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ASSAY FOR DOPAMINE β-HYDROXYLASE IN RAT SERUM AND ADRENAL MEDULLA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive method for the assay of dopamine β-hydroxylase in rat serum and in sample solution prepared from rat adrenal medulla is described which employs high-performance liquid chromatography with fluorescence detection. Octopamine, formed enzymatically from the substrate tyramine, is separated by Dowex 50W-X4 column chromatography and oxidized with periodate to *p*-hydroxybenzaldehyde, which is then converted into a fluorescent compound with 2,2'-dithiobis(1-aminonaphthalene). The derivative, after extraction with *n*-hexane—chloroform, is separated by normal-phase chromatography on Alox T. The limit of detection for octopamine formed enzymatically is 10 pmol. This method requires as little as 5 μl of rat serum.

INTRODUCTION

Dopamine β-hydroxylase [DBH, 3,4-dihydroxyphenylethylamine, ascorbate: oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1], catalyzes the conversion of dopamine to norepinephrine [1]. The enzyme is released into the blood stream from the peripheral sympathetic nerve endings and the adrenal medulla along with catecholamines [2—4], and its activity in serum has therefore become of interest as a possible index of the sympathetic nerve functions. But kinetic, pharmacological and biochemical studies of DBH in serum or tissues in small animals are required for the experimental clarification of the nerve functions, where a highly sensitive assay method for the enzyme is preferable.

Many assay methods for DBH in biological materials have been proposed: two-step enzymatic radiochemical [5, 6], one-step radiochemical [7], tritium

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release [8], spectrophotometric [9], dual-wavelength spectrophotometric [10], fluorimetric [11, 12], phosphorimetric [13] and high-performance liquid chromatographic (HPLC) [14—16] methods. They are sensitive enough to assay the DBH activity in human serum. DBH activity in rat serum, which is 1/50—1/100 times lower than that in human serum, can be assayed by the radioassays, dual-wavelength photometry, fluorimetry and phosphorimetry. The reaction conditions of the radioassays are not optimal for the enzyme. The dual-wavelength photometry and fluorimetry are not appreciably sensitive and so require relatively large amounts of rat serum (100 and 50 μl, respectively). The phosphorimetry permits the assay of DBH in 20 μl of rat serum, but requires cryogenic equipment.

We have developed a sensitive HPLC method with fluorescence detection for the assay of DBH in 5 μl of rat serum. This method is based on the determination of octopamine formed from substrate tyramine, under the optimum reaction conditions for the enzyme. Octopamine, after separation by ion-exchange chromatography, is oxidized with periodate to *p*-hydroxybenzaldehyde, which is then converted into a fluorescent compound by reaction with 2,2′-dithiobis-(1-aminonaphthalene) (DTAN), a selective fluorescent derivatization reagent for aromatic aldehydes [17]; the fluorescent product is 2-(*p*-hydroxyphenyl)-naphtho[1,2-*d*]thiazole [18]. The fluorescent product is extracted into a mixture of *n*-hexane and chloroform and separated by normal-phase HPLC on LiChrosorb Alox T (aluminium oxide). The assay for DBH in a preparation from rat adrenal medulla homogenate is also described.

EXPERIMENTAL

Reagents and materials

All chemicals were of reagent grade, unless otherwise noted. Deionized and distilled water was used. *p*-Hydroxybenzaldehyde was recrystallized from water. Hydrochlorides of tyramine and octopamine (both from Sigma, St. Louis, MO, U.S.A.) were purified by recrystallization from methanol—diethyl ether in the presence of a small amount of hydrochloric acid and stored in the dark. DTAN was prepared as described previously [17]. DTAN solution was prepared as follows. To 40 mg of DTAN dissolved in 60 ml of methanol, 0.1 ml of tri-*n*-butylphosphine was added. After the solution became clear on standing for several min, 25.0 ml of 6 *M* sulfuric acid were added, and the mixture was diluted with water to 100 ml. The solution was stable for at least one month when stored at 4°C.

Rat blood was obtained from male Donryu rats (2—126 days old). Serum was obtained by centrifugation of the blood at 10,000 *g* for 10 min at 5°C. The DBH preparation from rat adrenal medulla was the supernatant from its homogenate, which was prepared by the method of Kato et al. [10] with minor modification as follows. Male Donryu rats (2—126 days old) were stunned and exsanguinated, and the adrenal glands were immediately removed and chilled on ice. All further procedures were carried out at 0—5°C. The adrenal medulla (3—8 mg) was dissected out from the cortex and homogenized in 0.3 ml of 0.05 *M* potassium phosphate buffer (pH 7.4) containing 0.1% (w/v) Triton X-100. In the case of the 2-day-old rats, the adrenal glands were used without

dissection from the cortex because of their minute amounts (the glands weighed less than 1 mg per animal). The homogenate was gently mixed for 20 min and then centrifuged at 23,000 *g* for 30 min. The protein concentration was adjusted to ca. 2 mg/ml with the phosphate buffer, and measured by the method of Lowry et al. [19] using bovine serum albumin as a standard protein.

The ion-exchange column was prepared by packing 0.2 ml of Dowex 50W-X4 (H^+, 200—400 mesh, Dow Chem., Midland, MI, U.S.A.) in a glass tube (130 × 4 mm I.D.). The column was washed successively with 2 ml of 1 *M* sodium hydroxide (twice), 2 ml of water (three times), 2 ml of 6 *M* hydrochloric acid (twice) and finally 2 ml of water (three times). The used column can be regenerated by washing in the same way and is usable more than ten times. The column for HPLC was prepared by packing LiChrosorb Alox T (particle size, 5 μm; Japan Merck, Tokyo, Japan) into a stainless-steel tube (150 × 4 mm I.D.) by the slurry technique [20]. The column can be used for more than 1000 injections with only a small decrease in the theoretical plate number.

Apparatus

An Hitachi 635 A liquid chromatograph was used, equipped with a Rheo dyne 7125 syringe-loading sample injector valve (20-μl loop) and an Hitach 650-10S spectrofluorimeter fitted with a 20-μl flow-cell operating at an emis sion wavelength of 392 nm and an excitation wavelength of 345 nm. Un corrected fluorescence excitation and emission spectra were measured with ar Hitachi MPF-4 spectrofluorimeter in quartz cells (optical path-length, 10 × 1(mm); spectral band-widths of 5 nm were used in both the excitation and emis sion monochrometers. Mass spectra (electron-impact ionization) were mea sured with a JEOL JMS-01-SG mass spectrometer.

Procedure

Substrate—cofactor solution consisted of 20 μl each of 2.0 *M* acetate buffe (pH 5.0), 0.2 *M* tyramine hydrochloride and 0.15 *M* N-ethylmaleimide (an inactivator of endogenous inhibitors of DBH present in serum and tissues [21—23]), and 10 μl each of 0.3 *M* sodium fumarate (an accelerator in the reaction for DBH [1, 24]), 20 μ*M* cupric sulfate (an inactivator of endogenous inhibitors of DBH [21—23]), 10,000 units/ml catalase (used for the protection of DBH from hydrogen peroxide generated by enzymatic oxidation of ascorbic acid [1, 23]) and ascorbic acid (250 m*M* for DBH assay in rat serum and 80 m*M* for DBH assay in the preparation from adrenal medulla). The solution was placed in a 10-ml centrifuge tube containing 100 μl of water and 5 μl of rat serum or the enzyme preparation from rat adrenal medulla (total volume of the incubation mixture, 205 μl). The mixture was incubated at 37°C for 30 min with continual shaking. The reaction was stopped by the addition of 1 ml of 0.6 *M* trichloroacetic acid. The mixture was centrifuged at 1000 *g* for 10 min. The supernatant (1.0 ml) was poured on to a Dowex 50W-X4 column. The column was washed three times with 2 ml of water, and the adsorbed amines were eluted with 1.0 ml of 3 *M* ammonium hydroxide. Octopamine in the eluate was converted to *p*-hydroxybenzaldehyde by the addition of 100 μl of 0.15% (w/v) sodium periodate solution. This oxidation procedure was carried out at 0°C. The excess periodate was decomposed by the addition of 100 μl of 0.6% (w/v)

sodium sulfite solution. The mixture was neutralized with 0.3 ml of 5.0 *M* sulfuric acid. To the resulting solution, 2.0 ml of DTAN solution and 0.5 ml each of 0.05% (w/v) sodium sulfite solution and 15% (w/v) sodium phosphite pentahydrate solution (both freshly prepared; accelerators of the fluorescent derivatization reaction) were successively added. The mixture was allowed to stand at 37°C for 30 min. 2-Mercaptoethanol solution (20%, w/v; 0.5 ml) was added to stop the reaction. To the reaction mixture, 1.0 ml of *n*-hexane—chloroform (7:3, v/v) was added and the fluorescent product was extracted with shaking for 10 min. A 20-μl volume of the upper organic layer was injected into the chromatograph. The organic layer could be used for more than one day when stored in the dark.

The mobile phase comprised 37 m*M* acetic acid in *n*-hexane—chloroform (1:1, v/v) and the flow-rate was 2.0 ml/min (30 kg/cm^2). The column temperature was ambient (20—25°C).

For the blank, 5 μl of the enzyme sample were replaced with 5 μl of water and the same procedure was carried out. For the calibration curve, 100 μl of water in the procedure were replaced with 100 μl of octopamine standard solution (0.1—10 nmol per 100 μl) and the same procedure as for the blank was carried out.

The peak height in the chromatogram was used for the quantitation of octopamine.

RESULTS AND DISCUSSION

Ascorbic acid in the incubation mixture gave a maximum and constant activity of DBH at concentrations of 10—20 m*M* for DBH assay in rat serum and 3—6 m*M* for DBH assay in the enzyme preparation from adrenal medulla, with an observed K_m of 0.6 m*M* in each case; 12.2 and 3.9 m*M* were used for serum and adrenal medulla, respectively, in the standard procedure. The other conditions for the enzyme reaction are optimal [9, 11, 12].

The amount of octopamine formed was proportional to the sample sizes of both the enzyme preparations up to at least 15 μl. The enzyme activity was almost linear with time up to at least 50 min when incubated at 37°C.

The fluorescent compound can be extracted from the reaction mixture with *n*-hexane—chloroform (7:3, v/v). The recovery of the compound from the *p*-hydroxybenzaldehyde (an authentic sample of 2-(*p*-hydroxyphenyl)naphtho-[1,2-*d*]thiazole) added to the reaction mixture of the blank in the amount of 1.0 nmol was 96 ± 1% (mean ± standard deviation; n = 10). When the extract was allowed to stand in daylight for more than 7 h, the peak due to octopamine increased slightly in height for unknown reasons. Therefore the extract should be kept in the dark when left standing for long periods.

Fig. 1 shows typical chromatograms obtained with rat serum according to the procedure. The fluorescent compound and the blank can be completely separated within 3.5 min. The retention times for the blank (DTAN) and octopamine are 0.8 and 3.0 min, respectively. The eluate from peak 2 in Fig. 1a has fluorescence excitation (maximum 345 nm) and emission (maximum 392 nm) spectra identical with those of the authentic fluorescent compound dissolved in the mobile phase.

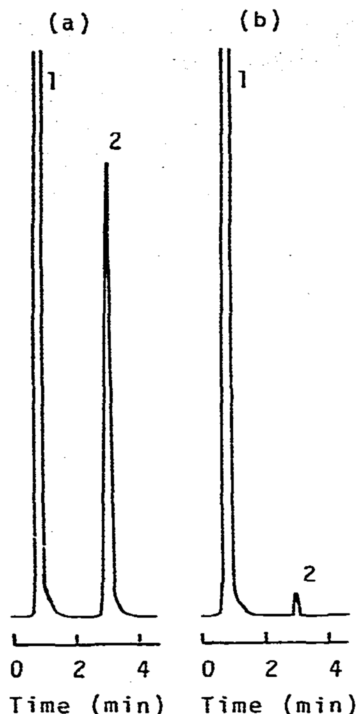

Fig. 1. Chromatograms obtained with (a) rat serum and (b) the blank, according to the procedure described. Peaks: 1 = blank; 2 = octopamine. The activity of DBH was 1.42 μmol of octopamine per min per liter of serum.

The eluate, after evaporating the mobile phase, has a mass spectrum [main peaks: m/e 277 (M^+, base peak), 249 ($M^+ - CO$) and 248 ($M^+ - CHO$)] identical with that of the authentic fluorescent compound.

A small peak in the chromatogram of the blank (peak 2 in Fig. 1b) has exactly the same retention time as that of octopamine and increases in height when the blank with the authentic fluorescent compound added was subjected to HPLC. This indicates that the peak is due to *p*-hydroxybenzaldehyde. This peak is also observed even when purified tyramine hydrochloride alone is treated as in the standard procedure, but not when treated without the oxidation procedure. Commercial tyramine hydrochloride, or purified amine hydrochloride, allowed to stand in daylight for long periods gives a larger peak. It is known that tyramine is converted to octopamine by irradiating tyramine with ultraviolet light [25]. Serum treated as in the procedure without tyramine gives no peak at the retention time of 3 min. These observations suggest that the *p*-hydroxybenzaldehyde results from octopamine present in the tyramine hydrochloride, and this octopamine can not be completely removed by recrystallization.

The peak height of the blank (Fig. 1b, peak 2) was dependent on the temperature in the oxidation procedure. At room temperature (20—25°C), the height was 30—60% greater than at 0°C. Thus the oxidation of octopamine should be carried out at 0°C.

Acetic acid in the mobile phase in a concentration of 20—70 m*M* provides a

good separation of octopamine and the blank; 35 m*M* was used in the procedure recommended. In its absence, the peaks were badly broadened. The magnitude of the *n*-hexane—chloroform ratio in the mobile phase has an effect on the retention time only for octopamine. With a mobile phase of higher ratios, the peak due to octopamine shifts to longer retention times (Fig. 2). A ratio of 1:1 was selected in the standard procedure as it resulted in rapid separation of the peak.

A linear relationship was obtained between the peak height of octopamine and the amount of the amine added in the range of 0.1—5.0 nmol to the enzyme reaction mixture. The recoveries of octopamine added to the enzyme reaction mixture of the blank in the amounts of 0.5 and 2.0 nmol were 96 ± 2% (mean ± standard deviation, n = 5 in each case).

The lower limit of detection for octopamine formed enzymatically was 10 pmol per assay tube (corresponding to DBH activity of 0.07 μmol of octopamine per min per liter of rat serum).

The precision was established with respect to repeatability. The coefficients of variation were 3.4% and 4.2% for mean activities of 1.14 μmol of octopamine per min per liter of rat serum and 1.53 nmol per min per mg of protein of the preparation from rat adrenal medulla, respectively (n = 8 in each case).

Fig. 3 shows the change with age in male Donryu rats of DBH activities in serum and the preparation from adrenal medulla. DBH activity in serum increased with age and reached a maximum value at 14 days of age, which was 4—5 times higher than that for adults (42—126 days of age), and then rapidly decreased to the adult value by 42 days. These values are in agreement with the

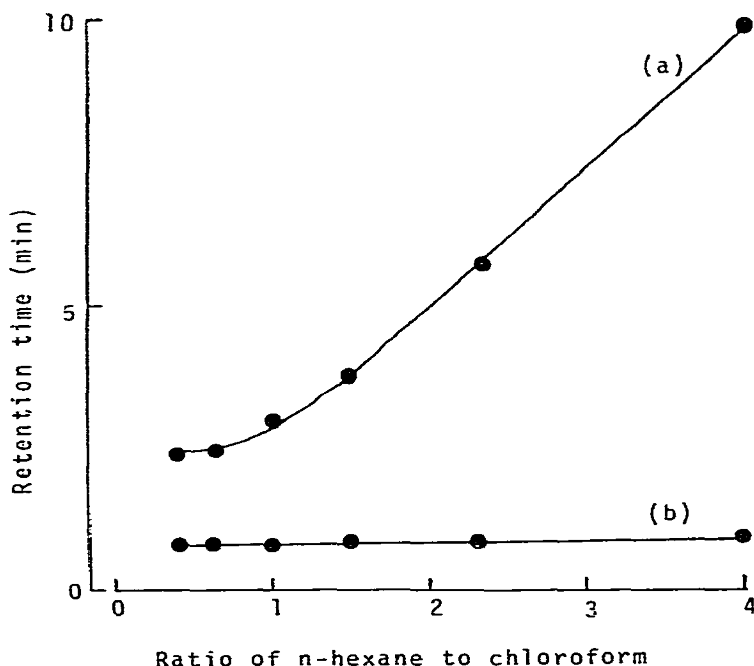

Fig. 2. Effect of the *n*-hexane—chloroform ratio in the mobile phase on the separation of the peaks in the chromatogram. Curves: a = octopamine; b = reagent blank.

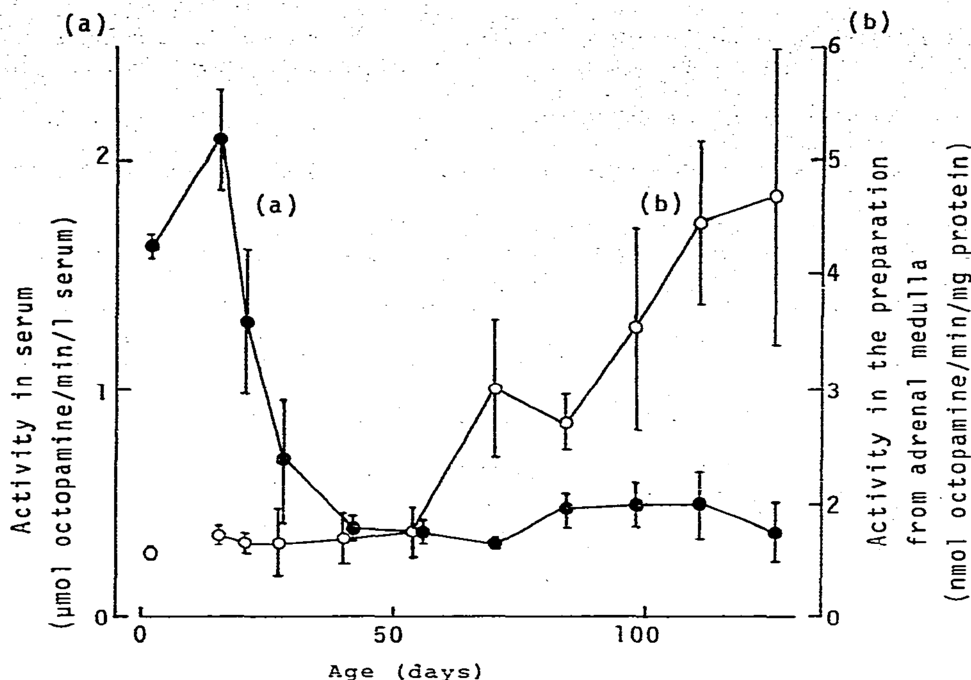

Fig. 3. DBH activity in (a) rat serum and (b) the preparation from adrenal medulla, from rats of different ages (mean ± standard deviation, n = 5 in each case). Curves: a = serum; b = adrenal medulla.

published data [26, 27]. DBH activity in the preparation from adrenal medulla reached a minimum by 56 days of age and then gradually increased with age.

This method is precise and highly sensitive and so requires only 5 μl of rat serum. The method should therefore be useful for biological and biomedical investigations of human diseases accompanying changes in the level of serum DBH activity (such as hypertension and renal and neurological diseases), where only a small amount of serum or tissue is obtainable.

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