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HIGHLY SENSITIVE ASSAY FOR TYROSINE HYDROXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A highly sensitive assay for tyrosine hydroxylase (TH) activity by high-performance liquid chromatography (HPLC) with amperometric detection was devised based on the rapid isolation of enzymatically formed DOPA by a double-column procedure, the columns fitted together sequentially (the top column of Amberlite CG-50 and the bottom column of aluminium oxide). DOPA was adsorbed on the second aluminium oxide column, then eluted with 0.5 *M* hydrochloric acid, and assayed by HPLC with amperometric detection. D-Tyrosine was used for the control. α-Methyldopa was added to the incubation mixture as an internal standard after incubation. This assay was more sensitive than radioassays and 5 pmol of DOPA formed enzymatically could be measured in the presence of saturating concentrations of tyrosine and 6-methyltetrahydropterin. The TH activity in 2 mg of human putamen could be easily measured, and this method was found to be particularly suitable for the assay of TH activity in a small number of nuclei from animal and human brain.

INTRODUCTION

Tyrosine hydroxylase (TH, EC 1.14.16.2) is a monooxygenase which catalyzes the formation of DOPA from L-tyrosine in peripheral and central catecholaminergic neurons and chromaffin cells of the adrenal medulla [1]; its assay is frequently required for physiological and pathological studies. Because the enzyme activity is extremely low, only radioassays [1—3] have been suitable for its measurement, especially in the brain. We have recently devised a sensitive fluorometric assay of TH activity which is widely applicable to any crude tissues, including human brain [4]. In this method, DOPA formed enzymatically from L-tyrosine was isolated rapidly from interfering substances by a sequential double-column procedure (the top column of Amberlite CG-50 and the bottom column of aluminium oxide) and was assayed by an improved hydroxyindole method. The limit of sensitivity was 100 pmol DOPA. Although

this fluorometric method was the first non-isotopic assay for TH widely applicable to any crude tissues, recent progress in the neurosciences requires an extremely sensitive method which permits the assay of TH activity in less than milligram quantities of brain nuclei obtained by punching techniques. This has been somewhat difficult even by using the most sensitive radioassays with carrier-free radioactive tyrosine as substrate.

In this study we have combined the simple and specific isolation of enzymatically formed DOPA by our double-column procedure [4] with the highly sensitive assay of DOPA by high-performance liquid chromatography (HPLC) with amperometric detection [5—7]. α-Methyldopa as an internal standard was added to each sample after TH incubation [8]. Both the double columns and the high-performance liquid chromatography (HPLC) permitted nearly complete isolation of DOPA, and thus the blank values became very low. The only interfering substance is endogenous DOPA in crude tissues and non-enzymatically formed DOPA from both L- and D-tyrosine, and this blank value can be completely cancelled by the control with D-tyrosine. Use of α-methyldopa as an internal standard made the assay very accurate. TH activity in less than 1 mg of a brain nucleus could be assayed by this method. An assay method for TH by HPLC with amperometric detection was first reported by Blank and Pike [9], but our present method is simpler and much more sensitive than their method.

EXPERIMENTAL

Materials

L-Tyrosine, D-tyrosine, and 2-mercaptoethanol were obtained from Wako Chemical Company (Osaka, Japan); 6-methyl-5,6,7,8-tetrahydropterin was from Calbiochem (Los Angeles, Calif., U.S.A.); α-methyldopa was from Sigma (St. Louis, Mo., U.S.A.); catalase was from Boehringer (Mannheim, G.F.R.); Amberlite CG-50 was from Rohm and Haas (Philadelphia, Pa., U.S.A.); and aluminium oxide was from Merck (Darmstadt, G.F.R.). All other chemicals used were of analytical grade.

Rat brain stem (including medulla oblongata, pons and midbrain) was dissected. Human putamen was dissected at autopsy from a patient without a history of neurological disorders. The brains were homogenized in 4 volumes of 0.25 *M* sucrose in a glass Potter homogenizer.

6-Methyl-5,6,7,8-tetrahydropterin was used as cofactor; the 10 m*M* solution was prepared in 1.0 *M* 2-mercaptoethanol and stored at —20°, protected from light and prepared once a week. The molar concentration of 6-methyltetrahydropterin was estimated from the extinction coefficient, 18,500 M^{-1} cm^{-1} at 264 nm in 2 *M* HCl. Amberlite CG-50 and aluminium oxide were treated as described previously [4].

Experimental procedures

All experimental procedures were carried out on the scale of one-fifth of the fluorometric procedures previously reported [4]. The standard incubation mixture consisted of the following components in a total volume of 100 μl (final concentrations in parentheses): 10 μl of 1 *M* acetate buffer pH 6.0 (0.2

M), 20 μl of 1 m*M* L-tyrosine in 0.01 *M* HCl (0.2 m*M*), 10 μl of 10 m*M* 6-methyl-5,6,7,8-tetrahydropterin (1 m*M*) in 1 *M* 2-mercaptoethanol (100 m*M*), 30 μl of 0.25 *M* sucrose (75 m*M*) containing enzyme, 10 μl of 1 mg/ml catalase (10 μg/100 μl) or 10 μl of 10 m*M* ferrous ammonium sulfate (1 m*M*), and water. For the blank incubation, D-tyrosine was used as substrate instead of L-tyrosine and 50 pmol or 100 pmol of DOPA were added to another blank incubation as an internal standard for DOPA.

Incubation was done at 37° for 10 min, and the reaction was stopped with 600 μl of 0.5 *M* perchloric acid containing 50 pmol or 100 pmol of α-methyldopa as an internal standard in an ice-bath. After 10 min, 20 μl of 0.2 *M* EDTA and 300 μl of 1 *M* potassium carbonate were added to adjust the pH to 8.0—8.5, and the mixture was centrifuged at 1600 *g* for 10 min at 4°. The clear supernatant was passed through the double columns, the upper column, containing 200 μl of Amberlite CG-50 (12.5 cm × 0.5 cm I.D..), and the bottom column, containing 100 mg of aluminium oxide (12.5 cm × 0.4 cm I.D.), fitted together sequentially. The effluent through both columns was discarded. Both columns were washed once with 1.5 ml of water, and the washings were discarded, DOPA and α-methyldopa were passed through the first Amberlite column and adsorbed on the second aluminium oxide column, which was separated and washed with 1.5 ml of water twice, and with 100 μl of 0.5 *M* HCl once. DOPA and α-methyldopa were eluted with 200 μl of 0.5 *M* HCl.

A 100-μl aliquot of the eluate was injected into the high-performance liquid chromatograph (Yanaco L-2000) with an Yanaco VMD-100 voltammetric detector and a column (25 cm × 0.4 cm I.D.) packed with Yanapak ODS (particle size 5 μm) (Yanagimoto Manufacturing Co., Fushimi-ku, Kyoto, Japan). The mobile phase was a 0.1 *M* potassium phosphate buffer (pH 3.5) with a flow-rate of 0.6 ml/min; the detector potential was set at 0.8 V against the Ag/AgCl electrode. Under these conditions the retention times were: solvent front, 1.8 min; DOPA, 3.8 min; and α-methyldopa, 5.5 min.

The DOPA formed enzymatically by TH was calculated by the equation

$$\frac{R\,(\mathrm{L}) - R\,(\mathrm{D})}{R\,(\mathrm{D}+\mathrm{S}) - R\,(\mathrm{D})} \times 50 \text{ pmol (or 100 pmol)}$$

where R is the ratio of peak heights (peak height of DOPA/peak height of α-methyldopa), R (L) being that from the L-tyrosine incubation, R (D) from the D-tyrosine incubation, and R (D + S) that of D-tyrosine plus DOPA (internal standard, 50 pmol or 100 pmol).

RESULTS

The voltammetric detector system provides high sensitivity for catechol compounds. Therefore DOPA, the product of TH reaction, can be assayed in the column eluate with extremely high sensitivity. Fig. 1 is the calibration curve showing the linear response of the peak height of the voltammetric detector for the amounts of DOPA injected from 500 fmol to 5 nmol.

The chromatographic pattern of the TH reaction with the homogenate of

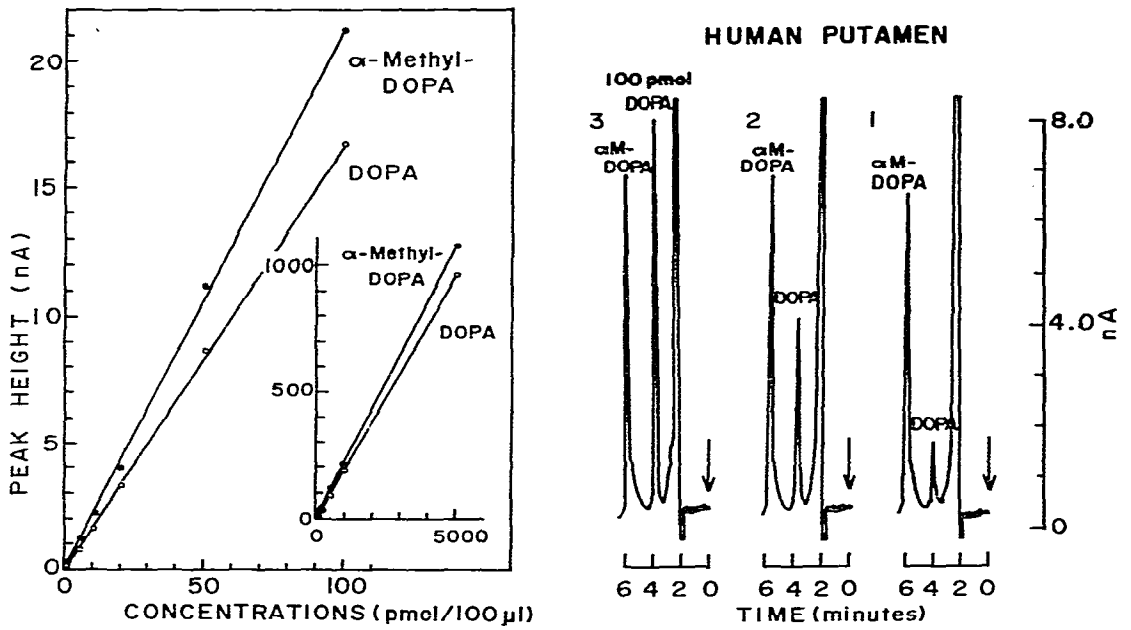


Fig. 1. Standard curves of DOPA and α-methyldopa in HPLC with voltammetric detection for the peak height. One hundred microliters of a sample containing various amounts (500 fmol to 5 nmol) of DOPA and α-methyldopa were injected into the column and detected by a voltammetric detector. The conditions are described in *Experimental procedures.*

Fig. 2. HPLC elution pattern of tyrosine hydroxylase incubation mixtures with the homogenate of human putamen as enzyme. The conditions are described in *Experimental procedures.* The incubation mixture contained 2 mg of human putamen and 10 µg of catalase. (1) Blank incubation with D-tyrosine. (2) Experimental incubation with L-tyrosine. (3) 100 pmol of DOPA were added as an internal standard to a blank incubation with D-tyrosine. 100 pmol of α-methyldopa (αM-DOPA) were added to each sample after incubation. Formation of 37.3 pmol of DOPA from L-tyrosine during 10 min incubation at 37° was calculated from the charts.

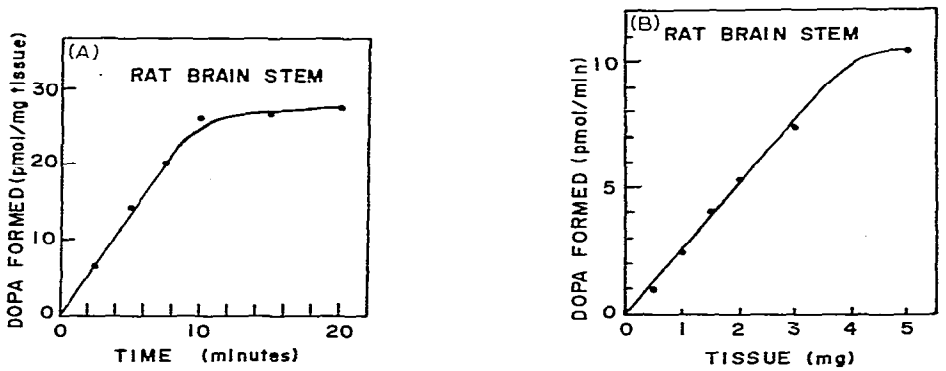
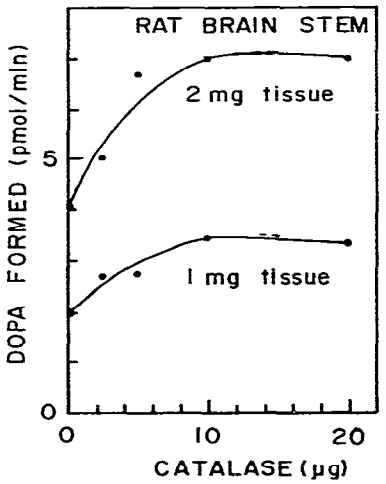


Fig. 3. (A) The rate of DOPA formation using an homogenate of rat brain stem as enzyme at 37°. Standard incubation system containing 10 µg of catalase was used as described under *Experimental procedures.* (B) Tyrosine hydroxylase activity in homogenates of rat brain stem as a function of enzyme concentration. The standard incubation system with 10 µg catalase was used and incubation was carried out for 10 min at 37°.

human putamen is shown in Fig. 2. The peak of DOPA in the blank incubation may be mainly due to its non-enzymatic formation.

With rat brain stem homogenate as enzyme in the presence of 10 μg catalase, the reaction proceeded linearly with time for 10 min at 37° (Fig. 3A). The reaction rate was linear up to 3 mg of tissue (Fig. 3B).

The presence of either catalase or Fe^{2+} ion was required for maximum activity. As shown in Fig. 4, 10 μg of catalase in the standard incubation system with rat brain homogenate gave maximum activity. Fe^{2+} ion was more effective for the stimulation of TH activity, especially for the assay in human brain homogenates. As shown in Fig. 5, the activity in the homogenate of 2 mg human putamen was stimulated by catalase only slightly, but 1—2 m*M* Fe^{2+} ion activated TH activity about three-fold.



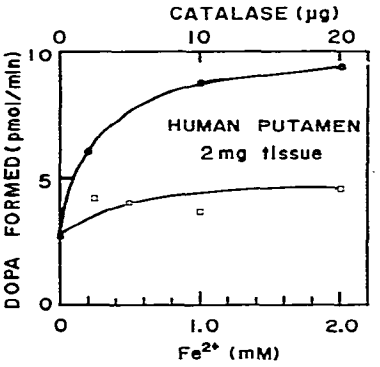


Fig. 4. Effects of catalase on tyrosine hydroxylase activity in the homogenate of rat brain stem. The standard incubation system was used with various concentrations of catalase and with the homogenate containing 1 mg or 2 mg tissue as enzyme. Incubation was carried out for 10 min at 37°.

Fig. 5. Effects of Fe^{2+} ion (•) and catalase (□) on tyrosine hydroxylase activity in the homogenate of human putamen. The standard incubation system was used with various concentrations of Fe^{2+} ion or catalase and with the homogenate containing 2 mg tissue as enzyme. Incubation was carried out for 10 min at 37°.

DISCUSSION

This assay of TH with the preliminary isolation of DOPA formed from tyrosine by a double-column procedure and subsequent assay by HPLC with a voltammetric detector has many advantages.

First, it is highly sensitive. The limit of sensitivity was about 5 pmol of DOPA formed enzymatically. The sensitivity was found to be even higher than that of various radioassays, in which the limit is about 10 pmol of DOPA formed even if the substrate concentration is reduced to increase the specific radioactivity of labelled tyrosine, and the V_{max} cannot be obtained. TH

activity could be assayed with less than 1 mg of a brain nucleus. With such a high sensitivity for DOPA, the sensitivity of the TH assay is determined solely by the blank value. In the present method DOPA could be completely and rapidly separated by the combination of the double-column procedure and HPLC. Therefore, the blank value with D-tyrosine, which is not the substrate for TH at all [1], derives either from DOPA formed by the non-enzymatic reaction or from DOPA contained in a crude enzyme preparation. Since endogenous DOPA in crude brain tissue is very low (less than 250 pmol per g tissue [10]), the blank is considered to be mainly derived from non-enzymatically formed DOPA. In this assay catalase gave a lower blank than Fe^{2+} ion, but Fe^{2+} ion was more or less essential for the assay of human brain enzyme. This confirms our previous results [4, 11].

Secondly, the method is simple and rapid, and the total time of the assay was 3 h.

Thirdly, it is economical since labelled substrate and a liquid scintillation spectrometer are not needed.

Fourthly, the incubation can be done in optimal conditions under saturating concentrations of L-tyrosine and a pterin cofactor; thus the V_{max} can be obtained. It is not necessary to measure tyrosine in a crude enzyme preparation to calculate the specific radioactivity of tyrosine as in radioassay.

Blank and Pike [9] first reported an assay of TH activity using HPLC with electrochemical detection. They used 4 nmol of dihydroxybenzylamine as internal standard and a batch method of aluminium oxide for the preliminary isolation of DOPA prior to HPLC. Our method is more sensitive and simpler. The recovery of dihydroxybenzylamine used in their study was found to be low. In contrast, α-methyldopa used as an internal standard in our study proved to be an excellent internal standard, because it showed a good recovery and was well separated from DOPA.

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