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Note

Assay of dopamine β-hydroxylase in human serum as a modification of the assay for the enzyme in rat serum by high-performance liquid chromatography

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Dopamine β-hydroxylase (DBH; 3,4-dihydroxyphenylethylamine, ascorbate : oxygen oxidoreductase (β-hydroxylating), EC 1.14.17.1] catalyzes the β-hydroxylation of dopamine, the last step in the biosynthesis of norepinephrine. The enzyme is released with catecholamines into the blood stream from the peripheral sympathetic nerve endings [1], and its activity in human serum may reflect the degree of diseases accompanying changes in the level of serum DBH activity, such as hypertension and renal and neurological diseases [2], and has therefore drawn much attention from clinical and biomedical investigators.

We reported recently a sensitive method for the assay of DBH in rat serum based on the following principle. 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) (DTAN). The compound, after extraction with *n*-hexane—chloroform, is separated by normal-phase high-performance liquid chromatography (HPLC) on LiChrosorb Alox T with fluorescence detection using *n*-hexane—chloroform containing a small amount of acetic acid as mobile phase [3]. DBH activity in human serum is much higher than that in rat serum; and the optimal conditions of the reaction with the enzyme in human serum were found to be slightly different from those with the enzyme in rat serum. Thus, the reported DBH assay method has been applied to the assay of the enzyme in a minute of human serum with some modifications.

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EXPERIMENTAL

Reagents, materials and apparatus

All chemicals, water, DTAN solution, Dowex 50W—X4 column, LiChrosorb Alox T column for HPLC, high-performance liquid chromatograph, fluorescence detector and mobile phase for HPLC were the same as those used previously [3]. The chromatograph and the detector were operated in the same way as described earlier [3].

Human blood was obtained from normal volunteers (22—51 years of age). Serum was obtained by centrifugation of the blood at 1000 *g* for 10 min at 5°C. The DBH activity in serum is stable for more than one month when stored at −20°C.

Procedure

Substrate—cofactor solution comprised 10 μl each of 80 m*M* ascorbic acid, 0.3 *M* sodium fumarate, 20 μ*M* cupric sulfate and 10,000 units/ml catalase, and 20 μl each of 0.2 *M* tyramine hydrochloride, 0.15 *M* N-ethylmaleimide and 2.0 *M* acetate buffer (pH 5.0). The solution (100 μl) was placed in a 10-ml centrifuge tube containing 100 μl of water and 0.1—2 μl of serum (the sample size of 2 μl is recommended for precise sampling). The mixture was incubated at 37°C for 10 min with continual shaking. After adding 1.0 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 with three 2.0-ml portions of water, and the adsorbed amines (octopamine and tyramine) were eluted with 1.0 ml of 3 *M* ammonium hydroxide. To the eluate chilled at 0°C, 100 μl of 0.15% (w/v) sodium periodate were added. After the addition of 100 μl of 0.6% (w/v) sodium sulfite to decompose the excess periodate, the mixture was netralized with 0.3 ml of 5 *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 and 15% (w/v) sodium hypophosphite pentahydrate 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 compound 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 24 h when stored in the dark.

For the blank, 0.1—2 μl of serum were replaced with 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

A maximum and constant activity of DBH was achieved in the presence of 2—5 m*M* ascorbic acid in the incubation mixture with observed K_m value at 0.6 m*M* and the acid at concentrations greater than 5 m*M* caused marked

inhibition of the enzyme; 4 mM ascorbic acid was therefore used. The amount of octopamine formed correlated well with the incubation time for the first 10 min, and then the correlation deviated slightly (Fig. 1); a 10-min incubation was thus used in the procedure. The other conditions for the enzyme reaction are optimal, and are identical to those used in the assay of DBH in rat serum [3].

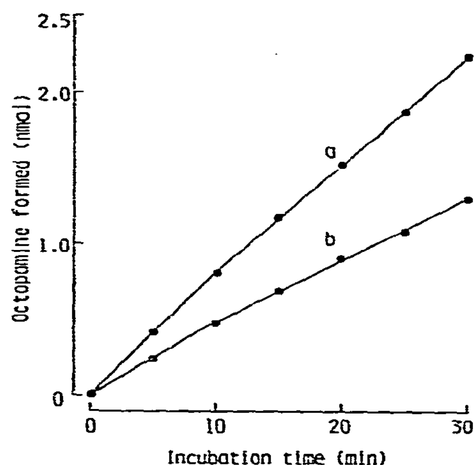

Fig. 1. Effect of incubation time on the amount of octopamine formed. Portions (2 μl) of serum were treated according to the procedure. DBH activity: (a) 40.4 and (b) 23.7 μmol of octopamine per min per liter of serum.

The calibration curve was linear. The amount of octopamine formed was proportional to the sample size of serum up to at least 5 μl. The pattern of the chromatograms obtained using the procedure was almost similar to that observed with the chromatograms for rat serum [3]; the retention times for the blank (DTAN) and octopamine were 0.8 and 3.0 min, respectively.

The recovery of octopamine added to the enzyme reaction mixture in the amounts of 0.5 and 2.0 nmol was 97 ± 2.5% (mean ± S.D., n=10 in each case). The lower limit of detection for octopamine formed enzymatically was 4 pmol per assay tube (corresponding to a DBH activity of 0.2 μmol of octopamine per min per liter of serum). This sensitivity is much higher than that obtained with the fluorimetric [4,5] and reversed-phase HPLC methods [6—8], and permits the assay of DBH in only 0.1 μl of human serum if the sampling is performed precisely, or using a water-diluted sample.

The precision of the method (serum sample of 2 μl) was established with respect to repeatability. The coefficient of variation was 3.6% for mean activity of 33.3 μmol of octopamine per min per liter of serum (n=10). DBH activities in normal sera assayed by the present method were 33.7 ± 12.5 μmol of octopamine per min per liter of serum (mean ± S.D., n=20). The data are in agreement with some previously published results [9,10].

This method is sensitive, precise and rapid, and should be useful in routine assays and in cases where only an extremely small amount of serum is obtainable.

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