported which allows the determination of the combined physiologically active forms of vitamin C, that is, ascorbic acid and dehydroascorbic acid. This paper reports an HPLC procedure for the rapid estimation of AA and TAA in beverages based on the observation of Hughes (1956), who showed that DL-homocysteine could rapidly and completely reduce DAA to AA.

METHODS AND MATERIALS

High-Performance Liquid Chromatography. Separation of ascorbic acid was achieved with a Waters Associates liquid chromatograph, ALC 202, equipped with a Model 6000A pump and a Model U6K injector. Column effluents were monitored at 244 nm, the UV absorbance maximum for AA in the solvent system described, with a Tracor Model 970A UV–vis variable-wavelength detector. A μBondapak NH_2 packed column, 4 mm i.d. × 30 cm (Waters Associates), efficiently separated AA isocratically by using a 50:50 (v/v) methanol–0.25% KH_2PO_4 buffer (pH 3.5) mobile phase. The flow rate was 0.8 mL/min. Sample injection volume was 20 μL.

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