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DETERMINATION OF PHENYLETHANOLAMINE-N-METHYLTRANSFERASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ON-LINE RADIOCHEMICAL DETECTION

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SUMMARY

A new assay technique for phenylethanolamine N-methyltransferase (PNMT) activity by high-performance liquid chromatography with on-line radiochemical detection is described. The method is based on the measurement of ^{14}C-labelled products of the substrate, normetanephrine, using S-adenosyl-L-[methyl-^{14}C]methionine as the methyl donor. The reaction products are determined from the reaction mixture after removal of protein by injecting an aliquot into the liquid chromatograph. The detection limit with 60% counting efficiency is 1.2 pmol of ^{14}C-labelled product. The method is suitable for assaying PNMT activity in the adrenal and in the brain tissue.

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; E.C. 2.1.1.28) catalyzes the transfer of a methyl group from S-adenosyl-L-methionine to a primary amino group of several β-hydroxylated monoamines, such as noradrenalin, normetanephrine and phenylethanolamine. The enzyme is most abundant in the adrenal medulla[1], but low activities are also present in other tissues and specific brain regions[2–4]. The main function of PNMT is to catalyze the formation of adrenalin from noradrenalin. Noradrenalin has been postulated to function as a neurotransmitter[5]. PNMT activity has also been related to hypertension[6]. These findings add increased pharmacological interest to the regulation of PNMT activity.

PNMT activity is usually determined by radiochemical methods, using [^{14}C]- or [^{3}H]methyl-labelled S-adenosyl-L-methionine as the radioactive marker. In these assays, the radioactive reaction product is isolated by extraction procedures[3,7] and quantitated by using conventional liquid scintillation techniques. However, this procedure does not allow reliable identification of the N-methylated products.

High-performance liquid chromatography (HPLC) with electrochemical detection provides a rapid and accurate means to assay PNMT activity, since all of the reaction products can be quantitated and identified. Since the first report by Borchardt *et al.*[8], Trocewicz *et al.*[9] have improved this method by increasing its sensitivity to assay low PNMT activity in the brain tissue. PNMT activity can also be assayed using HPLC with fluorescence detection. These methods are very sensitive, but they require rather elaborate derivatization procedures[10,11].

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By combining the resolving power of HPLC and the sensitivity and specificity of radiochemical detection, it has been possible to assay many enzymes involved in the metabolism of catecholamines[12–15]. This communication describes a fast and reliable method for determining the activity of PNMT in bovine adrenal medulla and rat brain by using HPLC with on-line radiochemical detection.

EXPERIMENTAL

Chemicals

Aqueous counting scintillant (ACS) and S-adenosyl-L-[methyl-^{14}C]-methionine (SAM), specific activity 55 mCi/mmol, were obtained from Amersham International (Little Chalfont, U.K.); normetanephrine (NMN), metanephrine (MN), noradrenalin (NA), adrenalin (A), tranylcypromine (TCP), dithiothreitol (DTT), pargyline, and tropolone were from Sigma (St. Louis, MO, U.S.A.); sodium hexane sulfonate was from Altex (Berkeley, CA, U.S.A.); Triton X-100, specially purified for membrane research, from Boehringer Mannheim (Penzberg, F.R.G.); methanol, HPLC grade, was from Orion Pharmaceutical (Espoo, Finland). All other reagents were of analytical grade and purchased from commercial sources.

Sample preparation

Fresh bovine adrenals were stored at −70°C until used. For the PNMT determination the adrenal medulla was homogenized in 0.9% potassium chloride (1 ml/100 mg tissue) using a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 47000 *g*, for 30 min. The supernatant was applied to a 50 mm × 15 mm prepacked Sephadex G-25 column (PD-10, Pharmacia, Uppsala, Sweden). The protein was eluted with 3.5 ml of 10 m*M* sodium phosphate (pH 7.4) containing 1 m*M* DTT. The eluate was divided into small aliquots and stored at −20°C.

Rats were killed with carbon dioxide, and the brains were removed and stored at −70°C until used. The brain tissue was homogenized in 0.9% potassium chloride (4 ml/g tissue) containing 0.2% Triton X-100. The homogenate was centrifuged and passed through the Sephadex G-25 column as described above. The protein eluate from the Sephadex G-25 was dialyzed in an Amincon ultrafiltration apparatus Model 8010, using a PM-10 membrane (Amincon, Danvers, MA, U.S.A.), and diluted with the elution buffer to a protein concentration of about 20 mg/ml. The enzyme solution could be stored at −20°C for two months without loss of activity. All of the above steps except Sephadex G-25 gel filtration were carried out at 4°C. The protein determinations were carried out using a Bio-Rad protein assay kit (Bio-Rad Labs., Richmond, CA, U.S.A.).

Assay

The standard incubation mixture consisted of the following components in a total volume of 250 μl: 100 μl of 0.5 *M* sodium phosphate (pH 7.9), 10 μl of 5 m*M* NMN, 10 μl of [^{14}C]SAM (0.25 μCi, 4.5 nmol, specific activity 55 mCi/mmol), 20 μl of adrenal extract or 100 μl of brain extract, and 110 μl of 30 μl of elution buffer, respectively. The blank was incubated without the substrate. The reaction was terminated after 30 min incubation at 37°C for adrenal PNMT and after 90 min incubation for brain PNMT by adding 25 μl of 4 *M* perchloric acid. The precipitated

protein was removed by centrifugation, and a 20-μl aliquot was injected into the liquid chromatograph.

Chromatography

The HPLC system consisted of a Kratos Model Spectroflow 400 pump (Kratos Analytical, Ramsey, NJ, U.S.A.), a Spark Holland HPLC autosample Model SpH 125 (Spark Holland Instruments, Emmen, The Netherlands) and a 150 mm × 4.6 mm, 5-μm Spherisorb ODS column, fitted with a direct-connect guard column (HPLC Technology, Macclesfield, U.K.). The mobile phase was composed of 0.1 *M* sodium phosphate (pH 2.6) and 2 m*M* hexane sulfonate in 5% methanol. The flow-rate was 1.5 ml/min. The detection of non-radioactive standards was carried out with a Waters Model 441 absorbance detector at 254 nm.

The on-line liquid scintillation counting was carried out with a Flo-One HS flow-through radioactivity detector (Radiomatic Instruments and Chemicals, Tampa, FL, U.S.A.), which was interfaced directly with the HPLC column. One part of column effluent was mixed with three parts of ACS. This produced a counting efficiency of 60% for ^{14}C. The counting efficiency was calculated as described previously[15].

RESULTS AND DISCUSSION

The chromatographic pattern of the ^{14}C-labelled reaction products from bovine adrenal PNMT assay is shown in Fig. 1. The blank (Fig. 1A), *i.e.* the incubation mixture without NMN, shows the [^{14}C]SAM peak being eluted at 8.8 min, an unknown impurity in SAM (t_R = 5 min), and a small amount of A at 2.8 min. The impurity was not S-adenosyl-L-homocysteine, since it was eluted at 28 min. Enzymatically formed MN (t_R = 12 min) is well separated from SAM, as shown in Fig. 1B. When the incubation was carried out in the presence of 0.4 m*M* TCP, a specific inhibitor of PNMT, about 70% inhibition in the enzyme activity was recorded (Fig. 1C). This was further evidence that the compound eluted at 12 min was formed from NMN by the action of PNMT.

Both Fig. 1B and 1C show a ^{14}C-labelled compound eluted at 3.5 min. The presence of 1 m*M* pargyline, which at this concentration is known to inhibit both forms of monoamine oxidase (MAO)[16], in the reaction mixture did not reduce the size of the peak. NMN, the substrate in this PNMT assay, is formed from NA by the action of catechol-O-methyltransferase (COMT). COMT is present in the adrenal tissue in large amounts[7], and since this enzyme also uses SAM as the methyl donor, it can interfere in this asay. When the incubation was performed in the presence of 0.1 m*M* tropolone, which is a known COMT inhibitor[17], the formation of the 3.5-min peak was prevented.

The amount of radioactive NMN in the blank (Fig. 1A) is insignificant, indicating that the adrenal preparation does not contain considerable amounts of NA. Commercial NMN contains 1–2% of NA, which seems to be enough to produce a small amount of radioactive NMN in this assay system (Fig. 1B).

The chromatographic pattern of ^{14}C-labelled reaction products from the rat brain PNMT assay is shown in Fig. 2. The blank (Fig. 2A) is similar to that of adrenal PNMT. The formation of MN from NMN is presented in Fig. 2B. The

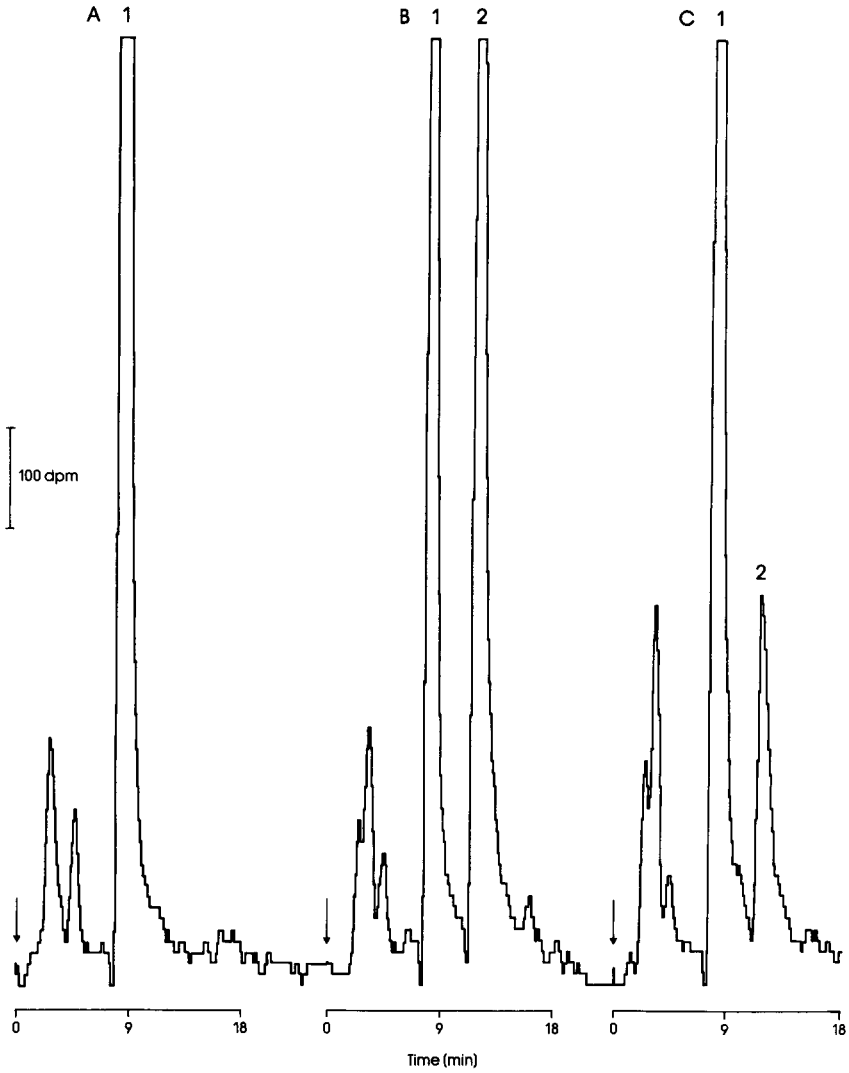

Fig. 1. Chromatographic profiles of ^{14}C-labelled reaction products from the bovine adrenal PNMT assay. (A) blank (*i.e.* the incubation mixture without the substrate), (B) standard assay, (C) assay in the presence of 0.4 m*M* TCP. Chromatographic conditions: mobile phase, 50 m*M* sodium phosphate (pH 2.6) and 2 m*M* hexane sulfonate in 5% methanol; flow-rate, 1.5 ml/min; injection volume, 20 μl; radiochemical detection sensitivity, 1000 dpm full scale; counting efficiency, 60%. Peaks: 1 = SAM, 2 = MN.

amount of MN is very small, but at the same time there is considerable increase in the amount of radioactive A. The inhibition of rat brain PNMT activity by 0.4 m*M* TCP is shown in Fig. 2C. The enzyme activity towards NMN is reduced about 60%, but there is no inhibition of A formation. Since PNMT has a much higher affinity for NA than for NMN[18], the formation of A is evident, if the enzyme preparation contains even small amounts of NA. As stated above commercial NMN contains NA, which drives the PNMT reaction towards formation of A. On the other hand,

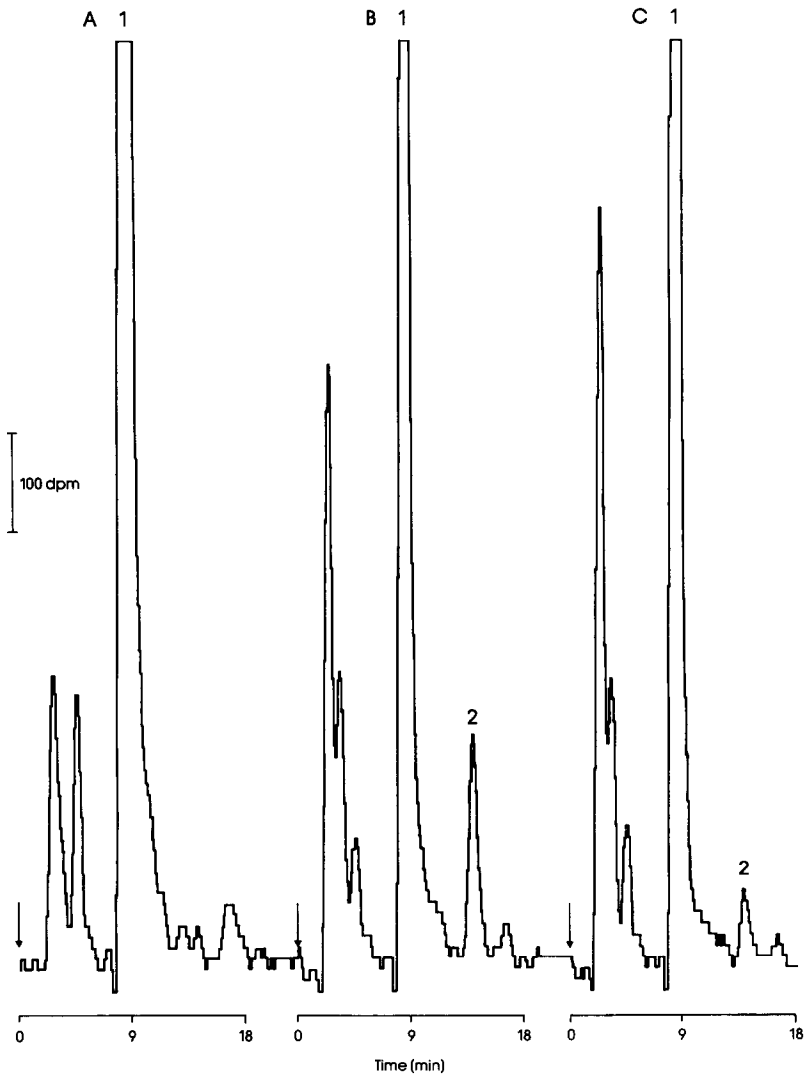

Fig. 2. Chromatographic profiles of ^{14}C-labelled reaction products from the rat brain PNMT assay. (A) blank, (B) standard assay, (C) inhibition of PNMT with 0.4 m*M* TCP. Other conditions, same as in Fig. 1.

NA acts as an inhibitor of PNMT if another β-hydroxylated monoamine is used as the substrate[18], so the formation of A by the action of PNMT is not affected by TCP.

The rate of MN formation was linear for 30 min for 0.1 mg of bovine adrenal PNMT ($y = 153x + 171$; y = dpm, x = time in min), and for 90 min for 2 mg of rat brain PNMT ($y = 31x + 42$). The amount of radioactive MN increased linearly with protein concentration up to 0.2 mg and up to 5 mg for adrenal and brain protein, respectively. The reproducibility of the standard assay for adrenal PNMT with 0.1

mg protein was 6% (n = 6), and for brain PNMT with 2 mg protein it was 10% (n = 6), expressed as coefficient of variation. The counting efficiency of 60% for ^{14}C under these chromatographic conditions allowed the reliable detection of 1.2 pmol of ^{14}C-labelled product, when the specific activity of [^{14}C]SAM was 55 mCi/mmol. The Michaelis–Menten constant (K_M) for NMN of bovine adrenal PNMT was 76 ± 9 μM. This is in close agreement with the K_M value obtained by using conventional radiochemical assay[18]. The K_M value for rat brain PNMT was not determined. The activity of PNMT in bovine adrenal medulla was calculated to be 2600 ± 156 pmol/h/mg protein and in the whole rat brain it was 12 ± 1.2 pmol/h/mg protein.

If the preparation was not passed through a Sephadex G-25 column, the adrenal extract did not have any PNMT activity for NMN. This procedure removes most of the small molecules, including NA, which even at very low concentrations inhibits PNMT activity, if NMN is used as the substrate[18]. However, the brain tissue, which has only trace amounts of PNMT activity, seemed to contain enough NA, even after gel filtration and membrane dialysis to drive the PNMT reaction towards the formation of A. In spite of this interference, it was possible to assay PNMT activity in the rat brain by using NMN as the substrate. However, it would be more advisable to determine the activity of PNMT in the brain by using NA as the substrate. This would require careful removal of NA and A from the preparation and the use of a proper blank, *i.e.* the incubation should be carried out in the presence of the enzyme preparation without the substrate. The presence of MAO inhibitor was not necessary, since MAO is localized in the outer mitochondrial membranes, which should not be present at significant amounts after high-speed centrifugation, gel filtration and membrane dialysis.

The use of HPLC with on-line radiochemical detection offers a sufficiently sensitive and reliable method for determining the activity of PNMT in different tissues. Its usefulness is emphasized when all of the reaction products must be quantitated, and when one must be sure that there are no overlapping components arising from experimental drugs or endogenous compounds.

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