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Note

A highly sensitive assay for non-specific N-methyltransferase activity in rat tissues by high-performance liquid chromatography with electrochemical detection

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Phenylethanolamine N-methyltransferase (PNMT) and non-specific N-methyltransferase (EC 2.1.1.28) catalyze the N-methylation of aromatic amines. PNMT is specific for phenylethanolamines such as noradrenaline (NA), and catalyzes the last step in catecholamine biosynthesis, forming adrenaline (AD) from NA. PNMT activity is high in adrenal gland [1,2], whereas non-specific N-methyltransferase is distributed in various tissues such as the lung [3]. Borchardt et al. [4] first reported a method to detect PNMT activity by high-performance liquid chromatography with electrochemical detection (HPLC–EICD), which could demonstrate the activity only in the adrenal medulla and hypothalamus. Recently, Trocewicz et al. [5] reported a highly sensitive assay method for PNMT using HPLC–EICD by which the activity in all regions of rat brains could be measured. The activity of non-specific N-methyltransferase in brain regions and peripheral tissues of the rat could be detected by a radioassay [6]. However, there has been no report on an assay method for non-specific N-methyltransferase using HPLC–EICD.

In this paper, we describe a highly sensitive assay procedure for the activity of non-specific N-methyltransferase by high-performance reversed-phase ion-pair chromatography with electrochemical detection. By this method the non-specific N-methyltransferase activity could be determined in various rat brain regions and peripheral tissues.

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EXPERIMENTAL

Materials

The chemicals used were as follows: pargyline HCl, dopamine (DA), N-methyldopamine (N-CH₃-DA) (Sigma, St. Louis, MO, U.S.A.), S-adenosyl-methionine hydrogen sulfonate (SAM) (Boehringer, Mannheim, G.F.R.), sodium N-pentanesulfonate (PSS) (Regis, Norton Grove, IL, U.S.A.), and 3,4-dihydroxybenzylamine (DHBA) (Eizai, Tokyo, Japan).

Sample preparation

The rats were decapitated, and brain and peripheral tissues were removed immediately. The brain regions were cut on glass plate placed over ice according to the method of Carlsson and Lindqvist [7]; hypothalamus, pons plus medulla oblongata, septum, cerebral cortex, striatum, cerebellum, olfactory lobe, and limbic brain were dissected out. The peripheral tissues examined were the lung, liver, stomach, heart, adrenal gland, and salivary gland. The brain and peripheral tissues were homogenized in 5 vols. of 0.32 M and 0.25 M sucrose, respectively, in a glass Potter homogenizer.

Apparatus

The chromatograph used was a Yanaco Model L-2000 with a Yanaco VMD-101 electrochemical detector and a column (25 cm × 0.4 cm I.D.) packed with Nucleosil 7 C₁₈ (particle size, 7.5 μm).

Experimental procedure

The HPLC column (stainless-steel, 25 cm × 0.4 cm I.D.) was packed with Nucleosil 7 C₁₈ using the slurry technique [8] with the slurry column packing apparatus Model 124 (Chemco Scientific, Osaka, Japan) as described before [5].

The incubation mixture for non-specific N-methyltransferase assay consisted of the following components in a total volume of 250 μl (final concentrations in parentheses): 10 μl of 0.01 M pargyline HCl (0.4 mM), 25 μl of 1 M Tris-HCl buffer, pH 8.0 (0.1 M), 20 μl of 0.3 mM SAM (24 μM), 25 μl of 0.2 mM DA (20 μM), 100 μl of 0.32 M sucrose for the brain or 0.25 M sucrose for the peripheral tissues, containing homogenized tissues as enzymes, and distilled water. No enzyme was added to the blank and standard, but the latter contained 30 pmol of N-CH₃-DA. The control was incubated without enzyme, which was then added after incubation.

After incubation for 60 min at 37°C, reaction was stopped by adding 600 μl of 0.42 M perchloric acid which contained 1.55 mg of Na₂EDTA, 3.12 mg of Na₂S₂O₈ and 30 pmol of DHBA as an internal standard. The reaction mixtures were left in an ice bath for 10 min, then 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, to adjust the pH of the solution at 8.0–8.5. The mixture was then centrifuged at 1900 g for 10 min at 4°C. The supernatant was loaded on a column containing 100 mg of aluminium oxide, then the column was washed with 4 ml of 0.05 M Tris-HCl buffer (pH 8.5), 5 ml of distilled water

twice, then 100 μl of 0.5 M HCl. The adsorbed product, N-CH₃-DA, and internal standard (DHBA) were eluted with 200 μl of 0.5 M HCl.

A 50- μl volume of eluent was injected into the high-performance liquid chromatograph equipped with an electrochemical detector and a column packed with Nucleosil 7 C₁₈. The mobile phase was 0.1 M sodium phosphate buffer (pH 2.6) containing 5 mM PSS and 0.25% (v/v) acetonitrile, at a flow-rate of 0.9 ml/min; the detector potential was set at 0.6 V vs. a Ag/AgCl electrode. The chromatography was performed at a temperature between 25°C and 28°C. Under these conditions the retention times were: solvent front 2.0 min, NA 4.4 min, AD 6.8 min, DHBA 8.0 min, DA 12.0 min, and N-CH₃-DA 16.4 min.

The N-CH₃-DA formed enzymatically by non-specific N-methyltransferase was calculated by the following equation:

$$\frac{R(E) - R(C)}{R(S) - R(B)} \times 30 \text{ (pmol)}$$

where R is the ratio of peak height (peak height of N-CH₃-DA/peak height of DHBA), $R(E)$ being that from the experimental incubation, $R(C)$ from the control incubation, $R(S)$ from the standard, and $R(B)$ from the blank.

RESULTS

The calibration curve indicated a linear relationship between the peak height and the amount of N-CH₃-DA from 0.1 to 10 pmol. The sensitivity was 1 pmol of N-CH₃-DA formed by the enzyme reaction. The chromatographic patterns of the non-specific N-methyltransferase reaction of rat whole brain is shown in Fig. 1. The experimental incubation with 10 mg of rat whole brain (Fig. 1A) showed significant formation of N-CH₃-DA during the reaction at 37°C for 1 h, as compared with a small peak in the control incubation without enzyme (Fig. 1B). Figs. 1C and D show no-enzyme blank without and with 30 pmol of N-CH₃-DA as a standard. The optimum pH for non-specific N-methyltransferase obtained using Tris-HCl buffer was pH 8.0. Enzyme activity was linear with the incubation period for at least 90 min (Fig. 2A) and with the amount of enzyme (Fig. 2B).

The kinetics of non-specific N-methyltransferase activity of the whole brain homogenate are shown in Fig. 3. Lineweaver-Burk plots [9] were obtained with DA (Fig. 3A) and with SAM (Fig. 3B) as substrates. The K_M values for DA and SAM calculated from the Lineweaver-Burk plots by Wilkinson's programme [10] were 32 μM and 14 μM , respectively. The maximum plateau activity was obtained when the concentration of SAM was between 20 and 40 μM , and the activity was inhibited when the concentration of SAM was higher than 40 μM (Fig. 3B).

Table I shows the activity of non-specific N-methyltransferase in brain regions and peripheral tissues of the rat. Non-specific N-methyltransferase activity can be detected in various brain regions and peripheral tissues. The highest activity in the brain was detected in the hypothalamus and cerebral cortex. Various peripheral tissues gave high activity.

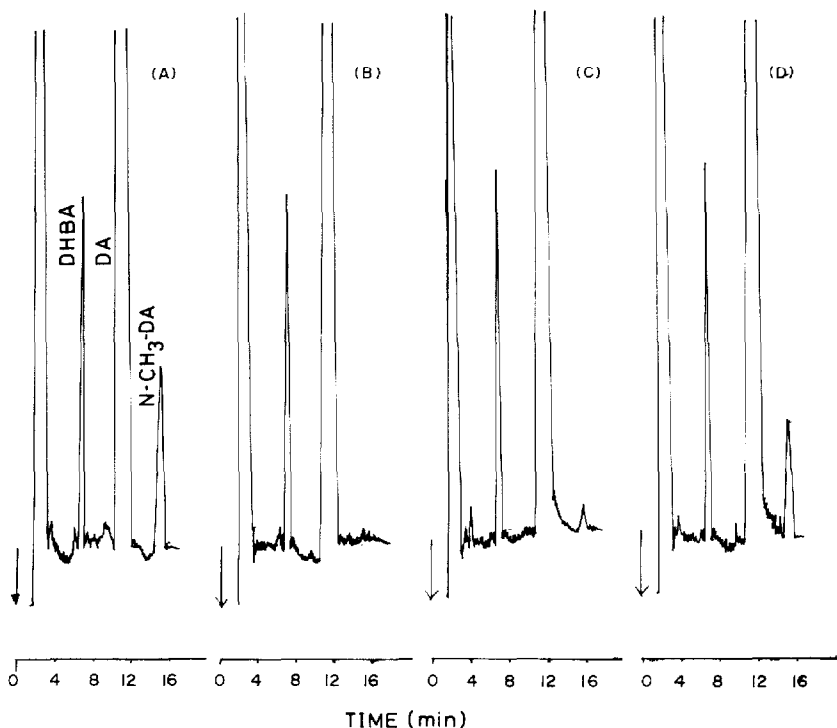


Fig. 1. A typical elution pattern of non-specific N-methyltransferase incubation mixtures with the homogenate of rat whole brain as enzyme. The conditions are described in Experimental procedure. The incubation mixture contained 10 mg of rat whole brain as enzyme and 20 μM dopamine (DA) and 24 μM S-adenosyl-L-methionine (SAM) as substrates. (A) Experimental incubation with homogenate of 10 mg of rat whole brain. (B) Control incubation without enzyme, and with addition of enzyme after incubation. (C) Blank without enzyme. (D) Standard without enzyme and with 30 pmol of N-methyl-dopamine (N-CH₃-DA). DHBA = dihydroxybenzylamine.

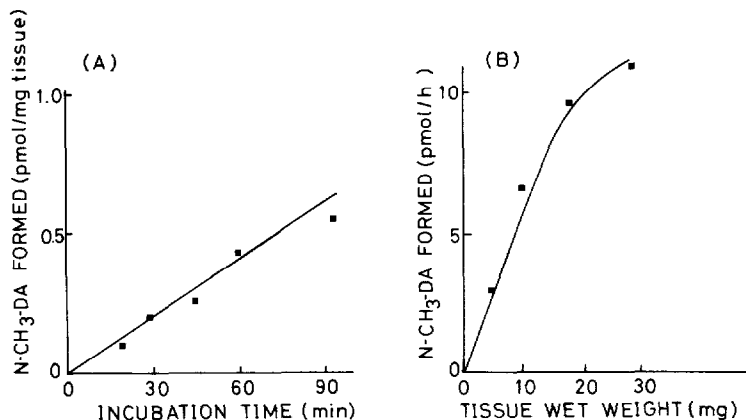


Fig. 2. (A) The rate of N-methyl-dopamine (N-CH₃-DA) formation using an homogenate of rat whole brain as enzyme at 37°C. Standard incubation system containing 10 mg of rat whole brain was used as described in Experimental procedure. (B) Relationship between the amount of N-CH₃-DA formed by non-specific N-methyltransferase and the tissue concentration as measured by HPLC-EICD assay. Incubations were carried out for 60 min at 37°C with increasing amounts of rat whole brain.

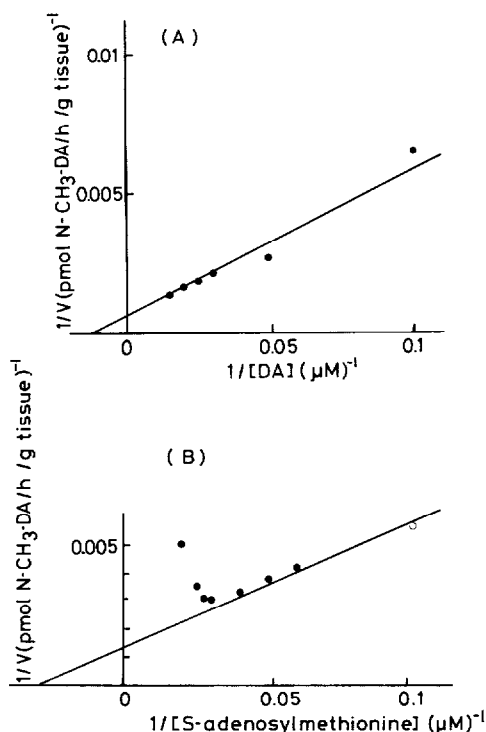


Fig. 3. Lineweaver-Burk plot of non-specific N-methyltransferase activity as function of (A) dopamine (DA) concentration and (B) S-adenosylmethionine (SAM) concentration. Standard incubation system containing $24 \mu M$ SAM or $20 \mu M$ DA and 10 mg of whole rat brain as enzyme was used as described in Experimental procedure.

TABLE I

NON-SPECIFIC N-METHYLTRANSFERASE ACTIVITY IN VARIOUS RAT BRAIN REGIONS AND PERIPHERAL TISSUES

Tissues were dissected out and processed as described under Materials. Results represent mean \pm S.E.M. for a group of five animals. Activity is expressed in pmol N-methyl dopamine formed per g of tissue per h of incubation.

	Non-specific N-methyltransferase activity (pmol per g per h)
Hypothalamus	736 \pm 97
Pons plus medulla oblongata	435 \pm 111
Septum	337 \pm 100
Cerebral cortex	661 \pm 178
Striatum	135 \pm 44
Cerebellum	322 \pm 126
Olfactory lobe	122 \pm 51
Limbic brain	754 \pm 31
Lung	699 \pm 162
Liver	1170 \pm 171
Stomach	276 \pm 108
Heart	719 \pm 154
Adrenal gland	885 \pm 224
Salivary gland	240 \pm 145

DISCUSSION

Saavedra et al. [6] reported the presence and properties of non-specific N-methyltransferase in the brain by a radioassay method using tryptamine and radiolabeled SAM as substrates. They found that the activity of non-specific N-methyltransferase is generally distributed throughout the rat brain, and that the regional and subcellular distribution of non-specific N-methyltransferase in rat brain was different from that reported for PNMT.

The present methods permit the activity of non-specific N-methyltransferase to be measured using DA as substrate and using HPLC—EICD. PNMT can also be measured simultaneously by the previously reported HPLC—EICD method [5]. The activity of non-specific N-methyltransferase was uniformly distributed in both brain and peripheral tissues, whereas PNMT activity was unevenly distributed [5]. These results agree with those by Saavedra et al. [6].

The present method is highly sensitive, simple and rapid. It is economical since a labeled substrate and liquid scintillation spectrometer are not needed. Since the endogenous substrate, DA, is used, this method should be useful to study changes in non-specific N-methyltransferase activity in physiological and pharmacological studies.

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