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Note

Simple and sensitive assay of dopamine β-hydroxylase in human cerebrospinal fluid by high-performance liquid chromatography with electrochemical detection

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Dopamine β-hydroxylase (DBH, E.C. 1.14.17.1) catalyses the hydroxylation of dopamine (DA) to norepinephrine (NE). The enzyme is released from nerve endings together with NE by exocytosis and appears in the blood [1, 2]. Therefore, many physiological and clinical studies on blood DBH levels have been reported. DBH activities have also been measured in cerebrospinal fluid (CSF) [3–7]. It has been suggested that DBH in CSF is of central origin [6], and its measurement is thought to be valuable for studying the central noradrenergic activity.

Several procedures are available for the assay of DBH [8]: radioassays [9–13], spectrophotometry [14–18], fluorometry [19, 20], gas chromatography–mass spectrometry [21], high-performance liquid chromatography (HPLC) [22, 23]. The limitations and advantages of these various procedures have been reviewed [8].

Recently, a number of methods using HPLC have been introduced in the assay of DBH. HPLC with fluorescence or ultraviolet absorbance detection using tyramine as substrate [22–26] and HPLC with electrochemical detection

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(ED) using DA as substrate [27—29] have been used for the assay of DBH activity.

In the present study, we developed a sensitive and simple HPLC method with electrochemical detection using tyramine as substrate. Under the optimal conditions, tyramine is enzymatically converted to octopamine which is then oxidized with periodate to *p*-hydroxybenzaldehyde. The latter compound is extracted into diethyl ether and determined by HPLC—ED. As our method has the advantages of sensitivity and simplicity, it can be applied to the measurement of very low DBH activity in CSF from children.

EXPERIMENTAL

Materials

Tyramine hydrochloride, octopamine hydrochloride, pargyline hydrochloride and N-ethylmaleimide were obtained from Sigma (St. Louis, MO, U.S.A.); isovanillin was from Tokyo Kasei (Tokyo, Japan), catalase from Boehringer (Mannheim, F.R.G.), and Dowex-50W-X4 from Dow Chem. (Midland, MI, U.S.A.). All other chemicals used were analytical grade.

Procedures

The incubation mixture contained (total volume 1.0 ml, each final concentration in parentheses): 500 μl of enzyme solution (CSF), 100 μl of 2 *M* sodium acetate buffer, pH 5.0 (0.2 *M*), 150 μl of 0.2 *M* N-ethylmaleimide (30 m*M*), 50 μl of 0.2 m*M* copper sulphate (10 μ*M*), 25 μl of aqueous solution (20 mg/ml) of catalase (500 μg, 25 000 I.U.), 25 μl of 40 m*M* pargyline hydrochloride (1 m*M*), 50 μl of 0.2 *M* ascorbic acid (10 m*M*), 50 μl of 0.2 *M* sodium fumarate (10 m*M*); 50 μl of 2 m*M* fusaric acid (100 μ*M*) were included for the blank (control). The reaction mixture was preincubated at 37°C for 5 min, and then the reaction was started by addition of 50 μl of 0.4 *M* tyramine hydrochloride (20 m*M*). After 45 min, the reaction was stopped by adding 1 ml of ethanol containing 1 m*M* fusaric acid in an ice-bath. The mixture was left at 0°C for at least 30 min and then centrifuged at 25 000 *g* for 10 min. The supernatant was immediately transferred to a small glass column (0.5 × 1.0 cm) of Dowex-50-X4 (H^+, 200—400 mesh, packed volume 0.2 ml). Octopamine was eluted with 1 ml of 3 *M* ammonia and converted to *p*-hydroxybenzaldehyde by addition of 20 μl of 2% sodium metaperiodate. The excess sodium metaperiodate was decomposed by addition of 20 μl of 10% sodium metabisulphite. The mixture was neutralized with 0.5 ml of 6 *M* hydrochloric acid and 1.0 nmol of isovanillin was added as an internal standard. Isovanillin and *p*-hydroxybenzaldehyde formed from octopamine were extracted with diethyl ether. After evaporating the diethyl ether, the residue was dissolved with 1.0 ml of the mobile phase and 50 μl were injected into the HPLC system (Yanaco PN-101) with a Yanaco VMD-101 electrochemical detector and with a column of Nucleosil 7 C_{18} (particle size 7.5 μm, 25 cm × 0.4 cm I.D.). The mobile phase was 0.05 *M* potassium phosphate buffer (pH 7.2) containing 20% methanol with a flow-rate of 1.0 ml/min. The detector potential was set at 0.9 V versus the Ag/AgCl electrode.

RESULTS

p-Hydroxybenzaldehyde and isovanillin (internal standard) can be measured with very high sensitivity by the present HPLC—ED system. A linear response of the peak height of the electrochemical detector for the injected amounts of *p*-hydroxybenzaldehyde was observed from 500 fmol to 10 nmol. The recovery of octopamine by cation-exchange resin, Dowex-50-X4, was 92 ± 2% (S.E.M.).

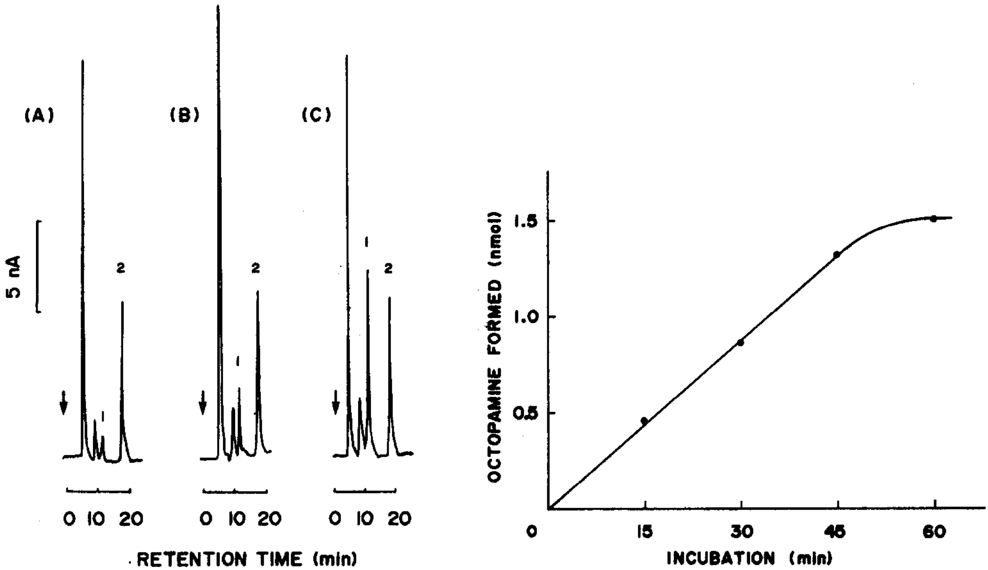

Fig. 1. Chromatographic patterns using DBH incubation mixture of human child CSF as enzyme. (A) Fusaric acid blank incubation; (B) experimental incubation; (C) 50 pmol of octopamine were added to the fusaric acid blank. Isovanillin (50 pmol) was added to each sample as internal standard. DBH activity was calculated to be 12.0 pmol per min per ml CSF. Peaks: 1 = *p*-hydroxybenzaldehyde; 2 = isovanillin.

Fig. 2. The rate of octopamine formation using human adult CSF (500 µl) as enzyme.

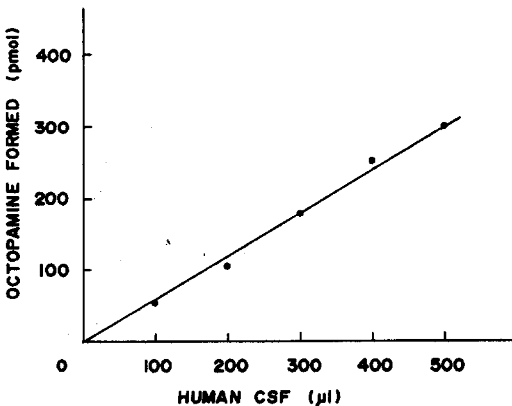

Fig. 3. Relation between the amount of octopamine formed in 45 min and the amount of human adult CSF as enzyme.

A typical chromatogram of the DBH reaction with human CSF as enzyme is shown in Fig. 1. The peak of *p*-hydroxybenzaldehyde (peak 1) derived from enzymatically formed octopamine in the experimental incubation (B) was 3.3 times higher than that in the blank incubation (A). The peak of the chromatogram in the blank incubation has almost the same retention time as that of *p*-hydroxybenzaldehyde and increases in height when the amount of tyramine is increased without enzyme sample. This peak may be due to *p*-hydroxybenzaldehyde, indicating that octopamine is present in commercial tyramine hydrochloride, as already mentioned by Nohta et al. [25]. The contamination of the commercial tyramine with octopamine was shown to be approximately 0.001% by the HPLC—ED assay.

The rate of octopamine formation using human CSF as enzyme proceeded linearly for 45 min, as shown in Fig. 2.

DBH activity in human CSF as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between human CSF in the range 100—500 μl and *p*-hydroxybenzaldehyde formed from tyramine.

The precision of this enzyme assay was tested with CSF as samples in ten simultaneous assays (within-assay) or in five consecutive assays (between-assay), the coefficients of variation being 9.0% and 9.5%, respectively.

Finally, we applied this method to measure DBH activity in CSF from children including epileptic patients. The results are summarized in Table I.

TABLE I

DBH ACTIVITIES IN CSF OF CHILDREN

Diagnosis	Sex	Age (years)	DBH activity (pmol per min per ml CSF)
Epilepsy	F	8 months	5.5
	F	8 months	5.3
	M	2	6.4
	F	2	43.5
	F	2	12.4
	F	5	15.2
	F	5	6.9
	F	5	37.9
	M	13	9.5
	F	14	8.5
Guillain-Barre syndrome	F	5	21.5
	F	5	23.3
Malignant lymphoma	F	9	28.8

DISCUSSION

The assay of DBH activity by HPLC—ED using tyramine as substrate has many advantages.

First, it is very sensitive. The limit of sensitivity for *p*-hydroxybenzaldehyde derived from octopamine and internal standard isovanillin is 500 fmol. Therefore, it is more sensitive than previously published methods and can be applied to measure very low DBH activities in CSF from children. Under such a high

sensitivity, the sensitivity of the assay is determined solely by the blank value. The blank peak appears at the same retention time as *p*-hydroxybenzaldehyde. It increased in height as the amount of the substrate tyramine was increased without an enzyme sample. Nohta et al. [25] also reported such a blank peak and speculated that it may be octopamine contaminating the commercial tyramine. We proved by HPLC—ED assay that tyramine was contaminated by approximately 0.001% of octopamine. Dowex-50 cation-exchange resin will retain other amines and metabolites endogenous to the CSF and present in the enzyme incubation mixture. These amines will be present with octopamine in a similar fashion as octopamine. The resulting products of this reaction are not shown in the chromatograms of Fig. 1. Perhaps the peak found in enzyme blanks, which co-chromatographs with *p*-hydroxybenzaldehyde, originates from a source other than octopamine. The blank peak, however, does not disturb the measurement of DBH activity in CSF. The limit of sensitivity of octopamine formed enzymatically was about 10 pmol.

Secondly, it is reproducible. The coefficient of variation in both simultaneous and consecutive assays was less than 10%.

Thirdly, it is simple and rapid, because this assay includes fewer preliminary steps before the HPLC—ED. Octopamine, which is also electroactive and capable of being detected by the HPLC—ED method, was not directly measured after enzymatic conversion from tyramine, because a large amount of tyramine, the substrate, is also extracted from the incubation mixture by the cation-exchange resin and interferes with the assay of octopamine in HPLC—ED. In the present method, tyramine can be almost completely removed prior to HPLC by converting octopamine to *p*-hydroxybenzaldehyde and by extracting it with organic solvent.

We applied this method to the measurement of low DBH activity in human CSF, especially from children. It has been suggested that DBH in CSF is of central origin [6], and its measurement is thought to be valuable for studying the central noradrenergic activity in physiological and pathological conditions. Recently, several authors reported the DBH activities in CSF of humans, but not of children. As an application of the present DBH assay, we measured DBH activities in CSF of children including epileptic patients. The DBH activities in CSF of children were lower than those of adults, as reported before [30]. Although no tendency towards changes in DBH activities in CSF could be found in epileptic children, this remains to be investigated further.

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