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OCCURRENCE OF AROMATIC L-AMINO ACID DECARBOXYLASE IN HUMAN PLASMA AND ITS ASSAY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH FLUORESCENCE DETECTION

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SUMMARY

A high-performance liquid chromatographic method using fluorescence detection for assessing the activity of aromatic L-amino acid decarboxylase in human plasma is described. Dopamine, formed enzymatically from L-DOPA, and isoproterenol (internal standard) are chromatographed on a small ion-exchange cartridge (Toyopak SP) and derivatized with 1,2-diphenylethylenediamine. The derivatives are separated by reversed-phase chromatography on an Ultrasphere ODS column. The detection limit for dopamine formed enzymatically is 0.6 pmol per 500 µl of enzyme reaction mixture. Aromatic L-amino acid decarboxylase in human plasma is very similar to that in rat kidney, with respect to optimum conditions for the enzyme reaction and gel chromatographic behaviour.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC; aromatic L-amino acid carboxylyase, EC 4.1.1.28) catalyses the decarboxylation of aromatic L-amino acids to the corresponding amines in the presence of pyridoxal phosphate. Since the enzyme has a high affinity, especially for L-DOPA and 5-hydroxytryptophan [1], it plays an important role in the biosynthesis of catecholamines (dopamine, norepinephrine and epinephrine) and serotonin, which function as neurotransmitters and hormones, and are involved in the regulation of neuronal functions, behaviour and emotions.

Many assays for AADC in biological materials have been reported: manometric [2], spectrophotometric [3, 4], fluorimetric [5, 6], radioisotopic [1, 7–9] and high-performance liquid chromatographic (HPLC) [6, 10–13]

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methods. The enzyme has been found in various mammalian tissues [1–7, 10, 11] and serum [6, 12], and has been identified in human liver [8, 10] and brain [9, 10, 13]. In contrast, the existence of AADC activity in human plasma has not been demonstrated, probably due to the lack of sensitivity of the assay methods.

We have previously reported a highly sensitive method for AADC in rat tissues and serum using reversed-phase HPLC with fluorescence detection [14], based on pre-column derivatization of dopamine formed enzymatically from substrate L-DOPA with 1,2-diphenylethylenediamine (DPE), a fluorogenic reagent for catechol compounds [15, 16].

This paper describes the occurrence of AADC in human plasma. The assay is a modification of the previously reported method for rat tissues [14]. AADC in human plasma is also compared with the enzyme activity in rat kidney determined using polyacrylamide gel electrophoretic and gel chromatographic techniques.

EXPERIMENTAL

Reagents, materials and apparatus

All chemicals were of reagent grade unless otherwise stated. Deionized, distilled water was used. DPE solution, the Toyopak SP cartridge, the Ultrasphere ODS column and the apparatus were the same as those described previously [14]. The chromatograph and the detector were operated in the same way as described previously [14].

Enzyme preparations

Human plasma. Venous blood from healthy volunteers (21–55 years of age) was collected in tubes containing EDTA · 2Na as an anticoagulant. The blood was centrifuged at 1500 *g* for 20 min at 4°C, and the plasma stored at −20°C until use. AADC activity in plasma remained stable for at least a month when stored at −20°C.

Rat kidney homogenate. Rat (Donryu, male, four weeks old, 180–200 g) kidney was prepared as described previously [14]. The tissue was homogenized with 10 vols. of 0.25 *M* sucrose and the protein concentration of the homogenate was 15 mg/ml.

Procedure for polyacrylamide gel electrophoresis

Analytical polyacrylamide gel electrophoresis was performed according to the methods of Williams and Reisfeld [17] and Davis [18], with the following minor modifications. The gel was polymerized with N,N,N′,N′-tetramethylethylenediamine in the presence of ammonium persulphate as a catalyst, and the electrode buffer was 0.05 *M* Tris–glycine (pH 8.1). Human plasma and rat kidney homogenate (50 μl each) were applied to each of the gels, which were analysed in parallel. The electrophoresis was carried out at a constant current of 2 mA per gel column. The gels were fractionated into 2-mm slices and the enzyme was eluted by immersing the slices in 0.3 ml of 0.2 *M* sodium phosphate buffer (pH 6.8) for 12 h or more at 0–2°C. AADC activities in the slices were estimated according to the procedure described below.

Procedure for gel chromatography

The column was prepared by packing 100 ml of Sepharose 6B (Pharmacia Fine Chemicals, Uppsala, Sweden) suspended in approximately 400 ml of 0.2 *M* sodium phosphate buffer (pH 6.8) into a glass tube (80 × 1.5 cm I.D.) using the standard technique. Human plasma (0.8 ml) or rat kidney homogenate (0.5 ml) was loaded on the column, which was eluted with the phosphate buffer at a flow-rate of 15 ml/h. Fractions (2 ml) were collected using a Toyo SF-100G fraction collector (Toyo Kagaku, Tokyo, Japan). The eluates were monitored at 254 nm with a Uvicon UV-254F spectrophotometer (Toyo Kagaku). All procedures were carried out at 0–2°C. AADC activities in the fractions were determined according to the procedure described below. The molecular weights of the components of the fractions having AADC activity were estimated from the standard curve, which was prepared by treating the standard proteins (Bio-Rad, Richmond, CA, U.S.A.) in the usual manner.

Assay procedure for AADC

To 100 μl of human plasma were added 200 μl of 0.2 *M* sodium phosphate buffer (pH 6.8) and 100 μl of 0.1 m*M* pyridoxal phosphate. The mixture was preincubated at 37°C for 10 min and then incubated for 30 min after addition of 100 μl of 8 m*M* L-DOPA. At the end of the incubation, 100 μl each of 3 *M* trichloroacetic acid and 0.2 nmol/ml isoproterenol (internal standard) were added. The mixture was centrifuged at 1000 *g* at 4°C for 10 min, and the supernatant was poured onto a Toyopak SP cartridge. The cartridge was washed successively with 10 ml of water, 2 ml of 0.2 *M* sodium phosphate buffer (pH 5.5) and 10 ml of water. The adsorbed amines were eluted with 2.0 ml of a mixture of ethanol–1.0 *M* sodium chloride (7:3). To the eluate, 100 μl each of the DPE 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 into the fluorescent compounds. The resulting mixture (100 μl) was chromatographed. For the blank, the enzyme preparation was carried through the same procedure except that the order of addition of L-DOPA and trichloroacetic acid was reversed and incubation was omitted. The preparation of the blank was unnecessary unless the chromatogram of the blank was investigated. The Michaelis constant (K_M) for L-DOPA was calculated from the Lineweaver–Burk plots.

RESULTS AND DISCUSSION

The conditions of the fluorescence derivatization and HPLC analysis were the same as described previously [14].

Fig. 1 shows a typical chromatogram of a sample of human plasma and that of the blank. The enzymatically formed dopamine (peak 1 in Fig. 1) had a retention time of 5.2 min. Its fluorescence excitation (maximum, 350 nm) and emission (maximum, 480 nm) spectra were identical to those of the authentic dopamine standard. All the peaks shown in the chromatograms (Fig. 1) were not observed when the DPE solution or potassium hexacyanoferrate(III) were omitted. Therefore, peaks 3 and 4 were believed to be catechol compounds [16]. Peaks 1 and 3 were not present when plasma was omitted in the

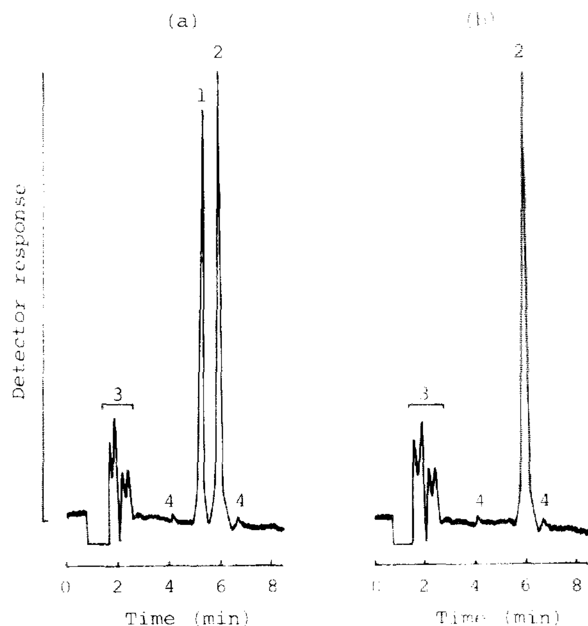

Fig. 1. Chromatograms obtained from (a) human plasma and (b) the blank. Portions (100 μl) of human plasma were treated according to the procedure. AADC activity 9.46 pmol/min/ml. Peaks: 1 = dopamine; 2 = isoproterenol; 3 and 4 = unknown.

enzyme reaction mixture; the same was true for peaks 1 and 4 when L-DOPA was omitted; peaks 3 can be attributed to catechol compounds in the plasma and peaks 4 to impurities in the commercial L-DOPA.

AADC in human plasma was most active at pH 6.8—7.0 in sodium phosphate buffer, and the buffer concentrations of 0.1—0.4 *M* gave a maximum and constant activity. Thus, a 0.2 *M* sodium phosphate buffer of pH 6.8 was used in the procedure.

L-DOPA gave a maximum and constant activity in a concentration range of 0.8—2.4 m*M* in the enzyme reaction mixture, with an observed K_M value (mean ± S.D., $n = 5$) of 0.16 ± 0.02 m*M*; 1.6 m*M* L-DOPA was used as a saturating concentration. The K_M value is comparable to those observed with various rat tissues (0.08—0.11 m*M*) [14].

Pyridoxal phosphate in the enzyme reaction mixture provided a maximum activity in a concentration range of 0.01—0.2 m*M*; 0.02 m*M* pyridoxal phosphate was selected in the procedure.

Dopamine can be deaminated in vivo by monoamine oxidase (MAO) [19] and is oxidized with oxygen as well as other oxidizing agents. L-DOPA is decarboxylated non-enzymatically in the presence of certain metal ions [20] and oxidizing substances [21, 22]. However, in the present procedure, cuprizone (0—0.1 m*M*) as a MAO inhibitor, ascorbic acid or dithiothreitol (0—0.6 m*M* each) as antioxidant, and β-mercaptoethanol and EDTA (1—500 μ*M* each) as inhibitors of the non-enzymatic decarboxylation reaction did not influence the amount of enzymatically formed dopamine. These results agree with those observed with various rat tissues [14].

3-Hydroxybenzylhydrazine and 3-hydroxybenzyloxyamine dihydrogen-

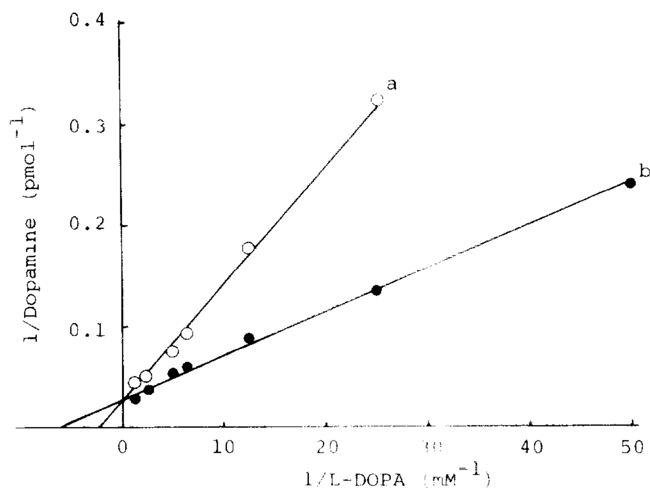

Fig. 2. Inhibition of human plasma AADC by 5-hydroxytryptophan. Portions (100 μl) of human plasma were treated as described in the procedure in (a) the presence (0.2 mM in the enzyme reaction mixture) and (b) the absence of 5-hydroxytryptophan. The data were analysed by linear regression analysis.

phosphate act as AADC inhibitors [23, 24]. At concentrations of 0.1, 0.5 and 5 μM in the enzyme reaction mixture, 3-hydroxybenzylhydrazine inhibited 80, 93 and 100% of the activity, respectively. At the same concentrations, 3-hydroxybenzyloxyamine dihydrogenphosphate inhibited 62, 83 and 100% of the enzyme activity, respectively.

Both 5-hydroxytryptophan and L-DOPA act as substrates for AADC, and these amines are thus competitive inhibitors of each other [3]. 5-Hydroxytryptophan inhibited AADC in human plasma in a competitive mode against L-DOPA (Fig. 2), with an observed inhibitory constant of 0.14 mM, which was obtained by the method of Dixon [25].

The formation of dopamine was not observed when D-DOPA was used instead of L-DOPA in the procedure.

All the above observations suggest that AADC exists in human plasma and the optimum reaction conditions for this enzyme are very similar to those for the enzyme in various rat tissues [14].

Fig. 3 shows AADC activities of the slices obtained by the gel electrophoretic procedure with human plasma and rat kidney homogenate. AADC activity in the two enzyme preparations was detected in slice numbers 1–3. However, in human plasma, AADC activities were not detected in slice numbers 11–13, whereas in rat kidney homogenate, the activities were also observed in these slice numbers. The difference between the electrophoretic patterns of human plasma and rat kidney homogenate remains unknown. AADC in pig kidney and human adrenal medulla has a subunit (molecular mass 40 000–50 000) that can be separated by gel chromatography [26]. Gel chromatograms obtained with both enzyme preparations using a Sepharose 6B column are shown in Fig. 4. Fraction numbers 27–31 in Fig. 4 corresponded to a molecular mass of ca. 45 000. This value is in good agreement with that of the subunit of AADC [1, 26–28]. The chromatogram suggests that AADC in

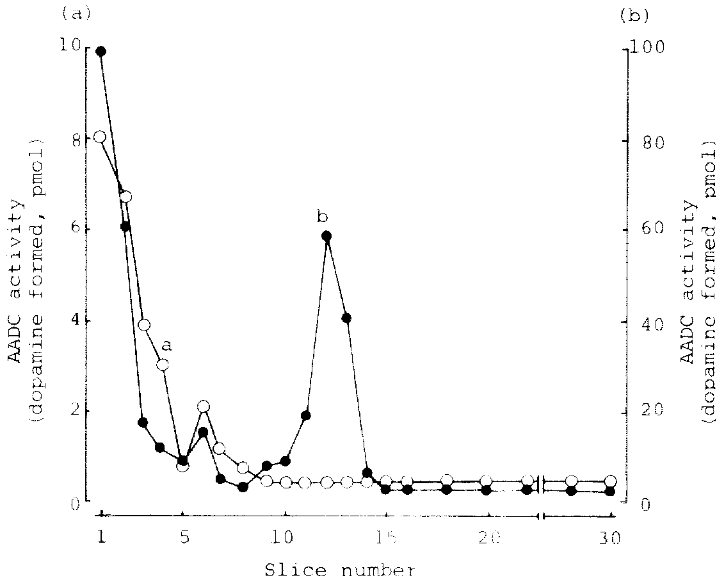

Fig. 3. Gel electrophoretic separation of (a) human plasma and (b) rat kidney homogenate. Both enzyme preparations were treated by the procedure for polyacrylamide gel electrophoresis.

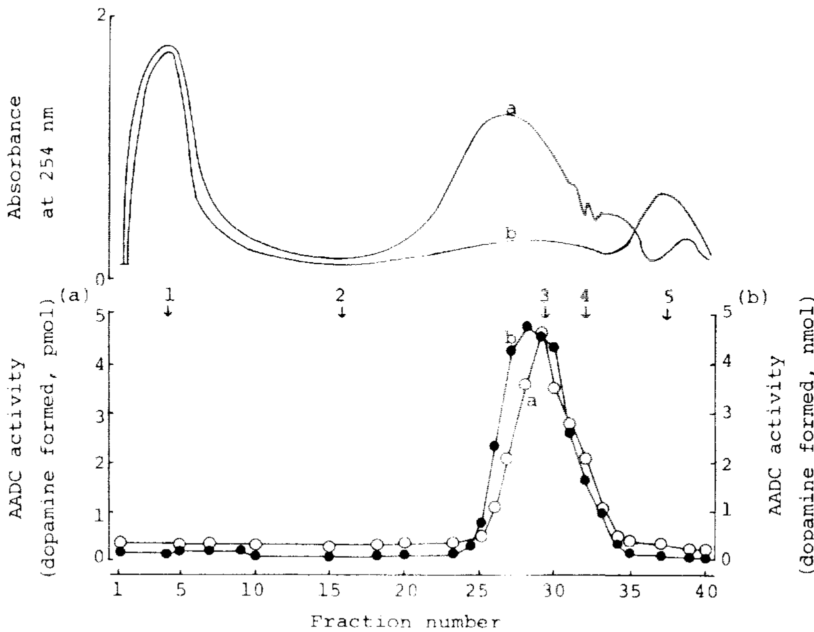

Fig. 4. Gel chromatograms of (a) human plasma and (b) rat kidney homogenate. Both enzyme preparations were treated by the procedure for gel chromatography. The arrows indicate the molecular mass of standard proteins: 1 = bovine thyroglobulin (670 000); 2 = bovine γ-globulin (158 000); 3 = ovalbumin (44 000); 4 = myoglobin (17 000); 5 = cyanocobalamin (1350).

human plasma is fairly similar to that of rat kidney with respect to molecular mass. The previous reports suggested that AADC existed as a dimer and had a molecular mass of approximately 100 000 [1, 27, 28], but the activity in fraction numbers 18–20, which corresponded to a molecular mass of 100 000, was almost nil. The reason for this remained unknown.

The amount of dopamine formed enzymatically in the assay procedure was proportional to the sample size of human plasma up to at least 150 μl; 100-μl samples were used in this procedure.

Enzyme activity was linear with time up to at least 70 min when incubated at 37°C; a 30-min incubation time was recommended.

A strong cation exchanger, Toyopak SP (H^+) cartridge was used for the clean-up of the incubated enzyme reaction mixture; the conditions for the clean-up are basically the same as those described previously [14]. Recoveries (mean ± S.D.) of dopamine and isoproterenol (20 pmol each) added to the incubated enzyme reaction mixture of the blank were 90.8 ± 0.2% and 92.1 ± 0.4% (n = 5), respectively.

A linear relationship was obtained between the peak-height ratios of dopamine and isoproterenol and the amount of dopamine added to the blank over the concentration range 1–100 pmol. The detection limit for dopamine formed enzymatically was 0.6 pmol per 500 μl of enzyme reaction mixture (15 fmol per 100-μl injection volume) at a signal-to-noise ratio of 2. The intra-assay coefficient of variation was 3.8% (n = 10) for a mean AADC activity of 6.17 pmol/min/ml.

Plasma AADC activities in healthy volunteers (21–55 years of age) were measured using this method (Table I). Human plasma AADC activity was approximately 50 times lower than that of rat serum [14]. A very low activity

TABLE I

PLASMA AADC ACTIVITIES IN HEALTHY VOLUNTEERS

Age	Sex*	AADC activity** (pmol/min/ml)
55	M	11.41
38	M	6.46
33	M	6.17
30	M	9.78
28	M	4.45
27	M	7.78
26	M	15.01
26	M	9.60
25	M	10.26
24	M	8.21
23	M	7.12
23	M	5.34
23	M	7.40
21	F	6.20
21	F	7.23
Mean ± S.D.		8.16 ± 2.69

*M = Male; F = female.

**Mean of duplicate determinations.

(ca. 1 pmol/min/ml) of the enzyme has been detected in human serum by the HPLC method using 5-hydroxytryptophan as a substrate [12], and the activity was 8 times lower than that obtained by the present method. This seems to be mainly caused by the difference in substrates [1, 12, 28].

This method is simple, precise and highly sensitive, and should, therefore, be useful for biological and biomedical investigations of catecholamine metabolism.

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