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Note

Determination of phenylethanolamine N-methyltransferase activity in rat brain by high-performance liquid chromatography with fluorometric detection

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Phenylethanolamine N-methyltransferase (PNMT, E.C. 2.1.1.28) catalyzes the formation of adrenaline (AD) from noradrenaline (NA). This enzyme is highly localized in adrenal medulla [1, 2], but can also be detected in specific brain regions of rats by an immunohistochemical method [3] and by highly sensitive radioassay [4]. The properties of PNMT from rat brain and adrenals, and those from rat, cat and chicken brains were compared [5, 6]. All of these previous investigations on brain PNMT have been carried out by the use of radioassay.

High-performance liquid chromatography with electrochemical detection (HPLC—ElCD) provides a rapid, sensitive and accurate technique for measuring PNMT activity. Borchard et al. [7] first reported an HPLC-ElCD assay for PNMT activity using cation-exchange resin as a column support. We have also reported a highly sensitive assay for PNMT activity by high-performance reversed-phase paired-ion chromatography with electrochemical detection [8].

AD can also be measured by HPLC with fluorescence detection (FD) by the post-column trihydroxyindole reaction [9]. In this paper, we describe a highly sensitive and specific assay for PNMT activity by high-performance reversed-phase paired-ion chromatography with trihydroxyindole fluorescence detection. The reversed-phase paired-ion chromatography permitted complete separation of AD from excess NA as substrate, which cannot be achieved by cation-exchange column chromatography. We used α-methylnoradrenaline (α-methyl-NA) as an internal standard in this HPLC—FD method. Although the post-column trihydroxyindole reaction is required, this HPLC—FD method is more specific and sensitive than the HPLC—ElCD method.

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EXPERIMENTAL

Materials

L-NA bitartrate and pargyline·HCl (N-methyl-N-benzyl-2-propynylamine) were obtained from Sigma (St. Louis, MO, U.S.A.), S-adenosyl-L-methionine hydrogen sulfate (SAM) was from Boehringer (Mannheim, G.F.R.) and sodium N-heptanesulfonate (SHS) from Chromato Research (Sagamihara, Japan). α-Methyl-NA was a kind gift from Dr. J. Daly (NIH, Bethesda, MD, U.S.A.). Commercially available L-NA was kindly purified by Dr. T. Katasawa (Dainippon Pharmaceutical Co., Osaka, Japan) to remove the contaminated AD according to the method of Tullar [10] by repeated recrystallization of L-NA bitartrate. All other chemicals were of analytical grade.

Apparatus

The chromatograph used was a Shimadzu LC-3A with a trihydroxyindole reaction system and a RF-500 spectrofluorometer (Shimadzu, Kyoto, Japan). A column (30 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} (particle size 7.5 μm; Macherey, Nagel & Co., Düren, G.F.R.) was used for HPLC.

Procedure

Rats were killed by decapitation, the brains were removed within 30 sec and dissected on a glass plate over ice. The brain tissues were immediately homogenized in 5 volumes of 0.32 *M* sucrose by a tissue sonicator (Ohtake Works, Tokyo, Japan).

The standard incubation mixture for measurement of PNMT activity consisted of the following components in a total volume of 250 μl (final concentrations in parentheses); 10 μl of 0.01 *M* pargyline (a monoamine oxidase inhibitor) in 0.01 *M* hydrochloric acid (0.4 m*M*), 50 μl of 0.5 *M* Tris—HCl buffer, pH 8.0 (0.1 *M*), 15 μl of 0.3 m*M* SAM (18 μ*M*), 20 μl of 0.2 m*M* NA (16 μ*M*), 50 μl of brain homogenate as the enzyme, and water to make up a total volume of 250 μl. The blank reaction mixture contained no enzyme or boiled (90°C for 5 min) enzyme. An amount of 15 pmol of AD was added to another blank incubation as a standard.

Incubation was carried out at 37°C for 60 min and the reaction was stopped by the addition of 600 μl of 0.42 *M* perchloric acid containing 1.55 mg of disodium EDTA, 3.12 mg of $Na_2S_2O_5$, and 10—30 pmol of α-methyl-NA as an internal standard. After stopping the reaction, the enzyme was added to the no-enzyme blank incubation. After 10 min in an ice bath, 200 μl of 0.8 *M* potassium carbonate were added to remove excess of perchloric acid, and 1 ml of 0.5 *M* Tris—HCl buffer (pH 8.5) was added to adjust the pH to 8.0—8.5. The mixture was centrifuged at 1600 *g* for 10 min at 4°C. The clear supernatant was passed through a column (0.4 cm I.D.) containing 100 mg of acid-washed aluminium oxide. The column was washed with 2 ml of 0.05 *M* Tris—HCl buffer (pH 8.5) containing 1% EDTA, 5 ml of water, and 100 μl of 0.5 *M* hydrochloric acid. All these washing solutions were cooled in ice before use.

Adsorbed NA, AD and α-methyl-NA were eluted with 200 μl of 0.5 *M* hydrochloric acid. A 50-μl aliquot of the eluate was injected into the chromatograph. The mobile phase was 0.05 *M* sodium phosphate buffer (pH 2.6),

containing 2 m*M* SHS and 1% of acetonitrile with a flow-rate of 0.8 ml/min, at room temperature. To this column effluent, the reagents for trihydroxyindole reaction — (1) 0.2 *M* potassium phosphate buffer (pH 6.5) containing 0.05% $K_3Fe(CN)_6$ and Brij 35 (0.002 mg/ml), (2) 0.05% ascorbic acid and 0.05% $Na_2S_2O_5$, and (3) 5 *M* sodium hydroxide — were pumped sequentially at a constant flow-rate of 0.3 ml/min using the proportioning pump. Finally the fluorescent products were detected fluorometrically with activation and emission maxima of 410 nm and 510 nm (uncorrected), respectively. Under these conditions, the retention times were: solvent front, 9.2 min; NA, 16.3 min; AD, 22.0 min; and α-methyl-NA, 27.0 min.

The AD formed enzymatically by PNMT was calculated by the equation:

$$\frac{R(\mathrm{E}) - R(\mathrm{B})}{R(\mathrm{B}+\mathrm{S}) - R(\mathrm{B})} \times 15 \text{ (pmol)}$$

where R is the ratio of peak heights (peak height of AD/peak height of α-methyl-NA), R(E) being that from the enzyme incubation, R(B) from the no-enzyme or boiled-enzyme incubation (blank), and R(B + S) from the no-enzyme or boiled-enzyme plus 15 pmol AD incubation (standard).

For comparison with the HPLC—ElCD method, 50 μl of the eluate from the aluminium oxide column were injected into a liquid chromatograph (Yanaco L-2000) with an Yanaco VMD-101 electrochemical detector (Yanagimoto Co., Kyoto, Japan) and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18} according to the method of Trocewicz et al. [8]. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.6) containing 5 m*M* sodium pentanesulfonate and 0.5% acetonitrile (v/v) at a flow-rate of 0.9 ml/min, and the detector potential was set at 0.6 V against a Ag/AgCl electrode.

RESULTS AND DISCUSSION

The peak height of AD showed a linear relationship with AD injected from 0.1 pmol to 100 pmol; $y = 0.87x$, where y is the relative fluorescence intensity and x is the concentration of standard adrenaline (pmol). The HPLC—FD assay for PNMT activity was developed using homogenate of rat pons plus medulla oblongata as the enzyme. Pargyline (0.4 m*M*) in the incubation mixture protected the NA substrate and enzymatically formed AD from oxidation by monoamine oxidase. The chromatographic pattern of the PNMT reaction with a homogenate of rat pons plus medulla oblongata is shown in Fig. 1. Enzymatically formed AD with 10 mg of rat pons plus medulla oblongata as enzyme during 60 min of incubation at 37°C (Fig. 1A) showed a significant increase of the peak height of AD as compared with a small amount of AD in a no-enzyme blank incubation (Fig. 1B). Fig. 1C shows blank incubation with 15 pmol of AD as a standard. In this chromatogram, only the peaks of solvent front, NA, AD, and α-methyl-NA appeared, and we could not observe any unknown peaks of interfering substances as in the HPLC—ElCD method [8]. Although time of the assay in this HPLC—FD method was longer than that in the HPLC—ElCD method due to the time for the trihydroxyindole reaction,

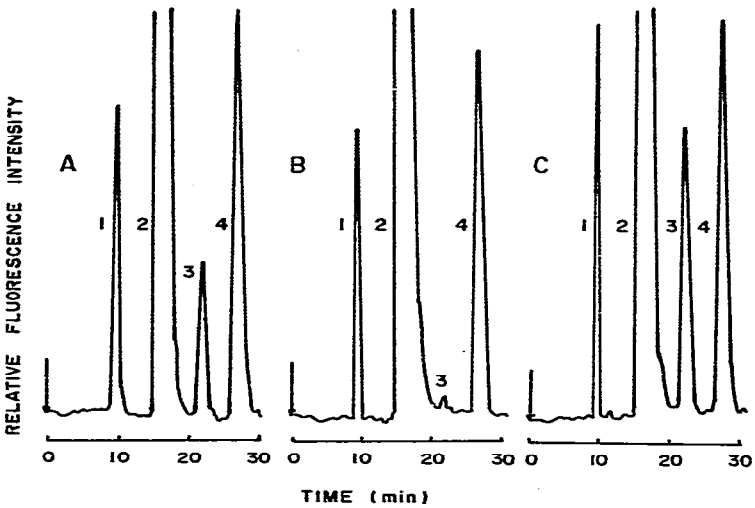

Fig. 1. Typical elution patterns of phenylethanolamine N-methyltransferase incubation mixtures with a homogenate of rat pons plus medulla oblongata as enzyme. The conditions are described in the *Procedure* section. (A) Incubation with a homogenate of 10 mg of rat pons plus medulla oblongata, 1 h incubation at 37°C. (B) Blank incubation without enzyme. (C) Another blank incubation with 15 pmol of adrenaline added as standard. Peaks: 1 = solvent front; 2 = noradrenaline; 3 = adrenaline; 4 = α-methylnoradrenaline.

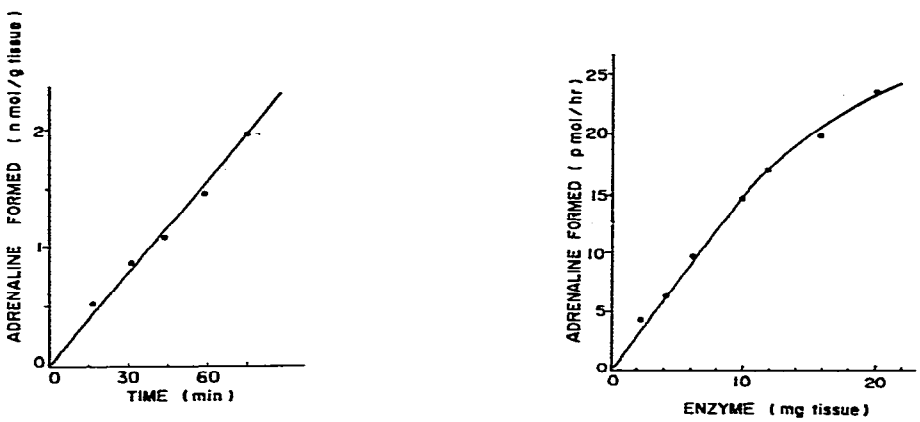

Fig. 2. Rate of adrenaline formation using a homogenate of rat pons plus medulla oblongata as enzyme at 37°C. Standard incubation system containing 10 mg of tissue was used as described in the *Procedure* section.

Fig. 3. Rate of adrenaline formation by rat brain phenylethanolamine N-methyltransferase as a function of tissue concentration. A homogenate of rat pons plus medulla oblongata was used as enzyme. Incubations were carried out for 1 h at 37°C as described in the *Procedure* section.

this assay method is more specific for catecholamines as compared with the HPLC—ElCD method.

The rate of AD formation using a homogenate of rat pons plus medulla oblongata as enzyme proceeded linearly for 75 min at 37°C, as shown in Fig. 2. A complete linearity was also observed between the amounts of ho-

mogenate from 2 mg to 10 mg tissue and enzymatically formed AD during 1 h incubation at 37°C (Fig. 3). Optimum pH was about 8.0, and this value is similar to those reported by Connet and Kirshner [11] and Yu [5]. The reproducibility of the assay with replicates of the same sample was 100 ± 10% (S.D. for five determinations). The sensitivity was 0.2 pmol AD formed enzymatically, and was higher than that of HPLC—ElCD method [8]. This blank value is mainly due to endogenous AD and contaminated AD in the NA used as substrate. Therefore, higher purification of the NA substrate to remove contaminated AD is necessary to increase the sensitivity.

We also studied kinetic properties of brain PNMT using a homogenate of pons plus medulla oblongata. The apparent K_m values calculated from the Lineweaver—Burk plots [12] by Wilkinson's program [13] were 16 ± 3 μM toward NA and 9 ± 1 μM toward SAM. These K_m values towards NA and SAM of rat brain PNMT agreed with those reported by Fuller and Hemrick [6] and with our values on the enzyme in human cerebral cortex [14].

We also found PNMT activity in rat bulbus olfactorius. Using both the HPLC—FD and HPLC—ElCD methods, we measured PNMT activity in rat bulbus olfactorius from five animals. The activities obtained by this HPLC—FD method and the HPLC—ElCD method were 363 ± 32 and 364 ± 32 pmol/h per g tissue (mean ± S.D.), respectively. The activity was comparable to that of septum or lower brain stem [8]. This suggests the presence of adrenergic terminals in rat bulbus olfactorius.

The present HPLC—FD method is the most sensitive and specific among various PNMT assay methods. Only one drawback is that it is somewhat time consuming, but this problem can be solved by using an auto-sampler for the HPLC—FD system, which can be completely automated. Therefore, this method may be useful for physiological and pharmacological studies. Vogel et al. [15] and Hobel et al. [16] reported the presence of PNMT activity in human plasma, and the activity was found to be low. This method has been found to be applicable to the assay of PNMT activity in human plasma, and therefore it may also be useful in clinical studies.

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