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Note

Assay of aromatic L-amino acid decarboxylase by high-performance liquid chromatography with radiochemical detection

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Aromatic L-amino acid decarboxylase (AADC; E.C. 4.1.1.28), also called DOPA decarboxylase, is the enzyme that catalyses, among various aromatic L-amino acids, the decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) and L-5-hydroxytryptophan (L-5-HTP) in the presence of cofactor pyridoxal phosphate to dopamine (DA) and 5-hydroxytryptamine (5-HT), respectively. The enzyme is present in various mammalian tissues including the brain [1]. The decarboxylated catecholamines DA, epinephrine and norepinephrine and the indoleamine 5-HT are biochemically and pharmacologically important monoamines, which function as neurotransmitters and hormones, and are involved in the regulation of neuronal functions, behaviour and emotions.

AADC activity can be measured by various methods. The most commonly used are high-performance liquid chromatography (HPLC) with UV detection [2] and with electrochemical detection [3, 4], or radiochemical determination of the released $^{14}CO_2$ [5, 6]. In order to study the low enzyme activity in the brain and the effect of various inhibitors on AADC activity, a sensitive and fast assay without interference is needed. HPLC with on-line radiochemical detection has been shown to be a suitable technique for the assay of enzymes involved in catecholamine metabolism [7—9].

This paper describes a fast and sensitive assay for AADC activity using [3-^{14}C] L-DOPA as the substrate, deproteinization with perchloric acid and quantitation of the radioactive product [^{14}C] DA after HPLC separation with on-line radiochemical detection.

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EXPERIMENTAL

Reagents

Aqueous counting scintillant (ACS) and L-3,4-dihydroxyphenyl[3-^{14}C]-alanine, specific activity 10.9 mCi/mmol, were obtained from Amersham International (Buckinghamshire, U.K.), DA, L-DOPA and pargyline from Sigma (St. Louis, MO, U.S.A.), pyridoxal-5′-phosphate (pyridoxal-5-P) from Boehringer (Mannheim, F.R.G.), sodium octanesulphonate from Eastman-Kodak (Rochester, NY, U.S.A.) and methanol (HPLC grade) was from Orion Pharmaceutical (Espoo, Finland). All other reagents were of analytical-reagent grade from commercial sources.

Sample preparation

Rats weighing about 200 g were killed with carbon dioxide. The brains were quickly removed and homogenized in 4 vols. of cold 0.32 *M* sucrose. The homogenates were centrifuged at 40 000 *g* for 30 min at 4°C and the supernatants were stored in small aliquots at −20°C until taken for assay. 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 contained, in a total volume of 250 μl, 30 m*M* sodium phosphate buffer (pH 7.2), 0.3 m*M* EDTA, 1 m*M* pargyline, 0.05 m*M* pyridoxal-5-P, 0.2 m*M* [^{14}C]L-DOPA, specific activity 2.1 mCi/mmol, and 0.5 mg of enzyme protein. The mixture was incubated for 30 min at 37°C except for the study of the time course. The reaction was stopped by the addition of 25 μl of cold 4 *M* perchloric acid. Precipitated protein was removed by centrifugation. A 20-μl aliquot was injected into the liquid chromatograph for on-line radiochemical analysis.

Chromatography

The HPLC system consisted of an Altex 110A pump equipped with pulse damper, a 5-μm Ultrasphere-ODS column (150 × 4.6 mm I.D.) fitted with a 45 × 4.6 mm I.D. pre-column (Beckman, Fullerton, CA, U.S.A.) and a Model 7125 injector with a 20-μl loop (Rheodyne, Cotati, CA, U.S.A.). Detection of the non-radioactive standards was carried out with a Waters Model 441 absorbance detector at 254 nm (Waters Assoc., Milford, MA, U.S.A.). The mobile phase was 10% methanol in 100 m*M* sodium phosphate containing 1 m*M* sodium octanesulphonate (pH 3.2) and the flow-rate was 1.5 ml/min.

Radioactivity detection

On-line liquid scintillation analysis was carried out using a Flo-One HS flow-through radioactivity detector (Radiomatic Instruments, Tampa, FL, U.S.A.), which was interfaced directly with the HPLC column. The effluent to scintillation fluid ratio was 1:3. This produced a 67% counting efficiency for ^{14}C. The radioactivity signal from the Flo-One HS was plotted as a histogram on a strip-chart recorder. The quantitation of ^{14}C was effected simultaneously with the results being printed out on the Flo-One HS printer. The enzyme activity

was calculated from the radioactivity of the product as nanomoles of [^{14}C]DA formed per minute per milligram of protein.

RESULTS AND DISCUSSION

The chromatographic profile of ^{14}C-labelled reaction products of AADC assay with [^{14}C] L-DOPA as the substrate are shown in Fig. 1. The blank (Fig. 1A), i.e. the incubation mixture without the enzyme preparation, shows only the L-DOPA peak (retention time, t_R = 4 min) with a minor impurity eluting at 2.5 min. The decarboxylated product [^{14}C]DA eluting at 8.5 min is clearly separated from the [^{14}C] L-DOPA peak, as shown in Fig. 1B. Both the substrate L-DOPA and especially the product DA are substrates for monoamine oxidase (MAO). The action of MAO on these compounds can be prevented by the use of MAO inhibitors, e.g. pargyline, which at the concentration used of 1 m*M* inhibits both forms of MAO [10]. DA may also be methylated to 3-methoxytyramine in the presence of magnesium ions and *S*-adenosyl-L-methionine (SAM) by the action of catechol-O-methyltransferase (COMT) [11]. However,

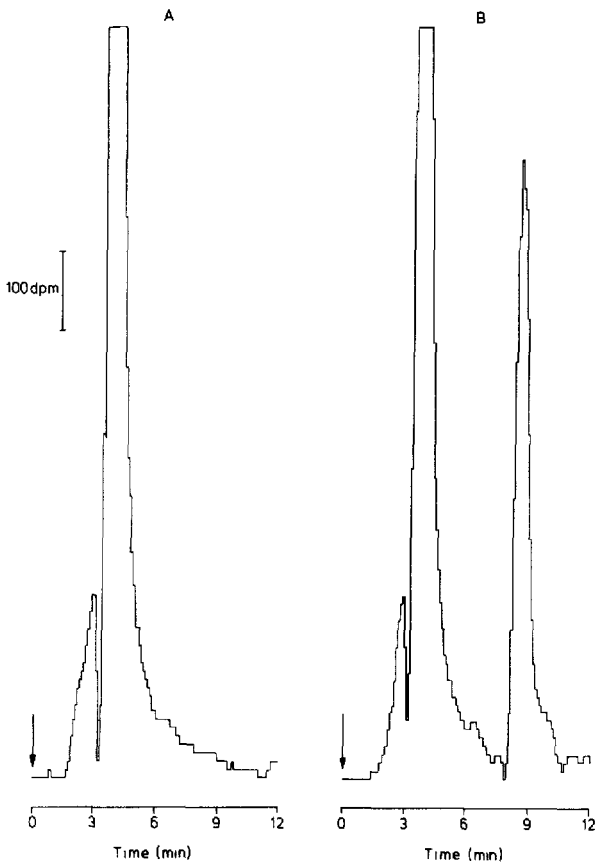

Fig. 1. Radioactivity patterns of AADC incubation mixtures with whole rat brain homogenate as enzyme: (A) blank, i.e., incubation mixture without enzyme; (B) homogenate incubated with 0.2 m*M* [^{14}C] L-DOPA. Chromatographic conditions: 5-μm Ultrasphere-ODS column (150 × 4.5 mm I.D.); mobile phase, 10% methanol in 100 m*M* sodium phosphate (pH 3.2) containing 1 m*M* sodium octanesulphonate; flow-rate, 1.5 ml/min; ACS mobile phase mixing ratio, 3:1; ^{14}C signal detection and print out at 0.5-min intervals; ^{14}C dpm values, 1000 dpm full-scale.

in this assay system the amounts of endogenous SAM and Mg^{2+} in the rat brain preparation do not provide enough of these cofactors for COMT activity.

Non-enzymatic decarboxylation of L-DOPA, which has been observed in the pH range 6.5—11 and thus reasoned to cause discrepancies in AADC determinations [12], was negligible in this assay system. It was found that the addition of 0.1 m*M* EDTA to the incubation mixture completely inhibited the non-enzymatic decarboxylation of L-DOPA. The addition of ascorbic acid or 2-mercaptoethanol to the assay mixture did not affect the results.

The rate of decarboxylation, expressed as an increasing amount of [^{14}C]DA in the on-line detection system, showed a linear relationship with incubation time up to 45 min ($y = 1.11x + 6.75$; $r = 0.999$; $n = 3$). Linearity was also obtained between the radioactivity in the product peak and the amount of enzyme protein up to 1 mg in the assay mixture during incubation for 30 min at 37°C ($y = 0.15x + 2.1$; $r = 0.999$; $n = 3$). The reproducibility of the assay under the described conditions was 5.4% expressed as the coeffient of variation ($n = 6$). This precision was achieved by counting about 7000 dpm with the flow-through liquid scintillation detector. The detection limit for ^{14}C was 20 pmol at the specific activity used of 2.1 pCi/pmol. By using higher specific activities the detection limit is lower.

AADC activity in whole rat brain ($n = 6$) with 0.2 m*M* L-DOPA as the substrate using the described HPLC—radiochemical assay system was 285 ± 15 pmol/min/mg protein. The Michaelis constant (K_m) for L-DOPA was 143 ± 7 μM ($n = 3$). These results are in good agreement with those obtained by $^{14}CO_2$ release assay [5] and HPLC—UV assay [2], but are higher than those obtained using voltametric assay [3].

AADC activity has recently been measured using oxygraphic assay [13]. However, this method requires coupling of the AADC with serum amine oxidase. The latter must first be purified and immobilized on Sepharose, after which this system is ready for continuous monitoring of decarboxylation reactions.

The HPLC method with radiochemical detection described here is precise and sensitive. It is expensive, but it allows the handling of about 40 samples per day, making it useful for rapid measurements of AADC activity in different tissues and for interference-free inhibition studies.

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