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# ASSAY FOR TYROSINE HYDROXYLASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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## SUMMARY

A sensitive assay method for tyrosine hydroxylase in rat brain and adrenal medulla by high-performance liquid chromatography with fluorescence detection is described. L-DOPA formed enzymatically from the substrate L-tyrosine and $\alpha$-methyldopa (internal standard), after clean-up with small cartridges of an activated alumina and a cation exchanger, Toyopak IC-SP M, are converted into the corresponding fluorescent compounds by reaction with 1,2-diphenylethylenediamine. The derivatives are separated by reversed-phase chromatography on TSK gel ODS-120T. The detection limit for L-DOPA formed enzymatically is 2 pmol per assay tube.

## INTRODUCTION

Tyrosine hydroxylase [TH, L-tyrosine, tetrahydropteridine: oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] is a monooxygenase that catalyses the tetrahydropterin-dependent conversion of L-tyrosine into L-DOPA [1], the rate-limiting step in the biosynthesis of catecholamines [2]. This reaction is believed to be regulated in vivo through feedback inhibition by catecholamine end-products [1,3]. Because TH activity is extremely low in the brain, a sensitive assay method is required for physiological and pathological studies.

Many assay methods for TH in biological materials have been reported: radiochemical methods using L-[3,5-$^{3}$H]tyrosine [4,5], L-[U-$^{14}$C]tyrosine [1,6] and L-[1-$^{14}$C]tyrosine [7,8] as substrates; fluorimetric methods [9–11]; high-performance liquid chromatographic (HPLC) methods coupled with electrochemical detection [12,13] and native fluorescence detection [14]; and enzyme immunoassay [15]. Of these, the radiochemical methods using L-[U-$^{14}$C]tyrosine and L-[1-$^{14}$C]tyrosine and the HPLC methods with electrochemical detection have been widely employed owing to their high sensitivity.

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We have previously reported a highly sensitive method for the determination of L-DOPA in human plasma and urine using reversed-phase HPLC with fluorescence detection [16], based on pre-column derivatization of the amine with 1,2-diphenylethylenediamine (DPE), a selective fluorescence derivatization reagent for catechol compounds [17,18]. This paper describes a highly sensitive HPLC method with fluorescence detection for the assay of TH in rat brain and adrenal medulla, based on the determination of L-DOPA formed from the substrate L-tyrosine under the optimum conditions for the enzyme reaction. L-DOPA formed enzymatically, after successive clean-up with small cartridges of an activated alumina and a cation exchanger, is measured by the reported HPLC method using $\alpha$-methyldopa as an internal standard.

## EXPERIMENTAL

### *Chemicals and reagents*

L-DOPA, $\alpha$-methyldopa and catalase (from bovine liver, 2890 units per mg of protein) were purchased from Sigma (St. Louis, MO, U.S.A.). DL-6-Methyl-5,6,7,8-tetrahydropterin dihydrochloride (4H-PT), D- and L-tyrosine and iron(II) ammonium sulphate hexahydrate were obtained from Nakarai Chemicals (Kyoto, Japan). Alumina (activated) and L-phenylalanine were from Wako (Osaka, Japan) and Takarakosan (Tokyo, Japan), respectively. All other chemicals were of reagent grade. Deionized and distilled water was used.

4H-PT solution (10 m*M*) was prepared in aqueous 1.0 *M* 2-mercaptoethanol solution and stored at $-20°C$ in the dark. DPE solution (0.1 *M*, pH 6.5–6.7) was prepared in 0.1 *M* hydrochloric acid. Toyopak IC-SP M (strong cation exchanger, sulphopropyl resin, $Na^+$ form; Toyo Soda, Tokyo, Japan) cartridge ($35 \times 10$ mm I.D.) was washed before use in the same manner as for Toyopak IC-SP S [16]. Activated alumina was prepared by the method of Anton and Sayre [19], and 100 mg of the alumina was dry-packed in a polyethylene tube ($35 \times 10$ mm I.D.) to form a cartridge.

### *Apparatus and HPLC conditions*

An Eyela LP-1 liquid chromatograph (Tokyo Rika, Tokyo, Japan) was used, equipped with a Rheodyne 7125 sample injector valve (100-$\mu$l loop) and a Shimadzu FLD-1 fluorescence detector (Shimadzu Seisakusho, Kyoto, Japan) fitted with a 14-$\mu$l flow-cell and an EM-4 emission filter. Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 850 fluorescence spectrophotometer (Hitachi, Tokyo, Japan) fitted with a 20-$\mu$l flow-cell. Spectral bandwidths of 5 nm were used both for the excitation and emission wavelengths. A TSK gel ODS-120T column (particle size 5 $\mu$m, $150 \times 4.6$ mm I.D.; Toyo Soda) was used. The column temperature was ambient (20–25°C). The mobile phase was a mixture of acetonitrile, methanol and 0.1 *M* acetate buffer (pH 5.0) (1:1:2, v/v) and the flow-rate was 1.0 ml/min.

### *Enzyme preparations*

Male Donryu rats (4 weeks) were decapitated and the brains and adrenal medullas were dissected. All further procedures were conducted at 0–4°C. The

brain was homogenized in 9 volumes of 0.25 *M* sucrose and the adrenal medulla in 49 volumes of the sucrose solution. The homogenates were stored at −20°C until use.

*Assay procedure*

To 100 μl of the enzyme preparation were added 200 μl of 0.5 *M* acetate buffer (pH 6.0) and 50 μl of 1 mg/ml catalase or 10 m*M* iron(II) ammonium sulphate. The mixture was preincubated at 37°C for 5 min and again incubated for 8 min after the addition of 100 μl of 5 m*M* L-tyrosine in 10 m*M* hydrochloric acid and 50 μl of 10 m*M* 4H-PT solution. At the end of the incubation, 100 μl each of 3 *M* perchloric acid and 10 μ*M* α-methyldopa (internal standard) were added. After cooling in ice-water for 5 min, 50 μl of 0.1 *M* EDTA disodium salt, 300 μl of 0.5 *M* Tris–HCl buffer (pH 8.5) and 120 μl of 2 *M* potassium carbonate (to adjust pH to 8.3–8.5) were added, and the mixture was centrifuged at 1000 *g* at 4°C for 10 min. The supernatant (1 ml) was poured on to an alumina cartridge. The cartridge was washed with 5 ml of water. The adsorbed catechol compounds were eluted with 1.0 ml of 0.2 *M* hydrochloric acid, and the eluate was passed through a Toyopak IC-SP M cartridge equilibrated with 0.2 *M* sodium phosphate buffer (pH 6.0). The cartridge was washed with 10 ml of water and the adsorbed amino compounds were eluted with 2 ml of a mixture of ethanol, 2 *M* sodium perchlorate and 2 *M* sodium hydroxide (7:3:0.4, v/v). To the eluate were added 200 μl of the DPE solution, 80 μl of 1 *M* sodium carbonate (to adjust the pH to 6.5–6.9) and 50 μl of 1 m*M* potassium hexacyanoferrate(III). The mixture was allowed to stand at 25°C for 20 min to derivatize the catechol compounds, and then 100 μl of 0.4 *M* sodium sulphite were added. The final mixture (100 μl) was subjected to HPLC. For the blank, 100 μl of the enzyme preparation denatured by heating at 90°C for 20 min were carried through the same procedure. Michaelis constant ($K_m$) values for L-tyrosine and 4H-PT were calculated from the Lineweaver–Burk plots.

## RESULTS AND DISCUSSION

The conditions of the fluorescence derivatization and HPLC were almost the same as described previously [16].

Typical chromatograms obtained with the rat brain and adrenal medulla preparations, and those of the blanks are shown in Fig. 1. The fluorescent compounds from L-DOPA and α-methyldopa (peaks 1 and 2, respectively) could be well separated from the other fluorescent compounds in all the cases. The retention times for peaks 1 and 2 were 3.6 and 4.7 min, respectively. The peak for L-DOPA was identified on the basis of its retention time and fluorescence excitation (maximum 350 nm) and emission (maximum 475 nm) spectra of the eluate in comparison with the standard compound and also by co-chromatography of the standard. The peaks for the oxidized form of 4H-PT and endogenous norepinephrine, epinephrine and dopamine (peaks 3, 5, 7 and 8, respectively) were identified by the same techniques.

The peaks for L-DOPA in the blanks (Fig. 1b and d) were considered to be

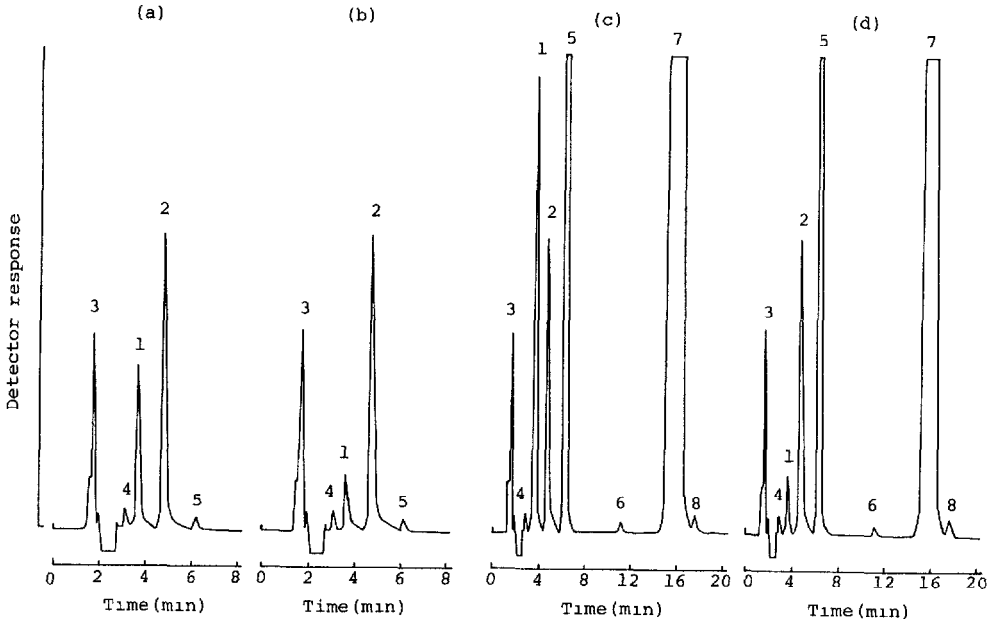


Fig. 1. Chromatograms obtained with the enzyme preparations from (a) brain, (c) adrenal medulla and (b and d) their blanks, respectively. Portions (100 $\mu$l) of the enzyme preparations were treated according to the procedure with catalase. Peaks: 1 = L-DOPA; 2 = $\alpha$-methyldopa; 3 = 4H-PT (oxidized form); 4 = unidentified; 5 = endogenous norepinephrine (pmol/mg tissue: a, ca. 0.3; c, ca. 17); 6 = unidentified; 7 = endogenous epinephrine (pmol/mg tissue: c and d, ca. 63 each); 8 = endogenous dopamine (pmol/mg tissue: c and d, ca. 0.9 each). TH activity (pmol/min/mg tissue): a, 2.1; c, 41.6.

mainly derived from non-enzymatic formation. Commercial L-tyrosine was found to contain L-DOPA as an impurity (ca. 0.2 pmol L-DOPA in 5 nmol L-tyrosine).

For the blank, four procedures were examined: (a) D-tyrosine was used instead of L-tyrosine; (b) the enzyme reaction was carried out without enzyme preparation; (c) the enzyme preparations denatured by heating at 90 °C for 20 min were used; and (d) the incubation for the enzyme reaction was omitted. The blank values for L-DOPA (pmol/tube) were (a) ca. 60, (b) ca. 70, (c) ca. 90 and (d) ca. 30. The denatured enzyme preparations (c) are recommended for use in the procedure because of their relatively high blank values, being derived mainly non-enzymatically. The other procedures may cause an overestimation of the enzyme activity.

TH reaction was accelerated by catalase or iron (II). As shown in Fig. 2a, 40–100 $\mu$g of catalase in the reaction mixture gave maximum activity for both the enzyme preparations. TH is an iron enzyme [20,21], and iron (II) in a concentration range 0.8–1.5 m*M* also gave maximum activity for both the enzyme preparations (Fig. 2b). TH activity in both the enzyme preparations could not be stimulated any more in the presence of both iron (II) (1 m*M*) and catalase (50 $\mu$g) in the enzyme reaction mixture. Therefore, either catalase or iron (II) was required for the maximum activity, and iron (II) was more effective; iron (II) gave ca. three times higher activity (see also Table I). However, catalase gave much lower and stable blank values [ca. one-fifth those given by iron (II)]. The high blank values

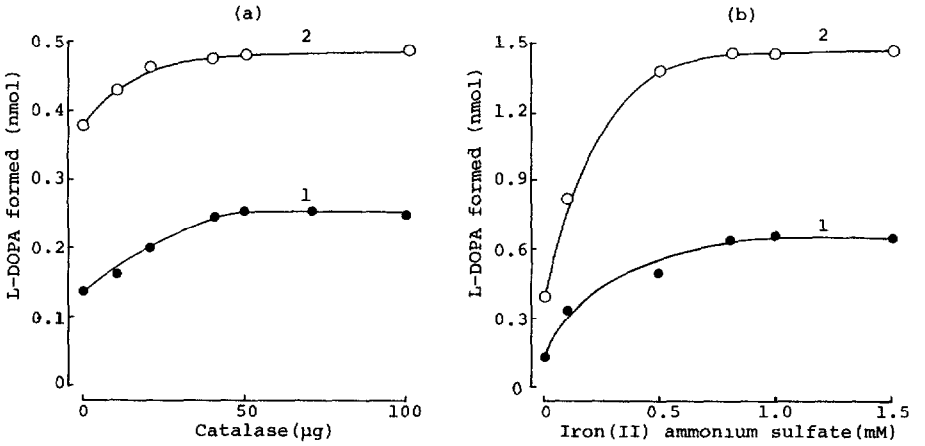


Fig. 2. Effects of (a) catalase and (b) iron(II) on the amount of L-DOPA formed. Portions (100 $\mu$l) of the enzyme preparations were treated as in the procedure at various concentrations of catalase or iron(II). Enzyme preparations: 1, brain; 2, adrenal medulla.

by iron(II) were ascribable to mainly non-enzymatic hydroxylation. Similar results were reported by other workers [13,22], though the role of iron(II) in the enzyme reaction has remained unknown [21]. Catalase (50 $\mu$g in the enzyme reaction mixture) can be recommended for this procedure.

TH in both the enzyme preparations was most active at pH 5.9–6.1 in acetate buffer and the buffer concentrations at 0.2–1.0 *M* gave a maximum and constant activity; 0.5 *M* acetate buffer of pH 6.0 was used. Phosphate (0.2 *M*, pH 6.0) and 2-(N-morpholino)ethane sulphonate (MES) (0.1 *M*, pH 6.0) buffers also gave the same activity as that obtained with the acetate buffer.

L-Tyrosine in a concentration range 0.4–2.0 m*M* gave a maximum and constant activity of TH in brain and adrenal medulla, with the $K_m$ values (mean ± S.D., $n=5$ each) of $33 \pm 4$ $\mu M$ in brain TH and $42 \pm 6$ $\mu M$ in adrenal medulla TH; 1 m*M* L-tyrosine was used as a saturating concentration for the enzyme reaction.

4H-PT in the enzyme reaction mixture gave maximum and constant activity in a concentration range 0.8–3.0 m*M* for brain and adrenal medulla TH, with

TABLE I

TH ACTIVITIES IN RAT BRAIN AND ADRENAL MEDULLA

| Rat tissue | TH activity* (pmol/min/mg tissue) | | *b/a* |
|---|---|---|---|
| | Catalase (*a*)** | Iron(II) (*b*)** | |
| Brain | 2.21 ± 0.70 | 5.90 ± 0.82 | 3.02 ± 0.71 |
| Adrenal medulla | 40.87 ± 9.19 | 110.78 ± 14.54 | 2.74 ± 0.55 |

*Mean ± S.D.; obtained from five rats (Donryu, male, 4 weeks old).
**Portions (100 $\mu$l) of the enzyme preparations were treated according to the procedure.

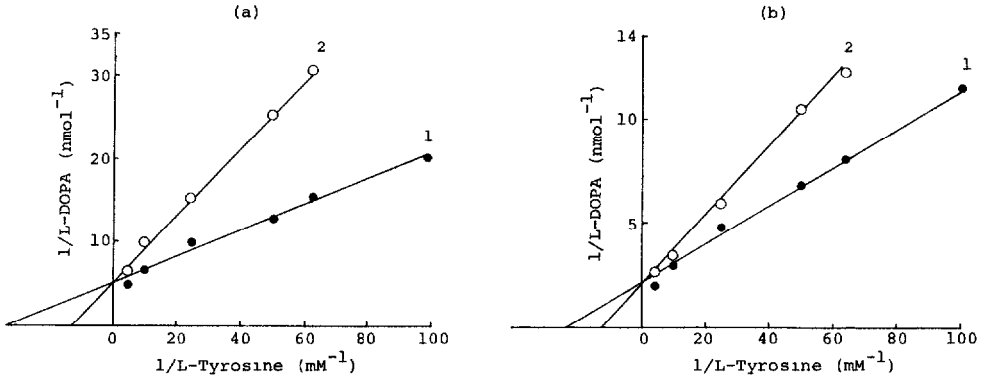


Fig. 3. Inhibition of (a) brain and (b) adrenal medulla TH by L-phenylalanine. Portions (100 $\mu$l) of the enzyme preparations were treated as in the procedure with catalase in (1) the absence and (2) the presence of L-phenylalanine (0.1 m$M$ in the enzyme reaction mixture). The data were plotted by linear regression analysis.

observed $K_m$ values (mean $\pm$S.D., $n=3$ each) of $31\pm1$ and $29\pm2$ $\mu M$, respectively; 1 m$M$ 4H-PT was selected for the procedure. 4H-PT reduced the TH activity markedly when the enzyme preparations were incubated in the absence of L-tyrosine. A similar result has been obtained by other workers [23]. Thus, in the procedure, the enzyme reaction was initiated by adding L-tyrosine and 4H-PT at the same time.

TH works on L-phenylalanine as well as L-tyrosine, which can be competitive inhibitors with each other [24]. L-Phenylalanine at 0.1 m$M$ in enzyme reaction mixture inhibited TH in brain and adrenal medulla in a competitive mode against L-tyrosine (Fig. 3), with observed inhibitory constant values of 67 and 110 $\mu M$, respectively, which were obtained according to the method of Dixon [25].

As L-DOPA can be converted into dopamine by aromatic L-amino acid decarboxylase [26] in many tissues, there is a possibility of degradation of L-DOPA formed enzymatically in the incubation mixture. However, an aromatic L-amino acid decarboxylase inhibitor, 3-hydroxybenzyloxyamine [27], had no effect on TH activities in either of the enzyme preparations in the concentration range 0.01–0.1 m$M$ in the enzyme reaction mixture.

With brain and adrenal medulla homogenates as enzyme, TH activity was proportional to the incubation time at 37°C up to 12 min (Fig. 4); the prescribed incubation time of 8 min provided enough L-DOPA to be precisely measured. The reaction rate was linear up to 20 mg of brain and up to 4 mg of adrenal medulla.

Cartridges of an activated alumina and a strong cation exchanger (Toyopak IC-SP M) were successively used for the clean-up of the incubated enzyme reaction mixture. The alumina served to eliminate 4H-PT, which interfered with the fluorescence derivatization and the cation exchanger to remove compounds other than amino compounds, including L-DOPA and catecholamines. Recoveries (mean $\pm$S.D.) of L-DOPA and $\alpha$-methyldopa (1 nmol each) added to the enzyme reaction mixture were $55.6\pm3.9\%$ and $62.7\pm4.4\%$ in brain, and those of L-DOPA

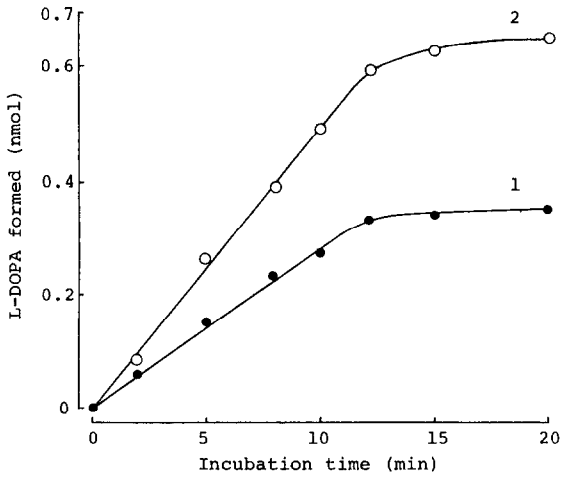


Fig. 4. Effects of the incubation time on the amount of L-DOPA formed in (1) brain and (2) adrenal medulla TH-mediated reactions. Portions (100 $\mu$l) of the enzyme preparations were treated according to the procedure with catalase for various incubation times. TH activities (pmol/min/mg tissue): 1, 2.7; 2, 49.2.

and $\alpha$-methyldopa (1 nmol each) were $58.9 \pm 4.1\%$ and $56.4 \pm 3.6\%$ in adrenal medulla ($n=5$ in each case).

For calibration curves, linear relationships were observed between the peak-height ratios of L-DOPA and $\alpha$-methyldopa and the amount of L-DOPA added to the blanks over the range from 2 pmol to 2 nmol. The detection limit for L-DOPA formed enzymatically was 2 pmol per assay tube (40 fmol per 100-$\mu$l injection volume) at signal-to-noise ratio of 2.

The coefficients of variation in intra-assay ($n=7$ each) were 6.4 and 8.3% for mean activities of 2.5 and 35.4 pmol/min/mg tissue in the brain and adrenal medulla preparations, respectively. TH activities in brain and adrenal medulla from rats (Donryu, male, 4 weeks old) were measured by the present method (Table I).

This method is sensitive enough to assay low TH activity in brain, and should be useful for biological and biomedical investigations.

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