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PLASMA B_6 VITAMER AND 4-PYRIDOXIC ACID CONCENTRATIONS OF MEN FED CONTROLLED DIETS

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SUMMARY

A rapid and sensitive procedure is described for the analysis of all the B_6 vitamers and 4-pyridoxic acid in human plasma utilizing reversed-phase high-performance liquid chromatography with ultraviolet and fluorometric detection. Pyridoxal phosphate values obtained by radiometric and chromatographic, ultraviolet and fluorometric, assays were highly correlated as were pyridoxine phosphate values determined using both detectors. Plasma B_6 vitamer and 4-pyridoxic acid concentrations of 22 men fed diets containing 0.75–0.98 mg of vitamin B_6 daily for eight weeks were in the range of reported values; pyridoxal phosphate was their predominant plasma B_6 vitamer. This methodology should be useful in the assessment of vitamin B_6 requirements.

INTRODUCTION

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 (P). 4-Pyridoxic acid (4-PA) is the major excretory catabolite. Microbiological assays, enzymatic assays and open-column cation-exchange (OCC) chromatography are some of the methods developed for the analysis of vitamin B_6 in human plasma. However, a recent inter-laboratory

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comparison study revealed large differences in the concentrations of B_6 vitamers and 4-PA measured by these techniques in a pooled plasma sample [1]. The physicochemical properties of the B_6 vitamers facilitate their assay by high-performance liquid chromatographic (HPLC) methodologies. The B_6 vitamer content of plasma from humans has been quantitated by HPLC techniques [2–10]. It would be quite advantageous to be able to detect nanogram quantities of each metabolite, thus providing a better understanding of the metabolism of vitamin B_6. Coburn and Mahuren [6] utilized a cation-exchange HPLC procedure for detecting nanogram quantities of all seven common vitamin B_6 metabolites in a variety of biological samples. Lui et al. [7] compared the cation-exchange and the OCC HPLC methods and the L-tyrosine apodecarboxylase (LTD; L-tyrosine carboxy-lyase; EC 4.1.1.25) assay in which case the coefficients of correlations for two of the three methods measuring plasma PLP were greater than 0.93 and between the two chromatographic methods quantifying PL and 4-PA, 0.82 and 0.63, respectively. In the present study a reversed-phase HPLC method utilizing UV and fluorometric detectors has been developed to detect nanogram quantities of the phosphorylated and non-phosphorylated forms of vitamin B_6 and its excretory catabolite 4-PA in plasma from young men having radiomonitored plasma PLP levels indicative of adequate vitamin B_6 status.

EXPERIMENTAL

Analytical instrumentation

The chromatographic separation was accomplished using a Waters Assoc. (Milford, MA, U.S.A.) HPLC system which consisted of the following components: a Model 730 data module, a Model 720 system controller, two Model 45 solvent delivery systems, a Model U6K universal injector, a column temperature control system, a Model 440 UV absorbance (254 nm) and a Model 420 E/AC fluorescence detector equipped with a mercury lamp (300-nm excitation and 375-nm emission filters). The analytical column was a μBondapak octadecylsilane (ODS) column (30 cm×3.9 mm I.D., 10 μm porous packing, Waters Assoc.) preceded by a guard column packed with μBondapak ODS.

TABLE I

GRADIENT-ELUTION CONDITIONS

Time (min)	Solvent A* (%)	Solvent B* (%)	Curve type**
0	0	100	Convex
5	25	75	Convex
8	75	25	Concave
12	40	60	Linear
16	0	100	Linear
20	0	100	

*Composition given in text.

**Ref. 11.

Mobile phase

The mobile phase for gradient elution consisted of methanol–water (85:15, v/v; solvent A) and a combination of the two paired-ion reagents PIC B-7 and B-8 (0.005 *M* heptane sulfonic acid and octane sulfonic acid in 1% acetic acid; solvent B). The flow-rate was 1.0 ml/min at ambient temperature. The mobile phase solvents were degassed by vacuum filtration through a 0.3-mm glass fiber filter (Gelman Science, Ann Arbor, MI, U.S.A.). The programmed gradient-elution conditions are given in Table I.

Reagents

The following reagents were obtained from Sigma (St. Louis, MO, U.S.A.): pyridoxal hydrochloride, pyridoxal 5′-phosphate, pyridoxamine dihydrochloride, pyridoxamine 5′-phosphate, pyridoxamine hydrochloride, 4-pyridoxic acid, 4-deoxypyridoxine (DPN) and potato acid phosphatase. Pyridoxine 5′-phosphate was synthesized according to the method of Peterson and Sober [12]. The identity of PNP was confirmed by nuclear magnetic resonance (NMR) as well as by HPLC.

Resolution of standards

After resolution of the individual standards (PLP, PNP, PMP, PL, PN, PM, 4-PA and the internal standard, DPN), a 100-μl aliquot of combined standards (containing 5–200 ng/ml) was injected onto the column which was maintained at 37°C. Peak identities were confirmed by retention times, comparison of the ratio of retention times of the vitamers to that of the internal standard, by spiking with standards, as well as off-line chromatographic spectrofluorometry on collected HPLC eluates. Using the gradient elution program, these standards were satisfactorily separated in 25–35 min; a representative chromatogram (standards) is given in Fig. 1. Minimum detectable quantities were 2–5 ng; calibration curves were linear to 50 ng. Theoretical plates (N) were approximately 3000, even after column use of over a year; whereas the α values ranged from 1.1 to 2.6. Likewise, the K values were 0.2, 0.3, 0.8, 1.0, 3.0, 3.0 and 4.0.

Blood sampling

Approximately 15 ml of blood was obtained between 7:00 and 8:30 a.m. in vacutainers containing EDTA from each fasting subject by a Registered Medical Technologist. The samples were kept in crushed ice and protected from light. Blood samples were centrifuged in a refrigerated rotor at 3000 *g* and 5°C for 10 min. Plasma samples were then frozen at −20°C.

Extraction of B_6 vitamers and 4-PA from human plasma

DPN (5 ng) was added to 2 ml of human plasma (fasting) along with 0.2 ml of 50% trichloroacetic acid; the mixture was incubated at 50°C for 15 min and cooled to 5°C. An equal volume of methylene chloride was added to the mixture followed by centrifugation at 7000 *g* for 20 min at 5°C. The supernatant was removed and an equal volume of freon amine was added, followed by recentrifugation, removal of the supernatant and freon amine treatment again, with recentrifugation. The

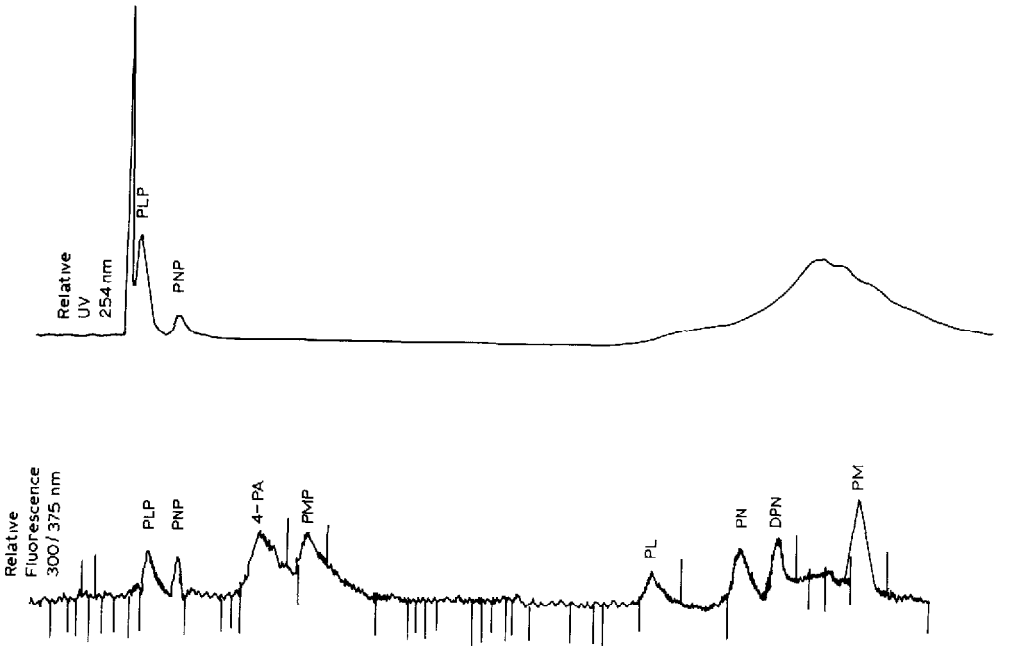

Fig. 1. Representative chromatogram of B_6 vitamers and 4-PA in combined standard solution containing 5 ng of each. The retention times were as follows: 1 0, 1.6, 4.2, 5.4, 14.0, 16.4, 17 3 and 19.4 min.

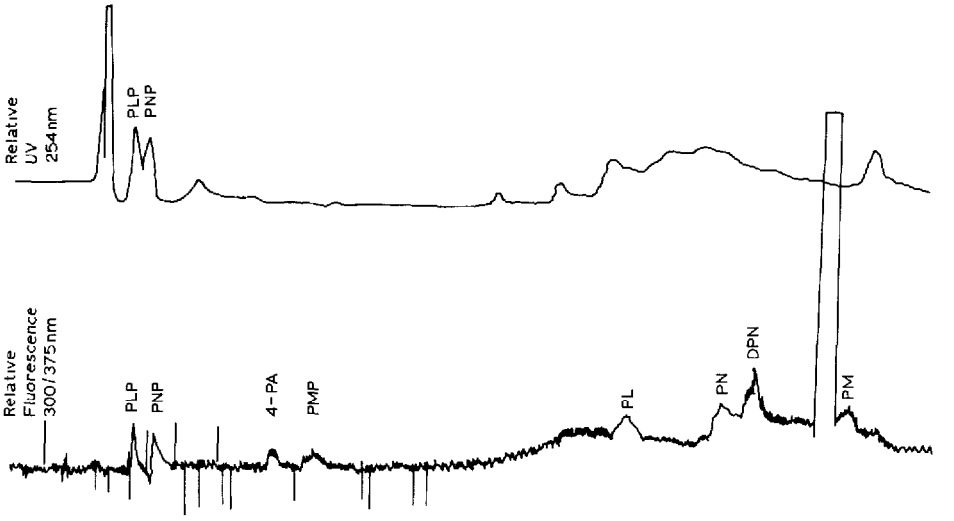

Fig. 2. Representative chromatogram of B_6 vitamers and 4-PA in human plasma. The retention times were as follows 1.0, 1.6, 4.4, 5.4, 13.8, 16.2, 17.2 and 19.5 min.

supernatant (at 50°C) was dried using nitrogen followed by reconstitution with 1 ml of solvent B; the sample was then adjusted to pH 2.9 and filtered through a 0.2-μm Acrodisc (Gelman) and then through a C_{18} Sep-Pak (Waters Assoc.). The sample filtrate (100–250 μl) was then injected into the HPLC system. A representative chromatogram of the B_6 vitamers and 4-PA in a human plasma

TABLE II

RECOVERY OF B_6 VITAMERS FROM PLASMA

Plasma was spiked with 10 ng of each vitamer per ml plasma ($n=3$).

Vitamer	Recovery (%)
PLP	95.2
PNP	97.1
4-PA	94.9
PMP	88.0
PL	94.6
PN	93 5
PM	90.6

extract is depicted in Fig. 2. Peak identities were confirmed as described for the B_6 standards.

Plasma samples were spiked before extraction with known quantities of each of the B_6 vitamers and 4-PA. The recoveries of the vitamers (Table II) ranged from 88 to 97%. The data were not corrected for percent recoveries. The coefficients of variation for B_6 vitamers and 4-PA concentrations of plasma samples that were extracted and analyzed on different days were around 5%. The plasma samples were also treated with potato acid phosphatase (orthophosphoric-monoester phosphohydrolase; EC 3.1.3.2) to convert the phosphorylated forms to the non-phosphorylated forms of the vitamin; 94–99% of the phosphorylated forms were recovered in this conversion as non-phosphorylated B_6 vitamers. B_6 vitamer and 4-PA concentrations of plasma from freshly drawn blood and plasma which had been frozen for as long as twenty months were compared using samples from three females. The concentrations of all B_6 vitamers and 4-PA in the frozen samples were within 5% of the values found in freshly drawn samples from these subjects. Thus, freezing does not appear to affect the separation and quantitation of the B_6 vitamers and 4-PA in human plasma.

B_6 Vitamer and 4-PA analyses of plasma from subjects

The subjects included adult males who were white, black or of other races (Ecuador, Sri Lanka, China, Philippines, Taiwan, South Korea and Bangladesh). The 22 subjects, 20–37 years of age, were in good health, non-smokers and within normal weight ranges. The subjects were on controlled diets for eight weeks at which time blood was collected; plasma was frozen at $-20\,^{\circ}C$ for future analyses. The mean daily B_6 content of these diets as analyzed using *Saccharomyces uvarum* was 0.75–0.98 mg; the mean protein content as analyzed by the Kjeldahl method were 80.8–84.5 g. Plasma PLP concentrations of the subjects were measured radioenzymatically. Subjects, diets and the radioenzymatic assay have been described previously in detail [13]. The B_6 vitamer and 4-PA levels present in plasma from these subjects were quantitated using the developed HPLC methodology.

Statistical analysis

All data were evaluated by analysis of variance and Duncan's multiple range test [14]. The general linear model procedure was utilized to evaluate race–dietary group interaction. Means and standard errors of the mean (S.E.M.) were calculated. Pearson r correlation coefficients were determined between data obtained by the various assay parameters.

RESULTS AND DISCUSSION

Twelve whites, three blacks and seven men of other races volunteered earlier as subjects. The ages, heights and weights of the subjects were statistically similar [13]. None of the variables were affected by race.

Plasma PLP measurements

Plasma PLP levels of the subjects as determined using radiometric, LTD [13], as well as HPLC, UV and fluorometric, methods are listed in Table III. The plasma PLP values of the subjects were in the range of HPLC-derived values reported by other researchers [2,4–6,10]. Values for plasma PLP concentrations obtained by LTD and HPLC, both UV and fluorometric, analyses were highly correlated ($r=0.80$, $P<0.001$; $r=0.87$, $P<0.0001$, respectively); high correlation ($r=0.86$, $P<0.0001$) was also observed between values obtained using the two HPLC detectors. Thus, similar data were obtained utilizing all three of these techniques.

The plasma PLP concentrations of all 22 of the subjects who consumed diets containing 0.75–0.98 mg (mean) of vitamin B_6 daily for eight weeks were well above levels suggested as being indicative of vitamin B_6 inadequacy [15–17]. Plasma PLP levels of approximately 75 nmol/l have been reported for humans consuming regular diets [18]. Plasma PLP values (mean ± S.D.) for 35 healthy

TABLE III

B_6 VITAMER AND 4-PA CONCENTRATIONS OF HUMAN PLASMA

Congener	Assay*	Number of subjects with detectable levels	Concentration** (mean ± S.E.M.) (nmol/l)
PLP	LTD	22	90.3 ± 3.5
	H-FL	22	88.0 ± 3.9
	H-UV	22	89.2 ± 3.8
PNP	H-FL	12	6.4 ± 1.6
	H-UV	9	6.1 ± 1.6
PMP	H-FL	14	12.2 ± 5.7
PL	H-FL	22	38.4 ± 5.0
PN	H-FL	22	41.4 ± 7.5
PM	H-FL	22	17 6 ± 2.0
4-PA	H-FL	16	39.3 ± 11.4

*LTD = radioisotopic tyrosine decarboxylase, H-FL = HPLC using fluorometric detection; H-UV = HPLC using UV detection.

**Non-detectable levels calculated as zeros

men who did not take supplements containing vitamin B_6 were 51.9 ± 19.3 nmol/l [15]. Steady-state plasma PLP levels reportedly are reached within three to four weeks following alteration of the dietary vitamin B_6 intake [19]. Hence, the subjects in the current study had been on the diets long enough to have steady-state plasma PLP levels; these levels, indicative of adequate vitamin B_6 status, were attained when the men had consumed diets analyzed to contain 0.75–0.98 mg (mean) of the vitamin daily, an amount less than the 1980 Recommended Dietary Allowance (R.D.A.) [20] of 2.2 mg for men.

Plasma PNP, PMP, PL, PN, PM and 4-PA measurements

Plasma B_6 vitamer and 4-PA levels of the subjects are given in Table III. A high correlation ($r=0.91, P<0.0001$) was observed between the subjects' plasma PNP concentrations as measured utilizing the two HPLC techniques. Twelve of the subjects in the current study had detectable plasma levels of PNP utilizing the HPLC–fluorometric method and nine of these twelve had detectable levels utilizing the HPLC–UV technique. To our knowledge, others have not reported detecting PNP concentrations in plasma from any of their subjects.

Plasma PMP values of the subjects in the current study were similar to those reported by Chauhan and Dakshinamurti [4] but were also in the range of values reported by others [5,6]. Mean plasma PL values were higher than reported values [2,6,7,10] but values were in the range of those of Coburn and Mahuren [6]. Plasma PN values were in the range of values reported by Coburn and Mahuren [6] and Lumeng et al. [2] although mean values were higher than their reported mean concentrations. Plasma PM values were in the range of those reported by Lumeng et al. [2]. Plasma 4-PA values of the subjects were similar to reported values [2,6,7]; two of the research groups [4,5] did not determine 4-PA concentrations. Overall, the B_6 vitamer and 4-PA values of subjects in this study were rather similar to those reported by others. However, it should be emphasized that the subjects of all the researchers including ourselves consumed varying quantities of the vitamin and each research group has a somewhat different HPLC method/system.

Significant correlation ($r=0.62$. $P<0.005$) was observed between plasma PN and 4-PA levels of the subjects as well as between plasma PN and PMP concentrations ($r=0.78, P<0.0001$). The B_6 vitamers but not 4-PA are easily interconvertible; however, 4-PA cannot be converted back to any of the B_6 vitamers [21].

B_6 Vitamer distribution

The plasma B_6 vitamer distribution of subjects in this study as quantitated by the HPLC–fluorometric method is depicted in Table IV; S.E.M. were quite large for all except PLP. Over half of the B_6 vitamers in the subjects' plasma was PLP. Other [1,2,6,10] but not all [4,5] researchers have reported PLP to be the predominant vitamer in plasma from seemingly healthy adults some of whom took supplements containing the vitamin. The men in the current study had practically equal plasma distributions of PL and PN; Coburn and Mahuren [6] also reported finding nearly equal distributions of PL and PN in plasma from their subjects. PNP was the B_6 vitamer with the lowest percentage distribution in plasma from men in the current study.

TABLE IV

B_6 VITAMER DISTRIBUTION IN HUMAN PLASMA

Vitamer	Distribution (mean ± S.E.M.) (%)
PLP	54.1 ± 2.1
PNP	3.4 ± 0.8
PMP	5.0 ± 1.5
PL	15.2 ± 1.8
PN	15.1 ± 1.7
PM	7.9 ± 1.0

This HPLC methodology provides a rapid, reproducible and sensitive procedure for determining plasma B_6 vitamer and 4-PA concentrations in humans. The methodology has potential for use in the assessment of vitamin B_6 status in humans.

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