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Note

Liquid chromatographic separation and quantitation of B_6 vitamers in selected rat tissues

J.A. PIEROTTI*, A.G. DICKINSON*, J.K. PALMER** and J.A. DRISKELL*,*

Departments of Human Nutrition and Foods, and Food Science and Technology**, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 (U.S.A.)*

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Vitamin B_6 is the collective term for the metabolically related 3-hydroxy,2-methyl pyridine compounds. Vitamin B_6 exists in three interconvertible forms: pyridoxine (PN, also known as pyridoxol), pyridoxal (PL), and pyridoxamine (PM), each of which has a corresponding 5'-phosphate. The physicochemical properties of the B_6 vitamers, in particular their ionogenic nature, facilitate their assay by high-performance liquid chromatography (HPLC). Vanderslice and co-workers [1—6] have reported separations and quantitations of the non-phosphorylated and phosphorylated B_6 vitamers in human plasma, animal tissues, urine, and selected foods. Their method utilized two anion-exchange columns and a spectrofluorometer which resulted in 1—2 h separations. Gregory and Kirk [7] have reported separation and quantitation of the non-phosphorylated B_6 vitamers in dehydrated food systems by reversed-phase HPLC using an octadecylsilane (ODS) column and an absorbance detector; the vitamers were eluted within 6 min. Gregory [8] used a similar HPLC system to determine PL, PN, and PM as well as pyridoxal 5'-phosphate (PLP) in non-fat dry milk and a fortified breakfast cereal by converting PLP to its semicarbazone derivative; a detection limit of less than 0.5 ng/g was reported. Gregory [9] has also compared the HPLC assay with the conventional microbiological method in analyzing the B_6 vitamer content of fortified breakfast cereal, and found the HPLC method to demonstrate higher accuracy and precision, to facilitate the analysis of larger numbers of samples, and to be a simple and sensitive method. Gregory et al. [10] have also reported quantitation of PL, PM, PLP, and PMP in a variety of animal tissues utilizing derivatization with semicarbazide, a reversed-phase ODS column, and fluorescence detection. The vitamers eluted within 12 min. Lim and co-workers [11, 12] have

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also developed an HPLC method with ultraviolet (UV) detection utilizing an ODS column which separated and quantitated the non-phosphorylated B_6 vitamers in about 11 min, and they applied the method to selected foods. Tryfiates and Sattsangi [13] have reported a reversed-phase ion-pair method for separating the non-phosphorylated and phosphorylated B_6 vitamer standards; the method employed UV detection and required about 40 min for separation of the vitamers.

This communication reports on the use of a reversed-phase ion-pair chromatographic method with fluorometric detection for the analysis of B_6 vitamers in rat liver, kidney, and brain tissues.

EXPERIMENTAL

Analytical instrumentation

A Waters Assoc. HPLC system (Milford, MA, U.S.A.) consisting of the following components was employed in this research: Model 730 data module, Model 720 system controller, two Model 45 solvent delivery systems, Model U6K universal injector, and Model 420-E/AC fluorescence detector equipped with a mercury lamp, 300-nm excitation filter and 375-nm emission filter. The analytical column was a μBondapak ODS column (30 cm × 3.9 mm I.D., 10-μm porous packing, C_{18}) preceded by a guard column (2 cm × 3.9 mm I.D.) packed with Bondapak ODS/Corasil (37—50 μm); both were obtained from Waters Assoc.

Reagents

Water and methanol, HPLC grade, were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.). Pyridoxal hydrochloride, pyridoxal 5′-phosphate, pyridoxamine dihydrochloride, pyridoxamine 5′-phosphate and pyridoxine hydrochloride were obtained from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5′-phosphate and 4-deoxypyridoxine hydrochloride were purchased from ICN Nutritional Biochemicals (Cleveland, OH, U.S.A.). The buffered ion-pairing reagent was purchased as PIC B-7 from Waters Assoc.

Mobile phase

A binary mobile phase was employed consisting of a mixture of methanol—water (850:150, v/v; solvent A) and PIC B-7 reagent (0.005 *M* heptane sulfonic acid in 1% acetic acid; solvent B). Prior to use, the mobile phase solvents were degassed by vacuum filtration through a 0.3-μm glass fiber filter (Gelman, Ann Arbor, MI, U.S.A.). The mobile phase was delivered at a rate of 1.5 ml/min at ambient temperature.

Separation and quantitation of standards

All handling of B_6 vitamers occurred under red lighting. Deoxypyridoxine (DPN) was selected as the internal standard. Following chromatography of the individual vitamers PL, PN, and PM as well as DPN, 250 μl of an aqueous combined standard solution (containing 625 ng of PL, 500 ng of PN, 250 ng of PM, and 1250 ng of DPN per ml) were injected onto the column. Satisfactory separation of the vitamers was accomplished in about 20 min (Fig.

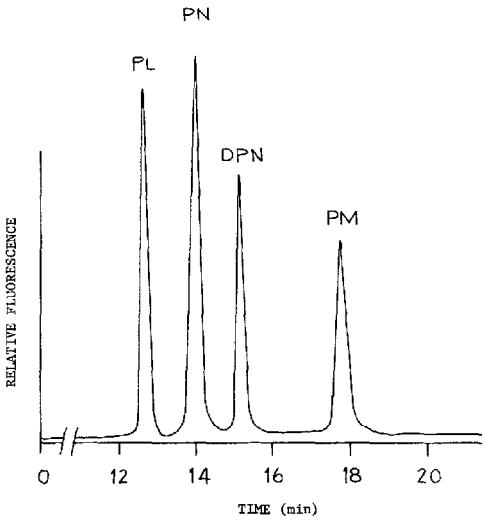

Fig. 1. Separation of B_6 standards by HPLC.

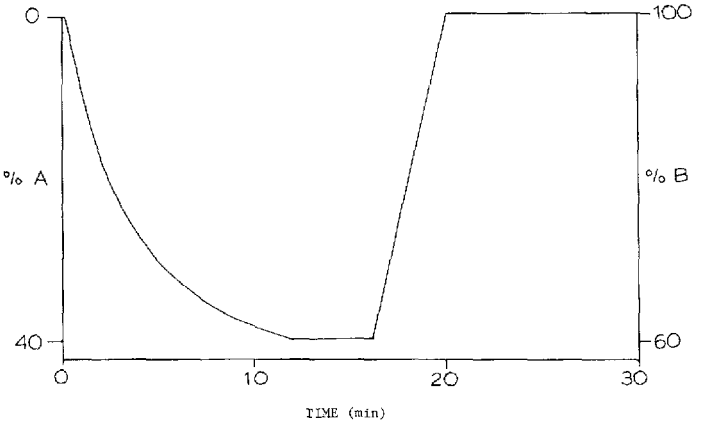

Fig. 2. Illustration of the gradient elution and column reequilibration program.

1) employing a gradient elution program (Fig. 2). Peak identity was confirmed by standard addition (spiking) as well as by extrachromatographic spectrofluorometry on collected HPLC eluates. Detection limits of 5 ng (200 ng/g tissue) for PN and PM and 10 ng (400 ng/g tissue) for PL were found with this method. The coefficient of variation of multiple injections of the same extract was found to be better than 5%. Peak areas, calculated by digital integration, were utilized for vitamer quantitation.

Extraction

B_6 extraction procedures were a modification of techniques reported by Gregory [14], Vanderslice et al. [3], and Thiele and Brin [15]. Samples and reagents were kept at 4° C in an ice—water—sodium chloride mixture until deproteinization was completed, in an effort to regard enzymatic activities. Approximately 1 g of liver, kidney, or brain (frozen, —20° C) was homogenized

with an aqueous solution of DPN (2.5 ml/g tissue) followed by immediate deproteinization with 8% perchloric acid (PCA; 2.5 ml/g tissue) and centrifugation at 12,000 *g* for 10 min at 4° C. An aliquot of the supernatant was adjusted to alkaline pH with 6 *M* potassium hydroxide to precipitate the PCA as insoluble potassium perchlorate; the pH was then adjusted to approximately 5.2 using 1 *M* hydrochloric acid. An equal volume of methylene chloride was added to remove non-polar impurities followed by vigorous shaking and centrifugation at 12,000 *g* for 10 min at 4° C. A 2-ml volume of the supernatant fluid was removed and combined with 2 ml of 0.055 *M* hydrochloric acid; the resulting solution was autoclaved for 5 h at 120° C and 1.04 bar to dephosphorylate the 5'-phosphates. After the solution had cooled to room temperature, the pH was adjusted to 5.2 with 6 *M* potassium hydroxide and dilute acetic acid and put through a 0.2-μm Gelman filter prior to injection into the HPLC system.

Animal treatment

Weanling Sprague-Dawley (Harlan-Sprague-Dawley, Madison, WI, U.S.A.) male albino rats were fed diets containing 15% protein and 7.2 mg of vitamin B_6 per kg of diet in the form of pyridoxine hydrochloride; these levels are considered to be nutritionally adequate [16]. After being on the experiment for almost four months, the animals were sacrificed by electrocution and the livers, kidneys, and brains excised immediately and frozen on dry ice. These organs were kept at −20° C until analyzed.

RESULTS AND DISCUSSION

A typical chromatogram of brain tissue extract is depicted in Fig. 3. Peaks obtained from brain extracts were adequate for quantitation. Satisfactory separation and quantitation of extracts from liver and kidney were also obtained. Peak identity was confirmed by comparison of standard retention times with sample retention times, use of relative retention time, spiking, and extrachromatographic spectrofluorometry of eluates.

Tissue homogenates (three of each tissue) were spiked with known quantities of PL, PN, and PM as well as their phosphorylated forms PLP, PNP, and PMP. B_6 vitamer recoveries were similar for all tissues, and were as follows: PL, 83%;

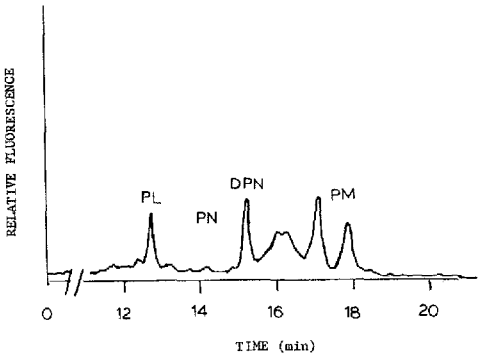

Fig. 3. Separation of B_6 vitamers in a representative brain extract by HPLC.

PN, 89%; PM 93%; PLP, 82%; PNP, 96%; PMP, 96%. The phosphorylated forms were recovered as non-phosphorylated vitamers; losses due to the dephosphorylation step were minimal. The loss of B_6 vitamers from inital homogenization of the tissues to HPLC separation and quantitation was considered acceptable and calculations were based on the recovery of the internal standard which ranged from 85 to 90%.

Vanderslice et al. [3] reported that the thawing, time of deproteinization, and buffer composition utilized with tissues during extraction affected the vitamer recoveries; these researchers observed some interconversions among the vitamers which may have been due to the activities of tissue oxidases. Enzyme-mediated interconversions during the extraction procedure used in the present study should have been minimal due to the low temperatures at which the samples and reagents were maintained throughout the procedure.

The B_6 vitamer concentrations of the three tissues are given in Table I. PM appeared to be the predominant B_6 vitamer in all three tissues with PL being the second most predominant. Vitamer data were in the range of values obtained by microbiological [10, 15, 17] and chromatographic [3, 10, 12, 18] techniques for these same tissues.

TABLE I

B_6 VITAMER CONCENTRATIONS OF RAT TISSUES

Values are given in μg/g and mean ± S.D. (S.D. refers to the range of values among individual rats and not precision). Phosphorylated forms of the vitamers are converted to their respective non-phosphorylated forms during extraction; values represent the sum of free and phosphorylated forms.

Tissue	No. of animals	PL	PN	PM	Total
Liver	10	4.25 ± 0.50	0.59 ± 0.21	6.91 ± 0.91	11.75 ± 1.02
Kidney	10	2.90 ± 1.34	0.62 ± 0.43	7.77 ± 1.74	11.29 ± 1.72
Brain	6	1.97 ± 0.01	nd*	2.45 ± 0.30	4.42 ± 0.30

*nd = not detectable.

The HPLC separation was rapid and the sensitivity adequate for determining physiological levels of the vitamers. Fluorometry provided not only high sensitivity but also selectivity, producing relatively clean chromatograms; spectrofluorometry was also useful in confirming sample vitamer peaks. The HPLC method offers potential for the quantitation of B_6 vitamers in a wide variety of matrices.

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