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DETERMINATION OF PHENYLETHANOLAMINE N-METHYLTRANSFERASE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A highly sensitive assay method for phenylethanolamine N-methyltransferase in rat adrenal medulla and brain is described which employs high-performance liquid chromatography with fluorescence detection. Epinephrine formed enzymatically from the substrate norepinephrine and isoproterenol (internal standard), after chromatography on a small cartridge of a cation exchanger, Toyopak SP, are converted into the corresponding fluorescent compounds by reaction with 1,2-diphenylethylenediamine, a selective fluorescence derivatization reagent for catechol compounds. The derivatives are separated by reversed-phase chromatography on TSK gel ODS-120T. The detection limit for epinephrine formed enzymatically is 0.66 pmol per assay tube.

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; phenylethanolamine:S-adenosylmethionine N-methyltransferase, E.C. 2.1.1.28) catalyses the transfer of the methyl group of S-adenosylmethionine to the amino groups of norepinephrine or other phenylethanolamines. PNMT activity is high in the adrenal gland[1], where epinephrine is synthesised. As PNMT activity also occurs in mammalian brain[2] and the enzyme has been implicated in the pathogenesis of experimental hypertension in animals[3], the role of PNMT in brain has attracted great attention.

Many assay methods for PNMT in biological materials have been reported. They are classified into two types, radiochemical methods[1,2,4–10] and high-performance liquid chromatographic (HPLC) methods coupled with electrochemical[11–13] and post-column fluorescence detection[14]. The radiochemical methods are sensitive but require complicated procedures and expensive radioactive substrates, and the HPLC methods with electrochemical detection require careful manipulation to attain high sensitivity and reproducibility. The HPLC method with fluorescence detection uses

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the trihydroxyindole method and thus requires a post-column derivatization apparatus.

We have developed a highly sensitive assay method for PNMT in rat adrenal medulla and brain involving HPLC with fluorescence detection, based on the determination of epinephrine formed from substrate norepinephrine under the optimum conditions for the enzyme reaction. Epinephrine and isoproterenol as an internal standard, after chromatography on a small cartridge of cation exchanger, are converted into fluorescent compounds by reaction with 1,2-diphenylethylenediamine, a fluorogenic reagent for catechol compounds[15–17]. The fluorescent compounds from the amines are separated by reversed-phase HPLC on TSK gel ODS-120T.

EXPERIMENTAL

Reagents and materials

Norepinephrine hydrogen tartrate, dopamine hydrochloride and glutathione (reduced form) were purchased from Wako (Osaka, Japan). Epinephrine hydrogen tartrate and isoproterenol hydrochloride were obtained from Nakarai Chemicals (Kyoto, Japan). 1,2-Diphenylethylenediamine and Good's buffers (bicine, TAPS, tricine and glycinamide hydrochloride) were obtained from Dojindo Labs. (Kumamoto, Japan). All other chemicals were of analytical-reagent grade. Deionized, distilled water was used. 1,2-Diphenylethylenediamine solution (0.1 *M*, pH 6.7) and Toyopak SP (strong cation exchanger, sulphopropyl resin, sodium ion form; Toyo Soda, Tokyo, Japan) cartridge for sample clean-up were prepared as described previously[17]. The cartridge was washed successively with 2 ml of 2 *M* sodium hydroxide solution, 5 ml of water, 2 ml of 2 *M* hydrochloric acid and 10 ml of water. The used cartridge can be regenerated by washing in the same way and is usable for more than five times.

Enzyme preparations

Male Donryu rats (4 weeks old) were decapitated and the adrenal medullae and brains were immediately removed and chilled on ice. All further procedures were carried out at 0–5°C. Adrenal medulla (5 mg) was homogenized with 1.25 ml of isotonic potassium chloride and the homogenate was dialysed at 4°C for 12–15 h against 5 m*M* phosphate buffer (pH 6.8) containing 0.1 m*M* reduced glutathione. Brain (500 mg) was homogenized with 2.0 ml of isotonic potassium chloride. The homogenates were stored at −20°C until used, and the amounts of protein were measured by the method of Lowry *et al.*[18] using bovine serum albumin as a standard protein.

HPLC apparatus and conditions

An Eyela PLC-10 liquid chromatograph (Tokyo Rika Kikai, Tokyo, Japan) was used, equipped with a Rheodyne 7125 syrine-loading sample injector valve (100-μl loop) and a Shimadzu FLD-1 fluorescence detector fitted with a 14-μl flow cell and an EM-4 emission filter. The column was TSK gel ODS-120T (250 × 4.6 mm I.D.; Toyo Soda). The column temperature was ambient (20–25°C). This column can be used for more than 2000 injections when washed with acetonitrile–methanol–water (52:3:45, v/v) at a flow-rate of *ca.* 1 ml/min for 25 min each day.

The mobile phase was acetonitrile–methanol–50 m*M* Tris–HCl buffer (pH 7.0) (52:3:45, v/v) and the flow-rate was 1.0 ml/min. Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi 850 fluorescence spectrophotometer fitted with an 18-μl flow cell, setting the spectral bandwidths at 5 nm in both the excitation and emission monochromators.

Assay procedure

Adrenal medulla PNMT. To 100 μl of the enzyme preparation from rat adrenal medulla were added 150 μl of 0.1 *M* bicine buffer (pH 8.5) and 50 μl of 0.7 m*M* S-adenosylmethionine. The mixture was pre-incubated at 37°C for 10 min and again incubated for 30 min after the addition of 50 μl of 0.3 m*M* norepinephrine. At the end of the incubation, 50 μl each of 3.0 *M* trichloroacetic acid and 2.0 μ*M* isoproterenol as an internal standard were added. The mixture was centrifuged at 1000 *g* at 4°C for 10 min and the supernatant (300 μl) was poured on to a Toyopak SP (H^+) cartridge. The cartridge was washed successively with 10 ml of water, 3 ml of 0.2 *M* phosphate buffer (pH 5.5) and 10 ml of water. The adsorbed norepinephrine, epinephrine and isoproterenol were eluted with 2.0 ml of ethanol–1.0 *M* sodium chloride (7:3, v/v). To the eluate, 100 μl each of the 1,2-diphenylethylenediamine solution and 15 m*M* potassium hexacyanoferrate(III) were added and the mixture was allowed to stand at 37°C for 40 min to derivatize the amines to the fluorescent compounds. The resulting mixture (100 μl) was injected into the chromatograph. For the blank, the enzyme preparation was carried through the procedure except that the order of the addition of norepinephrine and trichloroacetic acid was reversed, incubation being omitted. Michaelis constants (K_m) for norepinephrine and S-adenosylmethionine were calculated from the Lineweaver-Burk plots.

Brain PNMT. To 100 μl of the enzyme preparation from rat brain were added 100 μl of 0.1 *M* bicine buffer (pH 8.5) and 50 μl each of 0.7 m*M* S-adenosylmethionine and 1.0 m*M* pargyline. The mixture was pre-incubated at 37°C for 10 min and again incubated at 37°C for 60 min after the addition of 50 μl of 0.3 m*M* norepinephrine. At the end of the incubation, 50 μl each of 3 *M* trichloroacetic acid and 0.5 μ*M* isoproterenol were added. The mixture was then treated in the same way as for the adrenal medulla preparation.

RESULTS AND DISCUSSION

Fig. 1 shows typical chromatograms obtained with the adrenal medulla and brain preparations and those of the blanks. The fluorescent compounds for norepinephrine, epinephrine and isoproterenol (peaks 1, 2 and 3, respectively) can be well separated from the fluorescent components of the blanks within 14 min under the HPLC conditions used. The eluates from peaks 1 and 2 in Fig. 1 have fluorescence excitation (maxima 350 and 360 nm, respectively) and emission (maxima 470 and 480 nm, respectively) spectra that are identical with those for authentic norepinephrine and epinephrine, respectively. Peak 4 in Fig. 1c and d increases in height when authentic dopamine is added to the enzyme reaction mixtures for the test and blank, and the eluate from the peak shows fluorescence excitation (maximum 350 nm) and emission (maximum 480 nm) spectra identical with those for dopamine; the peak is ascribable to endogenous dopamine in brain. Peaks 5–8 in Fig. 1 increase in height

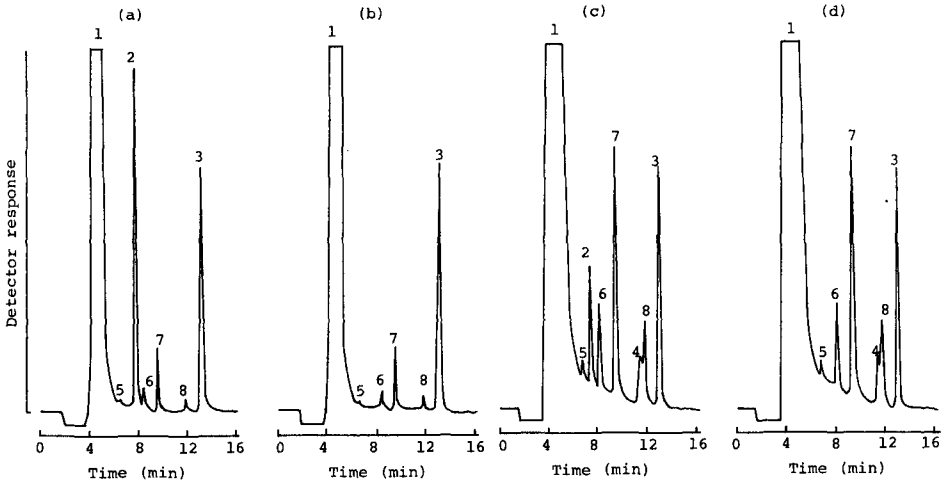

Fig. 1. Chromatograms obtained with the enzyme preparations from (a) adrenal medulla, (c) brain and (b) and (d) their blanks, respectively. Portions (100 μl) of the enzyme preparations were treated according to the procedure. Peaks: 1 = norepinephrine; 2 = epinephrine; 3 = isoproterenol; 4 = dopamine (endogenous); 5–8 = impurities in commercial norepinephrine. PNMT activity (pmol/min/mg protein): (a) 230; (c) 0.52. Detector sensitivity: (a) and (b) 2; (c) and (d) 8.

with increasing substrate concentration, and do not occur when the 1,2-diphenylethylenediamine solution or the norepinephrine solution is omitted from the procedure. The eluates from these peaks have fluorescence excitation (maxima around 350 nm) and emission (maxima around 460 nm) spectra that are fairly characteristic of 1,2-diphenylethylenediamine derivatives of catechol compounds[17]. These observations suggest that the peaks are due to some catechol compounds present as impurities in commercial norepinephrine. These peaks, however, do not interfere with the determination of epinephrine formed enzymatically and so further purification of norepinephrine is unnecessary.

The HPLC conditions are essentially the same as those described previously[17]. However, acetonitrile–methanol–50 m*M* Tris–HCl buffer (pH 7.0) was used as the mobile phase in order to obtain a complete seperation of the peaks for epinephrine and isoproterenol from those for dopamine and the impurities in norepinephrine.

Adrenal medulla contains a fairly large amount of epinephrine, which may interfere with the reproducible determination of the amine formed enzymatically; the endogenous epinephrine can be removed by dialysing the tissue homogenate at 4°C for 12 h or more against 5 m*M* phosphate buffer (pH 6.8) containing 0.1 m*M* reduced glutathione. PNMT in adrenal medulla is stable enough to be dialysed for at least 1 day.

PNMT in both the enzyme preparations is most active at pH 8.5–8.6 (Fig. 2) in bicine buffer and the bicine concentration at 0.1 *M* gives maximum activity. Although other buffers, *i.e.*, 0.1 *M* tricine, 0.1 *M* glycinamide, 0.1 *M* TAPS and 0.1 *M* Tris–HCl buffers, give maximum activities at pH 8.5, the activities did not exceed 90% of that given by the bicine buffer (pH 8.5–8.6) (Table I); 0.1 *M* bicine buffer of pH 8.5 was therefore used in the recommended procedure. Catecholamines are easily

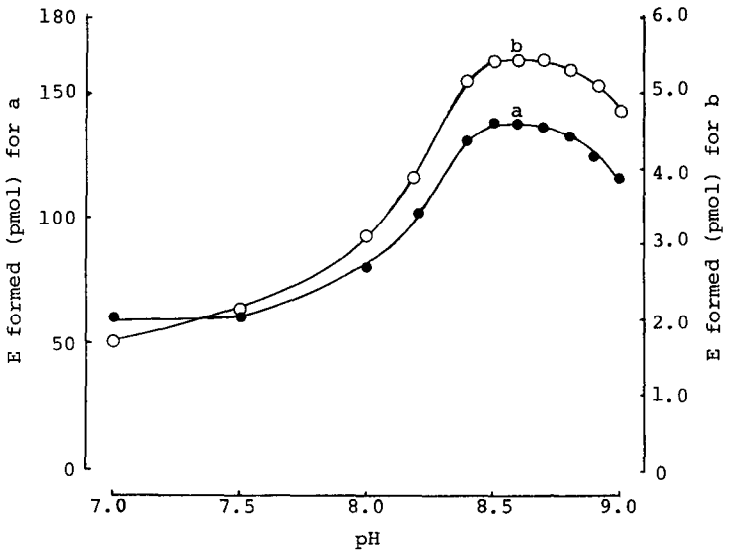

Fig. 2. Effect of pH of 0.1 *M* bicine buffer on the amount of epinephrine (E) formed in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portions (100 μl) of the enzyme preparations were treated according to the procedure at various pHs. PNMT activity (pmol/min/mg protein): (a) 220; (b) 0.51.

oxidized in alkaline media[19]. However, epinephrine and norepinephrine dissolved in the bicine buffer, provided that the enzyme preparations are added, are stable at 37°C for more than 90 min; the enzyme preparations may act as antioxidant(s).

Norepinephrine in the enzyme reaction mixture gives maximum and constant activity in the concentration ranges 30–70 μ*M* for adrenal medulla PNMT and 30–50 μ*M* for brain PNMT (Fig. 3), with K_m values for norepinephrine of 10.3 ± 1.2 and 13.8 ± 1.5 μ*M* (mean ± S.D., $n=5$ in each instance), respectively; 43 μ*M* norepinephrine was used as a saturating concentration for the enzyme reactions.

S-Adenosylmethionine in the concentration range 30–150 μ*M* in the enzyme reaction mixture of the adrenal medulla preparation gives a maximum and constant

TABLE I

EFFECT OF BUFFERS ON PNMT ACTIVITY

Portions (100 μl) of the enzyme preparations were treated as in the procedure with various buffers.

Buffer (0.1 M, pH 8.5)	*PNMT activity (epinephrine formed, pmol/min/mg protein)*	
	Rat adrenal medulla	*Rat brain*
Bicine	220 (100)*	0.51 (100)*
Tricine	190 (86)	0.46 (90)
Glycinamide	170 (77)	0.42 (82)
TAPS	160 (72)	0.45 (88)
Tris–HCl	160 (72)	0.28 (55)

* Relative activity in parentheses. The activity obtained with the bicine buffer was taken as 100.

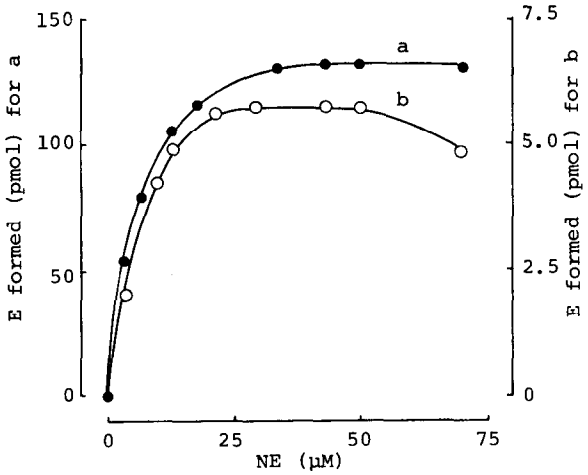

Fig. 3. Effect of the substrate concentration on the amount of epinephrine (E) in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portions (100 μl) of the enzyme preparations were treated according to the procedure at various norepinephrine (NE) concentrations. PNMT activity (pmol/min/mg protein): (a) 220; (b) 0.54.

activity with an observed K_m value of 5.4 ± 0.8 μ*M* (mean ± S.D., $n=5$). In the brain preparation, a weak PNMT activity, which corresponds to 10% of that given in the presence of 100 μ*M* S-adenosylmethionine in the enzyme reaction mixture, arises even when S-adenosylmethionine is not added to the enzyme reaction mixture. This may be due to an endogenous methyl donor (probably S-adenosylmethionine), and can be completely eliminated by dialysing the enzyme preparation against 5 m*M* phosphate buffer (pH 6.8) containing 0.1 m*M* reduced glutathione at 4°C for 12 h or more. This dialysis does not cause a loss of the enzyme activity. S-Adenosylmethionine in the concentration range 50–120 μ*M* gives maximum and constant activity in both the dialysed and non-dialysed brain preparations; dialysis was unnecessary for the assay. The K_m value for S-adenosylmethionine observed with the dialysed brain preparation is 9.1 ± 1.7 μ*M* (mean ± S.D., $n=5$), which is higher than that obtained with the adrenal medulla preparation. This supports the view that PNMT in brain is slightly different from that in adrenal medulla in its electrophoretic and gel chromatographic behaviours[9]. Hence 100 μ*M* S-adenosylmethionine was selected in the procedure.

As norepinephrine and epinephrine are deaminated and/or methylated *in vivo* by monoamine oxidase (MAO) and/or catechol O-methyltransferase (COMT) catalysed reactions[20,21], there is a possibility of deamination and/or O-methylation of epinephrine formed enzymatically and the substrate norepinephrine during the incubation period. The amount of epinephrine formed enzymatically is unaffected for the adrenal medulla preparation by the addition of the MAO inhibitor pargyline (0.05–0.5 m*M* in the enzyme reaction mixture). In the brain preparation, however, the PNMT activity in the absence of pargyline was only 28% of that obtained in its presence (0.05–0.5 m*M* in the enzyme reaction mixture); 0.14 m*M* pargyline was used in the assay for brain PNMT. The COMT inhibitor pyrogallol had no effect on

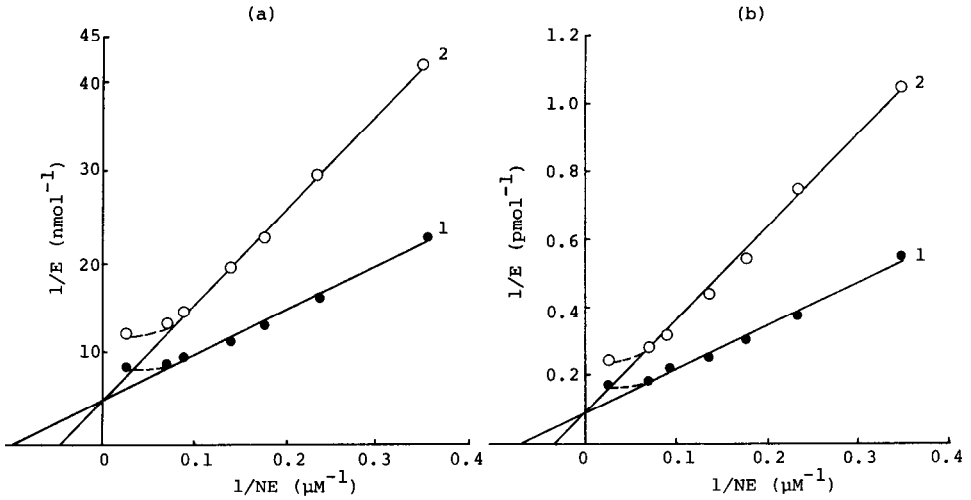

Fig. 4. Inhibition of (a) adrenal medulla and (b) brain PNMT by octopamine. Portions (100 μl) of the enzyme preparations were treated as in the procedure. Concentrations of octopamine in the enzyme reaction mixture: 1, nil; 2, 7.14 μ*M*. The data were plotted by linear regression analysis.

PNMT activity in either of the enzyme preparations in the concentration range 10 μ*M*–1.0 m*M* in the enzyme reaction mixture.

PNMT works on both octopamine and norepinephrine and these amines can be competitive inhibitors for each other[22]. Octopamine inhibited PNMT in adrenal medulla and brain in a competitive mode against norepinephrine (Fig. 4), with observed inhibitory constant (K_i) values of 6.3 and 6.2 μ*M*, respectively, which were obtained according to the method of Dixon[23]. The enzyme activity is inhibited by thiol blocking agents[21,24]. *p*-Chloromercuribenzoic acid added as a thiol blocking agent at cocentrations of 0.125 and 0.4 m*M* in the enzyme reaction mixture inhibits 28 and 100% of PNMT activity in the adrenal medulla preparation, respectively, and at concentrations of 0.125, 0.4 and 1 m*M* inhibits 17, 63 and 100% of the enzyme activity in the brain preparation, respectively. All the above observations suggest that epinephrine formed under the enzyme reaction conditions of the present procedure can be ascribed to the enzymatic N-methylation of norepinephrine.

The amounts of epinephrine formed enzymatically are proportional to protein amounts (μg per tube) of up to 200 (adrenal medulla) and 450 (brain), respectively; amounts (μg per tube) of approximately 20 (adrenal medulla) and 180 (brain) were used in the procedure.

PNMT activity in the adrenal medulla preparation is proportional to incubation time at 37°C up to 60 min and in the brain preparation up to 90 min (Fig. 5); incubation times of 30 and 60 min, respectively, are recommended.

For clean-up of the enzyme reaction mixture, a strong cation exchanger, Toyopak SP (H^+) cartridge was used under conditions the same in principle as those described previously[25]. Recoveries (%; mean ± S.D.) of epinephrine and isoproterenol (0.1 nmol each) added to the incubated enzyme reaction mixtures for the blanks were 77.7 ± 1.8 and 84.7 ± 1.3 in adrenal medulla, and those of epinephrine (5.0

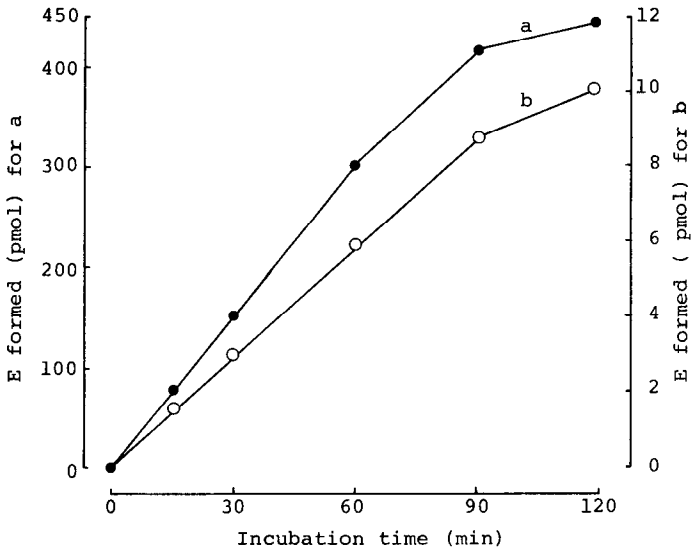

Fig. 5. Effect of the incubation time on the amount of epinephrine (E) formed in (a) adrenal medulla and (b) brain PNMT-catalysed reactions. Portion (100 μl) of the enzyme preparations were treated according to the procedure for various incubation times. PNMT activity (pmol/min/mg protein): (a) 240; (b) 0.58.

pmol) and isoproterenol (40 pmol) 71.3 ± 2.8 and 75.2 ± 1.6 in brain ($n=8$ in each instance), respectively.

A linear relationship was observed between the ratio of the peak height of epinephrine to that of isoproterenol and the amount of epinephrine added to the blank in each enzyme preparation over the range 1–100 pmol. The detection limit for epinephrine formed enzymatically was 0.66 pmol per tube (30 fmol per 100 μl injection volume) at a signal-to-noise ratio of 2. The precision was established with respect to repeatability. The coefficients of variations were 2.3 and 3.2% ($n=8$) for mean activities of 230 and 0.52 pmol/min/mg protein in the adrenal medulla and brain preparations, respectively.

PNMT activities in the adrenal medulla and brain preparations from rats (Donryu, male, 4 weeks old) were 233 ± 13 and 0.52 ± 0.06 pmol/min/mg protein (mean ± S.D., $n=5$), respectively. These values are in good agreement with reported data[14].

This method is sensitive enough to assay low PNMT activity in brain and precise, and should be useful for biological and biomedical investigations of catecholamines.

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