*Journal of Chromatography*, 434 (1988) 209–214
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4430

## Note

# Sensitive assay for determination of pyridoxal-5-phosphate in enzymes using high-performance liquid chromatography after derivatization with cyanide

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(First received April 19th, 1988; revised manuscript received July 4th, 1988)

Pyridoxal-5-phosphate (PLP) is a coenzyme of various kinds of enzyme, such as transaminases and amino acid decarboxylases. The concentration of PLP in the blood has been determined by use of high-performance liquid chromatography (HPLC) [1–4]. Detection of PLP in HPLC eluates is based on the measurement of its UV absorbance or of its native fluorescence. These methods are sensitive enough to determine PLP levels in the blood, serum or erythrocytes, but the lower limit of determination of PLP is ca. 100 ng or 400 pmol. For quantitative analysis of PLP in an enzyme, the sensitivity of the previous methods is so low that large amounts of purified enzymes are required. Determination of PLP in enzymes has been carried out by the enzymatic method [5], or by derivatization with cyanide [6,7] or with semicarbazide [8]. The enzymatic method is based on activation of tyrosine apodecarboxylase (L-tyrosine carboxy-lyase, EC 4.1.1.25) [5] or apotryptophanase [L-tryptophan indole-lyase (deanimating), EC 4.1.99.1] [9] by PLP, but this method requires a preparation of apoenzyme, which is not very stable. In addition, these enzymes are activated not only by PLP, but also by pyridoxamine phosphate [9]. The latter methods are based on derivatization of PLP into a fluorescent compound by reaction with cyanide

0378-4347/88/$03.50 

or by trans-Schiff base formation with semicarbazide. These methods were applied mostly to purified enzyme samples or after purification of samples by column chromatography [10]. Even then, quantitation of PLP in a crude sample has been difficult, because of the high blank value due to contaminating fluorescent compounds. Thus a sensitive method to determine PLP concentrations in crude samples is required.

This paper describes a sensitive HPLC assay for the quantitation of PLP crude samples, by separation and fluorimetric quantitation of a PLP-cyanide derivative. The method was applied to human brain homogenates, tissue-cultured cells and some purified enzymes.

## EXPERIMENTAL

### *Materials*

PLP was purchased from Nacalai tesque (Kyoto, Japan), and sodium 1-heptanesulphonate and phosphorylase-a (1,4-β-D-glucan:orthophosphate-β-glucosyltransferase, EC 2.4.1.1) purified from rabbit muscle from Sigma (St. Louis, MO, U.S.A.). Other chemicals were of analytical-reagent grade.

### *Samples*

Human brain was obtained at autopsy within 6 h after death from a control without any neurological diseases, and kept at −80°C until analysis. Gray and white matter from the frontal lobe were homogenized with 10 volumes per wet weight of 10 m*M* potassium phosphate buffer (pH 7.4). Clonal rat pheochromocytoma PC12h cells [11] were cultured as described previously [12]. L-3,4-Dihydroxyphenylalanine decarboxylase (DDC, aromatic-L-amino acid carboxylyase, EC 4.1.1.28) was purified from bovine adrenal medulla, according to the method reported previously [13].

### *Derivatization with cyanide*

Derivatization of PLP with potassium cyanide was carried out by the method reported by Adams [6], with some modifications. A PLP sample in 50 μl of 10 m*M* potassium phosphate buffer (pH 7.4) (25 μg to 20 mg protein for crude samples, or 0.5 to 10 μg protein for purified enzymes) was mixed with 50 μl of 10% trichloroacetic acid and incubated in an Eppendorf centrifuge tube at 50°C for 15 min. To the mixture, 35 μl of 3.3 *M* dipotassium hydrogenphosphate and 1 μl of 80 m*M* potassium cyanide were added and the mixture was incubated at 50°C for 25 min. To stabilize the fluorescent product, 12.5 μl of 28% phosphoric acid were added, then the mixture was centrifuged at 13 000 *g* for 10 min, and the supernatant was filtered through a Millipore HV filter (pore size, 0.45 μm).

### *Apparatus and chromatographic parameters*

An LC-3A HPLC apparatus (Shimadzu, Kyoto, Japan) was connected to a Shimadzu fluorescence spectrometer, RF-500LCA. A Shimadzu pre-packed reversed-phase column, STR ODS-H (150 mm×4 mm I.D.) of 5 μm particle size and 100 Å pore size was used. The mobile phase was 2 *M* potassium acetate buffer

(pH 3.75) (a 2 *M* acetic acid solution was adjusted to pH 3.75 with solid potassium hydroxide), and sodium 1-heptanesulphonate was added to 1 m*M*. The flow-rate was 0.8 ml/min. The fluorescence intensity at 418 nm was monitored with excitation at 318 nm. The peak area of the fluorescent peak was calculated with a Shimadzu Chromatopac, CR-3A. Quantitation of PLP was carried out by comparison with the peak area of standard PLP treated in the same way as samples. The excitation and emission spectra of the PLP-cyanide derivative were measured in a Shimadzu corrected recording spectrofluorophotometer, RF-502.

## RESULTS AND DISCUSSION

### *Properties of PLP-cyanide derivative*

As shown in Fig. 1, the excitation and emission maxima of the PLP-cyanide derivative were found to be at 318 and 418 nm, respectively. The maximum flu-

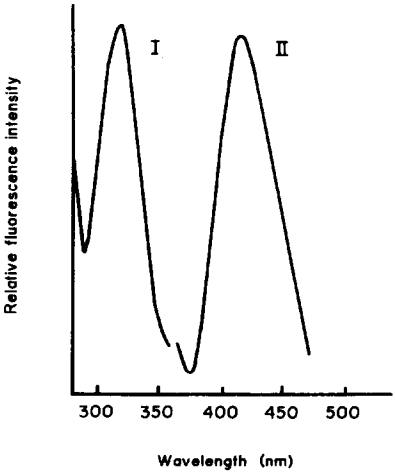


Fig. 1. Excitation and emission spectra of the PLP-cyanide conjugate. PLP (1 nmol) was treated with potassium cyanide in a ten-fold larger reaction mixture as described in Experimental, then the supernatant of the reaction mixture was diluted with nine volumes of 2 *M* potassium acetate buffer (pH 3.75) containing 1 m*M* sodium 1-heptanesulphonate. The excitation spectrum (curve I) was measured with the emission wavelength at 418 nm, and the emission spectrum (curve II) was measured with excitation at 318 nm.

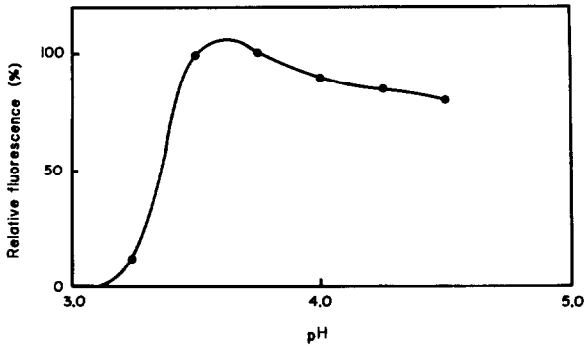


Fig. 2. Effect of pH on the fluorescence intensity of the PLP-cyanide derivative. PLP was treated as described in Fig. 1, and 2 *M* potassium acetate buffer from pH 3.25 to 4.5 was used.

orescence intensity of the derivative was found at pH 3.7, and it remained high up to pH 4.5 (Fig. 2). The fluorescent compound was stable for two or three days when stored in an ice-bath.

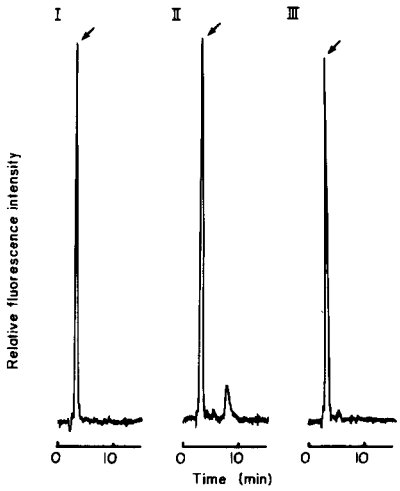


Fig. 3. HPLC patterns of the cyanide derivatives of standard PLP and PLP in gray and white matter of a human brain. Standard PLP (10 pmol) and gray and white matter of a human brain (300 and 325 $\mu$g protein, respectively) were treated as described in Experimental. A 5-$\mu$l volume of the reaction mixture was applied to the HPLC apparatus. (I) Standard PLP; (II) gray matter; (III) white matter.

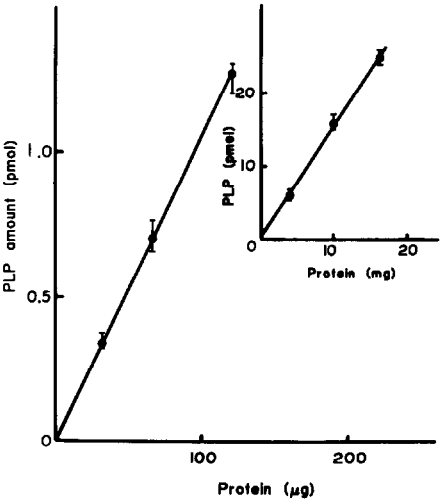


Fig. 4. Relation of protein amount of human brain gray matter to PLP amount. Homogenate of human brain gray matter (25 $\mu$g to 20 mg protein) was treated with potassium cyanide as described in the text. Each point represents the mean and S.D. of triplicate measurements of two experiments.

TABLE I

PLP CONTENTS OF HUMAN BRAIN GRAY AND WHITE MATTER, AND PHEOCHROMOCYTOMA PC12h CELLS

Each value is a result of triplicate measurements of three experiments.

| Sample | PLP content (pmol/mg of protein) |
|---|---|
| Gray matter | 17.1 ± 2.5 |
| White matter | 13.2 ± 0.4 |
| PC12h cells | 38.2 ± 7.8 |

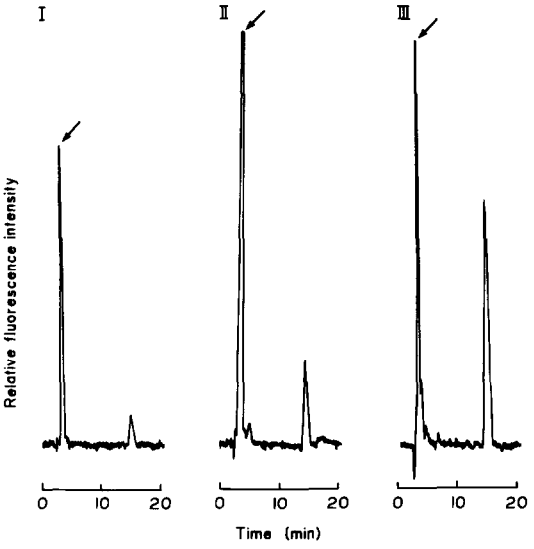


Fig. 5. HPLC patterns of standard PLP and the PLP-cyanide derivative from purified DDC and from PC12h cells. Purified DDC (1.05 μg protein) and PC12h cells (80 μg protein) were treated with potassium cyanide as described in the text. (I) Standard PLP; (II) purified DDC; (III) PC12h cells.

*Chromatography*

Fig 3 shows the HPLC patterns of the PLP-cyanide derivatives from standard PLP and homogenates of human brain gray and white matter. The peak of the PLP-cyanide derivative was well separated from other fluorescent peaks. Addition of sodium 1-heptanesulphonate was required to elute fluorescent compounds other than the PLP-cyanide derivative from the column. Fluorescent contaminations were eluted well after the peak of PLP-cyanide derivative. The sensitivity of the method was high enough to measure 50 fmol of PLP, when the limit of sensitivity was defined as a signal-to-noise rate of 5. The linearity of the fluorescence intensity to PLP amounts in the reaction mixture was confirmed up to 10 nmol. This assay procedure was applicable to the sample amounts containing from 100 μg to 20 mg of protein in the case of human brain gray matter, as shown in Fig. 4. Table I summarizes the PLP contents in human brain gray and white matter and in cells of a tissue-cultured cell line, clonal rat pheochromocytoma

PC12h. Fig. 5III shows the HPLC pattern of the PLP-cyanide derivative from PC12h cells.

Enzymes containing PLP as a cofactor were analysed for their PLP content. Only 1 $\mu$g of protein of DDC highly purified from bovine adrenal medulla was required to determine the PLP content (Fig. 5II). The PLP content of DDC was $20.2 \pm 2.5$ nmol/mg of protein, and the minimum molecular mass of DDC was calculated to be 49.6 kDa. The PLP content in phosphorylase-a was $3.28 \pm 0.35$ nmol/mg of protein, and the value was almost the same as that reported by Adams [6]: 3.41 nmol/mg of protein.

The method reported here is an HPLC application of the method reported by Adams [6]. The sensitivity for detection of the fluorescent derivative of PLP with cyanide is increased almost a thousand-fold, and interferences from other fluorescent compounds in crude samples can be removed. In addition, by reduction of the size of the reaction system, the amount of purified enzymes required for analysis of PLP concentration could be reduced markedly. This method should have a broad application to the analysis of PLP in minimal amounts of samples.

ACKNOWLEDGEMENT

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science and Culture, Japan.

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