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Assay of phenylethanolamine N-methyltransferase activity using high-performance liquid chromatography with ultraviolet absorbance detection

Corinne Beaudouin*

Laboratoire de Neuropharmacologie–CNRS UMR 105, Faculté de Pharmacie, Université Claude Bernard, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

Gisèle Haurat, Laurent Fraisse and Jérôme Souppe

Service de Chimie Enzymatique, Groupement de Recherches de Lacq, B.P. 34, 64170 Artix (France)

Bernard Renaud

Laboratoire de Neuropharmacologie–CNRS UMR 105, Faculté de Pharmacie, Université Claude Bernard, 8 Avenue Rockefeller, 69373 Lyon Cedex 08 (France)

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ABSTRACT

A simple and rapid method for measuring phenylethanolamine N-methyltransferase (PNMT) activity by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is described. This assay requires a partially purified PNMT preparation derived from bovine adrenals, with noradrenaline and S-adenosyl-L-methionine (SAM) as co-substrates. After incubation, the reaction is stopped by addition of acid and the reaction mixture is analysed directly by HPLC. The enzymatically formed S-adenosyl-L-homocysteine (SAH) is detected at 258 nm and determined. Under optimum conditions, the stability of SAH allowed automation of the HPLC detection. This assay was validated by the determination of the kinetic properties of PNMT. K_m values for noradrenaline and SAM defined in this assay (16 and 5.7 μM, respectively) are consistent with previously published values. This assay is simple enough to be used for large series of measurements of PNMT activity testing new methyl acceptors, potential inhibitors or PNMT activity in adrenal medulla.

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28) catalyses the final step of adrenaline biosynthesis. This reaction involves the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to noradrenaline to produce adrenaline and S-adenosyl-L-homocysteine (SAH) [1]. In the periphery, adrenaline is mainly synthesized in the adrenal medulla [1].

Several methods for the assay of PNMT using noradrenaline as a substrate have been reported previously [1–11]. They may be classified into two types: radiometric methods using either [^{3}H]SAM [2–4] or [^{14}C]SAM [1,5–7] and HPLC methods coupled with electrochemical [8,9] or fluorescence [10,11] detection. These methods are sensitive but generally require time-consuming and complicated procedures to isolate and mea-

* Corresponding author.

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sure the enzymatically formed adrenaline, such as extraction procedures (solvent extraction [1,6], techniques of separation by paper [7] or column chromatography [9], adsorption on alumina [2–4], precipitation steps [2,5]) or derivatization methods [10,11]. Moreover, radiometric methods, which are the most often utilized, require expensive radioactive products.

An alternative means of determining PNMT activity could be to measure the formation of SAH instead of adrenaline. HPLC methods coupled with UV detection have recently been used for the determination of adenosyl compounds such as SAH and SAM [12–18]. These methods use reversed-phase [12–14] or cation-exchange [15–18] columns with isocratic [12–14,18] or gradient [15–17] elution. As they are sensitive and require a simple one-step procedure [12–18], we sought to adapt one of these methods to the determination of PNMT activity.

In this paper, we describe a simple, rapid and convenient method to measure PNMT activity by HPLC with UV detection using noradrenaline as the methyl acceptor. This assay is based on the separation of substrates and products of the enzymatic reaction by elution on a reversed-phase column followed by the measurement of SAH detected at 258 nm. This assay may be applied to series of measurements because of the automation of the HPLC detection, and it is sensitive enough to measure PNMT activity in rat adrenal medulla homogenates.

EXPERIMENTAL

Materials

Adrenaline, L-noradrenaline, S-adenosyl-L-methionine *p*-toluenesulphonate salt (SAM), S-adenosyl-L-homocysteine (SAH), SAH hydrolase (EC 3.3.1.1), dithiothreitol (DTT) and sodium octanesulphonate were obtained from Sigma (St. Louis, MO, USA), 8,9-dichloro-2,3,4,5-tetrahydro-1*H*-2-benzazepine hydrochloride (LY 134046) from RBI, Bioblock Scientific (Illkirch, France), ethylenediaminetetraacetic acid (EDTA) disodium salt, and methanol from Merck (Darmstadt, Germany), sodium acetate, potassium dihydrogenphosphate and dipotassium hydrogenphosphate from Prolabo (Paris, France) and citric acid from Fluka (Buchs, Switzerland). Water was purified with a Milli-Q reagent water system (Millipore, Bedford, MA, USA).

Enzyme preparations

Commercial, partially purified PNMT preparation. Partially purified PNMT derived from bovine adrenals was obtained from Sigma. The enzyme was dissolved in water and dialysed for 16 h against 0.05 *M* phosphate buffer (pH 7.5) containing 0.5 m*M* DTT.

Homogenates of rat adrenal medulla. Animals used as sources of PNMT were male OFA rats weighing 400–500 g (Iffa Credo, St. Germain sur l'Arbresle, France). Rats were killed by decapitation and the adrenal medullas were taken and stored at −80°C. All the following procedures were carried out at 0–5°C. Tissues were homogenized in 0.5 m*M* aqueous DTT (1:20, w/v) using a Vibracell sonicator (Bioblock Scientific). The homogenates were centrifuged at 39 000 *g* for 15 min. The supernatants (cytoplasmic fraction) were dialysed for 16 h against 0.05 *M* phosphate buffer (pH 7.5) containing 0.5 m*M* DTT and were used without further purification.

Assay procedure

The reaction mixture consisting of 880 μl of 0.1 *M* phosphate buffer (pH 7.5), 10 μl of 1 m*M* noradrenaline, 10 μl of 1 m*M* SAM and 100 μl of enzyme preparation in a total volume of 1 ml was incubated for 10 min at 37°C in the dark. The reaction was stopped by the addition of 25 μl of glacial acetic acid and the reaction mixture (200 μl) was analysed directly by HPLC.

Depending on the experiment, two types of enzyme preparations were used: a solution containing 180 μg/ml of a commercial, partially purified PNMT, or a supernatant derived from rat adrenal medulla homogenates.

Protein determinations were carried out using the biuret procedure.

HPLC analysis

The chromatograph was a System Gold from Beckman (San Ramon, CA, USA). It consisted of a Model 507 automatic sample injector fitted with a 200-μl injection loop, a Model 126 two-pump system and a Model 167 UV detector. The whole system was controlled by the Gold software installed on an IBM PS Model 50 microcomputer.

The column used was an MOS Hypersil (5 μm) column (200 mm × 4.6 mm I.D.) (Hewlett-Packard, Palo Alto, CA, USA) fitted with a Spheri 5 RP-8 guard column (30 mm × 4.6 mm I.D.) (Brownlee Labs., Santa Clara, CA, USA).

The eluent (buffer A) consisted of 0.1 *M* sodium acetate, 0.02 *M* citric acid, 0.93 m*M* sodium octanesulphonate and 0.12 m*M* EDTA disodium salt (pH 4.6). The solution was filtered just before use through a 0.45-μm Millipore HA membrane filter. The mobile phase (flow-rate 1.0 ml min^{-1}, pressure 138 bar) was obtained by mixing, extemporaneously, buffer A with methanol (90:10, v/v) through the Model 126 two-pump system.

UV detection was applied at 258 nm, the wavelength of maximum absorption (λ_{max}) of adenosyl compounds (SAH and SAM). Chromatograms were recorded and treated by means of Gold software.

RESULTS AND DISCUSSION

Chromatographic analysis

A standard aqueous solution of noradrenaline, adrenaline, SAM and SAH was analysed under the chromatographic conditions described above. UV detection was applied at 279 nm (λ_{max} of the catecholamines) and at 258 nm (λ_{max} of the adenosyl compounds). Chromatograms are shown in Fig. 1A and B, respectively. The retention times obtained were SAH (peak a) 4.6 min, noradrenaline (peak b) 5.5 min, SAM (peak c) 6.0 min and adrenaline (peak d) 7.3 min. These results show that substrates (noradrenaline, SAM) and products (adrenaline, SAH) of the PNMT-catalysed reaction can be easily separated under the applied elution conditions.

As SAH detection at 258 nm (A_{258} = 14.6 l $mmol^{-1}$ cm^{-1}) is more sensitive than adrenaline at 279 nm (A_{279} = 2.6 l $mmol^{-1}$ cm^{-1}), we measured SAH in order to optimize the assay. This is a new method as the PNMT activity is generally determined by measurement of the adrenaline formed [1–11]. We established that the amount of SAH formed was stoichiometrically related to that of adrenaline; therefore, SAH should be a good reference for the determination of the PNMT activity (not shown). Further, this assay can be applied to studies of other methyl acceptors (*e.g.*, phenylethanolamine or normetanephrine, or for a search for new methyl acceptors) under the same conditions, which is an advantage compared with other methods [1–11].

The reaction mixture and a blank without enzyme were analysed directly by HPLC after incubation at 37°C for 10 min. Typical chromatograms obtained with UV detection at 258 nm are

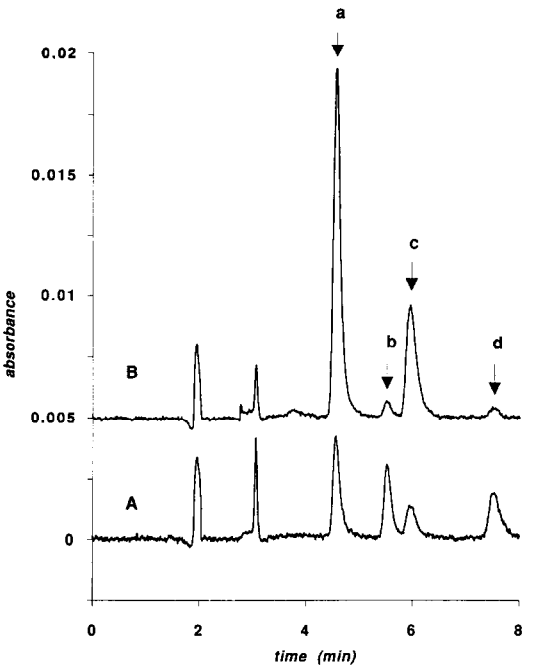

Fig. 1. Chromatograms of standard aqueous solution containing 1.5 μ*M* noradrenaline, adrenaline, SAM and SAH in a 200-μl injection. The column used was a MOS Hypersil (5 μm) column (200 mm × 4.6 mm I.D.). The mobile phase was a mixture of buffer A (see Experimental) and methanol (90:10, v/v). The flow-rate was 1.0 ml/min. UV detection was at (A) 279 nm (λ_{max} of catecholamines) and (B) 258 nm (λ_{max} of adenosyl compounds). Peaks: a = SAH; b = noradrenaline; c = SAM; d = adrenaline.

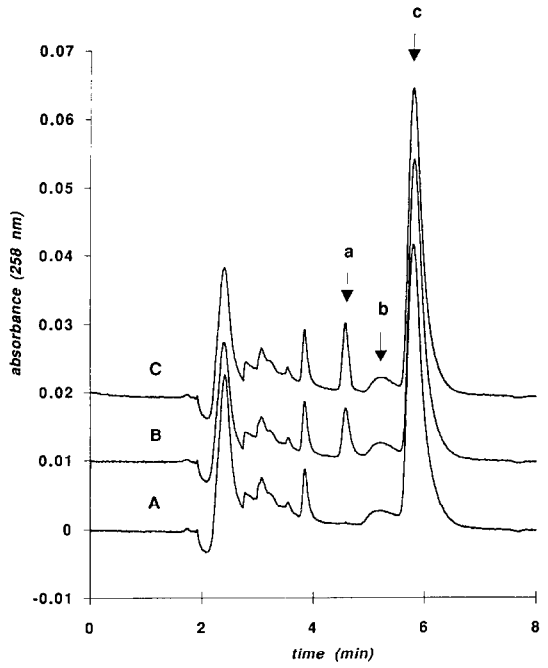

Fig. 2. Typical chromatograms of the reaction mixture containing partially purified PNMT from bovine adrenal medulla (Sigma) as enzyme source and noradrenaline and SAM and co-substrates. After incubation at 37°C for 10 min, the reaction mixture was injected directly into the HPLC system. Chromatographic conditions as in Fig. 1. UV detection at 258 nm. (A) Enzyme-free blank; (B) reaction mixture; (C) reaction mixture co-eluted with a standard solution of SAH added just before the injection. Peaks: a = SAH; b = noradrenaline; c = SAM. Under the conditions chosen for the PNMT assay, adrenaline formed enzymatically cannot be detected at 258 nm.

shown in Fig. 2A (blank) and B (assay). The peak corresponding to the enzymatically formed SAH was identified on the basis of its retention time (4.6 min), its co-elution with a standard solution of commercial SAH (Fig. 2C) and its disappearance in the presence of SAH hydrolase (EC 3.3.1.1), which catalyses the hydrolysis of SAH into adenosine and homocysteine [19]. The SAH hydrolase was added to the reaction mixture after incubation (results not shown). These results show that the SAH enzymatically formed by PNMT can be isolated by HPLC and determined with UV detection.

The detection limit for SAH at a signal-to-noise ratio of 3 was 5 pmol (for a 200-μl injection). In comparison, the detection limit for adrenaline at 279 nm was 40 pmol. The detection limits reported previously varied from 0.8 to 70 pmol [13,15–18]. Hence, the present detection method is among the most sensitive and allows the detection and titration of the PNMT activity in biological samples.

PNMT activity measurement

The amount of enzymatically formed SAH was determined by measurement of the peak area in comparison with a calibration graph for commercial SAH. The detector response was linear for SAH solutions with concentrations ranging from 0.05 to 10 μM.

The PNMT activity was expressed as nanomoles of SAH formed per hour per mg of protein for the partially purified PNMT and per pair of adrenal medulla for the rat adrenal medulla homogenates. When partially purified PNMT (*ca.* 18 μg of protein) was used as the enzyme source, the activity was 203 nmol h^{-1} (mg protein)$^{-1}$ under the present assay conditions (incubation for 10 min and injection of a 200-μl volume). This corresponds to 118 pmol of SAH per peak, a value 24 times higher than the detection limit.

The intra- and inter-assay coefficients of variation were 1.7% (n = 5) and 2.7% (n = 8), respectively.

After establishing that SAH was stable in an acidic solution for more than 24 h (enzymatic reaction was stopped by addition of acetic acid), we used the chromatograph with an automatic sample injector in order to analyse series of samples. This automation would not have been possible if adrenaline had been measured instead of SAH, because adrenaline is an unstable molecule which oxidizes easily into adrenochrome. Under the present chromatographic conditions, the reaction mixture was thoroughly eluted after 12 min and we decided to set up a period of 15 min between subsequent injections which allowed analyses of up to 95 samples per 24 h.

Determination of optimum conditions for the PNMT assay

Substrate concentrations of noradrenaline and SAM selected for the test were both 10 μM. This value is close to the K_m for each substrate. These

concentrations were chosen in order to avoid substrate inhibition by excess of noradrenaline (see below) or product inhibition by excess of SAH present in commercial SAM [20].

In an alkaline medium, SAM hydrolyses spontaneously with liberation of adenine [21]. As this loss would be very damaging in the present assay because of its low SAM concentration (10 μM), we therefore used an incubation buffer at pH 7.5 to avoid this decomposition. The pH was lower than the optimum pH for PNMT, which is 7.9 in phosphate buffer [22,23] or 8.5 in Tris buffer [10,24].

Under these conditions, the PNMT activity was linear for incubation times up to 12 min (Fig. 3); for longer periods of time, we observed a decrease in the reaction rate, which may have been due to inhibition of PNMT by the reaction products (SAH and adrenaline) [20,25] or to the low concentrations of both substrates, which are limiting factors for the enzymatic reaction. A standard incubation time of 10 min was therefore chosen.

The amount of enzymatically formed SAH was also linear up to 61 μg, depending on the protein added to the reaction mixture (Fig. 4). A standard amount of approximately 18 μg of protein [activity = 203 nmol h^{-1} (mg protein)$^{-1}$] was then selected.

Determination of kinetic parameters of partially purified bovine PNMT

The kinetic parameters for noradrenaline and SAM were determined from their Lineweaver–Burk plots.

With noradrenaline as the variable substrate, PNMT activity was determined for concentrations of noradrenaline ranging from 2 to 100 μM while a saturating concentration of SAM (100 μM) was used. The curve that was obtained represented a typical inhibition by excess of substrate (Fig. 5A). This result was consistent with

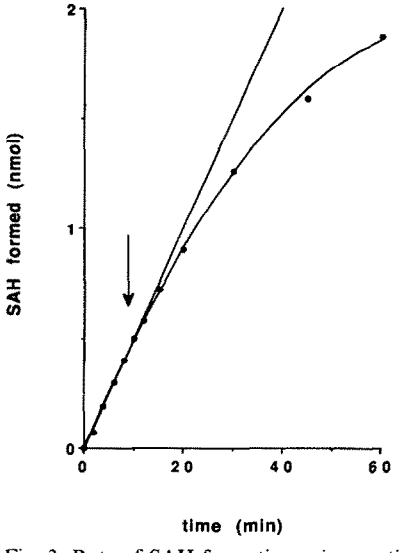

Fig. 3. Rate of SAH formation using partially purified PNMT from bovine adrenal medulla (Sigma) as a function of incubation time. The reaction mixture consisted of 10 μl of 1 mM noradrenaline, 10 μl of 1 mM SAM and 100 μl (*ca.* 18 μg of protein) of enzyme preparation. Incubation was carried out at 37°C for different times from 0 to 60 min. Each point represents the mean of three determinations. The amount of SAH formed is expressed in nanomoles. The arrow indicates the standard incubation time chosen for the assay (10 min).

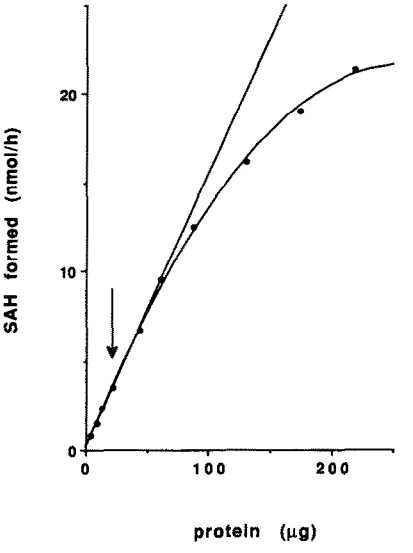

Fig. 4. Rate of SAH formation using partially purified PNMT from bovine adrenal medulla (Sigma) as a function of enzyme protein concentration. A 10-μl volume of 1 mM noradrenaline and 10 μl of 1 mM SAM were used as co-substrates. The incubation was carried out at 37°C for 10 min. Protein amounts ranging from 0 to 218 μg were used in the experiments. Each point represents the mean of three determinations. Results are expressed in nanomoles of SAH formed per hour. The arrow indicates the standard protein amount chosen for the assay (18 μg).

those reported by Fuller and co-workers [26,27] and Pohorecky and Baliga [23], who showed inhibition of PNMT by high concentrations of noradrenaline. Under our assay conditions the PNMT activity did not follow Michaelis–Menten kinetics for noradrenaline concentrations above 10 μM (Fig. 5A). We then determined the PNMT kinetic parameters in the noradrenaline concentration range 2–8 μM. Under these conditions, the K_m for noradrenaline was 16 μM and the V_{max} was 956 nmol of SAH formed per hour per mg of protein. The K_m value defined in our assay was higher than that determined with radiometric tests (3–7.7 μM) [25,27–31]. This discrepancy may result from the higher noradrenaline concentrations used to determine the K_m in those tests, since when a substrate-dependent inhibition is present the apparent K_m value decreases.

With SAM as the variable substrate, a fixed concentration of 25 μM of noradrenaline was used because of PNMT inhibition at higher concentrations. PNMT activity was determined for SAM concentrations ranging from 2 to 100 μM (Fig. 5B). K_m for SAM was 5.7 μM and V_{max} was

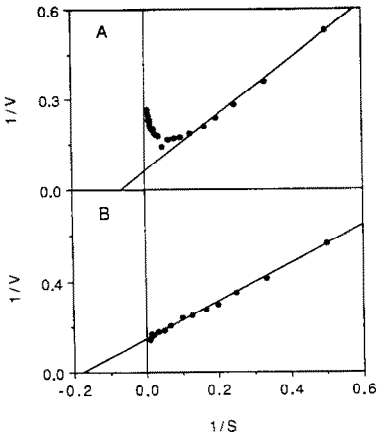

Fig. 5. Determination of kinetic parameters for noradrenaline and SAM. The enzyme preparation used was partially purified PNMT from bovine adrenal medulla (Sigma). Each point represents the mean of two determinations. (A) Lineweaver–Burk plot obtained when noradrenaline was the variable substrate. SAM concentration was fixed at 100 μM. V = PNMT activity expressed in nanomoles of SAH per hour and S = noradrenaline concentration (μM). (B) Lineweaver–Burk plot obtained when SAM was the variable substrate. Noradrenaline concentration was fixed at 25 μM. V = PNMT activity as in (A) and S = SAM concentration (μM).

382 nmol of SAH formed per hour per mg protein. The K_m value defined under our conditions was consistent with those reported previously (2.9–8.7 μM) [32–34], although the latter values were determined with tests using phenylethanolamine as substrate. However, Pendleton *et al.* [35], using partially purified PNMT from rabbit adrenal medulla, had previously observed that the K_m for SAM was unaffected by changing the methyl acceptor from noradrenaline to phenylethanolamine.

In order to validate the assay, we then studied the PNMT inhibition with LY 134046, a specific inhibitor of the enzyme [36]. The IC_{50} (concentration producing 50% inhibition of PNMT activity) determined in our assay was $0.8 \cdot 10^{-7}$ M. This value is consistent with that reported by Fuller *et al.* ($2 \cdot 10^{-7}$ M) [36].

Detection of PNMT activity in biological samples

The present assay was applied to the determination of PNMT activity in rat adrenal medulla homogenates. Each homogenate was dialysed against a phosphate buffer (see Experimental) to remove low-molecular-mass compounds, such as substrates or products of transmethylation reactions using SAM as the methyl donor, which could modify the PNMT enzymatic activity.

After incubation at 37°C for 10 min, the reaction mixture was analysed directly by HPLC (Fig. 6). The peak corresponding to the SAH was identified on the basis of its retention time, its co-elution with a standard solution of SAH and its disappearance induced by SAH hydrolase according to the same procedure as that described for the partially purified PNMT (results not shown).

Under these assay conditions (100 μl of homogenate supernatant, PNMT activity was linear for incubation times up to 15 min (not shown); for longer periods of time, we observed a decrease in this activity which may have resulted from enzymatic decomposition of SAH by endogenous SAH hydrolase. An incubation time of 10 min was then chosen.

The average activity of the rat adrenal medulla homogenates was 3.5 ± 0.4 nmol of SAH formed

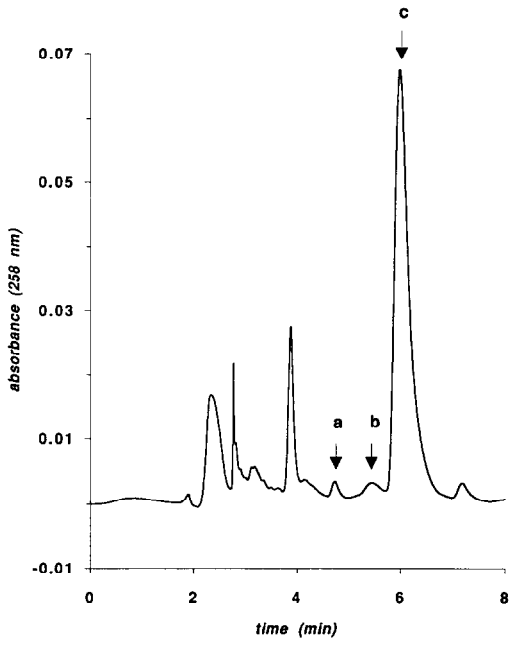

Fig. 6. Typical chromatogram obtained from the reaction mixture using rat adrenal medulla homogenates as source of enzyme. The reaction mixture consisted of 10 μl of 1 m*M* noradrenaline, 10 μl of 1 m*M* SAM and 100 μl of enzyme preparation. Incubation was carried out at 37°C for 10 min. Chromatographic conditions as in Fig. 1. UV detection at 258 nm. Peaks: a = SAH; b = noradrenaline, c = SAM.

per hour per pair of adrenal medulla (n = 6). This value was lower than those previously reported (6.2–13 nmol per hour per pair of adrenal medulla) [37–41] and may be due to the present assay conditions, which are very different from those in radiometric tests which have usually been used to measure PNMT activity in rat adrenals. In particular, the lower value obtained with the present assay procedure may be due to (i) the nature of the substrate, as PNMT activity in rat adrenal medulla was determined using noradrenaline as substrate instead of phenylethanolamine [37–39] or normetanephrine [40,41], which are usually used in radiometric tests, (ii) both substrate concentrations ($\approx K_m$) which were lower than those used in previous tests and (iii) the pH of the incubation buffer which was lower than the optimum pH for PNMT [1,20,24,25]. As explained above, this pH was chosen to prevent the non-enzymatic decomposition of SAM in an alkaline medium.

CONCLUSION

A method for measuring PNMT activity by HPLC with UV detection of SAH has been described. This assay is based on the measurement of the enzymatically formed SAH and, for this reason, represents an original approach, as most published PNMT assays are based on the determination of adrenaline formed. Such a method is possible because UV detection of SAH is more sensitive than UV detection of catecholamines (noradrenaline and adrenaline).

The chief advantages of this assay are (i) the equipment, which is simple and easy to use, (ii) the method is inexpensive compared with radiometric tests, which require expensive labelled compounds, (iii) the method is rapid because it does not need extraction procedures, which are usually time-consuming, (iv) the chromatographic procedure is automated, which allows the analysis of a large series of samples (made possible because of the stability of SAH in acidic media) and (v) it can be applied to other methyl acceptors for PNMT and other methyltransferase enzymes, as the assay is based on SAH titration.

This simple and rapid method was used to check the value of PNMT kinetic constants *versus* noradrenaline and SAM and was validated by testing LY 134046, a reference inhibitor of PNMT. Although this assay is aimed principally at routine measurements *in vitro* using commercial, partially purified PNMT, it is also sensitive enough to measure PNMT activity in adrenal homogenates. This test can therefore be applied to enzyme purification studies.

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