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ANALYSIS OF B_6 VITAMERS IN PLASMA BY REVERSED-PHASE COLUMN LIQUID CHROMATOGRAPHY

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SUMMARY

The seven vitameric forms of B_6 can be measured in biologic fluids by high-performance liquid chromatography. Use of a reversed-phase column, with optimized solvent and buffer conditions, combined with fluorometric detection, allowed linear detection of vitamers from 0.4 to 20 ng. All vitamers from plasma, urine or tissue extracts can be analyzed within 50 min. Reproducibility and recovery studies indicate a selective and sensitive procedure, which greatly enhances studies on vitamin B_6 metabolism.

INTRODUCTION

Vitamin B_6 undergoes several interconversions in the body and, as a result, can occur in as many as six vitameric forms in addition to its dead-end catabolite. Any comprehensive study involving the metabolism of vitamin B_6 requires the analysis of each of these forms. Such analyses, however, are hampered by the complexity of the biological matric and the low level at which the vitamers occur. Several high-performance liquid chromatographic (HPLC) procedures have been described which are capable of separating the standard vitamers and, to a limited extent, of analysing the vitamers from natural sources [1—6]. However, most suffer from inadequate resolution, lack of sensitivity of the complexity of the procedure.

This report describes a procedure developed in our laboratory for the study of the metabolism of vitamin B_6 in cirrhosis. It is based on the previously reported HPLC procedure of Tryfiates and Sattsangi [1]. Several modifications were incorporated into the procedure which, combined with its short analysis time and simplicity, make the present methodology attractive in its

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application to plasma, urine and tissue extracts. In addition, the new procedure features fluorescence detection, providing the necessary increased sensitivity.

EXPERIMENTAL

Materials

Pyridoxal 5′-phosphate (PLP), pyridoxamine 5′-phosphate (PMP), pyridoxic acid (PIC), pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM) and alkaline phosphatase were purchased from Sigma (St. Louis, MO, U.S.A.). Pyridoxine 5′-phosphate (PNP) was a gift from Dr. Alfred Merrill*. Acetonitrile, 4-ml amber sample vials, 1-octanesulfonate and 0.45-μm membrane filters were purchased from Scientific Products (Atlanta, GA, U.S.A.). Deionized, distilled water was prepared in our laboratory. All solvents were vacuum filtered.

Apparatus

The chromatographic system consisted of a Waters Model 840 high-pressure liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.) equipped with two Model 510 high-pressure pumps, a WISP® sample injector, two Autochrom solvent selectors, a Farrand System 3 spectrofluorimeter (Farrand Optical, Valhalla, NY, U.S.A.) equipped with a 10-μl flow cell and a 150-W xenon lamp and a Gilson Model 302 pump (Gilson Medical Electronics, Middleton, WI, U.S.A.) for post-column solvent delivery. Chromatography was carried out on a Waters μBondapak C_{18} reversed-phase column, 30 × 0.4 cm I.D., 10 μm average particle size. The spectrofluorimeter was set at an excitation wavelength of 330 nm and an emission wavelength of 400 nm. The bandwidths of the excitation and emission monochromators were set at 20 nm. Sensitivity range was 1000×.

Mobile phase

Solvent A was 4 m*M* 1-octanesulfonate in 0.09% acetic acid, solvent B was 20% acetonitrile in 0.09% acetic acid and solvent C was 0.5 *M* sodium phosphate buffer (pH 7.5) to which 1 mg/ml of sodium bisulphite was added just prior to use. A linear gradient from 100% solvent A to 50% solvent A and 50% solvent B is generated over 10 min. A further linear increase from 50% solvent A and 50% solvent B to 30% solvent A and 70% solvent B was carried out for another 10 min, followed by isocratic elution at this final concentration for an additional 10 min. The flow-rate was 1 ml/min. Solvent C, the post-column reagent, was introduced to the column effluent through a mixing tee at a flow-rate of 0.1 ml/min. Samples were dissolved or diluted in solvent A. A 10-min reverse gradient followed by a 10-min equilibration with solvent A was performed between injections.

Standards

PLP, PIC, PMP, PN, PL, PNP and PM were dissolved at a stock concentra-

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*Assistant Professor of Biochemistry, Emory University, Atlanta, GA, U.S.A. The material eluted as a single peak on HPLC and was quantitatively converted to pyridoxine by alkaline phosphatase.

tion of 1 mg/ml in water. A working reference solution containing all seven standards at 5 μg/ml in water was stored in 200-μl aliquots at −80°C in amber vials. On the day of analysis, an aliquot was removed and diluted to 40 ng/ml in solvent A. The vitamers were quantified using peak-area quantitation.

Sample work-up

All procedures were carried out in a darkened room under a hood equipped with yellow fluorescent lights. Blood was collected in ethylenediaminetetraacetate (EDTA) containing vacutainer tubes. Plasma was separated from cells after centrifugation at 1000 *g* for 15 min at 4°C. A 1-ml aliquot of plasma was mixed with 1 ml of 20% trichloroacetic acid (TCA) under vigorous vortexing, followed by centrifugation at 1000 *g* for 15 min. The supernatant was syringe-filtered through 0.45-μm membranes into a 15-ml glass-stoppered conical tube, and 10 ml of water-saturated diethyl ether were added. The tube was mixed for 5 min on a rocker shaker, after which the ether was removed and fresh ether added. The procedure was repeated for a total of three extractions. The aqueous phase was then transferred to a 4-ml amber sample vial and dried under nitrogen at 45°C. The dried extracts were reconstituted in 2.5 ml of solvent A and 500 μl (equivalent to 0.2 ml of plasma) were injected. Urines were analysed directly after a 1:100 dilution in solvent A followed by filtration. Both blood and urine samples were collected on ice and if not immediately analysed were stored at −80°C.

Conversion of PLP, PNP and PMP to PL, PN and PM

A 0.5-ml aliquot of 0.05 *M* phosphate buffer (pH 7.5) was added to the dried extract followed by 40 U of alkaline phosphatase and incubated at 37°C for 1 h. A 2-ml aliquot of solvent A containing 0.6% acetic acid was added and the sample was mixed and analysed.

Recovery experiment

Blood was collected from a normal volunteer. After centrifugation, the plasma was pooled and divided into twelve 1-ml aliquots. Six of the aliquots were spiked with the standard mixture and all samples were extracted as given in the text. The samples were divided into two sets consisting of three unspiked and three spiked samples. One set was analysed immediately and the other analysed after two weeks.

RESULTS AND DISCUSSION

A typical chromatogram of a standard mixture of the vitamers is given in Fig. 1. The order of elution is similar to that which has been shown using cation-exchange chromatography [2, 7, 8]. The use of 1-heptanesulfonate [1] did not improve retention or resolution over the use of 1-octanesulfonate alone, and therefore was not used in this method. The range of linearity is 0.4—20 ng for PLP, PL, PN and PMP, and 0.1—20 ng for PNP, PIC and PM (Fig. 2). However, values < 0.4 ng in sample extracts were rejected for all vitamers since they could not be distinguished from noise at the sensitivity employed. The precision of analysis is given in Table I. The results are based

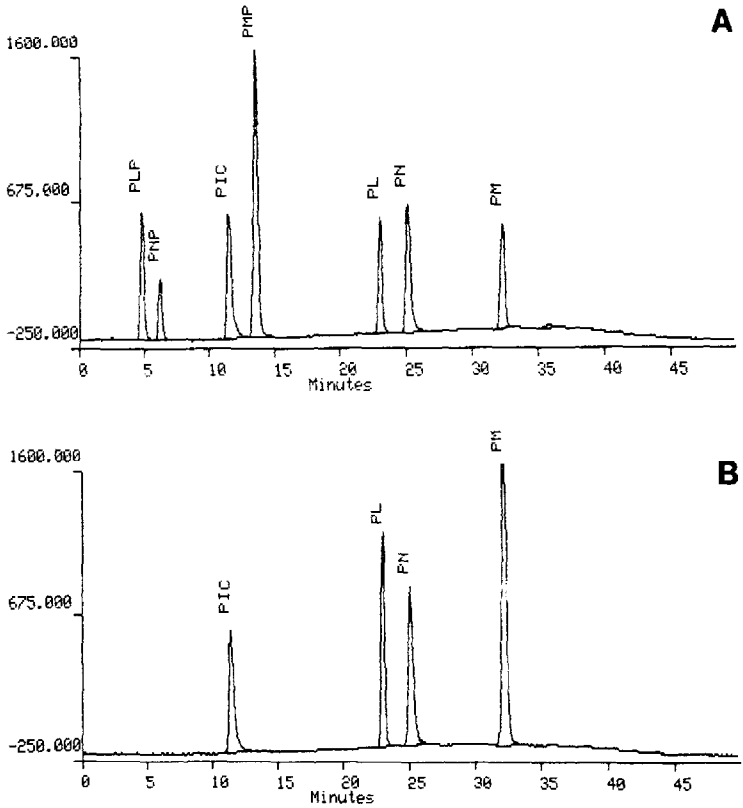

Fig. 1. (A) Mixture of vitamer standards containing 16 ng of pyridoxal phosphate (PLP), pyridoxamine phosphate (PMP), pyridoxal (PL) and pyridoxine (PN), 8 ng of pyridoxic acid (PIC) and pyridoxamine (PM) and 4 ng of pyridoxine phosphate (PNP). (B) Standard vitamer mixture after treatment with alkaline phosphatase. Conversion of the phosphorylated vitamers to the non-phosphorylated forms was > 98%.

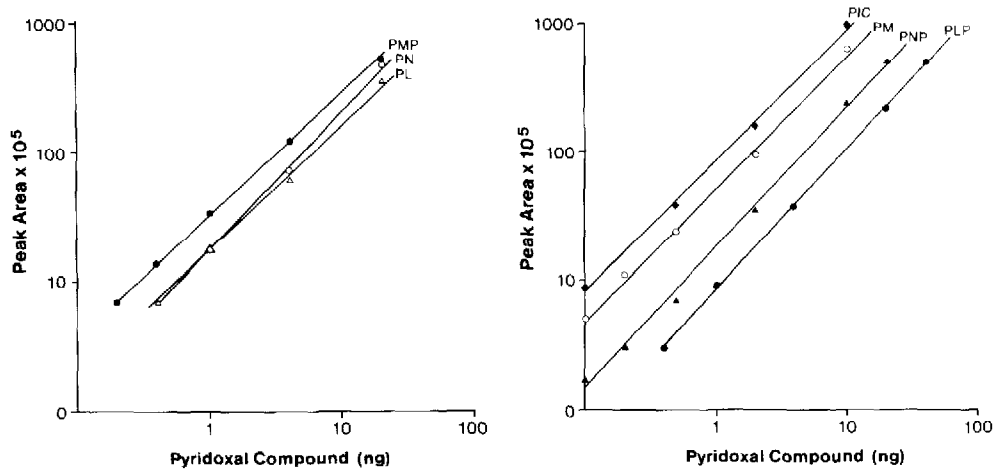

Fig. 2. Dose- response curves (log—log) of each of the vitameric compounds, showing linear relationship between the area of the peak and the amount injected. Left (•) pyridoxamine phosphate (PMP); (○) pyridoxine (PN); (△) pyridoxal (PL). Right: (■) pyridoxic acid (PIC); (○) pyridoxamine (PM); (▲) pyridoxine phosphate (PNP); (•) pyridoxal phosphate (PLP).

TABLE I

WITHIN-RUN PRECISION OF ANALYSIS BASED ON TEN REPLICATE INJECTIONS

Vitamer	Mean amount injected (ng)	Coefficient of variation (%)
PLP	3.89	2.8
PNP	4.12	3.6
PIC	2.23	4.5
PMP	4.01	2.2
PL	4.22	1.9
PN	4.52	4.6
PM	2.03	2.0

on ten replicate injections of the standard mixture. The total analysis time is 50 min from injection to injection. The phosphorylated vitamers were verified through disappearance of the peak after treatment with alkaline phosphatase and the quantitative appearance of a peak corresponding to the non-phosphorylated form (Fig. 1A and B). This step not only served to verify peak identity but also peak purity.

Alkaline phosphatase of highest purity was employed in order to decrease the possibility of reactions other than dephosphorylation due to contaminating enzymes. Forty units were found sufficient for > 98% conversion of up to 3 μg of the phosphorylated vitamers in 1 h.

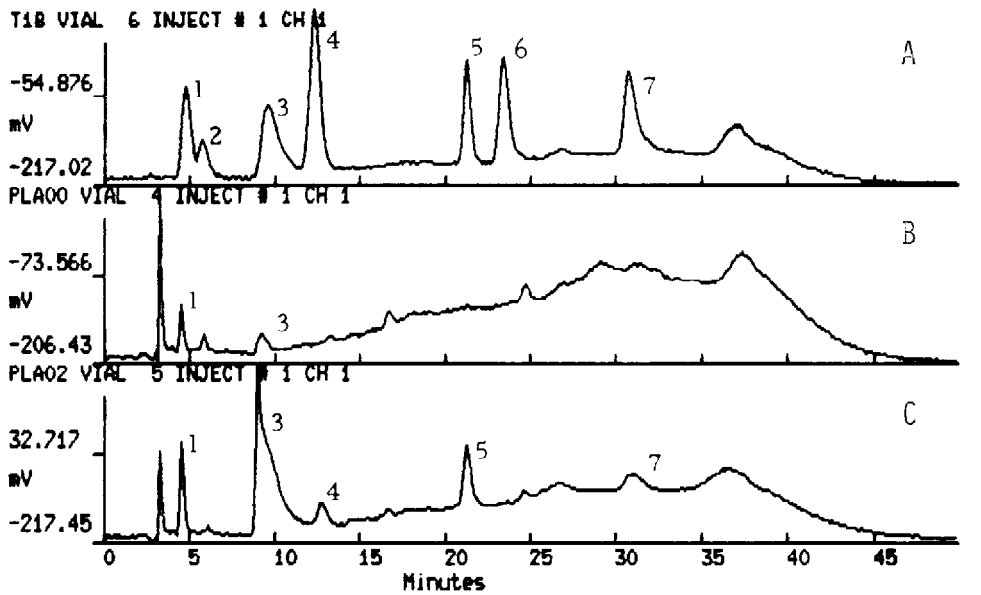

Fig. 3. Comparative plots of chromatograms produced by the vitamer standards and plasma extracts. (A) Vitamer standards consisting of 4 ng of pyridoxal phosphate (1), 1 ng of pyridoxine phosphate (2), 2 ng of pyridoxic acid (3), 4 ng of pyridoxamine phosphate (4), 4 ng of pyridoxal (5), 4 ng of pyridoxine (6) and 2 ng of pyridoxamine (7). (B) Extract of plasma from a fasting individual, 0.1 ml plasma equivalent. (C) Extract of plasma from the same individual 2 h post-ingestion of 25 mg of pyridoxine; peaks corresponding to the individual vitamer standards are similarly numbered.

TABLE II

RECOVERY OF VITAMERS ADDED TO PLASMA

Asssay 1 and assay 2 were analyzed two weeks apart. See text for details.

Vitamer	Concentration* (ng/ml)		Amount added (ng)	Concentration* (ng/ml)		Recovery (%)		Coefficient of variation (%)		
	Assay 1	Assay 2		Assay 1	Assay 2	Assay 1	Assay 2	Recovery	Intra-assay	Inter-assay
PLP	20.69 ± 0.94	21.98 ± 1.86	10.0	28.23 ± 1.55	31.65 ± 2.26	92	99	5.2	5.4	8.1
PNP	2.14 ± 0.54	1.62 ± 0.17	2.5	4.84 ± 0.81	4.12 ± 0.26	104.3	100	2.9	16.7	5.8
PIC	13.47 ± 2.71	11.23 ± 2.07	5.0	21.71 ± 0.99	18.89 ± 0.74	117.5	116.4	0.7	4.5	9.8
PMP	2.23 ± 0.08	2.36 ± 0.06	10.0	10.24 ± 0.48	11.12 ± 0.14	83.7	90.0	5.1	4.7	5.8
PL	4.81 ± 0.40	4.49 ± 1.89	10.0	16.68 ± 0.65	14.77 ± 0.36	112.6	101.9	7.0	3.9	8.6
PN	—	—	10.0	13.96 ± 0.52	14.89 ± 1.79	139.6	148.9	4.6	3.7	4.6
PM	—	—	5.0	8.08 ± 0.95	8.96 ± 0.52	161.6	179.2	7.3	10.7	7.3

*Mean ± S.D. of three replicate extractions.

The use of the buffer—bisulfite post-column reagent has been described previously [2]. The buffer raises the pH of the column effluent, and in this way enhances the fluorescence of all of the vitamers. The bisulfite enables the detection of PLP. The reagent is stable for at least 20 h.

The vitamers are stable for 24 h at room temperature in solvent A. Automatic sample injection allows routine overnight analyses of a maximum of twenty samples including standards. All of the vitamer standards have remained stable when frozen at −80°C at 1 mg/ml in water for up to six months and have tolerated several thawings and refreezings. The PLP and PIC standards were less soluble in water than the other vitamers. PLP required extensive vortexing and PIC formed a cloudy solution which cleared after the addition of 20—30 μl of 1 *M* sodium hydroxide to a 5-ml solution.

Representative chromatograms of fasting plasma and the profile 2 h after oral ingestion of 25 mg of pyridoxine are given in Fig. 3. PLP and PIC were the only vitamers consistently present at a significant level in the fasting state. PN and PM were not detected and PMP was below detection (2 ng/ml). In some samples, a peak is sometimes detected which occurs at the elution position of PNP. This peak is not converted to PN after alkaline phosphatase treatment, however. PL was only variably present but was always detected in plasma when PLP levels were > 15 ng/ml. The level of PL, however, did not correlate with the level of PLP.

The numerous extraneous fluorescent peaks found in urine samples (Fig. 4) emphasize the need for a procedure that provides high selectivity. We have found that the best way to monitor column selectivity over time is the reanalysis of a standard urine sample. Loss of resolution primarily affects the PIC to PMP region of the chromatogram.

The 4-ml sample vials require a sampling volume in excess of 1.5 ml. For this

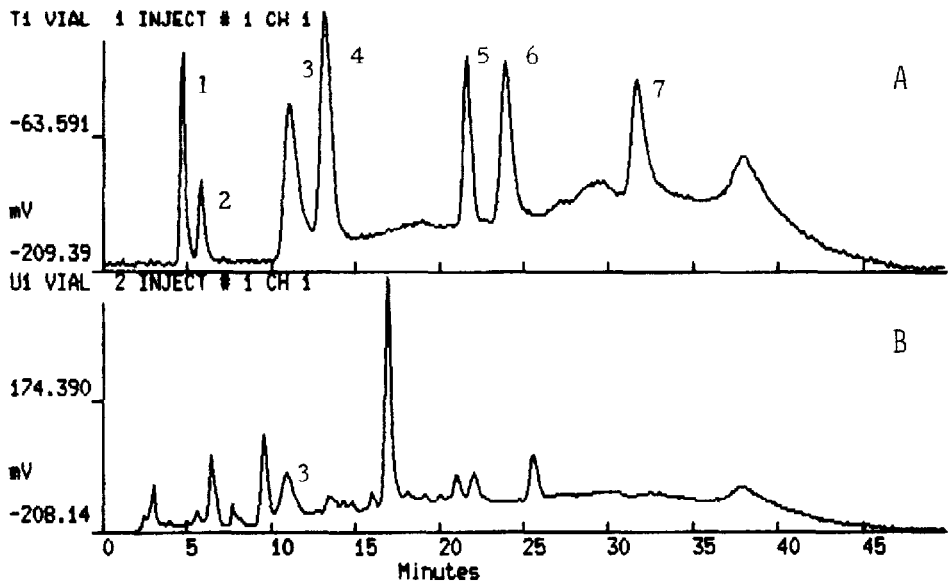

Fig. 4. Comparative plots of chromatograms produced by the vitamer standards (A) and urine (B). Concentrations and symbols are the same as in Fig. 3.

reason, all dried extracts were reconstituted with 2.5 ml of solvent A, allowing 2—500-μl injections. In practice, it was more convenient to dry, reconstitute and sample from the same vial. In the analysis of plasma from fasting subjects, the injection of aliquots equivalent to 0.2 ml of plasma was found adequate. In urine, the large dilution required to obtain a readable chromatogram limits the detection of the vitamers to 80 ng/ml.

The recovery of vitamers added to plasma prior to extraction is given in Table II. The recoveries of PN and PM are > 100%, which probably results from the additive effect of the baseline noise of the sample. This could not be quantified in the unspiked sample and so could not be subtracted out.

The fasting vitamer profile of ten normal individuals not taking vitamin supplements is given in Table III. The range of PLP is similar to previously reported values using the tyrosine decarboxylase assay [9, 10]. Some of the other vitamers are lower than have been reported using chromatography [2]. However, we feel that this is primarily due to greater resolution and exclusion of samples from individuals with a history of supplementary vitamin intake.

TABLE III

PYRIDOXYL COMPOUNDS IN HUMAN PLASMA

Pyridoxyl compound	Concentration (ng/ml)			
	This paper (n = 10)	Ref. 2* (n = 38)	Ref. 9**,*** (n = 12)	Ref. 10**,§ (n = 43)
Pyridoxal phosphate	15.2 ± 8.3	14 ± 6.4	17 ± 12.7	15.6 ± 4.0
Pyridoxine phosphate	Not detected	Not detected	Not determined	Not determined
Pyridoxic acid	4.6 ± 1.6	8.9 ± 3.5	Not determined	Not determined
Pyridoxamine phosphate	Below detection	2.3 ± 2.3	Not determined	Not determined
Pyridoxal	0.9 ± 1.5	4.7 ± 2.0	Not determined	Not determined
Pyridoxine	Not detected	3.9 ± 6.8	Not determined	Not determined
Pyridoxamine	Not detected	0.5 ± 0.5	Not determined	Not determined

*Data converted to ng/ml.
**Tyrosine decarboxylase assay.
***Data estimated from graph.
§Data averaged over sex and age groups.

Retention times decrease as the column ages. The column has a two-month life, i.e. over 300 injections. An additional 200–300 injections can be obtained by the tandem mounting of two such deteriorated columns. The chromatograms in Fig. 1 were obtained using a one-month-old column, while those in Figs. 2 and 3 were obtained with a four-month-old column. On older columns (three months), PIC elutes earlier with injection volumes > 100 μl. This is of no consequence for most plasma samples but in urine this results in coelution with an earlier eluting peak.

CONCLUSION

The procedure presented here is both highly selective and sensitive. It allows analysis of all the B_6 vitamers in a single sample within 50 min, with a minimum of interference from extraneous fluorescent peaks. Sensitivity is 0.4 ng on the column and the procedure is applicable to plasma, urine and tissue extracts. Using the dilutions given, the minimum detection of the vitamers in plasma is 2 ng/ml, and 80 ng/ml in urine.

ACKNOWLEDGEMENT

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