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HIGHLY SENSITIVE ASSAY FOR PHENYLETHANOLAMINE N-METHYLTRANSFERASE ACTIVITY IN RAT BRAIN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

This paper describes a new and highly sensitive assay for phenylethanolamine N-methyltransferase (PNMT) activity with noradrenaline as substrate in various rat brain regions by high-performance liquid chromatography with electrochemical detection. Commercially available noradrenaline contained about 0.27% of contaminating adrenaline, which was removed to reduce the blank value. Enzymatically formed adrenaline was adsorbed on an aluminium oxide column, eluted with 0.5 *M* hydrochloric acid, separated by high-performance reversed-phase paired-ion chromatography and measured with electrochemical detection. 3,4-Dihydroxybenzylamine was added to the incubation mixture as an internal standard after the reaction. This assay was very sensitive and 0.5 pmol of adrenaline formed enzymatically could be detected. This assay method was applied to measure PNMT activity in various rat brain regions. The highest activity was observed in the hypothalamus, pons plus medulla oblongata, septum, lower brain stem, and cerebral cortex; the lowest activity was in the striatum, hippocampus, cerebellum, and limbic brain.

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT), also referred to as noradrenaline N-methyltransferase (EC 2.1.1.28), is the enzyme that catalyzes the formation of adrenaline (AD) from noradrenaline (NA). The enzyme activity is high in the adrenal gland [1, 2], and recently the enzyme has also been detected in specific brain regions of rats by an immunohistochemical method [3] and by a highly sensitive radioassay [4]. PNMT activity has been also demonstrated in human brain regions by Nagatsu et al. [5] and by Vogel et al. [6] using radio-

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assay. Ray et al. [7] have compared the properties of this enzyme from rat, cat and chicken brains. All of the investigators used radioassay to measure PNMT activity in brain, which is a sensitive, but expensive, method.

High-performance liquid chromatography with electrochemical detection (HPLC—ECD) provides a rapid, sensitive and accurate technique for measuring PNMT activity. Moyer and Jiang [8] optimized isocratic conditions for analysis of catecholamines by reversed-phase paired-ion chromatography. Borchardt et al. [9] first reported a liquid chromatographic assay for PNMT activity using cation-exchange resin and electrochemical detection, but the assay was only sensitive enough to measure the enzyme activity in the adrenal medulla, and they were unable to detect PNMT activity in brain regions except in the hypothalamus. In this paper we describe a highly sensitive assay of PNMT activity by high-performance reversed-phase paired-ion chromatography with electrochemical detection, which permits the measurement of PNMT activity in all rat brain regions.

EXPERIMENTAL

Materials

Pargyline HCl (N-methyl-N-benzyl-2-propynylamine), L-NA bitartrate and L-AD bitartrate 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-pentanesulfonate was from Regis (Morton Grove, IL, U.S.A.). 3,4-Dihydroxybenzylamine (DHBA) was a gift from Eizai (Tokyo, Japan). L-NA bitartrate was also obtained from Wako (Tokyo, Japan).

These commercially available L-NA samples were found to contain 0.27% of AD; they were kindly purified by Dr. Karasawa (Dainippon, Osaka, Japan) to remove the contaminating AD by the method of Tullar [10] based on repeated recrystallization of L-NA bitartrate.

Nucleosil 7 C_{18} (particle size 7.5 μm) for reversed-pjase liquid chromatography was obtained from Macherey-Nagel (Düren, G.F.R.); aluminium oxide 90 was from Merck (Darmstadt, G.F.R.). All other chemicals used were of analytical grade.

The animals were decapitated, and the brains were removed within 30 sec. The whole brain was dissected on a glass plate over ice, according to the method of Carlsson and Lindqvist [11], into nine parts: limbic brain, hypothalamus, cerebellum, pons plus medulla oblongata, striatum, septum, cerebral cortex, hippocampus, and lower brain stem. The brain tissues were then homogenized in 5 volumes of 0.32 *M* sucrose in a glass Potter homogenizer.

Apparatus

The chromatograph used was a Yanaco L-2000 with a Yanaco VMD-101 electrochemical detector and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C_{18}.

Experimental procedure

The HPLC column employed was Nucleosil 7 C_{18}, which was packed using the slurry technique [12] in a 25 cm × 0.4 cm I.D. stainless-steel column. A

slurry column packing apparatus Model 124 (Chemco Scientific Co., Osaka, Japan) was used. A reversed-phase packing material in 12 ml of Slurry Solvent B (non-polar type, from Macherey-Nagel), 5 ml of methanol, 5 ml of dioxane, and 18 ml of carbon tetrachloride, was pumped into an empty column, the back-pressure being maintained up to 500 bars. After the pressure remained constant with no further adjustments of flow-rate, the pumping was continued with 50 ml of chloroform and the pressure was reduced during 15 min. The column was then washed with 100 ml of methanol prior to use in order to obtain a low background signal with electrochemical detection. The number of theoretical plates was calculated from the equation $N = 5.54\ (t_R/w)^2$, where t_R is the retention time of the component of interest and w is the width of the peak at half of the peak height. The calculated value of N for AD was 5500 under the mobile phase used. The mobile phase consisted of reagent-grade chemicals in deionized water: 0.1 *M* sodium phosphate buffer (pH 2.3—3.5), containing 5 m*M* sodium pentanesulfonate as counter-ion forming ion-pairs with ions of catecholamines, and various concentrations (%, v/v) of acetonitrile.

The standard incubation mixture 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* HCl (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 μM), 20 μl of 0.2 m*M* NA (16 μM), 100 μl of 0.32 *M* sucrose (128 m*M*) containing homogenized tissues as enzyme, and water. The blank reaction mixture was incubated either without enzyme or with boiled enzyme (90°C for 5 min). AD (15 pmol or 30 pmol) was added to another no-enzyme blank incubation as a standard.

Incubation was carried out at 37°C for 60 min, and the reaction was stopped with 600 μl of 0.42 *M* perchloric acid containing 1.55 mg of disodium EDTA and 3.12 mg of $Na_2S_2O_5$, and 15 pmol or 30 pmol of DHBA as an internal standard, in an ice-bath. After stopping the reaction, 100 μl of homogenate were added to the no-enzyme blank and no-enzyme standard tubes. After 10 min, 200 μl of 0.8 *M* potassium carbonate were added to remove excess 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 aluminium oxide. The column was washed with 2 ml of 0.05 *M* Tris—HCl buffer (pH 8.5) and 5 ml of water twice, then 100 μl of 0.5 *M* HCl. All of these washing solutions were previously cooled in ice before passing through the column. Adsorbed NA, AD and DHBA were eluted with 200 μl of 0.5 *M* HCl; 50 μl of the eluate were injected into the high-performance liquid chromatograph equipped with electrochemical detector and a column packed with Nucleosil 7 C_{18}. The mobile phase was 0.1 *M* sodium phosphate buffer (pH 2.6) containing 5 m*M* sodium pentanesulfonate and 0.5% (v/v) acetonitrile, at a flow-rate of 0.9 ml/min; the detector potential was set at 0.6 V vs. an Ag/AgCl electrode. The chromatography was performed at 21°C. Under these conditions, the retention times were: solvent front 2.2 min, NA 5.0 min, AD 8.0 min, and DHBA 9.8 min. The AD formed enzymatically by PNMT was calculated from the equation

$$\frac{R_{(E)} - R_{(B)}}{R_{(B+S)} - R_{(B)}} \times 15 \text{ or } 30 \text{ (pmol)}$$

where R is the ratio of peak height (peak height of AD/peak height of DHBA), $R_{(E)}$ being that from the enzyme incubation, $R_{(B)}$ from the no-enzyme or boiled-enzyme incubation, and $R_{(B+S)}$ from the no-enzyme or boiled-enzyme plus AD (internal standard, 15 or 30 pmol) incubation.

RESULTS

AD, NA and DHBA could be measured with very high sensitivity by the present HPLC—ECD method. The linear response of the peak height of the electrochemical detector for the amount of AD injected was observed from 0.1 pmol to 1 nmol. L-NA bitartrate as substrate obtained from Wako or Sigma contains about 0.27% of AD. This contaminating AD gave a high blank value, thus decreasing the sensitivity of the PNMT assay. Dr. Karasawa kindly purified the NA samples down to 0.012% of contaminating AD.

The assay method of PNMT by HPLC—ECD was developed with homogenates of rat brain as enzyme. Pargyline added to the standard incubation (0.4 mM) inhibited monoamine-oxidase activity to protect enzymatically formed AD. The chromatographic pattern of the PNMT reaction with the homogenate

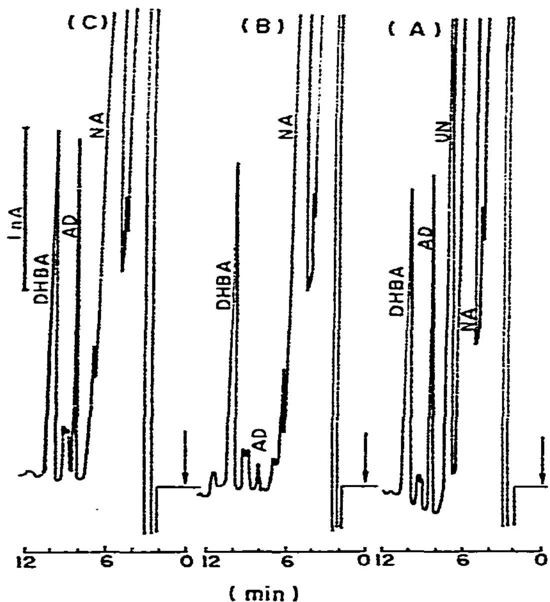

Fig. 1. A typical elution pattern of phenylethanolamine N-methyltransferase incubation mixtures with the homogenate of rat pons plus medulla oblongata as enzyme. The conditions are described in Experimental. The incubation mixture contained 10 mg of rat pons plus medulla oblongata as enzyme and 16 μM noradrenaline (NA) and 18 μM S-adenosyl-L-methionine (SAM) as substrates. (A) Experimental incubation with homogenate of 10 mg of rat pons plus medulla oblongata. (B) Blank incubation without enzyme. (C) 15 pmol of adrenaline (AD) were added to another blank incubation after stopping the reaction. Formation of 16.6 pmol of AD from NA during 60 min incubation at 37°C was calculated from the charts. DHBA = dihydroxybenzylamine (internal standard), UN = unknown peak.

of rat pons plus medulla oblongata is shown in Fig. 1. The experimental incubation with 10 mg of rat pons plus medulla oblongata (Fig. 1A) showed significant formation of AD during the reaction at 37°C for 1 h, compared with a small amount of AD in the blank incubation without enzyme (Fig. 1B). Fig. 1C shows no-enzyme blank with 15 pmol of AD as a standard. Between the peaks of NA and AD (Fig. 1A) an unknown peak appeared, probably a metabolite of NA. For the blank incubation, the reaction mixture was also incubated either without enzyme or with boiled enzyme, and both blank incubations were compared. However, there were no differences between these two blanks. When 0.1 *M* sodium phosphate buffer (pH 2.3) with 5 m*M* sodium pentanesulfonate and 0.5% acetonitrile was used as a mobile phase, the retention time of the unknown peak was the same as that of AD. After changing the pH of the mobile phase to 2.6, we obtained complete separation between the peak of AD and the unknown peak. This unknown peak was 3—4 times higher than the peak height of AD formed, and appears to be a catechol, since we did not observe this peak when the aluminium oxide column was substituted by an Amberlite CG-50 column for isolation of AD.

The rate of AD formation using an homogenate of rat hypothalamus as PNMT proceeded linearly for 60 min at 37°C as shown in Fig. 2. PNMT activity in rat hypothalamus homogenate as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between 2.5 and 12.5 mg of hypothalamus homogenate and those of AD formed from NA as substrate and using 18 μM SAM as the methyl-donating substrate.

The reproducibility of the assay with replicates of the same sample was 100 ± 5.6% (S.D. for five determinations).

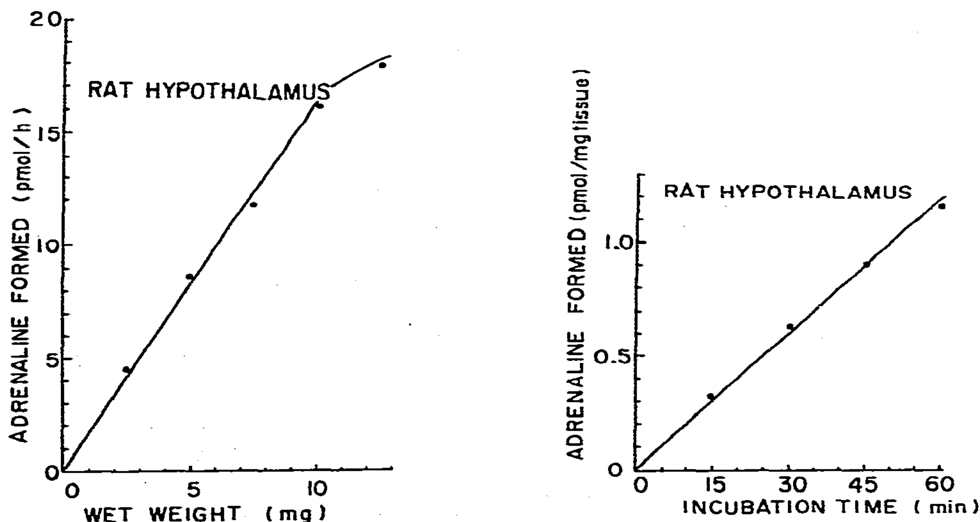

Fig. 2. The rate of adrenaline formation using an homogenate of rat hypothalamus as enzyme at 37°C. Standard incubation system containing 10 mg of tissue was used as described in Experimental.

Fig. 3. Relationship between the amount of adrenaline formed by phenylethanolamine N-methyltransferase and the tissue concentration as measured by HPLC—ECD assay. Incubations were carried out for 60 min at 37°C with increasing amounts of rat hypothalamus.

The activity of PNMT in rat brain was reported to be very low and could be found only by radioassay [4], and Borchardt et al. [9] reported that PNMT activity could be determined only in rat hypothalamus using their HPLC—ECD method. We have applied this newly established reversed-phase paired-ion liquid chromatography with electrochemical detection to measure PNMT activity in nine different parts of rat brain. As shown in Table I, the highest activity was found in the hypothalamus, pons plus medulla oblongata, lower brain stem, septum and cerebral cortex; the lowest activity was in the striatum, hippocampus, cerebellum and limbic brain.

TABLE I

PHENYLETHANOLAMINE N-METHYLTRANSFERASE (PNMT) ACTIVITY IN VARIOUS RAT BRAIN REGIONS

Brain samples were dissected out and processed as described under Materials. Results represent mean ± S.E.M. for a group of five animals. Activity is expressed in pmol of adrenaline formed per g of tissue per hour of incubation.

Brain region	PNMT activity
Hypothalamus	1517 ± 225
Pons plus medulla oblongata	861 ± 58
Septum	369 ± 145
Lower brain stem	224 ± 17
Cerebral cortex	168 ± 15
Hippocampus	78 ± 3
Limbic brain	61 ± 12
Striatum	42 ± 15
Cerebellum	33 ± 6

DISCUSSION

An assay procedure for PNMT activity using HPLC—ECD was first reported by Borchardt et al. [9]. They injected the supernatant of the deproteinized reaction mixture directly into the HPLC system. Although their method was very simple, the sensitivity was not enough to measure rat brain PNMT activity except in the hypothalamus. We have tried to increase the sensitivity of PNMT assay by HPLC—ECD. First, we isolated AD formed enzymatically with an alumina column. Secondly, a reversed-phase column was used instead of an ion-exchange column. This reversed-phase column has a high capacity, and good separation was obtained for AD. Thirdly, we used highly purified NA as a substrate. Therefore, the limit of the sensitivity in the present method depends only on endogenous AD in the crude enzyme. The content of endogenous AD is low in the brain and non-enzymatic reaction hardly occurs. Consequently, about 0.5 pmol of AD formed enzymatically could be detected in the present method.

We applied this highly sensitive method to the measurement of PNMT activity in rat brain regions. The activity was detected in all brain regions, as indicated in Table I, and the localization of PNMT activity was consistent with that reported by Saavedra et al. by the radiometric method [4]. The present method is simple and rapid. It is economical since a labelled substrate and liquid scintil-

lation spectrometer are not needed. Since the main endogenous substrate, NA, is used for the enzymatic reaction rather than phenylethanolamine, which is used in the radioenzymatic method [4], this method may be useful for physiological studies. The recovery from the alumina column can be corrected by the use of the internai standard, DHBA. Therefore, the activity can be calculated very accurately. These results clearly show that this newly established HPLC—ECD method is useful for the measurement of low PNMT activity in the brain regions, which is required for physiological and pharmacological studies.

This method should be useful to study changes in PNMT activity in animal models of various diseases such as spontaneously hypertensive rats [13], or in human brain tissues from patients at autopsy.

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