Journal of Chromatography, 374 (1986) 155—161
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2824

Note

Quantitation of B_6 vitamers in rat plasma by high-performance liquid chromatography*

THERESA E. HEFFERAN, BARBARA M. CHRISLEY and JUDY A. DRISKELL*

Department of Human Nutrition and Foods, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061 (U.S.A)

(First received March 22nd, 1985; revised manuscript received August 9th, 1985)

Vitamin B_6, a group of metabolically related 3-hydroxy-2-methylpyridine compounds, is required for the growth and development of normal tissues. This vitamin exists in three interconvertible forms, pyridoxine (PN, also known as pyridoxol), pyridoxal (PL), and pyridoxamine (PM), each of which has a corresponding 5′-phosphate (P). The physicochemical properties of these B_6 vitamers, particularly their ionic nature, facilitate their assay by high-performance liquid chromatographic (HPLC) methodologies. The B_6 vitamer content of animal and human tissues has been quantitated by HPLC techniques [1—8].

Thus, in view of the analytical capabilities of HPLC, the methods of Pierotti et al. [5] and Morrison and Driskell [7] were modified so that adequate sensitivity was obtained to detect and quantitate the B_6 vitamers in plasma from vitamin B_6 deficient and control rats. B_6 vitamer concentrations as determined by this reversed-phase ion-pair HPLC fluorometric method were compared to those obtained using microbiological assay.

EXPERIMENTAL

HPLC methodology

A Waters Assoc. (Milford, MA, U.S.A.) HPLC system equipped with a fluorescence detector (300 nm excitation, 375 nm emission) and a μBondapak

*Presented in part by B.M.C. at the Annual Meeting of the Federation of American Societies for Experimental Biology, St. Louis, MO, April 2, 1984 [Fed. Proc., Fed. Am. Soc. Exp. Biol., 43 (1984) 470 (Abstract)].

0378-4347/86/$3.50

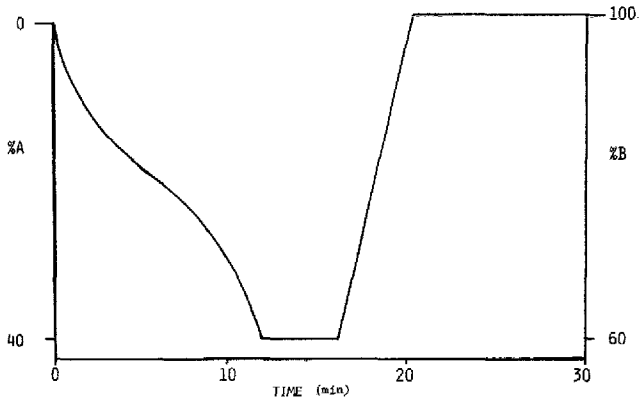

Fig. 1. Illustration of the gradient-elution and column-reequilibration program.

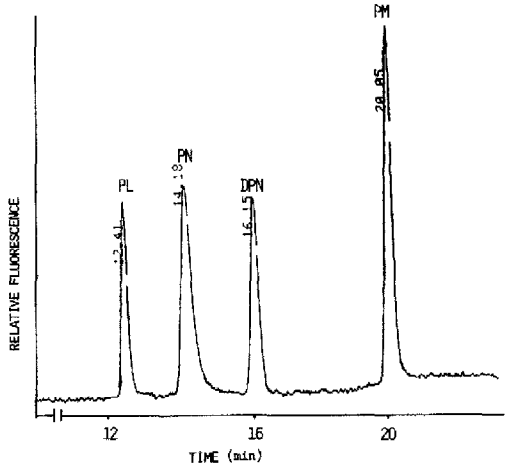

Fig. 2. Separation of B_6 vitamer standards by HPLC.

ODS column (30 cm × 3.9 mm I.D., 10 μm porous packing, C_{18}, Waters Assoc.) was utilized in this research. The vendors of reagents and instrumentation have been previously described [5]. A binary mobile phase, delivered at a flow-rate of 1.5 ml/min at ambient temperature, 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), was utilized as described previously [5]; the gradient elution and column reequilibration program was modified and is given in Fig. 1.

Deoxypyridoxine (DPN) was utilized as the internal standard. Following chromatography of the individual vitamers PL, PN and PM as well as DPN, a 250-μl aliquot of a combined standard solution containing 50 ng of each vitamer and 200 ng of DPN was injected into the column. Satisfactory separation of the vitamers was obtained in about 20 min (Fig. 2) using the gradient-elution program. Peak identity was confirmed by standard addition (spiking) as well as by extrachromatographic spectrofluorometry on collected HPLC eluates. Linear calibration curves for PL, PN, PM and DPN were obtained; minimum detectable quantities were 2 ng (about 8 ng/ml of plasma) for PL and 1 ng (about 4 ng/ml) for PN and PM.

Extraction of B_6 vitamers

The method of Morrison and Driskell [7] for extraction of B_6 vitamers from human milk was modified. DPN, 100 μl of a 2 mg/dl solution, was added to 2.5 ml plasma. Next, 0.5 ml of a 2 U/ml potato acid phosphatase (EC 3.1.3.2; orthophosphoric monoester phosphohydrolase; Sigma, St. Louis, MO, U.S.A.) in 0.2 *M* potassium acetate, pH 4.5, was added to the sample in order to hydrolyze the phosphate esters of the B_6 vitamers; samples were incubated for 1 h in a 37°C shaking water bath. The protein was precipitated by adding 0.2 ml of 100% trichloroacetic acid (TCA); the sample was vortex-mixed and incubated for 15 min in a 50°C water bath; TCA may also function in the hydrolysis of the phosphate esters. Cold methylene chloride (3 ml) was added and the sample shaken vigorously. Samples were centrifuged at 7000 *g* at 5°C for 20 min; resultant supernatants were adjusted to pH 5.5 with 33% sodium hydroxide and put through a 0.2-μm Gelman (Ann Arbor, MI, U.S.A.) acrodisc filter prior to injection into the HPLC system. A typical chromatogram of plasma extract is depicted in Fig. 3. Peak identity was confirmed by comparison of standard retention times with sample retention times, spiking, and extrachromatographic spectrofluorometry of HPLC eluates. Vitamer recoveries were determined by spiking the samples before extraction; the recoveries were as follows: PL, 98%; PN, 95%; PM, 95%; PLP (dephosphorylated to PL), 85%; PNP (dephosphorylated to PN), 95%; PMP (dephosphorylated to PM), 97%. Hence, vitamer values represent the sum of both the phosphorylated and unphosphorylated forms of the vitamer. The data were not corrected for percent recoveries. The coefficients of variation for B_6 vitamer concentrations of plasma samples that were extracted and analyzed on different days were around 5%.

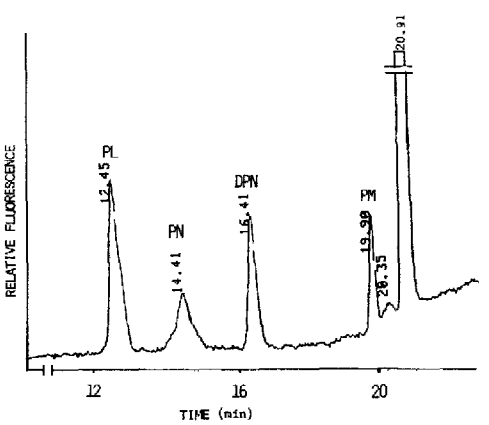

Fig. 3. Separation of B_6 vitamers in a representative plasma extract by HPLC.

Microbiological assay

The B_6 vitamer content of the plasma extract was determined by microbiological assay by a modification [7] of the AOAC procedure [9] using *Saccharomyces uvarum* (ATCC 9080, American Type Culture Co., Rockville, MD, U.S.A.). In order to check vitamer recoveries, plasma samples were spiked with each vitamer prior to extraction. The recoveries were as follows: PL, 94%;

PN, 95%; PM, 88%; PLP (dephosphorylated to PL), 89%; PNP (dephosphorylated to PN), 87%; PMP (dephosphorylated to PM), 88%. The data were not corrected for percent recoveries. The coefficients of variation of B_6 vitamer concentrations of plasma samples that were extracted and analyzed on different days were around 5%.

Animal treatment

The following preliminary experiment was performed in order to determine if differences in plasma B_6 vitamer levels of rats fed nutritionally adequate diets were observed when the animals were sacrificed in a fasting or non-fasting state. Weanling male albino rats (Harlan—Sprague—Dawley, Madison, WI, U.S.A.) were fed a nutritionally adequate diet containing 15% protein and 7.2 mg pyridoxine hydrochloride per kg diet for twelve weeks. Three rats (mean body weight 374 g) were fasted for 12 h and three animals (366 g) were not fasted before being electrocuted; blood was obtained via cardiac puncture; EDTA was utilized as the anticoagulant. The blood was kept in ice and in the dark until centrifugation at 3000 *g* at 5°C for 10 min. B_6 vitamer concentrations of the plasma samples were determined using HPLC and microbiological techniques. Mean plasma B_6 vitamer concentrations of the non-fasted rats (PL, 381.8 ng/ml; PN, 22.2 ng/ml; PM, 13.3 ng/ml) were similar to those which were fasted (PL, 364.0 ng/ml; PN, 20.1 ng/ml; PM, 14.5 ng/ml). The rats obtained their vitamin B_6 from the diet at a level near requirement recommendations [10—12] rather than from a supplement several times these recommendations.

Weanling male albino rats were randomly divided into two groups: a control group of fifteen rats and a deficient one of ten rats. The control animals were fed a nutritionally adequate diet containing 15% casein and 7.2 mg pyridoxine hydrochloride per kg diet. The deficient animals were fed the same diet except that it contained 0.0975 mg pyridoxine hydrochloride per kg diet; in that the current researchers had not yet perfected the HPLC method and the animals had begun losing weight, the rats in the deficient group received 20 g of the diet fed the control animals at weeks 5, 10 and 18. The animals had deficient or adequate (control) vitamin B_6 status when they were sacrificed after being on the diets for 27 weeks. The body weights of the ten rats in the deficient group (324 ± 20 g, mean ± S.D.) were significantly lower ($p < 0.001$) from those of the fifteen control animals (502 ± 37 g). The rats (non-fasted) were electrocuted and blood procured and plasma obtained as described above. Plasma from one rat or that pooled from two rats (equal quantities) represented a sample. B_6 vitamers were extracted from an aliquot of plasma from each sample within 2 h after death; the extract was utilized for immediate HPLC analysis and microbiological determinations of B_6 vitamer content. An aliquot of plasma from each sample was also frozen at −20°C for 55 weeks at which time B_6 vitamer content was determined by HPLC assay.

Statistical analysis

Analysis of variance procedures [13] were utilized to compare data obtained from the two groups of animals as well as that from the assay procedures. Means and standard deviations were also calculated. Pearson *r* correlation determined between data obtained for the various assay

RESULTS AND DISCUSSION

The B_6 vitamer concentrations of plasma for the deficient and control rats are given in Table I; similar PL, PN, PM and total B_6 vitamer values were obtained by HPLC as by microbiological assays. Phosphorylated vitamers were dephosphorylated and quantitated in their respective unphosphorylated forms. The following significant Pearson r correlation coefficients were observed between vitamer values obtained by HPLC and microbiological procedures: PL, $r = 0.996$, $p < 0.0001$; PN, $r = 0.74$, $p < 0.003$; PM, $r = 0.90$, $p < 0.001$; total B_6 vitamers, $r = 0.997$, $p < 0.0001$; the same plasma extracts were utilized for both assay procedures. Other researchers [6, 7, 14] have reported finding satisfactory agreement between total B_6 vitamer concentrations obtained by HPLC and microbiological assays.

TABLE I

B_6 VITAMER CONCENTRATIONS OF RAT PLASMA

Individual vitameric values represent the sum of both forms of the vitamer, the unphosphorylated and the phosphorylated.

Group	n*	Assay method	Concentration (mean ± S D..) (ng/ml)			
			PL	PN	PM	Total B_6 vitamers
Deficient	5	HPLC	10.5 ± 1.8**	10.1 ± 2.1***	68.0 ± 8.9	88.6 ± 8.8**
		Microbiological	11.8 ± 1.5**	9.9 ± 3.1***	69.1 ± 7.7	90.8 ± 6.5**
Control	9	HPLC	368.9 ± 46.3	20.1 ± 6.8	63.1 ± 27.2	452.1 ± 61.5
		Microbiological	362.2 ± 49.5	22.8 ± 5.2	69.3 ± 19.1	454.2 ± 55.0

*Plasma from one rat or pooled (equal quantities) from two rats represented a sample.
**Significantly different from values of control group at $p < 0.0001$.
***Significantly different from values of control group at $p < 0.01$.

The B_6 vitamer concentrations of plasma from deficient and control rats were statistically different from each other as indicated in Table I. The same p values were observed for HPLC-derived data as for the microbiological data. Plasma from control rats had significantly higher ($p < 0.0001$) sum PL (PL + PLP) values than that of deficient. The sum PN values were significantly higher ($p < 0.01$) for the control animals than the deficient. The sum PM concentrations of plasma from the two groups of rats were similar. The total plasma B_6 vitamer concentrations of control animals were significantly higher ($p < 0.0001$) than those of deficients. Morrison and Driskell [7] found that milk from women having adequate vitamin B_6 status had significantly greater quantities of PL, PM and total B_6 vitamers than samples from women classed as having inadequate vitamin B_6 status.

The PL, PN, PM and total B_6 concentrations of plasma samples as measured by HPLC techniques were similar for freshly obtained plasma and plasma from the same rats that had been frozen for 55 weeks. Freezing of the plasma samples did not seem to affect their vitamin B_6 content.

The B_6 vitamer distribution given by percent (mean) is depicted in Fig. 4; since no differences in concentrations of any of the vitamers were observed when measured by the two assay techniques, values given are means between the values obtained by both assay methods. The percentage of sum PL was

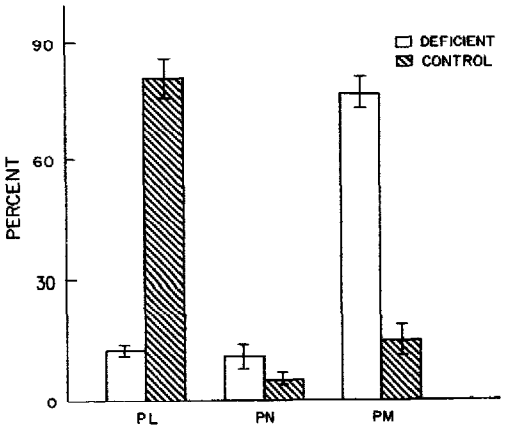

Fig. 4. Distribution of B_6 vitamers in plasma extracts from vitamin B_6 deficient and control rats. Values represent mean ± S.D.

significantly higher ($p < 0.0001$) and that of sum PM and sum PN significantly lower ($p < 0.0001$, $p < 0.001$) in plasma from control rats as compared to those deficient in the vitamin. The predominant B_6 vitamer in plasma from vitamin B_6 deficient rats was sum PM and that in plasma from control animals was sum PL. Lyon et al. [15] found that unphosphorylated derivatives constitute less than 10% of the total vitamin B_6 content of mammalian tissues; hence, one could assume that the predominant vitamers were actually PMP and PLP, respectively. Reynolds [16] reported that PLP was the predominant vitamer in plasma from adult men supplemented with vitamin B_6. Lui et al. [8], using HPLC techniques, found that PLP constituted a mean of 77.7% of the sum PL (PL + PLP) in plasma from nine healthy adults. Coburn et al. [6] found that PLP constituted a mean of from 63 to 95% of the sum PL (PL + PLP) in plasma from pigs, horses, calves, goats, sheep, dogs and cats as measured utilizing HPLC methods although there sometimes were some interfering peaks near those of PLP. The predominance of sum PM may be a way in which rats on severely restricted intakes of vitamin B_6 conserve the body's pool of this vitamin since PM can be oxidized to PL via PMP oxidase (EC 1.4.3.5; pyridoxamine phosphate:oxygen oxidase) and PL not PM is oxidized to the metabolite pyridoxic acid [17]. Perhaps PMP may also be the predominant B_6 vitamer in plasma from humans who are deficient in the vitamin.

The vitamer values for one of the deficient rats were not included in the statistical analyses in that the plasma sum PL value was found to be three times higher than the sum PL concentrations for other animals in the deficient group. The electrocution unit malfunctioned and the animal was exposed to a stressful situation. Cheney et al. [18] demonstrated that the injection of cortisone, an adrenal hormone, increased erythrocyte alanine aminotransferase (EC 2.6.1.2; L-alanine:2-oxoglutarate aminotransferase) activities. The stress incurred by the vitamin B_6 deficient rat in the current study may have induced synthesis of adrenal hormones such as cortisone, epinephrine and norepinephrine. PLP is needed for the synthesis of epinephrine and norepinephrine; hence, plasma PL concentration may have increased.

Plasma B_6 vitamer concentrations and distributions were found to be signifi-

cantly different in vitamin B_6 deficient and control rats. This HPLC methodology has potential for use in the assessment of vitamin B_6 status in humans.

ACKNOWLEDGEMENT

This research was supported in part by USDA/CRGO 59-2511-0-1-482-0.

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