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HIGHLY SENSITIVE ASSAY FOR CHOLINE ACETYLTRANSFERASE ACTIVITY BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A highly sensitive assay for choline acetyltransferase activity by high-performance liquid chromatography with electrochemical detection was devised. This assay method is based on the separation of acetylcholine and choline on a Develosil Ph-5 reversed-phase column (a phenyl column), followed by their enzymatic conversion to hydrogen peroxide through post-column reaction with acetylcholinesterase and choline oxidase. The sensitivity of the system is excellent and 5 pmol of acetylcholine enzymatically formed could be detected. The linearity between the peak height and the amount of acetylcholine was observed over the range of 5 pmol to 5 nmol. Some enzymatic properties were investigated by using a soluble fraction of bovine caudate nucleus as enyzme. The Michaelis constants of the enzyme for choline and acetyl coenzyme A were 0.3 m*M* and 0.03 m*M*, respectively. The enzyme exhibited the maximum activity over the pH range 7.4—9.5. The regional distribution of choline acetyltransferase activity in rat brain was examined. The order of the activity from the highest to the lowest agreed with the reported brain distribution of the enzyme: striatum, pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus, olfactory bulb and cerebellum.

INTRODUCTION

Choline acetyltransferase (acetyl-CoA : choline O-acetyltransferase, E.C. 2.3.1.6; CAT) is the enzyme which catalyses the formation of neurotransmitter acetylcholine (ACh) from acetyl-CoA and choline (Ch). CAT is recognized as a specific marker for cholinergic neurons and is thought to play an important role together with the action of acetylcholinesterase (AChE) in regulating the amount of ACh in the brain. Recently, it was reported that the amount of ACh is decreased significantly in the Meynert nucleus of the patients with senile

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dementia [1]. It is therefore conceivable that CAT activity is much affected in some cerebroneuronal disorders as well as in dementia or ageing.

The measurement of CAT activity has been performed by several methods. These include radiometric assay [2—4], colorimetric assay [5, 6], fluorometric assay [7], and enzyme cycling technique [8]. Because of the absence of a suitable detection system, a simple and sensitive assay of ACh and Ch by high-performance liquid chromatography (HPLC) could not be attained. However, Potter et al. [9] have recently developed a highly sensitive and simple procedure using HPLC with electrochemical detection (ED). The principle of the technique is based on the separation of ACh and Ch on a reversed-phase column, followed by their enzymatic conversion through post-column reaction with AChE and choline oxidase to hydrogen peroxide which is detectable electrochemically by a platinum electrode. This method was found to be simple and reproducible, but ACh was eluted as a somewhat broad and tailed peak owing to the strong adsorption of ACh to the octadecylsilane (ODS) even in the presence of 1.2 m*M* tetramethylammonium (TMA). We found that this problem was solved by employing a phenyl column, which is less hydrophobic than ODS. This modification provided a sharp and symmetrical elution pattern of ACh. Using this improved method, we first describe a highly sensitive and simple HPLC assay for CAT activity in brain tissues.

EXPERIMENTAL

Materials

Acetylcholine chloride, choline chloride, acetyl-CoA, eserine sulphate, AChE type V-S (electric eel), and choline oxidase (*Alcaligenes*) were obtained from Sigma (St. Louis, MO, U.S.A.); TMA chloride and sodium 1-octanesulphonate (SOS) were from Nakarai (Kyoto, Japan); ethylhomocholine (EHC) was a gift from Tokai Irika (Tokyo, Japan). A Develosil Ph-5 packed column (spherical silica chemically bonded with phenyl groups, particle size 5 μm, 250 × 4.6 mm I.D.) for reversed-phase HPLC was obtained from Nomura Chemical (Seto, Japan). A guard column (10 × 4 mm I.D.) was dry-packed with Develosil Ph with particles size of 15—30 μm. All other chemicals used were of analytical grade.

Bovine caudate nucleus was dissected from a fresh brain and stored at −20°C. Rats were decapitated, and the whole brain was dissected on a glass plate over ice into six parts: striatum, pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus, olfactory bulb, and cerebellum. The brain tissues were frozen and stored at −20°C.

The enzyme solution for the measurement of CAT activity was prepared from frozen brain tissues by homogenization in 12.5 ml of 25 m*M* sodium phosphate buffer, pH 7.4, per g of wet weight, using a Teflon homogenizer with twenty up and down strokes, followed by centrifugation at 20 000 *g* for 60 min at 4°C. The supernatant obtained was used as an enzyme solution.

A standard mixture of 19.2 μM ACh, 19.2 μM Ch and 23.1 μM EHC in 0.2 *M* perchloric acid was prepared daily from the stock solutions of 0.5 m*M* ACh, 0.5 m*M* Ch and 0.6 m*M* EHC in 0.01 *M* hydrochloric acid which were stored at 4°C.

Assay of CAT activity

The standard incubation mixture consisted of the following components [10] in a total volume of 200 μl (final concentrations in parentheses): 100 μl of substrate solution containing 10 m*M* choline chloride (5 m*M*), 0.4 m*M* acetyl-CoA (0.2 m*M*), 0.2 m*M* eserine sulphate (0.1 m*M*), 0.3 *M* sodium chloride (0.15 *M*), and 20 m*M* EDTA—2Na (10 m*M*) in 0.1 *M* sodium phosphate buffer, pH 7.4 (0.05 *M*), and 100 μl of enzyme solution in 25 m*M* sodium phosphate buffer, pH 7.4.

Incubation was done at 37°C for 20 min, and the reaction was stopped with 50 μl of 1 *M* perchloric acid in an ice-bath. After 10 min, 10 μl of 0.6 m*M* EHC in 0.01 *M* hydrochloric acid as an internal standard were added and the reaction mixture was centrifuged at 1600 *g* for 10 min at 4°C. A 150-μl aliquot of the clean supernatant was taken, and a 10-μl aliquot was injected into the HPLC system. For the control experiments, the enzyme solution was boiled at 95°C for 5 min, or either acetyl-CoA or Ch was omitted from the substrate solution.

The concentration of protein was determined using a Bio-Rad Protein Assay Kit (Bio-Rad Labs., Richmond, CA, U.S.A.) using γ-globulin as a standard.

Chromatographic conditions

Most of the chromatographic conditions used were the same as those reported by Potter et al. [9]. The HPLC system consisted of a PM-30A dual-piston pump, a LC-4B amperometric detector equipped with a TL-10A platinum electrode (Bioanalytical Systems, West Lafayette, IN, U.S.A.), a Rheodyne 7125 injector with a 200-μl sample loop (Berkeley, CA, U.S.A.), a Develosil Ph-15/30 guard column and a Develosil Ph-5 analytical column (250 × 4.6 mm I.D.). A 1-μm filter was placed between the injector and the guard column.

The mobile phase was 0.01 *M* sodium acetate—citrate buffer (pH 5.0) containing 0.4 m*M* TMA and 30 mg/l SOS, which was filtered through a 0.45-μm membrane filter (Toyo Roshi, Tokyo, Japan) and degassed with a water aspirator for a few minutes prior to use. The flow-rate was 0.8 ml/min.

AChE (2 U/ml) and choline oxidase (1 U/ml) in 0.2 *M* potassium phosphate buffer, pH 8.5, were pumped with a Minipuls 2 peristaltic pump (Gilson, Villiers Le Bel, France) at a rate of 0.5 ml/min. This enzyme reagent solution was mixed with the column effluent through the tee placed between the column and a reaction coil (Teflon tubing, 10 m × 0.5 mm I.D.). The enzymatic reaction was performed at room temperature in this coil, which was connected to the amperometric detector cell. The electrode potential was set to +0.5 V against an Ag/AgCl reference electrode for the detection of hydrogen peroxide. Under these conditions the retention times were: solvent front, 6.0 min; Ch, 12.6 min; EHC, 21.0 min; ACh, 25.8 min.

RESULTS

It was found that ACh, Ch and EHC could be measured with very high sensitivity by using the present HPLC—ED method. The linear response of

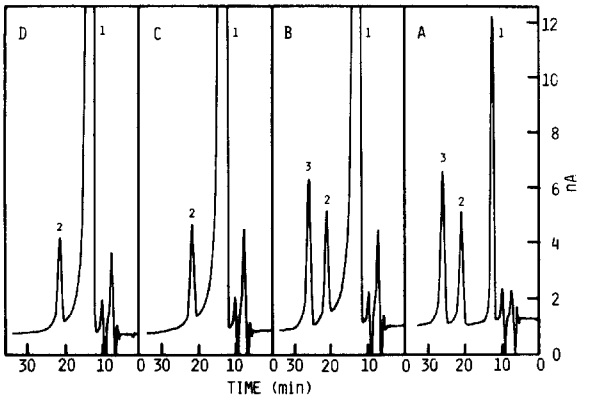

Fig. 1. HPLC elution pattern of the incubation mixtures for choline acetyltransferase with the soluble fraction of bovine caudate nucleus as enzyme. The conditions are described in the experimental section. The incubation mixture contained 40 μl (64 μg of protein) of the soluble fraction as enzyme and 5 m*M* choline and 0.2 m*M* acetyl-CoA as substrates. Internal standard, ethylhomocholine (6.0 nmol), was added in all cases after the enzyme reaction had been stopped by the addition of perchloric acid. (A) Standard samples in 0.2 *M* perchloric acid. Peaks: 1 = choline (192 pmol), 2 = ethylhomocholine (231 pmol), 3 = acetylcholine (192 pmol). (B) Experimental incubation with 40 μl of the soluble fraction of bovine caudate nucleus. (C) Control incubation with an inactivated enzyme. Soluble fraction of bovine caudate nucleus was boiled at 95° C for 5 min. (D) Control incubation without acetyl-CoA.

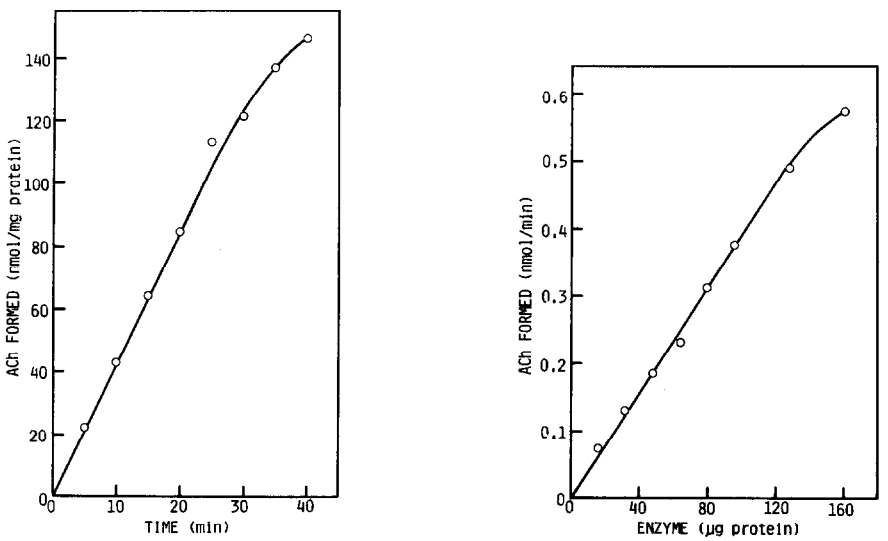

Fig. 2. The rate of acetylcholine formation by choline acetyltransferase using the soluble fraction of bovine caudate nucleus as the enzyme source at 37° C. Standard incubation mixture containing 40 μl (64 μg protein) of the enzyme solution was used as described under Experimental.

Fig. 3. Choline acetyltransferase activity in the soluble fraction of bovine caudate nucleus as a function of enzyme concentration. The standard incubation system was used and incubation was carried out for 20 min at 37° C.

the peak height of the electrochemical detector for the amounts of ACh injected was observed from 5 pmol to 5 nmol.

The chromatographic pattern of the CAT reaction with a soluble fraction of bovine caudate nucleus as enzyme is shown in Fig. 1. Fig. 1A shows the separation of standard samples of ACh, Ch and EHC. It should be noted that ACh is eluted as a sharp and symmetrical peak as those of Ch and EHC. The experimental incubation with 40 μl (64 μg of protein) of the enzyme solution from bovine caudate nucleus (Fig. 1B) shows the significant formation of ACh during the reaction at 37°C for 20 min. In the control incubation, in which the enzyme solution was boiled (Fig. 1C) or the substrate, acetyl-CoA was omitted from the reaction mixture (Fig. 1D), no formation of ACh was observed. These facts clearly indicate that the formation of ACh during the incubation can be ascribed to the enzymatic action of CAT present in the brain tissue.

The rate of ACh formation using the soluble fraction of bovine caudate nucleus as CAT proceeded linearly up to 30 min at 37°C as shown in Fig. 2. Accordingly, we selected 20 min as a standard assay condition. CAT activity as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between 16 and 128 μg of protein of the soluble fraction.

The reproducibility of the assay with replicates of the same sample was 100 ± 2.6% (C.V. for seven determinations).

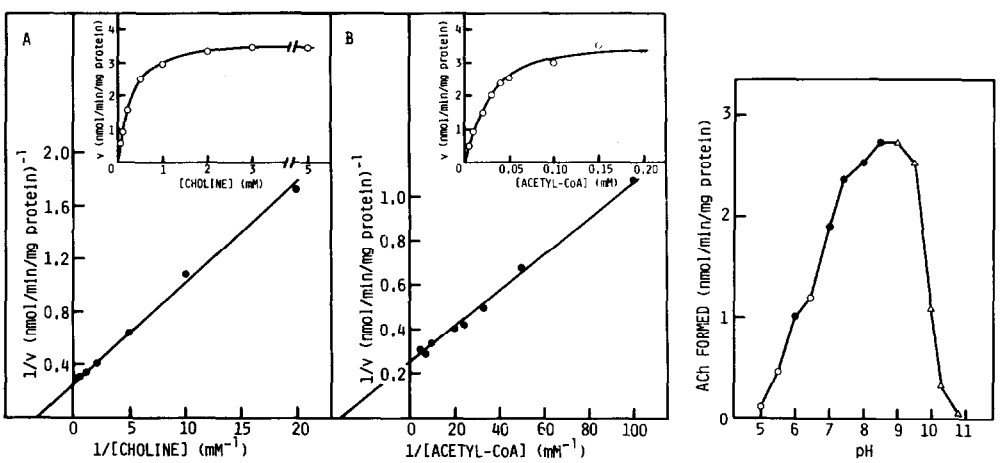

Fig. 4. Lineweaver—Burk and Michaelis—Menten plots for the evaluation of the kinetic parameters of choline acetyltransferase from the bovine caudate nucleus. The main panel shows Lineweaver—Burk plots and the inserted panel shows Michaelis—Menten plots. The standard incubation mixture containing 40 μl (64 μg of protein) of the soluble fraction was used, and incubation was carried out for 20 min at 37°C. (A) The concentration of choline was changed. (B) The concentration of acetyl-CoA was changed.

Fig. 5. Effect of pH on the formation of acetylcholine by choline acetyltransferase in the soluble fraction of bovine caudate nucleus. The incubation mixture consisted of 0.2 *M* buffer solution, 0.15 *M* sodium chloride, 5 m*M* choline chloride, 0.2 m*M* acetyl-CoA, 0.1 m*M* eserine sulphate, 10 m*M* EDTA—2Na, and 40 μl (64 μg of protein) of the soluble fraction as enzyme in a total volume of 200 μl. The incubation was carried out for 20 min at 37°C as described under Experimental. The following buffers were used; (○) sodium acetate buffer; (●) potassium phosphate buffer; (△) glycine buffer.

Fig. 4 shows the Lineweaver—Burk and Michaelis—Menten plots for Ch and acetyl-CoA. It was found that the CAT obeys simple Michaelis—Menten-type kinetics and the Michaelis constants (K_M) were 0.3 m*M* and 0.03 m*M* for Ch and acetyl-CoA, respectively. The same values for the maximum velocity (V_{max}) (230 pmol ACh formed per 20 min per injection; 0.0047 μmol per min per mg protein) were obtained from Fig. 4A and B, respectively.

The pH dependence of CAT activity in bovine caudate nucelus was measured by the present method. As shown in Fig. 5, a broad optimum was observed in the alkaline region and the enzyme exhibited maximum activity over the pH range 7.4—9.5. In the present method, we adopted pH 7.4 for the standard assay condition because it is near the physiological condition and has been used as the optimum condition by many investigators.

We have applied the present method to measure the CAT activity in different regions of rat brain. As shown in Table I, the highest activity was found in the striatum, followed by pons plus medulla oblongata, cerebral cortex, thalamus plus hypothalamus and olfactory bulb; the lowest activity was in the cerebellum.

TABLE I

CHOLINE ACETYLTRANSFERASE (CAT) ACTIVITY IN VARIOUS RAT BRAIN REGIONS

Brain samples were dissected out and processed as described under *Materials*. Results represent mean ± S.E. for a group of four animals. Activity is expressed in pmol of acetylcholine formed per min per mg protein.

Brain region	CAT activity
Striatum	4983 ± 487
Pons plus medulla oblongata	3245 ± 107
Cerebral cortex	2493 ± 134
Thalamus plus hypothalamus	2011 ± 90
Olfactory bulb	1085 ± 76
Cerebellum	249 ± 5

DISCUSSION

An assay method for CAT activity by HPLC—ED has been first reported in this paper. Potter et al. [9] first developed an assay method for ACh and Ch by HPLC—ED in brain tissues of rats sacrificed by microwave irradiation. However, in our experience on their original method using ODS as a column support, ACh was eluted as a broad and tailed peak compared with that of Ch. We examined different column supports, and found that Develosil Ph-5, a phenyl column, was most effective for the separation of ACh, Ch and EHC.

The present assay method for CAT activity has many advantages. First, it is highly sensitive. The limit of sensitivity was about 5 pmol of ACh formed enzymatically. The sensitivity was found to be even higher than that of various radiometric assays, in which the limit is about 100 pmol of ACh. The sensitivity is high enough to allow us to estimate the CAT activity in submilligram samples of brain tissue. Secondly, the procedure is simple and specific.

Deproteinized reaction mixture could be directly analysed by HPLC. Furthermore, there is no need to isolate ACh from the reaction mixture as in the case of radioassay. The specificity is excellent, because the method is based on HPLC—ED and two specific enzymatic reactions. Thirdly, it is very reproducible. Although the detector sensitivity decreases by about 50% after 8—10 h of successive operation of the system, due to possible contamination on the surface of the platinum electrode, it is easy to correct the decreased sensitivity by using the peak height of an internal standard, EHC, and to restore the sensitivity by washing the electrode with methanol. The coefficient of variation (C.V.) of 2.6% for the peak height of ACh was obtained with seven different incubations using the same enzyme solution.

In this study the K_M values of bovine caudate nucleus enzyme for Ch and acetyl-CoA were obtained as 0.3 m*M* and 0.03 m*M*, respectively. These values are in good agreement with those obtained by various radiometric assays (0.4—0.8 m*M* for Ch and 0.01—0.02 m*M* for acetyl-CoA) [11—13]. From the value of V_{max} obtained from Fig. 4, the specific activity of the enzyme in the soluble fraction of bovine caudate nucleus was calculated to be 0.0047 μmol ACh formed per min per mg protein, which is of the same order as the value of 0.0026 reported as the specific activity of the enzyme in the extract from bovine striatum [14].

CAT from bovine caudate nucleus has been reported to be inactive below pH 5.0 and showed a broad pH optimum from 7.5 to 10.0 [12]. A similar pH—activity curve was reported for the enzymes from human brain and placenta [15]. Our data (Fig. 5) agree well with these previous observations. On the other hand, Chao and Wolfgram [16] have demonstrated that the maximum activity was at about pH 7. The reason for this discrepancy is unclear.

From the data on the regional distribution of CAT activity in rat brain, the previous observation was confirmed that the enzyme activity is highest in the striatum and lowest in the cerebellum. It has been shown by immunohistochemical studies using cat brain [17] that cerebral cortex, olfactory bulb, and thalamus plus hypothalamus have no cholinergic cell bodies, although these regions are rich in cholinoceptive neurons. Therefore, the relatively high content of CAT in these regions is thought to be due to the nerve terminals of cholinergic neurons innervated from other parts of the brain.

Taking advantage of the high sensitivity of this method, it would be possible to study the changes in CAT activity in animal models of various diseases, or in human brain tissues from patients at autopsy.

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