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# Determination of vitamin $B_6$ vitamers and pyridoxic acid in biological samples

S. K. Sharma and K. Dakshinamurti

*Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba R3E 0W3 (Canada)*

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ABSTRACT

For the determination of vitamin $B_6$ vitamers (pyridoxal phosphate, pyridoxamine phosphate, pyridoxal, pyridoxine, pyridoxamine) and 4-pyridoxic acid in biological samples such as plasma, cerebrospinal fluid and rat brain regions, a sensitive micromethod using high-performance liquid chromatography (HPLC) with fluorescence detection in combination with post-column derivatization is described. Metaphosphoric acid tissue extracts with deoxypyridoxine as an internal standard were injected into the HPLC system with a binary gradient elution at a flow-rate of 1.2 ml/min. The excitation wavelength of the fluorescence detector was set at 328 nm and the emission wavelength at 393 nm with a 15-nm slit width for the photocell. This method allows the assay of vitamin $B_6$ vitamers within 30 min in one chromatographic run. The present method has been applied extensively for the measurement of vitamin $B_6$ vitamer levels in discrete brain regions of small animals, cells in culture and biopsy samples.

## INTRODUCTION

Vitamin $B_6$ occurs in biological tissues and fluids as three interconvertible 3-hydroxy-2-methylpyridine compounds [pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM)] and two phosphorylated coenzyme forms [pyridoxal phosphate (PLP) and pyridoxamine phosphate (PMP)]. The principal catabolite is 4-pyridoxic acid (4-PA). Accurate determination of all forms of vitamin $B_6$ at concentrations found in biological samples is essential for assessing nutritional and metabolic requirements. Microbiological, colorimetric, gas chromatographic and ion-exchange chromatographic procedures have been described for the analysis of vitamin $B_6$ vitamers [1]. Chauhan and Dakshinamurti [2] separated PL, PM, PN, PLP and PMP by ion-exchange chromatography and quantitated these compounds fluorimetrically as cyanohydrin derivatives, permitting a detection limit in the range 10–50 ng/ml. A minimum of 5 ml of serum was required per analysis, and one analysis required 6 h to be performed. These procedures are time-consuming and less sensitive than high-performance liquid chromatography (HPLC) with fluorescence detection [3–10].

Of the recent methods, Toshikatsu *et al.* [7] used an isocratic mode of elution and cyanide treatment for the determination of PLP. Sampson and O'Connor [4] have used a binary gradient and post-column derivatization of PLP with sodium bisulphite in 1 *M* potassium phosphate buffer, while Coburn and Mahuren [10] have used a ternary gradient system for the determination of both phosphorylated and non-phosphorylated vitamin $B_6$ vitamers in biological

*Correspondence to:* Dr. K. Dakshinamurti, Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Manitoba, 770 Bannatyne Avenue, Winnipeg, Manitoba R3E 0W3, Canada.

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samples. Taking into consideration the limitations of previously described procedures for the analysis of vitamin $B_6$ vitamers, we have now developed a micromethod for the determination of vitamin $B_6$ vitamers in biological samples.

## EXPERIMENTAL

PLP, 4-PA, PMP, PL, PN, 4-deoxypyridoxine (dPN) and PM were purchased from Sigma (St. Louis, MO, USA). Spectral-grade dichloromethane, isopropyl alcohol, metaphosphoric acid and phosphoric acid were procured from Mallinckrodt (Mississauga, Canada). All other reagents used were of HPLC- or spectral-grade quality.

### *Sample preparation*

All procedures were carried out in a dark room with dim light (to avoid photodegradation of PL and PLP) and in amber-colored microcentrifuge tubes. Sample preparation was carried out in an ice bath. Vitamin $B_6$ vitamers were extracted using 5% metaphosphoric acid for tissue samples and 10% metaphosphoric acid for cerebrospinal fluid (CSF) and plasma, essentially as described by Sampson and O'Conner [4]. A 10-mg sample of rat liver, 20 mg of rat cerebral cortex or 250 $\mu$l of rat (1:10 dilution) or human (1:2.5 dilution) plasma or human CSF (undiluted) was homogenized with 250 $\mu$l of 5 or 10% (w/v) metaphosphoric acid in a microcentrifuge tube using an ultrasonicator for 30 s at 300 W followed by centrifugation at 10 000 *g* for 20 min at 0–4°C. The supernatant was transferred to a microcentrifuge tube, 250 $\mu$l of dichloromethane (spectral grade) were added, and the mixture was vortex-mixed and centrifuged for 15 min at 0–4°C to remove lipids. The aqueous layer was filtered (Conz syringe filter, 0.22 $\mu$m) before injection into the column. In some cases, the extracts were stored at −70°C in dark containers before analysis.

For samples with very low vitamin $B_6$ vitamer levels a concentration step was included; 250-$\mu$l aliquots were dried in nitrogen and reconstituted in a known volume of 10% metaphosphoric acid for extraction and lipid removal before injection into the column.

### *Mobile phase*

Solvent A consisted of 0.033 *M* phosphoric acid containing 0.01 *M* 1-octanesulphonic acid, adjusted to pH 2.2 with 6 *M* potassium hydroxide. Solvent B consisted of 0.33 *M* phosphoric acid in 10% (v/v) 2-propanol (spectral grade), adjusted to pH 2.2 with 6 *M* potassium hydroxide.

### *Detector*

A Shimadzu RF-535 fluorescence monitor at fast response range maximum and high sensitivity, was used for detecting the fluorescence of vitamin $B_6$ vitamers. The excitation wavelength was 328 nm and the emission wavelength 393 nm, with the slit width fixed at 15 nm.

### *Post-column reagent*

To enhance the fluorescence of PLP, sodium bisulphite (1 mg/ml) in 0.1 *M* potassium phosphate buffer adjusted to pH 7.4 with 6 M potassium hydroxide was used.

### *Chromatographic conditions*

A Beckman Model 420 programmer was used for the binary gradient programming. A calibrated Beckman Model 050-169 gradient mixer was used for gradient mixing. Sample injection was followed by a linear gradient to 100% B for the first 10 min, followed by 100% B for the next 15 min. This was followed by a linear gradient to 100% A in 4.5 min and the column was equili-

TABLE I

GRADIENT ELUTION PROFILE FOR THE SEPARATION OF VITAMIN $B_6$ VITAMERS, COENZYMES AND 4-PYRIDOXIC ACID

| Time (min) | Solvent A (%) | Solvent B (%) |
|---|---|---|
| 0 | 100 | 0 |
| 0.01 | Inject sample | |
| 0.01–10 | 0 | 100 |
| 10–25 | 0 | 100 |
| 25–29.5 | 100 | 0 |
| 29.5–35 (column equilibration) | 100 | 0 |

brated for 5.5 min with 100% A. The gradient elution profile is given in Table I.

*Equipment.* A Waters U6K universal microinjector was used for injecting 25 μl of extracted and filtered samples into the HPLC column. Beckman Model 100A HPLC pumps were used throughout at 190–204 bar and a flow-rate of 1.2 ml/min. A Hewlett-Packard Model 2290A integrator was used for the quantitation of vitamin $B_6$ vitamer peaks.

*Column.* An Ultramex $C_{18}$ guard column (30 mm × 4.6 mm I.D., particle size 3 μm) and an Ultramex $C_{18}$ column (150 mm × 4.6 mm I.D., particle size 3 μm) (Phenomenex, Torrance, CA, USA) were used. The system was flushed after every thirty samples with HPLC-grade Millipore-filtered water at a flow-rate of 0.5 ml/min for 60 min and then with 100% 2-propanol at 0.2 ml/min for 60 min. Every morning the column was flushed for 1 h with HPLC-grade water at 0.5 ml/min. Two runs of standards were made to check inter-assay variability and to stabilize the retention time of PLP. Every day fresh mobile phase was prepared and solvents were changed using reduced flow-rates to avoid back-pressure, to stabilize the retention time and peak resolution and to avoid baseline drifts.

*Data analysis.* Data were analysed using peak areas of standards of known concentrations. Vitamin $B_6$ vitamer concentrations relative to peak areas of the internal standard dPN were calculated to correct for vitamer loss during sample preparation and manipulation. Twelve-point standard curves were prepared by injection of vitamers in the following ranges: PLP and PN, 0.5–10 pmol; PMP, PM and 4PA, 0.25–5 pmol; PL, 1–10 pmol.

*Vitamer recovery*

The recovery of vitamin $B_6$ vitamers was evaluated in human plasma, CSF, rat liver and cerebral cortex as representative tissues. Ten samples each of spiked and unspiked tissues were used for the analysis. Spikes of 12.5–62.5 pmol for plasma and 75–120 pmol for tissues were added to samples before homogenization. Recoveries were calculated as $(S - U)/S_p \times 100$, where $S$ and $U$ are analysed vitamer concentrations in the spiked and unspiked samples, respectively, and $S_p$ is the amount of added spike.

Detection and quantitation limits for each vitamer, expressed as pmol per injection, were calculated according to the following formulae: detection limit = $Y_B + 3\ S_y/X$ and quantitation limit = $Y_B + 10\ S_y/X$, where $Y_B$ is the $y$-intercept and $S_y/X$ is the standard deviation from the regression for each analyte's standard curve. The precision of each method was determined at two concentrations of each vitamer from ten replicate injections of standard solution. Precision was calculated as the percentage coefficient of variation of the ten replicates. Two different concentrations of standards were used to test the precision at vitamer concentrations near and above the quantitation limits (as defined above). We have compared vitamin $B_6$ vitamer concentrations in rat liver and plasma obtained with our method to values reported in literature [4,10–13].

## RESULTS

Representative chromatograms including standards (a), human plasma (b), rat liver (c), and rat cerebral cortex (d) are shown in Fig. 1. The order of elution was as follows: PLP, 4-PA, PMP, PL, PN, dPN and PM. The PLP peak was enhanced five-fold by post-column derivatization with sodium bisulphite. Vitamin $B_6$ vitamer concentrations varied from region to region in the rat brain. In rat tissues, phosphorylated derivatives such as PLP and PMP were higher in concentration (Table II). Resolution of both phosphorylated (PLP and PMP) and non-phosphorylated (PL, PM and PN) vitamers, as well as the excretory metabolite 4-PA and the internal standard dPN, was seen in all tissues analyzed. PLP and PL peaks were smaller in CSF than in plasma extracts. Concentrations of PLP and PL in human plasma were five to ten times lower than in rat plasma. A representative chromatogram of vitamin $B_6$ vitamers of human CSF is given in Fig. 2. It is possible to determine the levels of vitamin $B_6$ vitamers using as little as 250 μl of CSF which makes possible the investigation of PN transport in children with seizures.

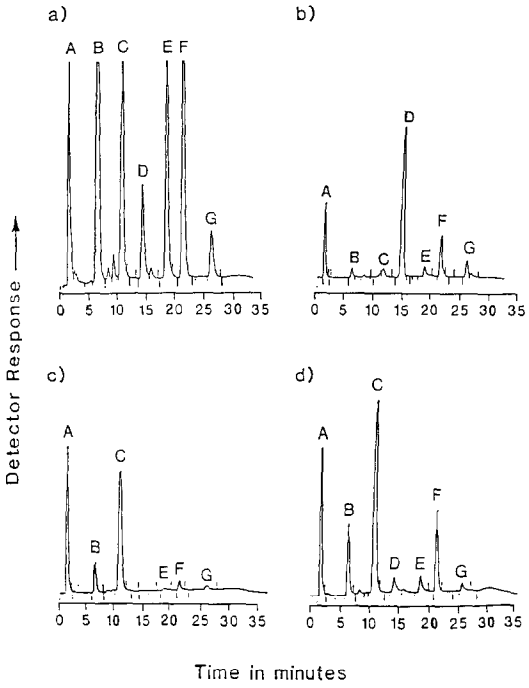


Fig. 1. (a) Representative chromatogram of standard vitamin $B_6$ vitamers, 4-deoxypyridoxine and 4-pyridoxic acid. Peaks: A = PLP (8 pmol); B = 4-PA (16 pmol); C = PMP (5.5 pmol); D = PL (10 pmol); E = PN (10 pmol); F = dPN (12 pmol); G = PM (6 pmol). Vitamin $B_6$ vitamer profiles of (b) human plasma, (c) rat liver and (d) rat cerebral cortex. Retention times (in min): PLP, 2.04 ± 0.2; 4-PA, 6.2 ± 0.1; PMP, 10 ± 0.03; PL, 13.6 ± 0.2; PN, 18.5 ± 0.12; dPN, 20.5 ± 0.3; PM 25.2 ± 0.35.

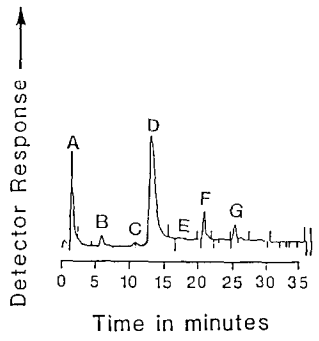


Fig. 2. Typical chromatogram of vitamin $B_6$ vitamers and 4-PA from a human CSF sample. (Analysis was done as described in the text.)

*Limit of detection*

For each vitamer, the limit of detection varied from 0.3 to 1.5 pmol per injection. Coburn and Mahuren [10] have reported background noise below 5 ng per injection. We have obtained resolved peaks and a stable baseline even at concentrations as low as 0.2–0.5 ng per injection. The resolution of vitamin $B_6$ vitamers in this procedure was better, owing to the use of 3-$\mu$m-size column packing. Limits of detection and precision are reported in Table III.

*Quantitation and recovery*

Quantitation limits ranged from 2.5 to 10 pmol for different vitamers. Our results on the analysis of vitamin $B_6$ vitamers in rat liver were similar to those reported by Sampson and O'Connor [4] and Coburn and Mahuren [10]. We have used dPN as an internal standard for determining vitamer recovery during sample preparation. The

TABLE II

VITAMIN $B_6$ VITAMER LEVELS IN BIOLOGICAL RAT SAMPLES

| Vitamer | Concentration (mean ± S.D., $n$ = 5) | | | |
|---|---|---|---|---|
| | Plasma (nmol/l) | Liver (nmol/g) | Kidney (nmol/g) | Cerebral cortex (nmol/g) |
| PLP | 693 ± 78 | 21 ± 0.67 | 7.5 ± 1.12 | 6.8 ± 1.12 |
| 4-PA | 9 ± 3 | 1.3 ± 0.45 | 0.3 ± 0.04 | 1.3 ± 0.51 |
| PMP | 10 ± 3 | 12 ± 2.23 | 23 ± 5.14 | 13 ± 1.34 |
| PL | 607 ± 56 | 3 ± 0.33 | 2 ± 0.67 | 1.0 ± 0.27 |
| PN | 60 ± 19 | 1.3 ± 1.12 | 0.2 ± 0.13 | 0.5 ± 0.08 |
| PM | 20 ± 8 | 1.2 ± 0.67 | 14 ± 1.80 | 0.1 ± 0.11 |

TABLE III

PRECISION OF FIVE REPLICATE INJECTIONS OF STANDARDS

| Vitamer | Injected (pmol) | Detected (mean ± S.D., $n$ = 5) (pmol) | Precision (C.V., %) |
|---|---|---|---|
| *Concentrations near quantitation limits* | | | |
| PLP | 2.5 | 2.4 ± 0.02 | 0.83 |
| 4-PA | 1.25 | 1.22 ± 0.03 | 2.45 |
| PMP | 1.25 | 1.30 ± 0.04 | 3.07 |
| PL | 4.10 | 4.5 ± 0.30 | 6.66 |
| PN | 2.60 | 2.7 ± 0.20 | 7.40 |
| PM | 1.30 | 1.2 ± 0.10 | 8.33 |
| *Concentrations above quantitation limits* | | | |
| PLP | 5.0 | 6.5 ± 0.30 | 4.60 |
| 4-PA | 3.20 | 2.5 ± 0.10 | 4.00 |
| PMP | 3.50 | 2.8 ± 0.06 | 2.14 |
| PL | 10.50 | 9.5 ± 0.12 | 1.26 |
| PN | 7.50 | 7.2 ± 0.20 | 2.77 |
| PM | 2.60 | 2.7 ± 0.15 | 5.50 |

vitamer recovery from various biological samples is given in Table IV.

*Thermal stability of vitamin $B_6$ vitamers*

Although vitamin $B_6$ vitamers are generally stable in acid, Sampson and O'Conner [4] have reported that vitamer standards in 5% metaphosphoric acid held at 4°C degrade at 0.70 ± 0.09%/h for 40 h and then remain stable up to 233 h. Calculated vitamer concentrations were not affected because the internal standard, dPN, degrades at the same rate [4]. However, uneven degradation could impair accurate assay, because limits of quantitation might be reached. To minimize degradation, samples that were not immediately analysed were stored at −70°C until use.

The vitamin $B_6$ profile of human plasma determined by our method has been compared with data on human plasma/serum reported in the literature (Table V) using other HPLC procedures [4,10–13]. The values for PLP are close to those of Chrisley *et al.* [11] and Sampson and O'Connor [4] and within the range of means (51–88 nmol/l) reported by others [4,10–13]. The values for the other $B_6$ vitamers (PMP, PNP, PL, PN and PM) obtained by our method are compara-

TABLE IV

PERCENTAGE RECOVERIES OF VITAMIN $B_6$ VITAMERS AND 4-PYRIDOXIC ACID

| Vitamer | Recovery (mean ± S.D., $n$ = 10) (%) | | | |
|---|---|---|---|---|
| | Plasma | CSF | Liver | Cortex |
| PLP | 90 ± 22 | 88 ± 13 | 100 ± 10 | 92 ± 13 |
| 4-PA | 93 ± 6 | 94 ± 10 | 100 ± 6 | 94 ± 10 |
| PMP | 100 ± 10 | 98 ± 6 | 103 ± 10 | 99 ± 13 |
| PL | 104 ± 16 | 100 ± 6 | 92 ± 16 | 102 ± 16 |
| PN | 98 ± 10 | 100 ± 10 | 103 ± 13 | 100 ± 10 |
| PM | 102 ± 6 | 103 ± 10 | 104 ± 13 | 104 ± 10 |

TABLE V

PLASMA CONTENT OF $B_6$ VITAMERS AND PYRIDOXIC ACID: A COMPARISON OF VARIOUS METHODS

N.D. = not detected; N.D.T. = not determined. A = present method; B = Chrisley *et al.* [11], C = Sampson and O'Connor [4]; D = Hollins and Henderson [12]; E = Coburn and Mahuren [10]; F = Shephard *et al.* [13].

| Vitamer | Content (mean ± S.D.) (nmol/l) | | | | | |
|---|---|---|---|---|---|---|
| | A ($n$ = 8) | B ($n$ = 22) | C ($n$ = 4) | D ($n$ = 10) | E ($n$ = 38) | F ($n$ = 27) |
| PLP | 80 ± 21 | 88 ± 18 | 75 ± 24 | 62 ± 34 | 57 ± 26 | 51 ± 25 |
| PNP | N.D. | N.D. | N.D. | N.D.T. | N.D. | N.D. |
| 4-PA | 75 ± 20 | 39 ± 53 | 90 ± 37 | 25 ± 9 | 49 ± 19 | 30 ± 21 |
| PMP | 7 ± 3 | 12 ± 27 | 5 ± 2 | N.D. | 8 ± 8 | 0.35 ± 0.7 |
| PL | 90 ± 10 | 38 ± 23 | 85 ± 12 | 4 ± 7 | 23 ± 10 | 7.4 ± 6 |
| PN | 12 ± 3 | 41 ± 35 | 10 ± 4 | N.D. | 19 ± 33 | 0.48 ± 1.5 |
| PM | 4.5 ± 2 | 18 ± 9 | 3 ± 2 | N.D. | 3 ± 2.4 | 0.4 ± 0.4 |

ble to the data reported by Sampson and O'Connor [4]. There is no explanation for the lower levels of these other vitamers reported by others [10,12,13]. No attempt has been made to correlate $B_6$ vitamer levels with the nutritional status of the subjects.

## DISCUSSION

To achieve better resolution and enhance sensitivity, the method described here involves several modifications of the procedure described by Sampson and O'Connor [4] such as type of guard and analytical columns (3 μm particle size *versus* 7 μm particle size), higher concentration of the ion-pairing agent octanesulphonic acid (0.01 *versus* 0.009 *M*), sensitivity of the detector (Shimadzu RF 535 fluorescence detector *versus* spectrofluorimeter] and post-column derivatization of PLP with sodium bisulfite in 0.1 *M* potassium phosphate buffer at pH 7.4.

The use of sulphosalicylic acid as protein precipitant [9] can interfere with the fluorescence of vitamin $B_6$ vitamers because it is highly fluorescent. Sulphosalicylic acid can be removed from the samples by using an ion-exchange column but this dilutes the sample and also removes 4-PA, a metabolite of interest. These problems were avoided by extracting samples with metaphosphoric acid.

We have used binary gradient elution, which eliminates the need for a third mobile phase and the associated hardware used by others [9,10]. Using 10% propanol in solution B with a gradient program, we obtained a good separation of vitamin $B_6$ vitamers without the intermediate mobile phase of Gregory and Feldstein [9] that contained 8 m*M* octanesulphonic acid in 2.5% (v/v) propanol. Coburn and Mahuren [10] also used ternary gradient elution to separate vitamin $B_6$ vitamers, but suggested that their method could be adapted to a binary HPLC system by addition of a valve to one solvent line. An advantage of the method described here is that the binary HPLC method can be used without modification to separate all vitamin $B_6$ vitamers.

We have used an Ultramex $C_{18}$ reversed-phase 3 μm analytical column for the separation of vitamin $B_6$ vitamers. During development of this method we tried other columns, such as an Ultrasphere-ODS 5-μm column, an Upchurch Scientific $C_{18}$ AXXI-Chrom ODS 5-μm column and a Waters $C_{18}$ radial compression μBondapak 5-μm column, and concluded that the Ultramex $C_{18}$ reversed-phase 3 μm analytical column gave the best vitamer resolution.

The detection and quantitation limits for the method described here appear to be equal to or better than the limits reported for other HPLC methods [3,4,10,14]. Several methods reported in

the literature do not permit measurement of all forms of vitamin $B_6$ commonly encountered in biological fluids and tissues. The method of Driskell *et al.* [15] involves dephosphorylation before analysis. Hamaker *et al.* [16] developed a method for analysis of vitamin $B_6$ in human milk but did not obtain resolution between PMP and PM or between PLP and PL. The methods of Ubbink *et al.* [5] and Gregory and Litherland [17] which are modifications of the procedure described by Schrijver *et al.* [18] can be used to measure PLP and PL only, and thus are restricted in use as many tissues contain PMP as well. The method of Tryfiates and Sattsangi [14] is not sensitive enough for analysis of human plasma, as it involves injection of 40 ng of PLP and PL, which corresponds to about 40 ml of plasma. Ang *et al.* [19] recently reported a reversed-phase method for analysis of PN vitamers in chicken meat but did not obtain complete resolution of PL from an unidentified peak or PMP from PM.

The objective of the present study was to develop a method that could be applied to small volumes of biological fluids such as CSF, small amounts of tissues such as discrete brain areas of small animals, cultured cells and biopsy samples. Samples were extracted with metaphosphoric acid in small-volume extractions. Under conditions where vitamin $B_6$ vitamer levels were low, the samples were concentrated by drying in nitrogen and reconstituted in a known dilution of 5% metaphosphoric acid for vitamer extraction. This procedure allows the determination of low concentrations of vitamin $B_6$ vitamers in biological samples such as CSF.

In summary, the method permits the analysis of vitamers (including PLP, PL, PMP, PM and PN) and the metabolite 4-PA in plasma, CSF and tissues using reversed-phase HPLC with binary gradient elution. The method is sensitive enough for samples that contain low concentrations of vitamin $B_6$ vitamers such as CSF or for small amounts of tissue samples such as discrete areas of rodent brain.

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