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High-performance liquid chromatographic procedure for the simultaneous determination of aromatic L-amino acid decarboxylase activity towards 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan

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ABSTRACT

A high-performance liquid chromatographic method with electrochemical detection has been developed and applied in the simultaneous determination of aromatic L-amino acid decarboxylase activity with respect to L-3,4-dihydroxyphenylalanine and 5-hydroxytryptophan as substrates. Both substrates are included in the incubation mixture, and the decarboxylated products, dopamine and serotonin, respectively, are detected. In contrast to several earlier claims, we found that the ratio of the decarboxylase activity to L-3,4-dihydroxyphenylalanine and 5-hydroxytryptophan is quite constant across several different rat tissues, supporting the notion that there is only one major single enzyme rather than two. We also observed that an erroneous ratio with respect to the L-3,4-dihydroxyphenylalanine/5-hydroxytryptophan decarboxylation activities, is obtained if these activities are assessed under non-linear kinetic conditions.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) catalyzes the decarboxylation of L-3,4-dihydroxyphenylalanine (L-DOPA) and 5-hydroxytryptophan (5-HTP) to dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT), respectively, two major neurotransmitters in various mammalian tissues [1,2]. There has been controversy as to whether a single decarboxylase is responsible for decarboxylating these two substrates as opposed to two enzymes, namely L-DOPA decarboxylase and 5-HTP decarboxylase, being responsible. Earlier investigations showed that AADC exhibited different kinetic properties and ratios of its activity towards L-DOPA and 5-HTP in various tissues [3–5], brain regions [6] and subcellular fractions [7]. Although two different enzymes have been considered to be involved, the single enzyme purified from pig kidney [8,9], guinea pig kidney [10] and rat kidney [11], for example, catalyzed the decarboxylation of both L-DOPA and 5-HTP, and antibodies to AADC reacted equally well to enzymes exhibiting L-DOPA and 5-HTP activities [8,12,13]. This suggests that a

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single enzyme acts on both substrates. More recently Southern blot analysis has revealed, and further confirmed, that it is a single gene that encodes for AADC [14,15]. If there is only one single AADC in different tissues, then the ratio of the enzyme activities with regard to L-DOPA and 5-HTP should be closely similar in these tissues. It is of course possible that a post-translational modification or the presence of modulatory factors to the enzyme may influence the expression of the enzyme activities, and in this regard it has been shown that pyridoxal phosphate or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and 1-methylphenylpyridininium (MPP^+) can apparently affect the expression of AADC activities towards L-DOPA and 5-HTP [16,17].

AADC activities are measured primarily by radioenzymatic or high-performance liquid chromatographic–electrochemical detection (HPLC–ED) procedures, and separate experiments are required to measure the AADC activity towards the L-DOPA and 5-HTP substrates. In this paper we describe an HPLC procedure which measures the decarboxylation of both L-DOPA and 5-HTP simultaneously. Such a method not only simplifies the procedure, but it also greatly reduces inter-experimental errors. We have investigated the distribution of AADC towards both L-DOPA and 5-HTP in eight different rat tissues. The validity of the method and the importance of linear enzyme kinetics in the assay have been demonstrated.

EXPERIMENTAL

Chemicals

L-DOPA, 5-HTP, DA, 5-HT, pargyline and pyridoxal phosphate were obtained from the Sigma (St. Louis, MO, U.S.A.); all other chemical reagents were of analytical grade and were obtained from commercial sources.

Preparation of tissue homogenates

Male Wistar rats (200–220 g) were decapitated, and the liver, kidney, adrenals, brain, heart, lung, small and large intestine quickly removed and homogenized in five volumes of chilled 0.05 *M* phosphate buffer (pH 7.4) using a Polytron homogenizer. The tissue homogenates were used immediately in the enzyme assays. Hog kidney AADC was partially purified as previously described [18].

AADC assay

Enzyme activities towards L-DOPA and 5-HTP, in various tissues, were determined on the basis of the measurement of DA and 5-HT, the products of the reaction, using an HPLC–ED procedure as previously described [19]. The standard incubation mixture contained 0.02 m*M* pyridoxal phosphate, 0.1 m*M* pargyline, $2 \cdot 10^{-4}$ *M* L-DOPA, $1 \cdot 10^{-4}$ *M* 5-HTP, 0.2 *M* phosphate buffer (pH 7.5) and enzyme in a total volume of 200 μl. In each experiment at least three concen-

trations of the tissue homogenates were used for the assay. Following incubation at 37°C for 30 min the reactions were terminated by addition of 800 μl of chilled 0.1 *M* perchloric acid (PCA) containing $1 \cdot 10^{-4}$ *M* sodium metabisulphite and $2 \cdot 10^{-4}$ *M* EDTA. The mixture was shaken using a vortex mixer and then centrifuged at 13 750 *g* for 10 min. A 200-μl aliquot of supernatant was transferred to a small vial, and from this vial aliquots were taken and subjected to HPLC analysis.

High-performance liquid chromatography

HPLC separation was conducted as described previously [20], at room temperature using a 250 mm × 4.6 mm I.D. Ultrasphere I.P. analytical column packed with octadecyl-bonded spherical 5-μm silica particles (Backman, Toronto, Canada). An SSI precolumn filter (0.5-μm filter elements, Terochem, Rexdale, Canada) and a 30 mm × 4.6 mm I.D. Brownlee MPLC RP-18 SPHER-5 guard column (Technical Marketing Assoc., Calgary, Canada) were installed between the Waters WISP 710B automated sample injector and the analytical column. An aliquot (10 μl) of each prepared sample was injected onto the column. The mobile phase, comprising 75 m*M* monobasic sodium phosphate, 1 m*M* sodium octylsulphate, 500 μM EDTA and 13% (v/v) acetonitrile, with the final pH adjusted to 2.75 with phosphoric acid, was pumped through the column at 1.0 ml/min using an SSI 222B solvent delivery system (State College, PA, U.S.A.). For the electrochemical detection an amperometric system at 0.75 V *versus* an Ag/AgCl reference electrode (Mandel, Rockwood, Canada) was used. Quantitation of the signals from the detector were by peak-area integration using a Spectra-Physics SP-4290 integrator.

Protein concentrations were measured according to Bradford [21] with bovine serum albumin as a reference standard.

RESULTS AND DISCUSSION

As can be seen in Fig. 1A, L-DOPA, 5-HTP, DA and 5-HT are well separated from each other under the described chromatographic conditions. The retention times are 4.1, 6.3, 7.3 and 12.6 min, respectively. After incubation of both L-DOPA and 5-HTP with the enzyme, the corresponding products DA and 5-HT are readily detected (see Fig. 1C). In the blank (*i.e.* after incubation of the substrates in the absence of enzyme) only the L-DOPA and 5-HTP substrate peaks are seen (see Fig. 1B). In this series of studies an external standard mixture (see Fig. 1A) was inserted after every tenth sample and from these it was shown that the DA and 5-HT peak heights remained constant throughout the whole study, indicating that these amines are stable in the PCA solution which contains reducing agents. The detection limit for the amine product was at least 2 pmol per injection defined at a signal-to-noise ratio of at least 2. The optimal pH ranges for the decarboxylation of L-DOPA and 5-HTP have been estimated to be 6.5–7.0

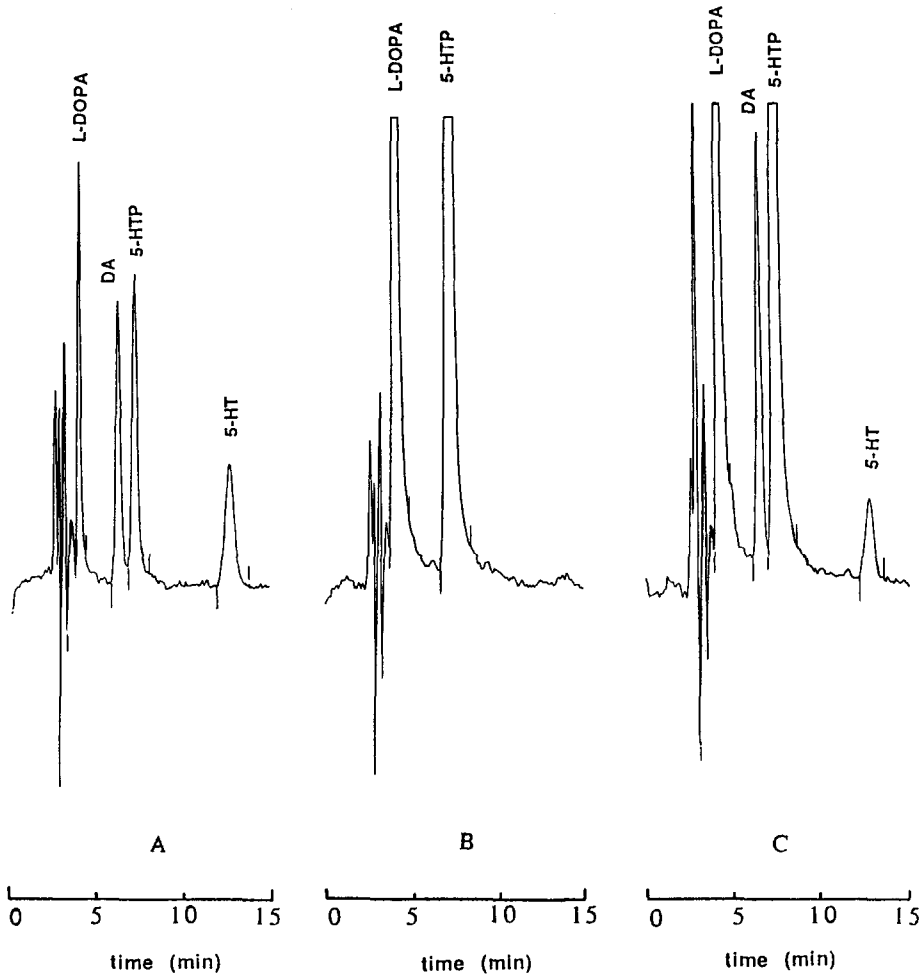

Fig. 1. HPLC traces of the decarboxylation of a mixed-substrate (L-DOPA and 5-HTP) assay. (A) Standard mixture containing L-DOPA, 5-HTP, DA and 5-HT (5 ng of each); (B) blank AADC assay in the absence of hog kiney AADC; (C) AADC assay in the presence of both L-DOPA and 5-HTP as substrates.

and 7.5–8.0, respectively. Under the present experimental condition, *i.e.* at pH 7.5, 82 and 96% of maximal activities are produced with respect to L-DOPA and 5-HTP as substrate.

AADC from rat kidney or liver is known to exhibit a somewhat higher Michaelis constant (K_M) for L-DOPA than for 5-HTP [13,22]. In the present mixed-substrate assay, near K_M concentrations for L-DOPA and 5-HTP were used. This permits us to assess not only the relative total activities towards the two substrates in a single assay, but also to detect any variation in activity due to a modification of the enzyme affinities (*i.e.* changes in K_M values).

In order to ensure a linear kinetic measurement of AADC activity both the

time course of the reaction and the linear relationship to the amount of enzyme have been established. In the routine analysis of AADC activity, we included at least three different enzyme concentrations in each assay. Fig. 2 shows a few typical results as obtained using homogenates of rat liver, brain, heart and small intestine. As can be seen in Fig. 2, linear relationships were obtained at lower enzyme concentrations; at higher levels of AADC, such as homogenates of liver or small intestine (Fig. 2C and D), a linear relationship was only found at relatively low enzyme concentration limits. This is probably due to depletion of substrates or some unknown enzyme inhibitory compound(s) which might be present in the tissue homogenates. In this study, therefore, AADC levels in different tissues (Table I) were estimated and derived over a limited linear range of enzyme concentrations.

Our results confirm that AADC activities vary considerably in different tissues [4,5,14], being higher in the liver and kidney and lower in the heart and lung. Interestingly, as revealed by the closely similar ratios, the distribution of 5-HTP decarboxylase activity was quite similar to that of L-DOPA decarboxylase activity in the different tissues (Table I). We also showed that the L-DOPA/5-HTP decarboxylation ratios in different rat brain regions were similarly quite constant

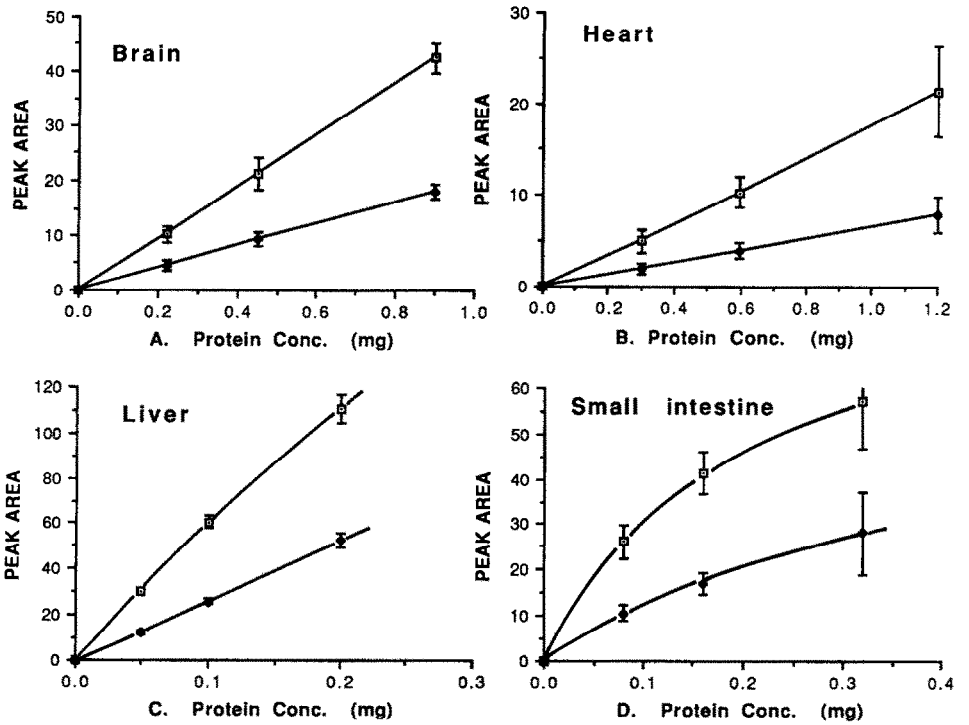

Fig. 2. AADC activities in different rat tissues. Various enzyme concentrations in homogenates of rat liver, brain, heart and small intestine were incubated with as mixture of L-DOPA and 5-HTP. The products DA (□) and 5-HT (◆) were determined by HPLC–ED. Each point represents the mean ± S.D. from five rats.

TABLE I

DISTRIBUTION OF AADC ACTIVITY TOWARD L-DOPA AND 5-HTP IN DIFFERENT RAT TISSUES USING A SIMULTANEOUS HPLC PROCEDURE

Values are the mean ± S.D. for eleven rats, each homogenate being assayed in triplicate.

Tissue	AADC activity (nmol min^{-1} (mg protein)$^{-1}$)		Ratio of L-DOPA/5-HTP activities
	L-DOPA as substrate	5-HTP as substrate	
Liver	4.013 ± 0.920	0.855 ± 0.191	4.5 ± 0.25
Kidney	4.025 ± 0.412	0.910 ± 0.128	4.5 ± 0.32
Adrenals	1.881 ± 0.783[a]	0.424 ± 0.173[a]	4.3 ± 0.42
Brain	0.569 ± 0.209[a]	0.139 ± 0.073[a]	4.5 ± 0.62
Heart	0.175 ± 0.049[a]	0.040 ± 0.013[a]	4.5 ± 0.67
Lung	0.335 ± 0.135[a]	0.073 ± 0.036[a]	4.5 ± 0.65
Small intestine	1.699 ± 0.371[a]	0.376 ± 0.112[a]	4.5 ± 0.58
Large intestine	1.444 ± 0.387[a]	0.342 ± 0.195[a]	4.2 ± 0.68

[a] $p < 0.05$ in comparison to liver.

(results not shown.) These findings confirm those of Kuntzman *et al.* [23], but are at variance with several others [3–7]. Results from these latter reports were rather inconsistent, probably because there was no clear indication or trend about the L-DOPA/5-HTP decarboxylation ratios in the various tissues. It is possible that the methodology used for the assay of the AADC activity was responsible for the ratio variations, and it is quite clear that the assessed values are misleading if they are obtained from a non-linear kinetic assessment.

Several immunological studies, such as those on monoclonal antibodies from rat kidney cross-reacting with AADC from other rat tissues [13] and from bovine brain AADC cross-reacting with bovine adrenal AADC [24], support the notion that AADC is a single enzyme and responsible for decarboxylating both L-DOPA and 5-HTP. The most convincing evidence, however, for the existence of a single AADC comes from molecular biological data. Hybridization analysis using a cDNA probe complementary to bovine adrenal AADC mRNA indicates the presence of a single mRNA species in bovine liver, kidney and adrenal as well as rat liver, brain and pheochromocytoma cells [14]. Human full-length cDNA-encoding human AADC has also been isolated [15] and transferred to COS cells [25]. This enzyme again catalyzes both L-DOPA and 5-HTP.

There seems little doubt, therefore, that AADC is a single enzyme, although post-translational modifications to its activity may be caused by endogenous factors, since it is known that decarboxylation of 5-HTP has a lower affinity to pyridoxal phosphate than does L-DOPA [16,17], and intracisternal administration of 6-hydroxydopamine can induce a selective decrease of the decarboxylation activity towards L-DOPA and 5-HTP in the rat brain [26]. This finding has

not been confirmed [27]. Another study indicated that electrolytic lesions to both the rat medial and dorsal raphe nuclei apparently also caused differential effects on AADC activity towards both L-DOPA and 5-HTP in the striatum [28].

In conclusion, the application of a newly developed procedure for the simultaneous determination of L-DOPA and 5-HTP decarboxylation along with appropriate selection of substrate concentrations chosen from a detailed knowledge of enzyme kinetics clearly demonstrates that a constant proportion of L-DOPA and 5-HTP decarboxylase activity exists in different rat tissues, and this supports the notion that there is only a single AADC. This method will be useful in future studies on the modulation and expression of AADC activity.

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