

A fluorimetric method for the measurement of pyridoxal and pyridoxal phosphate in human plasma and leucocytes, and its application to patients with sideroblastic marrows

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SUMMARY A highly sensitive fluorimetric assay for the measurement of pyridoxal and pyridoxal phosphate in biological tissues is described. The method involves the enzymic hydrolysis of pyridoxal phosphate to pyridoxal. The pyridoxal (free or total) is separated on an anion-exchange column, concentrated by cation-exchange chromatography and reacted with potassium cyanide under slightly alkaline conditions to form 4-pyridoxolactone, a highly fluorescent compound. The method is applied to the measurement of pyridoxal, pyridoxal phosphate and total pyridoxal in plasma and neutrophils from control subjects and patients with sideroblastic marrow and identified the patient with pyridoxine-responsive sideroblastic anaemia.

Pyridoxal 5'-phosphate, the coenzyme form of pyridoxine (vitamin B₆) plays an important role in numerous biochemical reactions. The pyridoxal content of tissues is regulated by a number of factors including plasma membrane transport,¹ phosphorylation of free pyridoxal² and by binding of the coenzyme to apoprotein.^{3,4} Pyridoxal phosphate concentrations are controlled by pyridoxal phosphate phosphatases^{5,6} which hydrolyse unbound pyridoxal phosphate to pyridoxal.

Although pyridoxine deficiency has been implicated in a number of diseases, including sideroblastic anaemia,^{7,8} alcoholism^{9,10} and liver disease¹¹ detailed investigations of the plasma and tissue pyridoxal and pyridoxal phosphate concentrations are not generally carried out. This is, in part, due to the lack of a suitable assay system which can be easily established in a clinical laboratory. Methods involving the spectrophotometric measurement of pyridoxal¹² lack sensitivity and are unsuitable for use with unpurified tissue homogenate. Sensitive methods have been developed for the assay of pyridoxal phosphate using radiometric assays with apotyrosine decarboxylase^{13,14} or apotryptophanase.¹⁵ However, these assays are technically quite difficult and do not permit the assay of free pyridoxal in the samples. The most sensitive method available for measuring both pyridoxal and

pyridoxal phosphate in biological samples is the microbiological assay method using *Lactobacillus casei*.¹⁶ Although the method is highly sensitive, it is very time-consuming and requires specialised expertise and equipment not available to the majority of laboratories.

This paper presents a sensitive fluorimetric assay for the estimation of pyridoxal and pyridoxal phosphate in biological samples. The assay is based on hydrolysis of pyridoxal phosphate by acid phosphatase followed by ion-exchange chromatography and oxidation of the pyridoxal to fluorescent 4-pyridoxolactone.

Material and methods

MATERIAL

Pyridoxal hydrochloride, pyridoxal phosphate and acid phosphatase (type II from potato), were purchased from Sigma London Ltd. Cation-exchange resin AG50W-X2, 100-200 mesh, H⁺ form, and anion exchange resin AG1-X8, 100-200 mesh, Cl⁻ form, were purchased from Bio-Rad Laboratories Ltd. All other reagents were of Analar grade.

METHODS

Preparation of ion-exchange columns

Anion-exchange column Anion-exchange resin AG1-X8 was washed with 20 vol 1 M NaOH, equilibrated with 2 vol 1 M acetic acid and

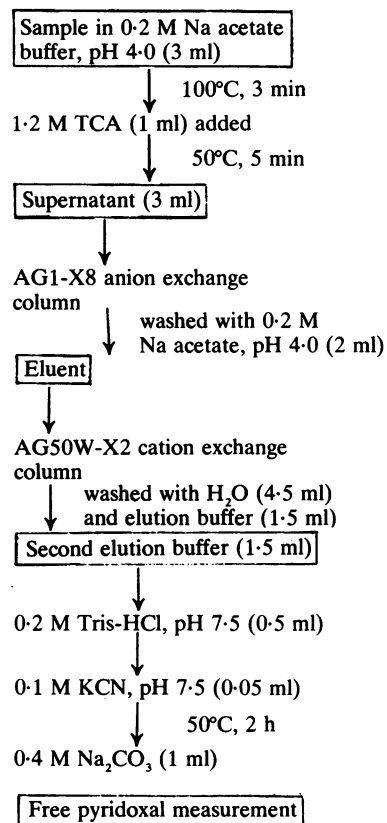
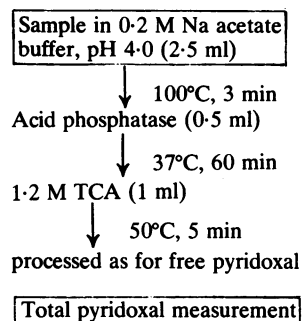
Pyridoxal*Pyridoxal phosphate*

Fig. 1 Flow diagram of assay for measurement of free and total pyridoxal. Pyridoxal phosphate was determined by difference. Further details in methods section. TCA = trichloroacetic acid.

repeatedly washed with distilled water until the effluent was less than pH 4.0. A 5 cm column was prepared in a Pasteur pipette (ID 5 mm) plugged with a 4 mm glass bead.

Cation exchange column Cation-exchange resin AG50W-X2 was equilibrated with 1 M NaOH and repeatedly washed with distilled water until the effluent was less than pH 7.0. A 2 cm column was prepared in a Pasteur pipette as described for the anion-exchange column. Sodium acetate buffer, 1 M, pH 4.0, was passed through the column until the effluent was pH 4.0.

Preparation of samples

Human polymorphonuclear leucocytes were isolated by dextran sedimentation and Ficoll-Hypaque centrifugation from 50 ml blood as previously described.¹⁷ The plasma was removed, boiled for 3 min to destroy enzyme activities and retained for analysis. The isolated cells were resuspended in 4.5 ml 0.2 M Na acetate-acetic acid buffer pH 4.0, and

disrupted in a Dounce homogeniser by 30 strokes of a tight fitting (type B) pestle. An aliquot was retained for protein determination and the remainder boiled for 3 min.

Estimation of free pyridoxal

Boiled plasma or neutrophil homogenate (2 ml) was mixed with 0.2 M Na acetate buffer, pH 4.0 (1 ml), and 1 ml 1.2 M trichloroacetic acid. The samples were heated at 50°C for 5 min to ensure complete precipitation of the protein. After centrifugation at 800 g for 5 min, the supernatant was removed and retained for analysis.

Estimation of total pyridoxal

Boiled plasma or neutrophil homogenate (2 ml) was added to 0.2 M Na acetate buffer pH 4.0 (1 ml) containing 0.34 milliunits of acid phosphatase and incubated at 37°C for 60 min. This hydrolysed all the pyridoxal phosphate in the sample to pyridoxal. After cooling, 1 ml 1.2 M trichloroacetic acid was

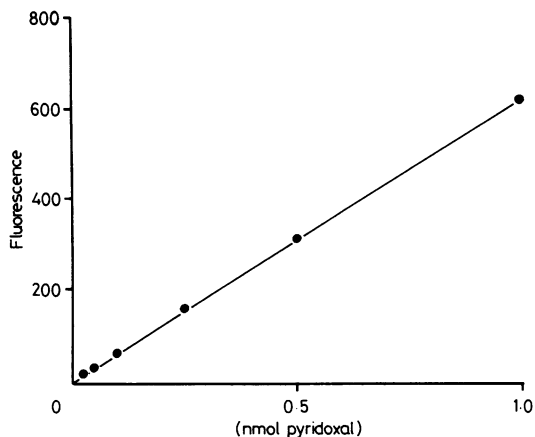


Fig. 2 Calibration curve for fluorescent assay of pyridoxal.

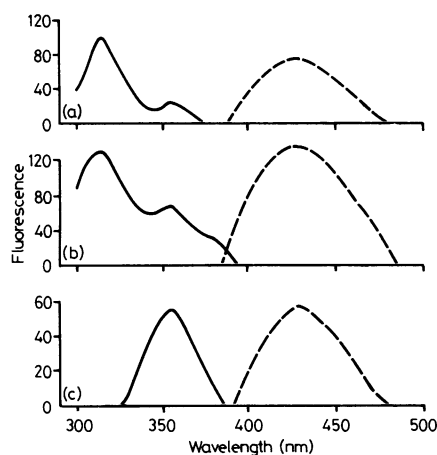


Fig. 3 Excitation (—) and emission (---) spectra for (a) neutrophil extract; (b) plasma extract; (c) authentic pyridoxal. The spectra were recorded after treatment with alkaline potassium cyanide.

added and the samples treated exactly as for the estimation of free pyridoxal. The difference between total and free pyridoxal was pyridoxal phosphate.

Fluorimetric determination of pyridoxal in tissue extracts

The procedure for the measurement of pyridoxal in tissue extracts is outlined in Fig. 1. A previously prepared 5 cm anion-exchange column was mounted above each cation-exchange column so that the effluent from the first column ran directly onto the second column. An extract of plasma or neutrophils, 3 ml, was loaded onto the anion exchange column and eluted with 2 ml 0.2 M Na acetate buffer, pH 4.0. The eluent from the first column then passed directly onto the cation-

exchange column. At this stage the first column was removed and discarded and the cation-exchange column eluted sequentially with 4.5 ml distilled water and with two portions of 1.5 ml 20 mmol Tris-HCl containing 0.1 M KCl, pH 9.0 (elution buffer). The second 1.5 ml eluate was retained for analysis. To the standards and unknown samples of pyridoxal (0.025–1.0 nmol in 1.5 ml elution buffer) were added 0.2 M Tris-HCl, pH 7.5 (0.5 ml) and 0.1 M KCN in 0.1 M Tris-HCl, pH 7.5 (0.05 ml). After thorough mixing, the tubes were heated in the dark for 2 h at 50°C in a Tecam dry bath. After cooling, 0.4 M Na₂CO₃ (1 ml) was added to each of the tubes and the fluorescence determined with 355 nm for excitation and 430 nm for emission wavelengths. A typical standard curve for the determination of pyridoxal is shown in Fig. 2. Suitable blanks were prepared with each batch of assays.

Fig. 3 compares the excitation and emission spectra of the derivitised authentic pyridoxal and plasma and leucocyte extracts processed for the assay of total pyridoxal. Authentic pyridoxal gives an excitation maximum of 355 nm with an emission maximum at 430 nm. The processed plasma and leucocyte extracts give excitation maxima at 315 nm and 355 nm. The emission maximum when excited at 355 nm was 430 nm. The nature of the compound excited maximally at 315 nm is unclear. However, it shows an emission maximum at 400 nm and clearly does not interfere with the fluorimetric measurement of pyridoxal. Similar conclusions were reached for estimation of both free and total pyridoxal in both plasma and neutrophil extracts.

Results

Hydrolysis of pyridoxal phosphate by acid phosphatase

A UV-visible spectrum of the conversion of pyridoxal phosphate to pyridoxal by acid phosphatase is shown in Fig. 4. After 60 min incubation all the pyridoxal phosphate had been hydrolysed to pyridoxal. This sample contained approx 25-fold the level of pyridoxal phosphate usually found in the tissue extracts.

Anion-exchange column

The purpose of the anion-exchange column was to remove contaminating substances in the tissue extracts which could interfere with the anion exchange column or the fluorimetric determination of pyridoxal. The major contaminant in the extracts was the trichloroacetic acid added to precipitate the protein. A series of tissue extracts were prepared and adjusted to pH 1.4. Each extract was loaded onto anion exchange columns of varying length and

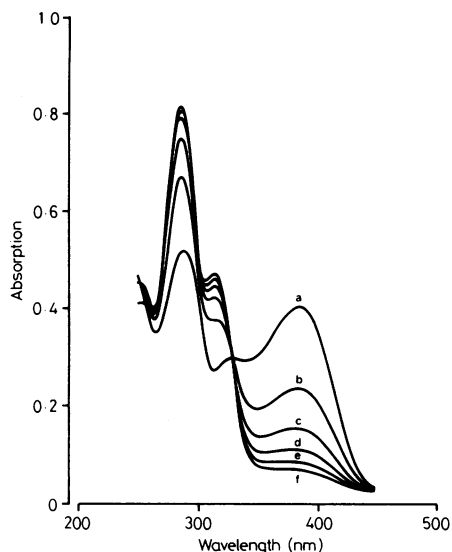


Fig. 4 Sequential UV-visible spectra of pyridoxal released from pyridoxal-5'-phosphate by acid phosphatase. Pyridoxal-5'-phosphate, 0.25 nmol, was incubated at 37°C with 0.34 milliunits of acid phosphatase in 0.2 M Na acetate-acetic acid buffer, pH 4.0. Spectra determined in a Perkin Elmer, model 557, dual beam spectrophotometer at (a) prior to addition of the enzyme, and after (b) 15 min, (c) 30 min, (d) 45 min, (e) 60 min and (f) 75 min.

the pH of the eluent measured. All columns less than 4 cm in length failed to remove the trichloroacetic acid and the pH of the effluents remained less than 4.0. A 5 cm column removed all trichloroacetic acid from the sample and the effluent (pH 4.0) was suitable for direct loading onto the cation exchange column.

Cation-exchange column

Under the conditions of assay pyridoxal forms a highly fluorescent product when oxidised with alkaline potassium cyanide. Unfortunately, pyridoxal phosphate, under the same conditions, also gives rise to a fluorescent product with an emission intensity one-sixth of that produced by pyridoxal. Therefore, in order to accurately assay pyridoxal a separation procedure was required. At pH 4.0 pyridoxal binds to the cation exchange resin whilst pyridoxal phosphate does not. The UV-visible spectra of a typical elution are shown in Fig. 5. All (99%) the pyridoxal was eluted in the second 1.5 ml of elution buffer with no further material present in the subsequent 1.5 ml of elution buffer. The recovery of pyridoxal over the entire procedure was 98-105%. There was no increase in recovery of pyridoxal after the addition of pyridoxal phosphate at the fluorescent step.

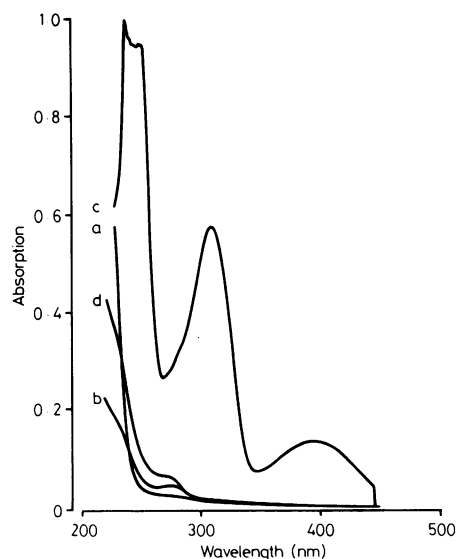


Fig. 5 UV-visible spectra of eluents from cation-exchange column. Pyridoxal, 0.5 nmol was loaded onto the dual anion-cation exchange columns as described in the text. The cation exchange resin was eluted sequentially with: (a) 4.5 ml H₂O; (b) 1.5 ml elution buffer (first aliquot); (c) 1.5 ml elution buffer (second aliquot); (d) 1.5 ml elution buffer (third aliquot). Spectra determined in a Perkin Elmer, model 557, dual beam spectrophotometer.

Levels of pyridoxal and pyridoxal phosphate

Using this assay, the level of pyridoxal and pyridoxal phosphate was measured in plasma and neutrophils from control subjects and a small number of patients with sideroblastic marrow (Table). Note that in plasma pyridoxal is largely present as the free vitamin whereas in neutrophils pyridoxal phosphate is twice the level of pyridoxal. In the patient with pyridoxine-responsive sideroblastic anaemia, the concentrations of both forms of the vitamins in plasma and neutrophils were reduced when compared to control subjects. However, in patients with a sideroblastic marrow associated with alcoholism or preleukaemia who did not respond to pyridoxine therapy the concentrations in neutrophils, with the exception of the pyridoxal in one patient, were normal. In plasma the concentrations of pyridoxal and pyridoxal phosphate varied widely. All sideroblastic patients were treated with pyridoxine (100 mg daily) and their plasma and neutrophil levels re-estimated after one month of treatment. In all patients the concentrations of pyridoxal and pyridoxal phosphate were raised, although the increase in neutrophils was not as striking as that found in plasma.

Concentrations of pyridoxal and pyridoxal phosphate in plasma and neutrophils from control subjects and patients with sideroblastic marrow

Patient	Haematological response to pyridoxine	Plasma			Neutrophils		
		Pyridoxal	Pyridoxal phosphate	Total	Pyridoxal	Pyridoxal phosphate	Total
<i>Pretreatment</i>							
A	Yes	<0.5	3.45	3.45	4.19	3.6	7.79
B	No	23.7	<0.5	23.7	8.14	15.7	23.8
C	No	2.16	19.5	21.6	8.69	13.0	21.7
D	No	17.5	8.21	25.7	<1.0	16.9	16.9
<i>On treatment</i>							
A		133	382	515	16.4	34.8	51.5
B		304	51.8	356	34.2	27.7	61.9
D		46.2	23.6	69.6	26.3	30.0	56.3
Control subjects (mean \pm SD) (5)		14 \pm 9.6	5.60 \pm 6.7	20.0 \pm 6.6	7.6 \pm 2.5	13.4 \pm 8.1	20.6 \pm 8.6
Normal range		5.5-30.0	0-16.4	10.0-30.0	4.0-10.3	3.2-23.2	12.6-33.5

Concentrations in plasma are expressed as pmol/ml and in neutrophils as pmol/mg protein.

Discussion

The present study has shown that pyridoxal and pyridoxal phosphate concentrations can be reliably measured in plasma and neutrophils by this fluorimetric technique. The assay is highly sensitive and can be established in any laboratory without the need for specialised equipment or techniques and is clearly applicable to the study of pyridoxal and pyridoxal phosphate metabolism in a variety of clinical conditions. Although the importance of pyridoxal phosphate in metabolism is well established, little is known about its role in disease. As well as being implicated in certain patients with sideroblastic anaemia,^{7,8,18} a deficiency of pyridoxal phosphate has also been associated with such diverse conditions as arteriosclerosis,¹⁹ convulsions,²⁰ toxæmia of pregnancy,²¹ alcoholism^{9,10} and liver disease.¹¹ A simple method for investigating the concentrations of both plasma and tissue pyridoxal and pyridoxal phosphate would be of considerable use in the further investigation of these conditions. In this preliminary study of patients with sideroblastic marrows it seems likely that this could be useful in the diagnosis of responsive and non-responsive sideroblastic anaemia.

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