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Fluorimetric Assay for Dopamine β-Hydroxylase in Rat Plasma

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A sensitive fluorimetric method for the assay of dopamine β-hydroxylase in rat plasma is described. Octopamine, formed enzymatically from the substrate tyramine, is separated by chromatography on a Dowex 50W-X4 column and oxidized with periodate to *p*-hydroxybenzaldehyde, which is then quantitated by means of the fluorimetric method for selective determination of aromatic aldehydes with 2,2′-dithiobis(1-aminonaphthalene). The method is readily performed with good precision and is suitable for the assay of many samples simultaneously.

Keywords——dopamine β-hydroxylase; rat plasma; octopamine; tyramine; fluorimetry; *p*-hydroxybenzaldehyde determination; 2,2′-dithiobis(1-aminonaphthalene)

Dopamine β-hydroxylase[2] (DBH) catalyzes the conversion of dopamine to norepinephrine in the presence of oxygen and ascorbate. The enzyme is released in the bloodstream from the peripheral sympathetic nerve endings along with catecholamines,[3] and its activity in plasma (or serum) has therefore become of interest as a possible index of the sympathetic nervous functions.

DBH also mediates the β-hydroxylation of tyramine and phenylethylamine.[4] This reaction is accelerated by fumarate.[4] N-Ethylmaleimide and the cupric ion enhance the reaction by inactivating endogenous inhibitors of DBH present in plasma and tissues.[5,6] Catalase protects the enzyme from hydrogen peroxide generated by nonenzymatic oxidation of ascorbate.[7]

Several assay methods for DBH in biological materials have been proposed so far; two-step enzymatic radiochemical,[8] one-step radiochemical,[9] tritium release,[10] spectrophotometric,[11] dual-wavelength spectrophotometric,[12] fluorimetric,[13] high-performance liquid chromatographic-fluorimetric[14] and phosphorimetric[15] methods. They are sufficiently sensitive to assay the DBH activity in human serum. DBH activity in rat plasma, which is much lower than in human serum, can be assayed only by the radioassays,[8–10] dual-wavelength photometry[12] and phosphorimetry.[15]

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We have developed a fluorimetric method for the assay of DBH in rat plasma based on the determination of octopamine formed from the substrate tyramine. Octopamine, after separation by ion-exchange chromatography, is oxidized with periodate to *p*-hydroxybenzaldehyde, which is then measured fluorimetrically by the reported method for selective and sensitive determination of aromatic aldehydes with 2,2′-dithiobis(1-aminonaphthalene)[16] (DTAN).

Experimental

Reagents and Solutions——All chemicals were of reagent grade, unless otherwise noted. Double-distilled H_2O was used. DTAN was prepared as described previously.[16] Hydrochlorides of tyramine and octopamine (Sigma) were purified by recrystallization.

DTAN solution: DTAN (40 mg) was dissolved in 6.0 ml of methanol, then 1.0 ml of methanolic 80 mg/ml tri-*n*-butylphosphine and 25.0 ml of 30% H_2SO_4 were added, and the mixture was diluted with H_2O to 100 ml. The solution is stable for at least 1 week when stored at 4°.

β-Mercaptoethanol solution: Distilled *β*-mercaptoethanol (5.0 ml) was added to 33.5 ml of 30% H_2SO_4 and diluted with H_2O to 50 ml. This solution is usable for 3 days.

Ion Exchange Column——Dowex 50W-X4 (H^+, 200—400 mesh, 50 ml; Dow Chemical Co.) was washed successively with approximately 500 ml each of 1 M NaOH and 6 M HCl. The resin (0.2 ml) was packed into a glass tube (4 mm i.d., 130 mm long) and washed successively with 2 ml of 1 M NaOH (twice), 2 ml of H_2O (3 times), 2 ml of 6 M HCl (twice), and finally 2 ml of H_2O (3 times). The used column can be regenerated by washing in the same way and is usable more than 10 times.

Rat Plasma——Heparinized blood (50 units of heparin in 50 μl of saline/3—5 ml blood) was obtained from male Donryu rats (3 and 10 weeks of age, 50—70 and 330—400 g, respectively). Plasma was obtained by centrifugation of the blood at $10000 \times g$ at 5° for 10 min. The plasma can be stored at −20° for more than 2 weeks without loss of DBH activity.

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-4 spectrofluorimeter using quartz cells of 10×10 mm optical path-length. The slit widths in terms of wavelength were set at 10 nm in both the exciter and analyzer. The fluorescence spectra are uncorrected. pH was measured with a Hitachi-Horiba M-7 pH meter at 25°. Centrifugation at low temperature was carried out using a Hitachi 05 PR-22 refrigerated centrifuge.

Procedure——Substrate-cofactor solution consisted of 200 μl of 1.0 M acetate buffer (pH 5.0) and 100 μl each of 0.3 M tyramine·HCl, 0.15 M Na_2 fumarate, 0.15 M N-ethylmaleimide, 10 μM $CuSO_4$ and 3500 units/ml catalase (total volume, 700 μl). To this solution, 50 μl of rat plasma and 150 μl of H_2O were added. The mixture was preincubated at 37° for 5 min, then incubated again after addition of 100 μl of 0.1 M ascorbic acid (freshly prepared) at 37° for 45 min in air with continual shaking. The reaction was stopped by the addition of 200 μl of 3 M trichloroacetic acid. The mixture was centrifuged at $3000 \times g$ for 10 min. The supernatant (1.0 ml) was poured into a Dowex 50W-X4 column. The column was washed 3 times with 2 ml of H_2O, and the adsorbed amines were eluted with 1.0 ml of 3 M NH_4OH. Octopamine in the eluate was converted to *p*-hydroxybenzaldehyde by the addition of 100 μl of 0.15% (w/v) $NaIO_4$ solution (at room temperature, 20—30°). The excess $NaIO_4$ was decomposed by the addition of 100 μl of 0.75% (w/v) Na_2SO_3 solution. The mixture was neutralized with 0.3 ml of 5 M H_2SO_4. To the resulting solution, 2.0 ml of DTAN solution and 0.5 ml each of 0.05% (w/v) Na_2SO_3 solution and 15% (w/v) $Na_2HPO_3 \cdot 5H_2O$ solution (both freshly prepared; accelerators of the fluorescence reaction) were successively added. The mixture was allowed to stand at 37° for 30 min to develop the fluorescence. *β*-Mercaptoethanol solution (1.0 ml) was added to stop the fluorescence reaction.

For the blank, the same procedure was carried out except that the ascorbic acid solution was added after the addition of trichloroacetic acid. To prepare a standard curve, 150 μl of H_2O in the procedure was replaced with 150 μl of octopamine standard solution (0.5—5.0 nmol) and incubation was omitted.

The fluorescence intensities were measured at 460 nm with excitation at 372 nm. DBH activity (unit) was expressed as μmol of octopamine/min/l of plasma at 37°.

Results and Discussion

The optimum pH for the enzyme reaction was at 5.0, and the maximum activity in acetate buffer was attained at concentrations of 25—200 mM in the incubation mixture; 100 mM was employed for convenience. A constant and maximum activity was achieved in the presence of 20—40 mM tyramine and 8—13 mM ascorbic acid with observed K_m values at 3.3 mM for

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tyramine and 1.2 mM for ascorbic acid; 30 mM tyramine and 10 mM ascorbic acid were used as saturating concentrations for the enzyme reaction. Fumarate at a concentration greater than 7.5 mM resulted in an activity of DBH 1.3 times higher than that in its absence; 15 mM was used in the standard procedure. N-Ethylmaleimide gave a maximum and reproducible activity in the concentration range of 10—30 mM; 15 mM was selected as the optimum. Cupric sulfate provided maximum activity at 1 μM in the presence of 15 mM N-ethylmaleimide (Fig. 1)

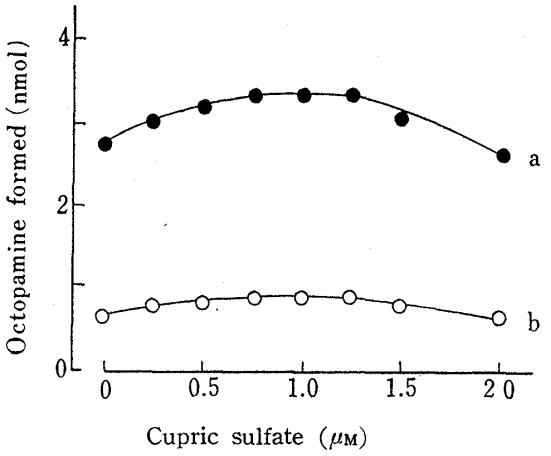

Fig. 1. Effect of Cupric Sulfate Concentration in the Incubation Mixture on DBH Activity in the Presence of 15 mM N-Ethylmaleimide

a, 1.51 units; b, 0.40 units palsma. Each plot represents the mean values of 5 determinations. The coefficient of variation in each case did not exceed 5.5%.

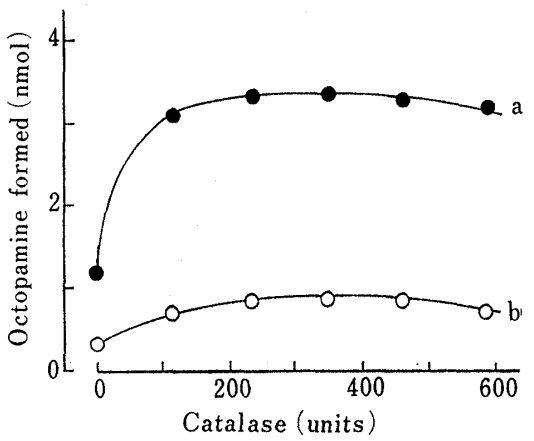

Fig. 2. Effect of Catalase Concentration in the Incubation Mixture on DBH Activity

a, 1.51 units; b, 0.40 units plasma. Each plot represents the mean values of 5 determinations. The coefficient of variation in each case did not exceed 6.5%.

though the optimum concentration varied slightly with each plasma used in the absence of N-ethylmaleimide. Catalase enhanced the enzyme reaction approximately 3 times in the range of 250—450 units in the incubation mixture (Fig. 2); 350 units was used in our procedure.

Pargyline, a monoamine oxidase inhibitor, was previously used at a concentration of about 1 mM in DBH assay procedures[8–12,14] to suppress extraneous consumption of the substrate with the generation of hydrogen peroxide by monoamine oxidase present in DBH preparations. Pargyline showed neither enhancement nor suppression of the DBH reaction at concentrations of 0.2—4 mM when examined on rat plasmas with monoamine oxidase activity[17] of 1—3 units, and thus was not used in our procedure.

The amount of octopamine formed was proportional to the rat plasma sample size up to 100 μl (Fig. 3). The enzyme activity was almost linear with time up to at least 45 min when incubated at 37°.

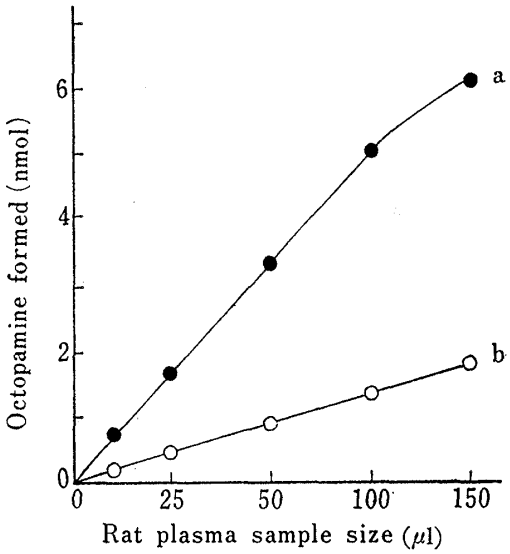

Fig. 3. Effect of Rat Sample Size on the Amount of Octopamine Formed

a, 1.51 units; b, 0.40 units plasma. Each plot represents the mean values of triplicate determinations. The coefficient of variation in each case did not exceed 6.5%.

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Octopamine was separated together with tyramine from the incubated reaction mixture by chromatography on a Dowex 50W-X4 column.[9,11,12,14,15] Octopamine (0.5—5.0 nmol) was completely eluted with 1 ml of 3 M ammonium hydroxide solution. Recovery of the amine was 97±3% (mean±SD, $n=18$). An incomplete elution was obtained with ammonium hydroxide solutions (1 ml) of less than 2.5 M.

Octopamine (0.5—5.0 nmol) in the eluate was oxidized quantitatively to *p*-hydroxybenzaldehyde with sodium periodate solution of 0.08% or greater concentration; 0.15% solution was used in this procedure. Recovery of the aldehyde was 98±2% (mean±SD, $n=18$). Excessive periodate interfered with the development of fluorescence from the aldehyde with DTAN, and was therefore decomposed with sodium sulfite. The resulting mixture was neutralized with sulfuric acid. The *p*-hydroxybenzaldehyde in the mixture was successfully determined by the fluorimetric method with DTAN.

The fluorescence excitation (maximum, 372 nm) and emission (maximum, 460 nm) spectra for the final solution in the assay were identical with those for the final solutions obtained through the procedure with octopamine standard solutions. The standard curve was linear up to at least 5.0 nmol of octopamine and passed through the origin. The blank prepared with plasma in this method was identical with the blank obtained from heated plasma (at 95° for 5 min).

The recovery of octopamine (1.0 and 3.0 nmol) added to the incubated reaction mixture was 95±3% (mean±SD, $n=10$ each). The lower limit of detection for octopamine was 0.33 nmol (corresponded to 0.15 units of DBH), which gave a fluorescence intensity of twice the blank value. The within-day precision was examined using a rat plasma with a mean DBH activity of 1.56 units. The coefficient of variation was 5.7% ($n=11$). The day-to-day precision was obtained by repeating the assay for 10 days on a rat plasma stored at −20° with a mean DBH activity of 1.32 units. The coefficient of variation was 6.5% ($n=20$).

DBH activities in 3- and 10-week-old rat plasmas were 1.32±0.12 and 0.34±0.09 units (mean±SD, $n=25$ each), respectively. The values are in agreement with the data obtained by the phosphorimetric[15] and dual-wavelength spectrophotometric[12] methods.

This method also permits the assay of DBH in only 5 μl of human serum (or plasma). This study provides the first fluorimetric DBH assay method which measures the amount of octopamine formed from the substrate tyramine. The entire procedure takes less than 4 hr and more than 20 samples can be assayed simultaneously. This method is sensitive, precise and relatively simple, and should be useful for biomedical investigations.

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