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A Simplified Method for Determination of Pyridoxal Phosphate in Biological Samples

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ABSTRACT

A new method for the determination of pyridoxal phosphate in plasma and in cerebrospinal fluid is described. It is based on the generally accepted apotyrosine decarboxylase method, but, in stead of following the decarboxylation rate using ^{14}C- och ^{3}H-labelled tyrosine, a high performance liquid chromatography (HPLC) method for separation of tyrosine and tyramine is used.

This procedure is recommended as it is less time consuming and avoids utilizing radioactive material.

INTRODUCTION

The level of pyridoxal phosphate in plasma and in cerebrospinal fluid lies in lower nanomolar range per liter, hence sensitive assay procedures must be utilized. These have most often been based on using pyridoxal phosphate as coenzyme in a pyridoxal phosphate dependent enzymatic reaction. The apoenzyme has been prepared and the conversion of the substrate traced. By adding varying amounts of pyridoxal phosphate the rate of enzymatic reaction has been calculated and a calibration curve obtained.

The enzyme used has often been tyrosine decarboxylase, the apoenzyme of which is rather easily prepared. The first description of this enzyme reaction for this purpose was by Boxer and al. in 1957 (2), who followed the reaction rate by measuring the carbon dioxide produced in a Warburg equipment. The method was made more sensitive by using tyrosine-1-^{14}C (3), later changed to a mixture of tyrosine-1-^{14}C and ^{3}H-tyrosine, determining the ^{14}C and ^{3}H activity at different time intervals in a dual channel liquid scintillation counter. In other methods the $^{14}CO_2$ has been collected in potassium hydroxide or hyamine and the radioactivity determined (5). The aim of this paper is to describe a method for determining the tyrosine decarboxylase reaction rate by using high

performance liquid chromatography (HPLC) for the separation of tyrosine and tyramine. This method for pyridoxal phosphate is compared with the one using tyrosine-1-^{14}C and tyrosine-^{3}H.

MATERIAL AND METHODS

The material used is EDTA-blood plasma and cerebrospinal fluid. The proteins of the samples are first precipitated by adding 100 µl trichloroacetic acid to 800 µl sample. The precipitate is centrifuged by 800 g for 10 minutes. The trichloroacetic acid is extracted from the supernatant with 5 ml ethyl ether to 0.3-0.6 ml supernatant. After three extractions the supernatant is used for determination of pyridoxal phosphate.

Tracer method

To 150 µl of sample with an expected concentration of pyridoxal phosphate 0-200 nmol/l is added 150 µl apotyrosine decarboxylase (about 10 mg/ml). This mixture is shaken for about 30 minutes at 30°C. Then a mixture of tyrosine-^{3}H, tyrosine-1-^{14}C, tyrosine and buffer is added and exact time taken. After 10, 20 and 30 minutes samples of 200 µl are withdrawn and poured into testtubes kept in water at 100°C. 25 µl samples from the tubes are added to 10 ml scintillation fluid (POP and POPOP toluene) and the radioactivity of ^{14}C and ^{3}H determined in a liquid scintillation counter. The ^{3}H activity is not changed by the enzyme reaction, whereas the ^{14}C activity decreases due to the decarboxylation. The rate of decarboxylation is correlated to the concentration of pyridoxal phosphate in the sample. More details about this method are given in reference (4).

High performance liquid chromatography method

In this method the increase of tyramine is determined instead of the decrease in tyrosine. The preparation of the samples is performed in the same way as in the tracer method described, but no radioactive tracers are added. After the reaction is stopped in the heated testtubes, samples of 60 µl are injected into a HPLC equipment (LDC Constametric III with LDG dynamic gradient mixer and with Water 710A WISP sample delivery system). Columns MPLC RP-18.3 cm and 10 cm are used. The extraction solution is 0.07 molar sodium acetate buffer pH 3.75. We use a Perkin Elmer spectrofluorometer MPF-3 with excitation wavelength 280 nm and emission wavelength 353 nm. A separation of tyrosine and tyramine is achieved as shown in Fig. 1.

In the chromatogram are found peaks for tyrosine and tyramine and a third peak which is recognized as serotonin. The computer connected to the system makes possible the determination of the tyrosine and tyramine.

A calibration curve for the content of pyridoxal phosphate in the sample is drawn as shown in Fig. 2.

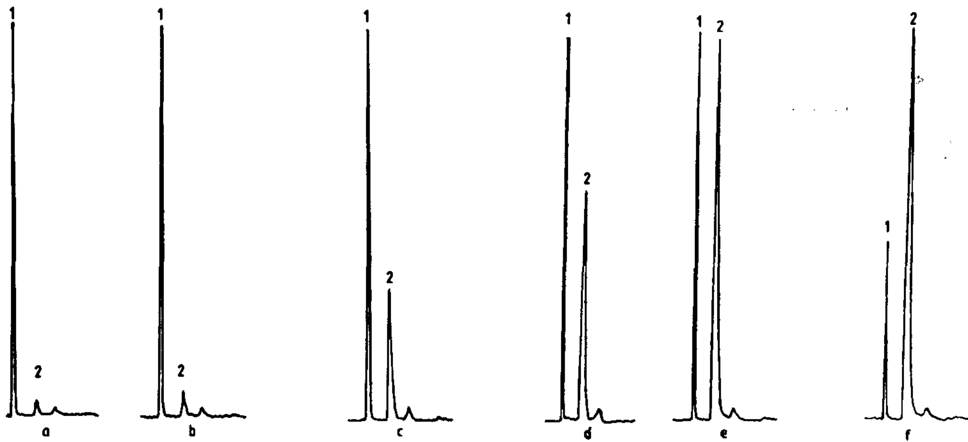

Fig. 1. Samples with pyridoxal phosphate levels 0, 20 and 200 nmol/l give tyrosine and tyramine peaks as shown in a–f. The first peak is tyrosine, the second tyramine. In a and b with no pyridoxal phosphate added to the reagents are shown the curves after incubation for 15 and 30 minutes. In c and d with pyridoxal phosphate 20 nmol/l, and in e and f 200 nmol/l, incubation time 15 and 30 minutes respectively.

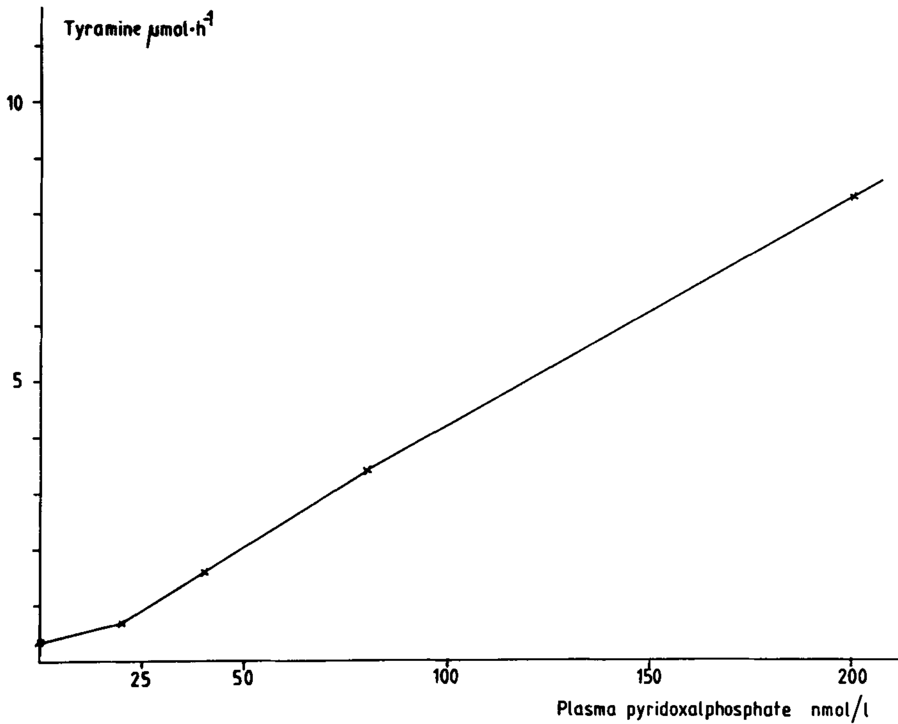

Fig. 2. Calibration curve for the HPLC method.

RESULTS

A comparison between the tracer method and the HPLC method is shown in Fig. 3.

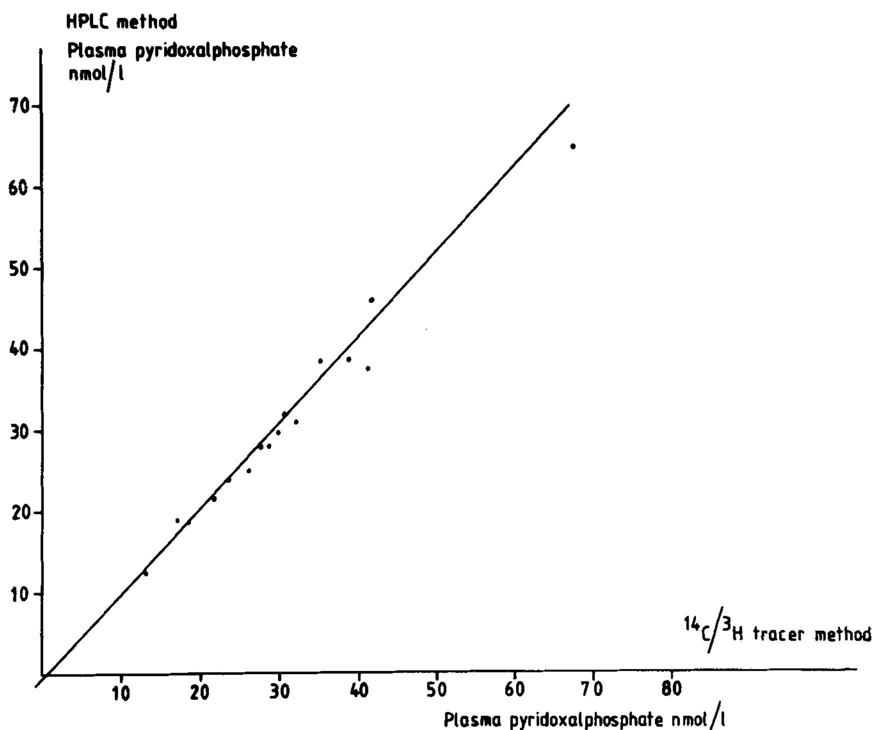

Fig. 3. Correlation between the method using HPLC and the tracer method for pyridoxal phosphate.

The correlation coefficient is r = 0.9864. The coefficient of variation (CV %) for the tracer method is 9.1 (mean value 32.4 nmol/l pyridoxal phosphate in 50 double determinations in plasma from healthy blood donors) and for the HPLC method 5.4 (mean value 32.1 nmol/l in 37 double determinations).

DISCUSSION

Although vitamin B_6 has been known for 50 years, not until recently its importance has been fully understood. Thus it is desirable to have an accurate and not too time-consuming method for determination of the active part of vitamin B_6, i.e. pyridoxal phosphate. The method described here is rather simple and, in addition, avoids using radioactive material. The HPLC stage in the analysis is easily automatized with computerized calculations. This makes the determination more exact and more convenient

for concentration analysis in systems with still lower levels than in blood, e.g. cerebrospinal fluid. The levels of pyridoxal phosphate in plasma and in spinal fluid from patients with neurological symptoms utilizing the method described here are given in reference 1.

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