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

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SUMMARY

A highly sensitive assay for acetylcholinesterase (AChE) activity was devised by high-performance liquid chromatography with electrochemical detection. It is based on the separation of acetylcholine and choline on an octadecylsilane reversed-phase column, followed by their enzymatic conversion into hydrogen peroxide through the post-column reaction with immobilized AChE and choline oxidase. The system is highly sensitive, and the relationship between the peak height and the amount of choline is linear over the range 5 pmol to 5 nmol. When homogenate of bovine caudate nucleus was used as enzyme, the Michaelis constant of the enzyme for acetylcholine was 0.4 m*M*. The regional distribution of AChE activity in rat brain was examined, and the order of the activity from the highest to the lowest agreed with the reported brain distribution of AChE: striatum, thalamus plus hypothalamus, pons plus medulla oblongata, cerebral cortex, olfactory bulb, and cerebellum.

INTRODUCTION

Acetylcholinesterase (acetylcholine acetylhydrolase, EC 3.1.1.7; AChE) is the enzyme that catalyses the hydrolysis of the neurotransmitter acetylcholine to choline and acetic acid. The enzyme exists primarily in nerve cells involving cholinergic synaptic transmission, but it is also found in a variety of other neuronal and non-neuronal cells. The measurement of AChE activity is one of the essential biochemical indices as that of choline acetyltransferase (EC 2.3.1.6; CAT) activity for the studies on the function of cholinergic neurons.

Several methods have been reported for the assay of AChE activity, including radiometry [1—6], colorimetry [7, 8], fluorometry [9], titrimetry using a pH-stat [10, 11] and electrometry [12]. Among these, the colorimetric

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method using acetylthiocholine as a substrate [7] has been widely accepted as a routine assay of the enzyme activity. However, it is still desirable to develop a new assay system with high sensitivity using a natural substrate, acetylcholine, especially for the assay in a very small amount of brain tissue in neurochemical studies.

Recently, Potter et al. [13] reported a highly sensitive and simple assay of acetylcholine and choline by high-performance liquid chromatography with electrochemical detection (HPLC—ED). The principle of the method is based on the separation of acetylcholine and choline on a reversed-phase column, followed by their enzymatic conversion, through a post-column reaction with AChE and choline oxidase, into hydrogen peroxide which is detectable electrochemically by a platinum electrode. We have developed a highly sensitive assay for CAT activity by applying this HPLC—ED method to the measurement of enzymatically formed acetylcholine [14], but the method is expensive. Because the post-column reaction is carried out in a flow system, relatively large amounts of these expensive enzyme reagents (i.e. AChE and choline oxidase) are required. To overcome this problem, Yao and Sato [15] have recently developed an immobilized enzyme column of AChE and choline oxidase with high enzymatic activity and stability, and have demonstrated the usefulness of this enzyme column as a reactor in the analysis of acetylcholine and choline by HPLC—ED. We have used this improved HPLC—ED method with immobilized enzymes to develop a highly sensitive and simple HPLC assay for AChE activity in brain tissues. Choline oxidase alone can fulfil the purpose of AChE activity determination, and the immobilized AChE is not necessary. However, the immobilized AChE did not interfere with the AChE assay, and the immobilized enzyme column of both choline oxidase and AChE is useful not only for the present AChE activity determination, but also for the previously reported CAT activity determination [14].

EXPERIMENTAL

Materials

Acetylcholine chloride and choline chloride were obtained from Sigma (St. Louis, MO, U.S.A.); tetramethylammonium chloride (TMA) and sodium 1-octanesulphonate (SOS) were from Nakarai Chemicals (Kyoto, Japan); ethylhomocholine (EHC) was a gift from Yanagimoto (Kyoto, Japan). A Yanapak ODS-120T packed column (particle size 10 μm, 250 × 4.6 mm I.D.) for reversed-phase HPLC and a guard column (10 × 4 mm I.D.) were obtained from Yanagimoto. All other chemicals used were of analytical-reagent grade. Glass-distilled water was further purified by using a Milli-QII system (Millipore, Bedford, MA, U.S.A.).

Bovine caudate nucleus was dissected from a fresh brain and stored at −20°C. Male Sprague—Dawley rats (body weight ca. 200 g) 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 immediately frozen on carbon dioxide dry ice and stored at −20°C.

The enzyme solution for the measurement of AChE activity was prepared

from frozen brain tissues by homogenization in 12 ml of 25 m*M* potassium phosphate buffer (pH 7.0) per g of wet weight, using a Teflon homogenizer with twenty up-and-down strokes. The homogenate was diluted from 50 to 100 times with the above buffer and used as an enzyme solution.

A standard mixture of acetylcholine, choline and EHC, each at a concentration of 1 m*M* in water, was stored at 4°C.

Assay of AChE activity

The standard incubation mixture consisted of the following components [2] in a total volume of 150 μl (final concentrations in parentheses): 50 μl of 0.15 *M* potassium phosphate buffer, pH 7.0 (0.05 *M*) containing 0.3 *M* sodium chloride (0.1 *M*) and 0.06 *M* magnesium chloride (0.02 *M*); 50 μl of 6 m*M* acetylcholine in water (2 m*M*); and 50 μl of enzyme solution in 25 m*M* potassium phosphate buffer, pH 7.0. To prepare the above reaction buffer, 0.153 *M* potassium phosphate buffer, pH 7.0, containing 0.306 *M* sodium chloride was stored at 4°C separately from a stock solution of 3 *M* magnesium chloride, and then the two solutions were mixed together freshly at 0°C. This solution is stable for a day, but easily forms crystals of magnesium phosphate. Therefore, it is also recommended to use 0.15 *M* N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) buffer, pH 7.0, instead of 0.15 *M* potassium phosphate. The same AChE activities were observed when either phosphate or HEPES buffer was used. Since the amount of non-enzymatically formed choline is proportional to the concentration of acetylcholine as the substrate, and also becomes significant at an alkaline pH, we used a substrate concentration of 2 m*M* and pH 7.0 as a standard condition, under which the blank value was almost negligible at a routine detector sensitivity (more than 128 nA full scale).

The reaction was started by the addition of the substrate solution and carried out at 37°C for 15 min, and then stopped with 40 μl of 5% metaphosphoric acid in an ice-bath. After 10 min, 10 μl of 2 m*M* EHC in water were added as an internal standard, and the reaction mixture was centrifuged at 1600 *g* for 10 min at 4°C. A 100-μl aliquot of the clear supernatant was taken, and a 5—10 μl aliquot was injected into the HPLC system. For the control experiments, (1) the enzyme solution was boiled at 95°C for 5 min, or (2) an inhibitor, eserine sulphate (final, 10 μM), was added, or (3) the enzyme was omitted from the reaction mixture and added after stopping the reaction. Concentration of protein was determined by the method of Lowry et al. [16] using bovine serum albumin as a standard.

Chromatographic conditions

Most of the chromatographic conditions used were the same as those reported by Potter et al. [13], as described previously [14]. The HPLC system consisted of a Yanaco L-3200 V liquid chromatograph with a platinum electrode (Yanagimoto), a Rheodyne 7125 injector with a 100-μl sample loop (Berkeley, CA, U.S.A.), and Yanapak ODS-120T guard and analytical columns. A 1-μm filter was placed between the injector and the guard column.

The mobile phase was 0.01 *M* sodium acetate buffered to pH 5.0 with citric acid, containing 1.2 m*M* TMA and 15 mg/l SOS, which was passed 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 1.0 ml/min.

The second solution, 0.2 *M* potassium phosphate buffer (pH 8.5), was filtered and degassed as described above, and pumped with a Model DHCR80HP dual-piston pump (Japan Servo, Tokyo, Japan) at a flow-rate of 0.6 ml/min. This second buffer was mixed with the column effluent through the tee placed between the column and a mixing coil (Teflon tubing, 10 m × 0.5 mm I.D.). Acetylcholine, choline and EHC in the effluent from the coil were then enzymatically converted into hydrogen peroxide at room temperature (15—25°C) by passing through the LiChrosorb-NH_2 (Merck, Darmstadt) column (20 × 2 mm I.D.) on which both AChE and choline oxidase were immobilized covalently [15]. The efficiency of the conversion of this enzyme column was 100% for AChE and 90% for choline oxidase. The enzyme column was stored in 0.2 *M* potassium phosphate buffer (pH 8.0) containing 0.02% sodium azide at 4°C after use. 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, 3.8 min; choline, 6.0 min; EHC, 8.5 min; and acetylcholine, 12.0 min.

RESULTS

Choline, acetylcholine and EHC can be measured with very high sensitivity by the present HPLC—ED system employing an immobilized enzyme column. The response of the peak height of the electrochemical detector for the amounts of choline injected was linear in the range from 5 pmol to 5 nmol.

The assay method of AChE by HPLC—ED was developed with a homogenate of brain tissue as enzyme. The chromatographic pattern of the AChE reaction with the homogenate of bovine caudate nucleus as enzyme is shown in Fig. 1. Fig. 1A shows the separation of standard samples of choline, acetylcholine and EHC. The experimental incubation with 50 μl (3.94 μg of protein) of the enzyme solution from bovine caudate nucleus (Fig. 1B) shows the significant formation of choline during the reaction at 37°C for 15 min. In the control incubation, in which the enzyme solution was boiled (Fig. 1C), or eserine sulphate, a potent inhibitor of AChE, was added in the incubation mixture at a concentration of 10 μM (Fig. 1D), no formation of choline was observed. These facts clearly indicate that the formation of choline during the incubation results from the enzymatic hydrolysis of acetylcholine by AChE present in the brain homogenate.

The rate of choline formation using the homogenate of bovine caudate nucleus as AChE proceeded linearly up to 30 min at 37°C (Fig. 2). We selected 15 min incubation as a standard assay condition, because this enzyme has high molecular activity and the present detector system also provides high sensitivity. AChE activity as a function of enzyme concentration is shown in Fig. 3. Complete linearity was observed between 2 and 40 μg of protein of the homogenate.

The reproducibility of the assay with replicates of the same samples was 100 ± 1.7% (coefficient of variation for seven determinations).

It was found that the AChE obeys simple Michaelis—Menten kinetics with a slight substrate inhibition at high concentrations of acetylcholine. The values of K_m and specific activity for the enzyme in the homogenate of bovine caudate nucleus were 0.4 m*M* and 0.526 μmol/min/mg of protein.

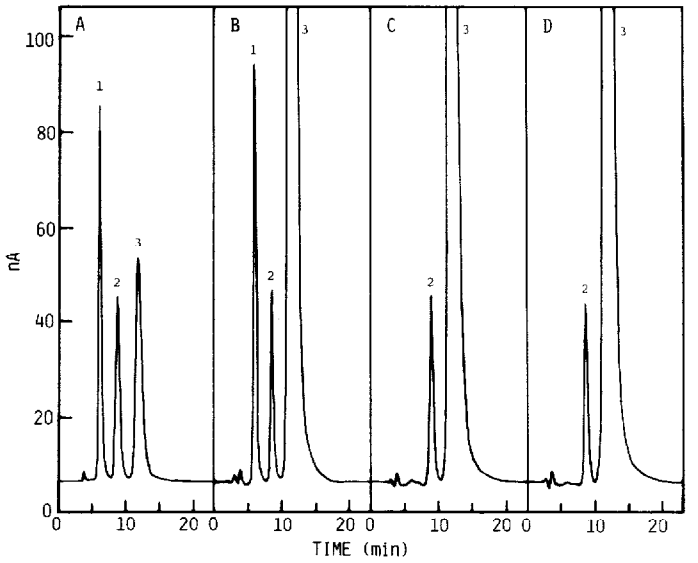


Fig. 1. HPLC elution pattern of the incubation mixtures for AChE with a homogenate of bovine caudate nucleus as enzyme. The conditions are described in Experimental. The incubation mixture contained 50 μl (3.94 μg of protein) of a diluted (× 100) homogenate of bovine caudate nucleus as enzyme and 2 m*M* acetylcholine as substrate. Ethylhomocholine (20 nmol) was added as an internal standard in all cases after the enzyme reaction had been stopped by the addition of 5% metaphosphoric acid. (A) Standard samples (500 pmol each) dissolved in water. (B) Experimental incubation for 15 min at 37°C with 50 μl of an enzyme solution. (C) Control incubation with inactivated enzyme. The enzyme solution was boiled at 95°C for 5 min. (D) Control incubation in the presence of an AChE inhibitor. The reaction mixture was preincubated with 10 μ*M* eserine sulphate at 0°C for 10 min, then acetylcholine (2 m*M*) was added. Peaks: 1 = choline; 2 = ethylhomocholine; 3 = acetylcholine.

TABLE I

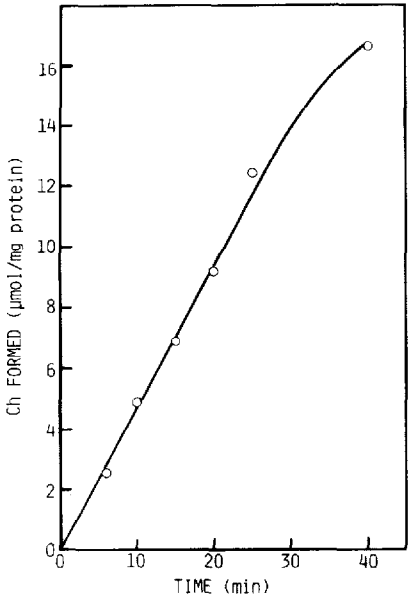
ACETYLCHOLINESTERASE ACTIVITY IN VARIOUS RAT BRAIN REGIONS

Brain samples were dissected out and processed as described under *Materials*. Results represent mean ± S.E. for a group of six animals.

Brain region	AChE activity		
	nmol hydrolysed per min·mg protein	μmol hydrolysed per h·g wet tissue	μmol hydrolysed per h·g wet tissue*
Striatum	228 ± 28	2139 ± 261	2080 ± 94
Thalamus plus hypothalamus	86 ± 5	811 ± 45	–
Pons plus medulla oblongata	80 ± 5	755 ± 48	657 ± 55
Cerebral cortex	38 ± 3	353 ± 24	753 ± 8
Olfactory bulb	27 ± 1	249 ± 12	–
Cerebellum	23 ± 2	216 ± 19	311 ± 27

*Data from Stavinoha et al. [20].

We have applied the present method to measure the AChE activity in different regions of rat brain. As shown in Table I, the highest activity was found in the striatum, followed by thalamus plus hypothalamus, pons plus medulla



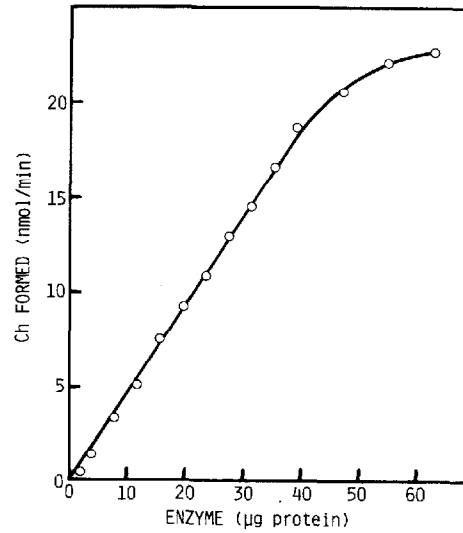


Fig. 2. The hydrolysis rate of acetylcholine by AChE using a homogenate of bovine caudate nucleus as an enzyme source at 37°C. Standard incubation mixture containing 50 μl (3.94 μg of protein) of the enzyme solution was used as described under Experimental. Ch = choline.

Fig. 3. AChE activity in the homogenate of bovine caudate nucleus as a function of enzyme concentration. The standard incubation system was used and incubation was carried out for 15 min at 37°C. Ch = choline.

oblongata, cerebral cortex, and olfactory bulb; the lowest activity was in the cerebellum.

DISCUSSION

An assay method for AChE activity by HPLC—ED has been reported for the first time in this paper. This method has many advantages.

First, it is highly sensitive. The limit of sensitivity is ca. 50 pmol of choline formed enzymatically. The sensitivity was found to be comparable with that of the most sensitive radiometric assays, in which the limit of detection is ca. 30—100 pmol of choline [1—6]. AChE activity can be assayed with less than 0.1 mg of brain tissue. With such a high sensitivity for choline, the sensitivity of AChE assay is determined solely by the blank value. Since the blank values that were obtained by incubation with a heat-inactivated enzyme preparation or by incubation without enzyme solution did not differ appreciably, endogeneous choline contained in a very small amount of crude enzyme preparations could be almost negligible and did not interfere with the assay. Accordingly, the blank is considered to be mainly derived from spontaneous hydrolysis of acetylcholine during the incubation and from trace amounts of choline contained in the substrate, acetylcholine.

Secondly, the procedure is simple and specific. Deproteinized reaction mixtures can be directly analysed by HPLC. Therefore, there is no need to isolate choline from the reaction mixture as in the case of radioassay. The HPLC con-

ditions are adjusted so that the product choline to be assayed elutes prior to the substrate, acetylcholine; in this way the large peak of acetylcholine does not interfere with the assay of choline. We employed metaphosphoric acid to stop the enzyme reaction and to precipitate proteins. This reagent was essential for the assay of AChE activity, because other deproteinizing agents examined, such as 0.2 *M* trichloroacetic acid, 0.2 *M* perchloric acid, 1% sodium phosphotungstate and 0.03 *M* barium hydroxide—zinc sulphate produced significant amounts of choline due to the non-enzymatic hydrolysis of acetylcholine. The specificity is excellent, because the method is based on separation by HPLC and two specific enzymatic reactions for ED.

Thirdly, the method is reproducible. The coefficient of variation of 1.7% for the peak height of choline was obtained with seven different incubations using the same enzyme solution.

Fourthly, it is very economical as AChE and choline oxidase are immobilized covalently onto a solid support and can be used repeatedly. The two enzymatic activities of the immobilized enzyme column were found to be unchanged over three months when stored at 4°C.

The Michaelis constant for acetylcholine with AChE for various different preparations and conditions has been reported to be between 0.28 and 5 m*M* [17]. In the present study with the homogenate of bovine caudate nucleus as enzyme, the K_m value was calculated as 0.4 m*M*, in good agreement with previously reported values.

It is generally considered that there is some relationship in the distribution between AChE and CAT activities in the mammalian brain [18, 19]. When the distribution of AChE activity was compared with that of CAT activity in rat brain reported in our previous study [14], the highest activities of both enzymes were found in the striatum and the lowest in the olfactory bulb and cerebellum. AChE activity in several regions of rat brain presented here was compared with literature values [20] using a radiometric assay (Table I). It was found that these data agreed reasonably well with each other. The discrepancy between the activities in the cerebral cortex may be due to the different way of brain dissection. It is well known that various mammalian brains contain cholinesterase (EC 3.1.1.8; ChE), which hydrolyses butyrylcholine faster than acetylcholine, besides AChE. In order to measure AChE activity and ChE activity separately, it may be necessary to employ specific substrates such as β-methylacetylcholine for AChE and butyrylcholine for ChE and/or specific inhibitors such as 1,5-bis(4-allyldimethylammoniumphenyl)pentan-3-one dibromide (BW 284 C 51) for AChE and ethopropazine for ChE [5, 21]. ChE activity in rat brain was reported to be of the order of about one tenth of AChE activity [5]. However, ChE in rat brain did not hydrolyse acetylcholine as a substrate, unlike the enzyme in plasma [5]. Therefore, the value obtained here does not seem to involve any non-specific ChE activity.

The present method is simple, rapid and economical. Since endogeneous substrate, acetylcholine, is used for the enzymatic reaction rather than pseudo-substrates, this method may be useful for physiological studies with a minute amount of enzyme preparations.

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