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NEW AND HIGHLY SENSITIVE ASSAY FOR L-5-HYDROXYTRYPTOPHAN DECARBOXYLASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY—VOLTAMMETRY

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SUMMARY

This paper describes a new, inexpensive and highly sensitive assay for aromatic L-amino acid decarboxylase (AADC) activity, using L-5-hydroxytryptophan (L-5-HTP) as substrate, in rat and human brains and serum by high-performance liquid chromatography (HPLC) with voltammetric detection. L-5-HTP was used as substrate and D-5-HTP for the blank. After isolating serotonin (5-HT) formed enzymatically from L-5-HTP on a small Amberlite CG-50 column, the 5-HT was eluted with hydrochloric acid and assayed by HPLC with a voltammetric detector. N-Methyldopamine was added to each incubation mixture as an internal standard. This method is sensitive enough to measure 5-HT, formed by the enzyme, 100 fmol to 140 pmol or more. An advantage of this method is that one can incubate the enzyme for longer time (up to 150 min), as compared with AADC assay using L-DOPA as substrate, resulting in a very high sensitivity. By using this new method, AADC activity was discovered in rat serum.

INTRODUCTION

Aromatic L-amino acid decarboxylase (AADC, EC 4.1.1.28) [1] is the enzyme which catalyzes the decarboxylation of L-DOPA and L-5-hydroxytryptophan (L-5-HTP) to dopamine and serotonin (5-HT), respectively, in mammalian tissues. The enzyme was homogeneously purified from hog kidney and was shown to decarboxylate various aromatic L-amino acids including L-DOPA and L-5-HTP [2]. Enzymatic decarboxylations of L-DOPA and L-5-HTP lead to the biosynthesis of catecholamines (dopamine, noradrenaline and adrenaline) and indoleamines (serotonin and melatonin), respectively. Since

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monoamines are physiologically very important, the assay of AADC using both L-DOPA and L-5-HTP are frequently required. However, the activity of AADC in some tissues such as human brain is very low, and the presence of AADC in human brain has even been questioned because of the possibility of nonenzymatic decarboxylation [3—5].

In order to resolve the above discrepancies on the presence of AADC in human brain, we had already established a specific method to measure L-DOPA decarboxylase activity by using high-performance liquid chromatography (HPLC) and voltammetry [6]. Since L-5-HTP is also an important substrate for AADC, the assay of the activity using L-5-HTP is also necessary, and it has some advantages over L-DOPA as substrate. We have therefore developed a new, specific and highly sensitive method for the measurement of L-5-HTP decarboxylase activity by using HPLC and voltammetry of 5-HT [7—9], as in our AADC assay using L-DOPA as substrate [6].

EXPERIMENTAL

Materials

L-5-HTP, D-5-HTP, 5-HT, pargyline HCl and N-methyldopamine were obtained from Sigma (St. Louis, MO, U.S.A.); pyridoxal phosphate was from Katayama Chemicals (Osaka, Japan); Amberlite CG-50 was from Rohm and Haas (Philadelphia, PA, U.S.A.). All other chemicals were of analytical grade.

Rats were killed by decapitation. Immediately after the decapitation cerebral cortex was dissected. Rat serum was also collected. Human cerebral cortex was also dissected at autopsy from patients without a history of neurological disorders. The brains were homogenized with 0.32 *M* sucrose solution (1 part tissue plus 9 parts 0.32 *M* sucrose solution) in a Potter glass homogenizer. Amberlite CG-50 (type 1, 100—200 mesh) was activated by washing with 2 *M* HCl, 2 *M* NaOH and finally with water, equilibrated with 1 *M* potassium phosphate buffer (pH 6.5), and stored in the same buffer.

Experimental procedures

The standard incubation mixture for L-5-HTP decarboxylase contained (total volume 400 μl, final pH 8.3) : 30 m*M* sodium phosphate buffer (pH 9.0), 0.01 m*M* pyridoxal phosphate, 1.0 m*M* L-5-HTP (or D-5-HTP for the blank), 0.1 m*M* pargyline HCl, and the enzyme. Incubation was done at 37°C for 20—120 min, and the reaction was stopped by adding 80 μl of 3 *M* trichloroacetic acid. After 10 min 1.82 ml of water and 100 μl of 0.01 *M* HCl containing 100—500 pmol of N-methyldopamine as an internal standard were added, and the mixture was centrifuged at 1600 *g* for 10 min. The supernatant was passed through a column (packed volume 0.5 ml) of Amberlite CG-50 (Na^+) equilibrated with 0.1 *M* potassium phosphate buffer (pH 6.5). The resin was washed twice with 4.5 ml of the buffer and with 200 μl of 1 *M* HCl. The 5-HT adsorbed was eluted with 1.4 ml of 1 *M* HCl. A 100-μl aliquot (or 50 μl) of the eluate was injected into the high-performance liquid chromatograph (Yanaco L-2000) with a Yanaco VMD-101 voltammetric detector and a Yanapak ODS-T reversed-phase column (particle size 10 μm, 25 cm × 0.4 cm I.D.) (Yanagimoto, Kyoto, Japan). The carrier buffer for the liquid chromatography was 0.1 *M* potassium phosphate buffer containing 10% methanol, pH 3.2, with a flow-rate

of 0.5 ml/min. The detector potential was set at 0.8 V against the Ag/AgCl electrode. The peak height of 5-HT was measured and converted to pmol from the peak height of N-methyldopamine added as an internal standard. The retention times under these conditions were: N-methyldopamine, 5.0 min; 5-HT, 9.25 min; and 5-HTP, 10.0 min.

RESULTS

This HPLC—voltammetry system for the measurement of 5-HT and 5-HTP was found to be very sensitive. The standard curves of 5-HT and N-methyldopamine (internal standard) showed linearity from 200 fmol to 70 pmol.

Among the brain regions of rats, cerebral cortex has the lowest enzyme activity. Therefore, for the development of this method, rat cerebral cortex homogenate was used as the enzyme source. Fig. 1 shows the chromatographic pattern of the 5-HTP decarboxylase activity in rat cerebral cortex. The experimental incubation with L-5-HTP (Fig. 1A) showed significant formation of 5-HT in contrast to a small amount in the incubation with D-HTP (Fig. 1B). In this method 5-HTP was completely removed from the Amberlite CG-50 column. Even if very little 5-HTP is present in the Amberlite eluate, it can be

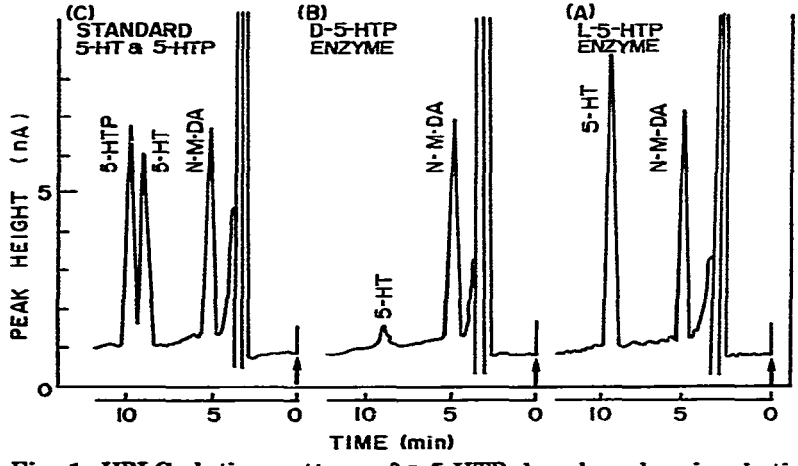

Fig. 1. HPLC elution pattern of L-5-HTP decarboxylase incubation mixtures with homogenate of rat cerebral cortex as enzyme. The conditions were as described under Experimental procedures. The standard incubation mixture contained 5 mg of rat cerebral cortex. (A) Experimental incubation with L-5-HTP; (B) blank incubation with D-5-HTP; 250 pmol of N-methyldopamine (N-M-DA) were added to each sample after incubation. (C) Standard mixture of 50 μl, containing 17.5 pmol, each of L-5-HTP, 5-HT and N-M-DA.

separated from 5-HT by HPLC. Fig. 1C shows the complete separation of the mixture of standard L-5-HTP and 5-HT.

L-5-HTP decarboxylase activity in a homogenate of rat cerebral cortex as a function of enzyme concentration is shown in Fig. 2A. Complete linearity was observed for plots of the amounts of homogenate from 1 to 15 mg tissue against those of 5-HT formed from L-HTP. Fig. 2B shows the rate of formation of 5-HT using rat cerebral cortex homogenate as enzyme. The reaction proceeded linearly up to 2 h.

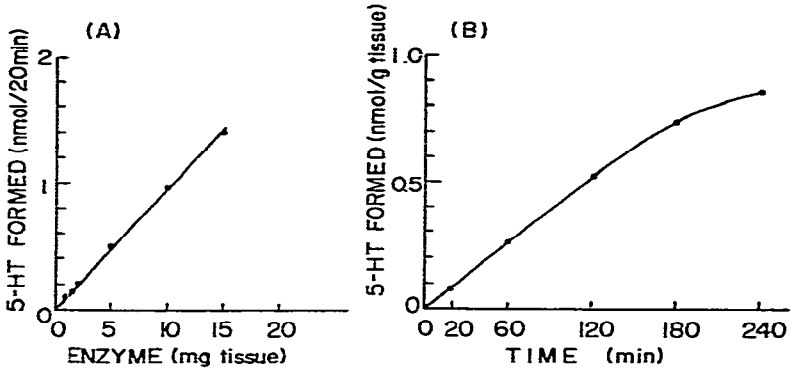

Fig. 2. (A) L-5-HTP decarboxylase activity in the homogenate of rat cerebral cortex as a function of enzyme concentration. The standard incubation mixture was used and incubation was for 20 min at 37°C. (B) Rate of 5-HT formation using the homogenate of rat cerebral cortex as enzyme at 37°C. Standard incubation mixture containing 1 mg of rat cerebral cortex was used as described under Experimental procedures.

Lineweaver—Burk plots illustrating the effect of the concentration of L-5-HTP on the rate of formation of 5-HT by a homogenate of rat cerebral cortex as enzyme are shown in Fig. 3. The Michaelis constant (K_m) toward L-5-HTP and the maximum velocity (v_{max}) values were calculated to be $9.5 \cdot 10^{-6}$ M and 3.27 nmol/min per gram wet tissue, respectively.

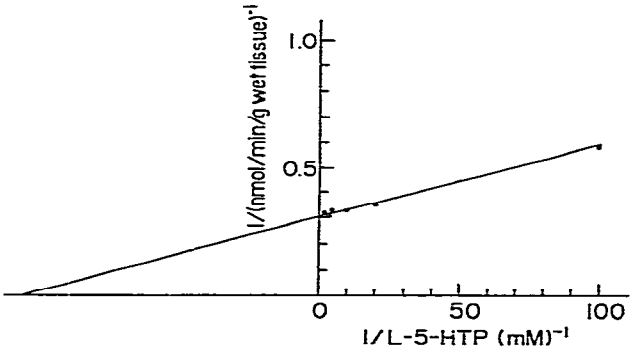

Fig. 3. Lineweaver—Burk plots illustrating the effect of the concentration of L-5-HTP on the rate of 5-HT formation by the homogenate of rat cerebral cortex as enzyme. The K_m and $v_{max.}$ values were calculated to be $9.5 \cdot 10^{-6}$ M and 3.27 nmol/min per gram wet tissue, respectively.

Since 5-HTP decarboxylase activity is found to be very low in human brains and only a small amount of data is available [10], we used this method for the measurement of the enzyme activity in the human cerebral cortex, which has the lowest activity. As shown in Table I, 5-HTP decarboxylase activity in human brains was variable and very low. Rat cerebral cortex had about 40-fold higher activity than human cerebral cortex.

The enzyme activity was discovered in rat serum by our method, and the activity was about 50 pmol/min per millilitre serum. The activity in human serum appears to be very low as compared with rat serum, about 1 pmol/min per millilitre serum, which is close to the limit of sensitivity by this method.

TABLE I

AROMATIC L-AMINO ACID DECARBOXYLASE ACTIVITY WITH L-5-HTP AS SUBSTRATE IN HUMAN AND RAT CEREBRAL CORTEX

Sample	AADC activity* (pmol/min/g wet tissue)	*n*
Rat cerebral cortex	3254 ± 53	4
Rat serum**	48.5 ± 9.4	3
Human cerebral cortex	89.3 ± 89.5	6
Range	21.2—246.0	

*The assay was carried out as described under Experimental procedures. Values are given as Mean ± S.E.M.
**pmol/min/ml of serum.

DISCUSSION

Many assay procedures have been reported on the activity of AADC: spectrophotometric [11,12], spectrofluorimetric [1], gas chromatographic [13], and radiometric [2,3,14]. Amongst these methods, the radiometric method using L- or DL-[1-^{14}C]DOPA as substrate to measure $^{14}CO_2$ formed [2,3] may be most widely used, since the method is simple and sensitive. However, as CO_2, not dopamine, is the product measured by this radioassay, non-enzymatic decarboxylation cannot be distinguished from enzymatic decarboxylation. We had established a highly sensitive and specific assay for AADC activity using L-DOPA as substrate and D-DOPA for the blank by HPLC—voltammetry [6]. This method is more sensitive than radioassay and can only measure the enzymatic decarboxylation of L-DOPA.

Since AADC forms not only dopamine from L-DOPA but also 5-HT from L-5-HTP as substrate, we have also tried to establish a new assay for AADC using L-5-HTP as substrate by HPLC—voltammetry. 5-HT was found to be assayed by voltammetry with a high sensitivity (limit of sensitivity, 200 fmol).

The present assay has many advantages. Firstly, it is very sensitive. The limit of sensitivity was about 1 pmol of 5-HT formed enzymatically. This method can measure $v_{max.}$ with saturated substrate concentration. This method is even more sensitive than our HPLC—voltammetry method using L-DOPA [6], because the reaction with L-5-HTP proceeds linearly for longer time (2 h) than that with L-DOPA. The sensitivity of the present AADC assay is determined solely by the blank value using D-5-HTP as substrate. The blank is derived either from 5-HT formed by the non-enzymatic decarboxylation or from 5-HT contained in a crude enzyme preparation. Secondly, this method is specific, because it only measures enzymatically formed 5-HT from L-5-HTP. Thirdly, it is economical. Also the maintenance of the glassy carbon electrode of the electrochemical detector is easy. Fourthly, as N-methyldopamine is used as internal standard in each incubation mixture, this method is very accurate.

It should be noted that AADC activity was for the first time found in rat serum by the present method. This method is considered to be useful to measure AADC activity using L-5-HTP as substrate and a small amount of brain

nucleus as enzyme source. Also, the assay of AADC in serum by this method would be useful for physiological and pathological studies of aromatic amino acid metabolism.

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