Journal of Chromatography, 146 (1978) 485—489
Biomedical Applications
© Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 214

Note

New method for quantitative analysis of pyridoxal-5'-phosphate in biological material

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(Received April 10th, 1978)

The chemical methods in use for determination of pyridoxal-5'-phosphate (PLP) in biological samples are all based on initial rate studies of enzyme-catalyzed reactions where PLP is necessary as a co-factor. By using well-resolved apoenzyme preparations and proper reaction conditions it is possible to determine quite low PLP concentrations from the linear dependence usually obtained between reaction rate and PLP concentration. In the most commonly used method, the rate of $^{14}CO_2$ formation from (L)-(-)-tyrosine-$^{14}C_1$ under the influence of PLP-stimulated tyrosine apodecarboxylase is estimated by trapping of the liberated $^{14}CO_2$ and liquid scintillation counting [1—5]. A method recently published by Suelter et al. [6] makes use of apotryptophanase and UV determination of liberated *o*-nitrothiophenolate from a synthetic chromogenic substrate, S-*o*-nitrophenyl-L-cysteine.

In connection with investigations concerning the purification of tyrosine apodecarboxylase (TAD) from *Streptococcus faecalis* [7], a need arose for a simpler and more reliable method for enzyme activity determinations. Because the enzyme was known to exhibit activity towards L-3,4-dihydroxyphenylalanine (L-DOPA) [8], a quantification of the dopamine produced by the reaction by means of chromatographic separation and amperometric detection (LCEC) [9], was considered as favourable in view of the extreme sensitivity of

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the electrochemical detector [10,11]. In this paper we present some results which clearly demonstrate certain advantages of this method over the previously used radioenzymatic one.

MATERIALS AND METHODS

Conditions for the enzymatic decarboxylation

Enzyme preparation. In all experiments the reaction mixture was completely homogeneous. For this purpose a TAD preparation, obtained using the prepurification procedure previously described [7] for the commercially obtained cell material (No. T 4629; Sigma, St. Louis, Mo., U.S.A.) was used throughout. This apoenzyme was shown to possess very little decarboxylase activity in the absence of any added PLP, indicative of the high resolution achieved.

Sample preparation. The standard curves were generated by the use of PLP-solutions obtained by dilutions (1:4 to 1:16) of a common stock solution containing 96 ng/ml. Plasma (1.5 ml) was deproteinized with 75% trichloracetic acid (150 μl) and centrifuged. A 700-μl sample of the supernatant was diluted with 800 μl of 0.1 *M* sodium acetate buffer pH 5.5 and 1.0 ml was diluted to 2 ml with 1 *M* acetate buffer pH 6.5. The PLP standards were treated similarly.

Kinetic procedure. The reaction was carried out in small, stoppered centrifuge tubes at 30.0°. Each tube was first supplied with 700 μl of the TAD-preparation and 300 μl of the PLP-containing sample. After 1 h of pre-incubation, 400 μl of a 4 m*M* solution of L-DOPA in 0.1 *M* acetate buffer was quickly added and a chronometer started. At the time t, 200 μl of the reaction mixture was rapidly quenched with 800 μl of 0.3 *M* perchloric acid. After centrifugation this solution was then subjected to LCEC analysis. If the analysis was not carried out immediately, the acid quenching solution was permitted to contain a small amount of antioxidant such as bisulphite or mercaptoethanol.

Chromatographic procedure

Instrumentation. The LCEC equipment was constructed from an Altex Model 100 constant-flow solvent pump, a Rheodyne Model 7120 injection valve provided with a 20 μl loop, an Altex 250 × 4.6 mm stainless-steel column, slurry-packed with 10 μ Nucleosil SA, a spherical, surface-porous cation exchanger, an electrochemical detector cell packed with silicone-oil-based graphite paste and equipped with a 50 μ PTFE spacer, a reference electrode compartment, an operational amplifier capable of converting 1nA to 1V and a Linear Model 264 potentiometric recorder. The detector and amplifier parts were obtained from Bioanalytical Systems (West Lafayette, Ind., U.S.A.). The column was separated from the pump by means of a 5 m × 1/16 in. O.D. PTFE coil and the potentiostat was operated at 0.55 V vs. the Ag—AgCl-reference electrode. The column, detector and amplifier were all contained within a Faraday-cage and carefully grounded in order to minimize electrical noise [11].

Chromatographic conditions. A citrate—acetate buffer of pH 5.2 was used throughout. This was prepared from 23.0 g of citric acid, 16.6 g of anhydrous sodium acetate, 4.20 ml of acetic acid and 9.6 g of sodium hydroxide dissolved in 4000 ml of permanganate-distilled water. During chromatography the buffer was heated to 45° in order to avoid air-bubble formation in the detector

cell. Flow-rate was set at 0.60 ml/min in all cases, unless otherwise stated.

Chemicals. All chemicals used were of analytical grade quality. The buffer chemicals were obtained from Merck (Darmstadt, G.F.R.) and pyridoxal-5'-phosphate and 3,4-dihydroxyphenylalanine from Sigma (St. Louis, Mo., USA).

RESULTS AND DISCUSSION

The chromatographic result from a typical kinetic run in which a plasma sample provided the PLP source is shown in Fig. 1. When the dopamine (DA) peak heights were plotted against the reaction time a perfectly straight regression line was obtained (Fig. 2.). The least-squares-fit showed a correlation coefficient of 0.9980. From Fig. 1 it is quite evident that the corresponding loss in L-DOPA concentration is less than 5%, which means that the reaction is essentially of pseudo-zero order.

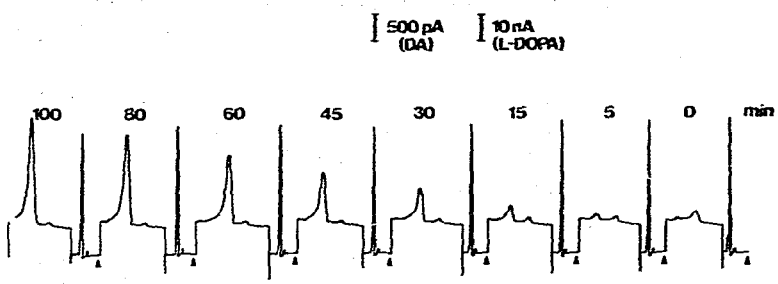

Fig. 1. Chromatographic recordings showing the progress of DA (left) formation in a plasma PLP-stimulated enzymatic decarboxylation of L-DOPA (right). The small, middle peak of each chromatogram corresponds to a hitherto unidentified component from the plasma.

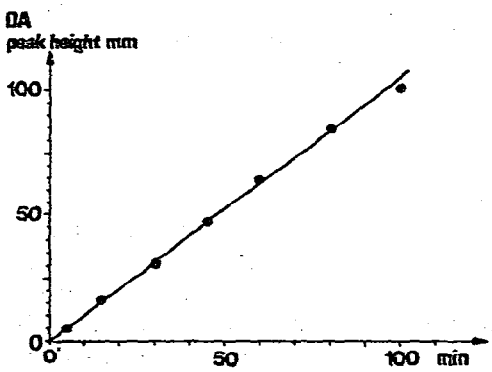

Fig. 2. Linearity obtained by graphic evaluation of the results shown in Fig. 1, illustrating the precision of the method for the initial rate determinations.

In another run the reactions were quenched at a single reaction time of 45 min (Fig. 3). A plot of the DA peak height obtained against the concentration of the standard PLP solutions used, gave a linear standard curve. Linear regression by the least squares method gave a correlation coefficient of 0.9992. This standard curve covers the normal range of plasma PLP which has been found by earlier investigators [1,3] to be within 5—20 ng/ml. The relative standard

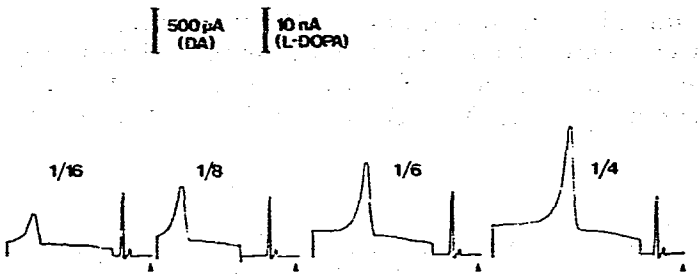

Fig. 3. Chromatographic recordings showing the relative amounts of DA formed at different PLP concentrations after a fixed reaction time of 45 min. Reaction conditions were otherwise identical.

deviation obtained upon repeated analyses of PLP from the same plasma pool with the use of a single reaction time (45 min) amounts to 6%. The recovery of PLP added to plasma was not investigated, because it has been shown earlier [1,3] to be ca. 90%.

Under the conditions used the retention volumes for L-DOPA and DA are 4.8 ml and 10.9 ml, respectively, which means that at a flow-rate of 0.60 ml/min one chromatogram will require ca. 20 min. We have found, however, that the flow-rate can be increased considerably, to speed up the analysis, with no other disadvantage than the accompanying rise in pressure.

It should be emphasized that because of the very small amounts required for each injection and the dilution made upon the quenching, all volumes described in the sample preparation and kinetic procedures can be scaled down considerably to suit the particular needs of a micro-method.

The sensitivity of the method, however, is highly dependent upon the quality of the apoenzyme. In our investigation we have found that under the conditions used, an over-all volume reduction will permit the analysis of 100 µl plasma, i.e. the method is sensitive enough for 1 ng of PLP and even less.

CONCLUSION

The use of high-performance liquid chromatography with LCEC for the monitoring of PLP-dependent, enzymatic decarboxylation of L-DOPA to DA, has been shown to provide an excellent method for the quantitative determination of PLP in biological fluids, such as plasma or serum. The method makes use of resolved and partially purified tyrosine apodecarboxylase (E.C. 4.1.1.25) from *Streptococcus faecalis*, a PLP enzyme which is sufficiently active towards L-DOPA as a substrate to permit initial rate determinations to be carried out with high precision, even at very low PLP concentrations. Because of the great selectivity and sensitivity inherent in the LCEC procedure, the reaction product, DA, can be quantitated in very low amounts, a fact which obviates the earlier need of a radiolabelled substrate. A further advantage of the method over the previously used radioenzymatic procedure is found in the very easy handling and control of the reaction mixture prior to analysis, which permits samples to be withdrawn and quenched at very precise time intervals, a prerequisite for accurate kinetic studies.

Our results have shown that this method for PLP determination is reliable, uncomplicated and easy to perform with a high degree of precision. In our opinion it is in many respects superior to the radioenzymatic procedure. It may also be suggested that the LCEC technique should be a very valuable tool for the study of selected enzyme-catalyzed steps in the area of tyrosine as well as tryptophan metabolism. Work in this field has recently been reported [12] and is also in progress in our laboratory.

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