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ASSAY FOR DOPAMINE β-HYDROXYLASE IN HUMAN PLASMA AND RAT SERUM BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORIMETRIC DETECTION

MYUNG-KOO LEE, HITOSHI NOHTA and YOSUKE OHKURA*

Faculty of Pharmaceutical Sciences, Kyushu University 62, Maidashi, Higashi-ku, Fukuoka 812 (Japan)

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SUMMARY

A highly sensitive assay for dopamine β-hydroxylase activity in human plasma and rat serum by high-performance liquid chromatography with fluorimetric detection is described. Norepinephrine, formed enzymatically from the substrate dopamine, and epinephrine (internal standard), after clean-up with a cation-exchange cartridge (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-80TM. The detection limit for norepinephrine formed enzymatically is 5 pmol per assay tube.

INTRODUCTION

Dopamine β-hydroxylase [DBH; 3,4-dihydroxyphenylethylamine,ascorbate:oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] is a monooxygenase and catalyses the hydroxylation of dopamine to norepinephrine [1]. This enzyme is localized in the chromaffin granules of the adrenal medulla and in the catecholamine-containing storage vesicles of central and peripheral catecholaminergic neurons. The enzyme is released into the blood stream from the peripheral sympathetic nerve endings and adrenal medulla together with catecholamines [2]. Therefore, DBH activity in plasma (or serum) has drawn much attention from physiological and clinical investigators.

Many assay methods for DBH in biological materials have been reported: two-step enzymatic radiochemical [3], one-step radiochemical [4], tritium release [5], spectrophotometric [6], phosphorimetric [7], fluorimetric [8] and high-performance liquid chromatographic (HPLC) [9–14] methods. Of these, the radiochemical and HPLC methods generally permit the assay of low DBH activ-

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ity in plasma (or serum) from small experimental animals such as rats and guinea pigs.

We have developed a highly sensitive HPLC method with fluorimetric detection for the assay of DBH in human plasma and rat serum, based on the determination of norepinephrine formed from a substrate dopamine under the optimum conditions for the enzyme reaction. Norepinephrine and epinephrine (as an internal standard), after chromatography on a small cartridge of cation-exchanger, are converted into fluorescent compounds by reaction with 1,2-diphenylethylenediamine (DPE), a fluorogenic reagent for catechol compounds [15]. The fluorescent compounds from the amines are separated by reversed-phase HPLC on TSK gel ODS-80TM.

EXPERIMENTAL

Reagents and materials

Norepinephrine bitartrate, epinephrine bitartrate, dopamine hydrochloride, N-methyldopamine hydrochloride and catalase (from bovine liver, 2890 U/mg of protein) were purchased from Sigma (St. Louis, MO, U.S.A.), L-ascorbic acid, sodium fumarate and copper(II) sulphate from Nakarai Chemicals (Kyoto, Japan), and N-ethylmaleimide and tyramine hydrochloride from Wako (Osaka, Japan). All other chemicals were of reagent grade. Deionized and distilled water was used. DPE solution (0.1 *M*, pH 6.5–6.7) was prepared by dissolving 212 mg of DPE in 10 ml of 0.1 *M* hydrochloric acid.

A Toyopak IC-SP M (strong cation exchanger, sulphopropyl resin, Na^+ form; Toyo Soda, Tokyo, Japan) cartridge (35×10 mm I.D.) was washed before use as described previously [16].

Enzyme preparations

Human plasma was obtained by centrifugation at 1000 *g* for 20 min at 4°C of the heparinized blood from healthy volunteers (21–39 years of age). The plasma was stored at −20°C until assay, and it was diluted four times with 0.25 *M* sucrose prior to the analysis. Rat serum was obtained by centrifugation of the blood from male Donryu rats (four weeks of age) at 1000 *g* for 20 min at 4°C and stored at −20°C until assay. The DBH activity in the plasma and serum is stable for more than one month when stored at −20°C.

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-μl loop) and a Hitachi 650-10 LC spectrofluorimeter fitted with 20-μl flow cell. The spectral bandwidths in the excitation and emission monochromators were both 5 nm. Uncorrected fluorescence excitation and emission spectra of the eluate were measured with a Hitachi 850 fluorescence spectrophotometer fitted with a 20-μl flow cell. Spectral bandwidths of 5 nm were used both for the excitation and emission wavelengths. A TSK gel ODS-80TM column (particle size 5 μm, 150×4.6 mm I.D., Toyo Soda) was used. The column temperature was ambient (20–25°C). The mobile phase

was acetonitrile–methanol–0.1 *M* acetate buffer, pH 5.0 (4:2:5, v/v/v) and the flow-rate was 1.0 ml/min. Peak heights were used for quantification.

Assay procedure

To 20 μl of the enzyme preparation were added 90 μl of 0.5 *M* acetate buffer (pH 5.0), 20 μl each of 0.3 *M* N-ethylmaleimide and 10 μ*M* copper(II) sulphate, and 10 μl each of 10 mg/ml catalase, L-ascorbic acid (60 m*M* for human plasma DBH, 0.25 *M* for rat serum DBH) and 0.3 *M* sodium fumarate. The mixture was preincubated at 37°C for 5 min and again incubated for 15 min for human plasma DBH and 30 min for rat serum DBH, after the addition of 20 μl of 80 m*M* dopamine. At the end of the incubation, 700 μl of 0.5 *M* trichloroacetic acid and 100 μl of 4 μ*M* epinephrine (internal standard) were added, and then the mixture was centrifuged at 1000 *g* at 4°C for 10 min. The supernatant (100 μl) was poured on to a Toyopak IC-SP M cartridge. The cartridge was washed successively with 10 ml of water, 2 ml of 0.2 *M* sodium phosphate buffer (pH 6.0), 2 ml of water and 2 ml of aqueous 50% acetonitrile. The adsorbed amines were eluted with 2 ml of a mixture of acetonitrile–0.6 *M* potassium chloride (1:1, v/v). To the eluate were added 200 μl of the DPE solution and 10 μl of 0.3 *M* potassium hexacyanoferrate(III). The mixture was allowed to stand at 37°C for 40 min to derivatize the amines to the fluorescent compounds. The final mixture (100 μl) was analysed by HPLC. For the blank, the enzyme preparation was carried through the procedure except that the order of addition of dopamine and trichloroacetic acid was reversed and incubation was omitted. The Michaelis constant (K_M) for dopamine was calculated from the Lineweaver–Burk plots.

RESULTS AND DISCUSSION

The conditions of the fluorescent derivatization and HPLC analysis were essentially the same as described previously [16].

Typical chromatograms obtained with the human plasma and rat serum preparations, and those of the blank samples are shown in Fig. 1. Peak 1 (retention time, 4.2 min) had fluorescence excitation (maximum 350 nm) and emission (maximum 475 nm) spectra identical with those for the norepinephrine standard. The peak of norepinephrine in the blank may be mainly due to its non-enzymatic formation. The same result has been reported by other workers [11]. For the blank, no incubation for the enzyme reaction with the deproteinized enzyme preparations and the incubation without enzyme preparations were investigated, and no difference was observed between the formation rates of norepinephrine in the two blanks: the former procedure was recommended for convenience.

A single peak has been observed in the chromatogram of the DPE derivative of dopamine at concentrations of 10 nmol per tube or less, or endogenous dopamine in plasma [16], urine [17], erythrocytes [18] or platelets [18]. However, when a large amount of dopamine (more than 10 nmol per tube) was used as the substrate for the DBH-catalysed reaction, two peaks (peaks 3 and 4; retention times, 10.4 and 2.5 min, respectively) were observed. Although the former peak

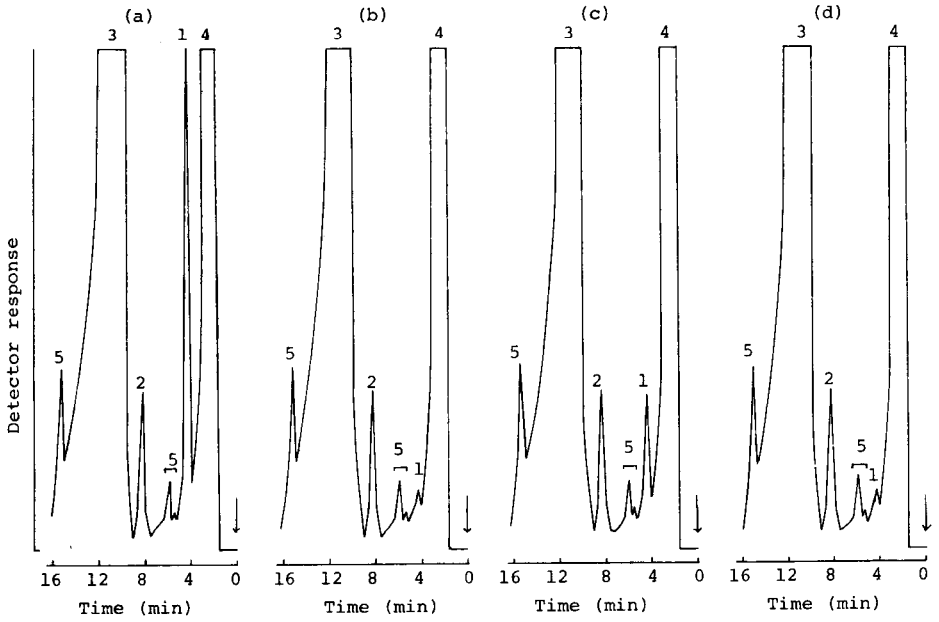

Fig. 1. Chromatograms obtained with enzyme preparations of (a) human plasma and (c) rat serum, and (b and d) their blanks, respectively. Portions (20 μl) of the enzyme preparations were treated according to the procedure. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = dopamine; 4 = by-product from dopamine; 5 = unidentified. DBH activities (nmol/min/ml): a, 15.8; c, 0.34.

showed fluorescence excitation (maximum 350 nm) and emission (maximum 480 nm) spectra that were identical with those of dopamine at low concentrations, the spectra (excitation and emission maxima, 335 and 450 nm, respectively) for the latter peak were quite different from the former. This suggests that the component of peak 4 should be a by-product of the DPE reaction. These peaks, however, did not interfere with the determination of norepinephrine formed enzymatically.

Epinephrine concentrations were extremely low in human plasma and rat serum [16,19], and the peak of endogenous epinephrine could not be detected under the present HPLC conditions. Epinephrine has been employed as an internal standard in DBH assay by HPLC with electrochemical detection [11] and with fluorimetric detection [14]. Thus, epinephrine was also recommended as an internal standard in the present procedure. N-Methyldopamine could also be used as an internal standard (Fig. 2), but it had a much longer retention time (28.2 min) than that of epinephrine (8.2 min).

DBH in both the enzyme preparations was most active at pH 5.0 in sodium acetate buffer and the buffer concentrations of 0.2–1.0 *M* gave the maximum activity; 0.5 *M* acetate buffer (pH 5.0) was used in the recommended procedure.

The activity of dopamine in the enzyme reaction mixture was maximal and constant in the concentration range 6.0–10.0 m*M* for both enzyme preparations. The K_M values for dopamine were 2.6 m*M* for human plasma DBH and 2.9 m*M* for rat serum DBH; 8.0 m*M* dopamine was used in the procedure.

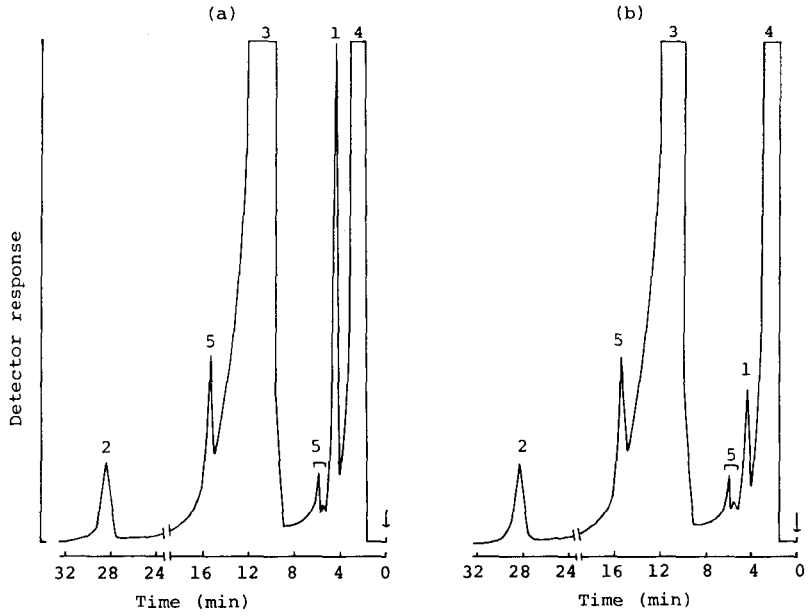

Fig. 2. Chromatograms obtained with enzyme preparations of (a) human plasma and (b) rat serum. Portions (20 μl) of the enzyme preparations were treated according to the procedure using N-methyldopamine (8 nmol/ml) as an internal standard. Peaks: 2 = N-methyldopamine; 1 and 3-5 as in Fig. 1. For DBH activities, see Fig. 1.

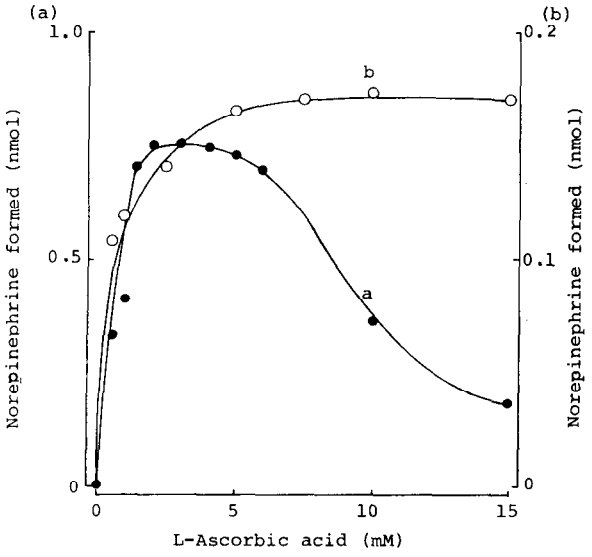

Fig. 3. Effect of L-ascorbic acid concentration on the amount of norepinephrine formed. Portions (20 μl) of enzyme preparations of (a) human plasma and (b) rat serum were treated according to the procedure at various concentrations of L-ascorbic acid. DBH activities (nmol/min/ml): a, 9.9; b, 0.32.

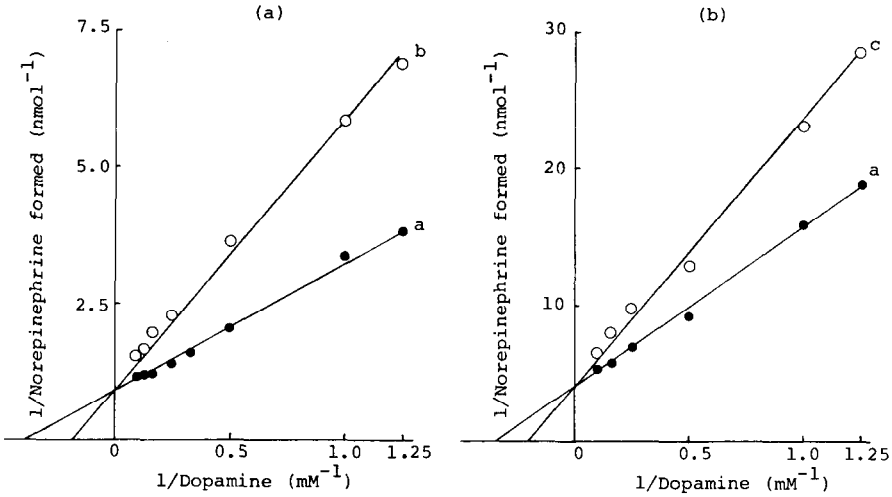

Fig. 4. Inhibition of (a) human plasma and (b) rat serum DBH by tyramine. Portions (20 μl) of enzyme preparations were treated in the presence of tyramine according to the procedure. Tyramine concentrations (mM) in the enzyme reaction mixture: a, nil; b, 2; c, 4. The data were plotted by linear regression analysis.

An almost maximal and constant DBH activity was obtained in the presence of 2–4 mM ascorbic acid for human plasma and 8–15 mM for rat serum (Fig. 3); 3 mM for human plasma and 12.5 mM for rat serum were used.

DBH is enhanced by N-ethylmaleimide and copper(II) ion, which inactivate endogenous inhibitors of DBH, mostly sulphydryl compounds [20,21]. N-Ethylmaleimide at a concentration greater than 10 mM was required for a maximum

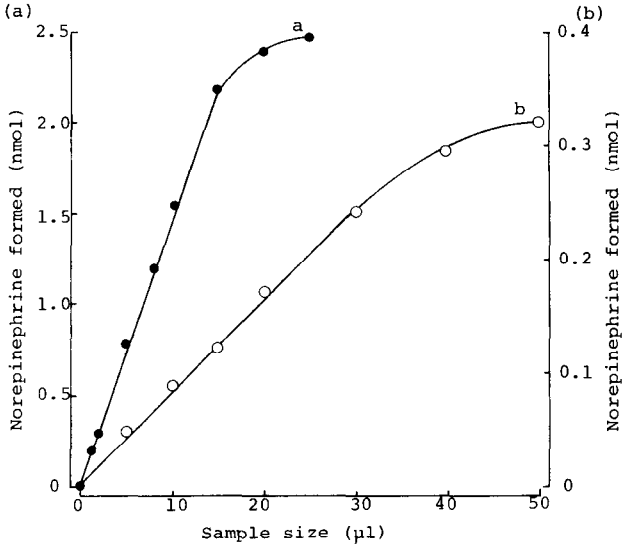

Fig. 5. Effect of the size of the enzyme preparation on the amount of norepinephrine formed. Portions of the enzyme preparations of (a) human plasma and (b) rat serum were treated according to the procedure. DBH activities (nmol/min/ml): a, 11.3; b, 0.28.

activity in both the enzyme preparations. Copper(II) sulphate provided a maximum activity at 0.8–1.5 μM and 0.8–2.0 μM in human plasma and rat serum DBH, respectively. Thus, 15 mM N-ethylmaleimide and 1 μM copper(II) sulphate were recommended in the procedure.

Catalase has been used for the protection of DBH from hydrogen peroxide generated by non-enzymatic oxidation of ascorbic acid [22]. Catalase at a concentration above 50 μg per assay tube gave a maximum activity in both enzyme preparations; 100 μg (280 U) was used in the procedure.

Sodium fumarate, which acts as an accelerator in the reaction [23], gave a maximum activity in both enzyme preparations in the concentration range 10–20 mM; 15 mM was adopted in the enzyme reaction.

DBH works not only on dopamine but also on tyramine [3,23]. Tyramine inhibited DBH in human plasma and rat serum in a competitive mode against dopamine (Fig. 4), with observed inhibitory constant values of 1.8 and 5.5 mM, respectively, which were obtained according to the method of Dixon [24].

The amount of norepinephrine formed enzymatically was proportional to human plasma preparation up to 15 μl and to rat serum preparation up to 30 μl (Fig. 5). The DBH activity was linear with time up to 30 min in human plasma DBH and up to 40 min in rat serum DBH, when incubated at 37°C.

For clean-up of the enzyme reaction mixture, a strong cation-exchange cartridge, Toyopak IC-SP M, was used under the same conditions in principle as described previously [16]. Recoveries (mean ± S.D., $n=5$) of norepinephrine and epinephrine (0.4 nmol each) added to the enzyme reaction mixture were 72.4 ± 2.8% and 87.7 ± 2.2% (human plasma) and 76.2 ± 1.6% and 87.0 ± 2.3% (rat serum), respectively.

A linear relationship was observed between the ratio of the peak height of norepinephrine to that of epinephrine and the amount of norepinephrine added to the blank in each enzyme preparation over the range from 5 pmol to 1 nmol. The detection limit for norepinephrine formed enzymatically was 5 pmol per assay tube (20 fmol per 100-μl injection volume) at a signal-to-noise ratio of 2. The relative standard deviations ($n=8$ each) were 7.6% and 6.2% for mean DBH activities of 11.2 and 0.33 nmol/min/ml in human plasma and rat serum, respectively.

DBH activities (mean ± S.D., $n=8$) in the plasma from healthy volunteers (21–39 years old) and serum from rats (Donryu, male, 4 weeks old) were 14.5 ± 4.2 and 0.35 ± 0.15 nmol/min/ml, respectively. The mean DBH activity in human plasma was slightly lower than that previously reported [8,25], but was in good agreement with the results from another group [26]. The mean rat serum DBH activity agreed well with reported data [7,13].

This assay method can use dopamine, a natural substrate for DBH, and is sensitive enough to assay low DBH activity in a small amount of rat serum. Furthermore, the method is simple and rapid to be carried out because it requires only a one-step clean-up; the entire procedure for ten samples takes less than 5 h. Therefore, the method should be useful for biological and biomedical investigations of catecholamines and their metabolism.

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