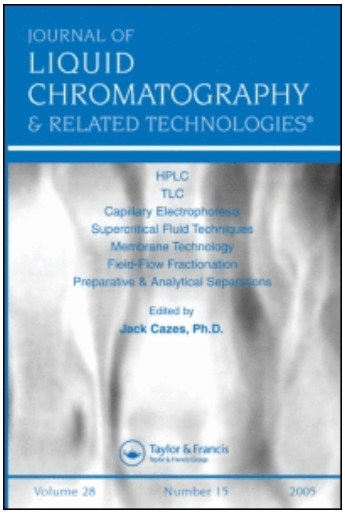

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DETERMINATION OF ACETYLCHOLINE AND CHOLINE IN RAT BRAIN TISSUE BY LIQUID CHROMATOGRAPHY/ELECTROCHEMISTRY USING AN IMMOBILIZED ENZYME POST COLUMN REACTOR

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ABSTRACT

Following separation by conventional LC, acetylcholine and choline are converted to hydrogen peroxide in a packed bed reactor consisting of covalently bound acetylcholinesterase and choline oxidase. Hydrogen peroxide, the ultimate enzymatic product, is detected amperometrically at a platinum electrode thin-layer cell. This method is simple and highly sensitive to the detection of acetylcholine and choline, with detection limits of about 100 femtomoles. The immobilized enzyme columns were stable for at least 60 days and conserved precious and expensive supplies of enzyme relative to continuous addition schemes. The apparatus is generally applicable to other enzymatic reactions which yield electroactive products.

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INTRODUCTION

Acetylcholine (ACh) is one of the major neurotransmitters in the central nervous system. It is distributed in the cerebellum, striatum, cerebral cortex and several other regions in the mammalian brain. Determination of ACh in the brain is very important to the identification of certain neurological disorders, and differences in choline acetyltransferase activity have been implicated in Alzheimer's disease.

The determination of ACh has been carried out by many methods including bioassay (1, 2) radiometric assays (3), gas chromatography (4), pyrolysis gas chromatography (5), and gas chromatography-mass spectrometry (6).

Recently, Potter et al. (7) published an LC method using electrochemical detection (LCEC) after enzyme reaction with acetylcholinesterase (AChE) and choline oxidase (COD). This method was based on the separation of ACh and Ch by a reverse phase column, adding a solution of AChE and COD via a separate pump, post-column mixing tee, and teflon reaction coil. Electrochemical detection of the peroxide, which was produced from both endogeneous Ch as well as Ch generated from ACh in the reaction coil, was carried out in a thin-layer cell utilizing a platinum electrode.

In the present report, we describe a new and simple method for LCEC determination of ACh using an immobilized enzyme column and thin-layer electrochemical cell which proved very stable for ACh and Ch detection. The method was selective for ACh and Ch in biological samples due to the column separation and the use of two specific enzymes (see Fig. 1). The detection limit was 100 fmoles for ACh and Ch.

MATERIALS AND METHODS

We used an LC-304 (Bioanalytical Systems, W. Lafayette, IN, USA) with an automatic sample injector (KSST-601, Kyowa Seimitsu

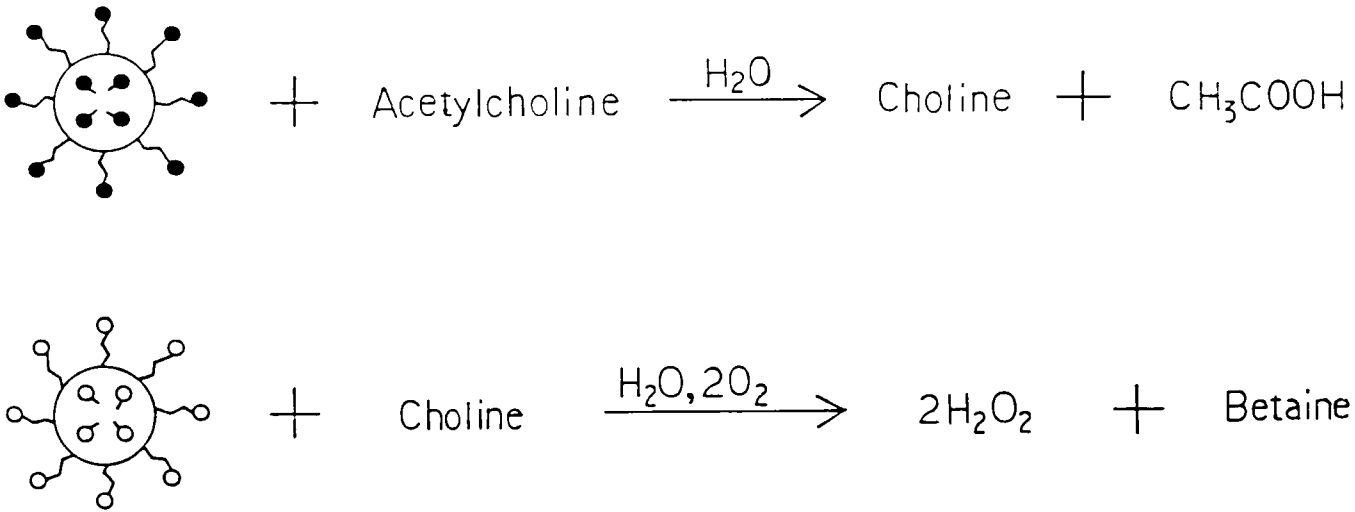

Fig. 1. Schematic of enzyme reactions with the immobilized enzyme for ACh and Ch.

Co, Tokyo, Japan), a column heater (LC-22/23A, Bioanalytical Systems), and a chromatogram processor (7000B, System Instrument Inc., Tokyo, Japan). The chromatographic column was 150 mm long, 4.6 mm I.D and filled with C18 (Nucleosil, Macherey-Nagel GMBH & Co, Duren, West Germany). A guard column (Nucleosil, Macherey-Nagel) was connected between the automatic injector and chromatographic column. The Degasser (Erma Optical Work Ltd, Tokyo, Japan) was installed between the mobile phase and reaction buffers and the two pumps (i.e., one consisting of a model 64, Knauer, Berlin, West Germany and the other a model PM-30A, Bioanalytical Systems).

After ACh and Ch were separated from each other using a separation column (see Fig. 2), the effluent was mixed at a teflon tee with a phosphate buffer (pumped by the model 64 Knauer) and then passed through the immobilized enzyme column. Hydrogen peroxide generated from the immobilized enzyme column then entered the electrochemical cell (LC-4B/17 Bioanalytical Systems), which contained a platinum electrode (TL-10A, Bioanalytical Systems)). The applied potential to the electrode was set at +500 mV vs. Ag/AgCl for detection of hydrogen peroxide.

Mobile Phase

The mobile phase was prepared as follows: 1.36 g of sodium acetate, 3.72 g of Na_2EDTA, 25 mg of sodium octyl sulfate (SOS) and 1.2 mmoles tetramethylammonium chloride (TMA) were dissolved in 900 mL of distilled deionized water. The solution was adjusted to pH 5.0 with 0.2 M citric acid and diluted to 1.0 L, which was then filtered through a 0.2 μm membrane filter (Schleicher & Schuell, Keene, NH, USA). Flow rate was 0.8 mL/min.

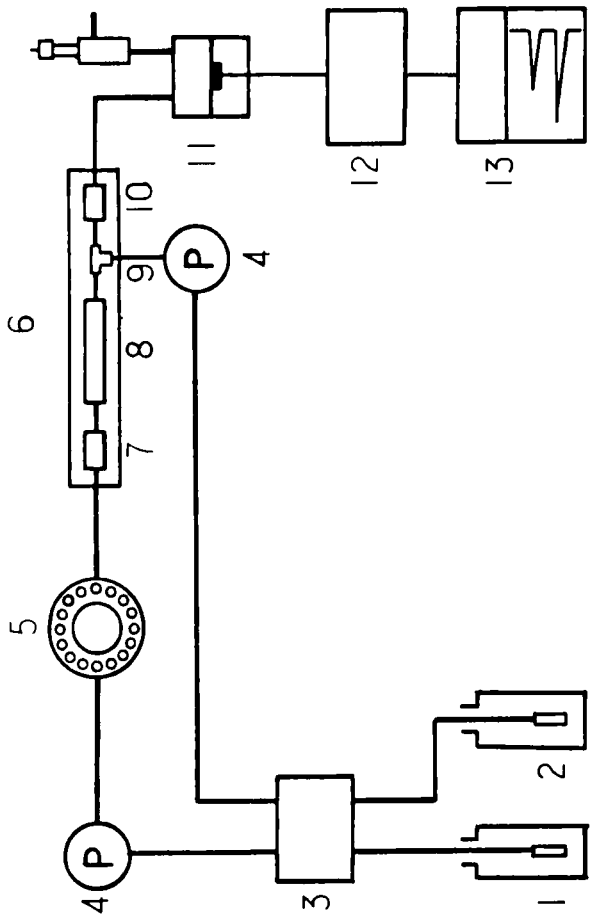

Fig. 2. Schematic of instrumentation for ACh and Ch assay.
1: Mobile phase
2: Reaction buffer
3: Degasser
4: Pumps
5: Autosampler
6: Column heater
7: Guard column
8: Analytical column
9: Mixing tee
10: Immobilized enzyme column
11: Platinum electrode
12: Electrochemical detector
13: Integrator

Reaction Solution

Disodium hydrogen phosphate dodecahydrate (71.6 g) and Na_2EDTA (0.372 g) was mixed in 900 mL of distilled, deionized water. The pH was adjusted to 8.5 with sodium dihydrogen phosphate and made to 1.0 L before being filtered through a 0.2 μm membrane filter (Schleicher & Schuell). Flow rate was 0.5 mL/min.

Reagents

ACh chloride, AChE type III (EC.3.1.1.7), Ch chloride and COD (EC.1.1.3.17) were purchased from Sigma Chemical Co (St. Louis, MO USA). TMA was purchased from Tokyo Kasei Co (Tokyo, Japan). SOS was obtained from Bioanalytical Systems (USA). 3-aminopropyltriethoxysilane was purchased from Aldrich Chemical Co (Milwaukee, WI USA). Porous glass beads, 200-400 mesh with a pore size of approximately 400 Å, were purchased from Electronucleonics (Fairfield, NJ USA). All other chemicals were reagent grade.

The standard solution contained ACh, Ch and ethylhomocholine (EHC, used as internal standard (7)) which was prepared daily in 0.02 M citrate-phosphate buffer, pH 3.5.

Method of Immobilization

Porous glass beads (500 mg) were heated in 5% (V/V) nitric acid, and then washed thoroughly with distilled water, dried and refluxed in anhydrous 10% (V/V) 3-aminopropylethoxysilane in toluene overnight (8).

COD and AChE were immobilized onto the alkylamino glass beads with glutaraldehyde through Schiff base formation. The enzymes (COD: 16.6 mg, AChE: 0.45 mg) were dissolved in 200 μL of 0.05 M phosphate buffer solution (pH 7.0) containing 10 mM

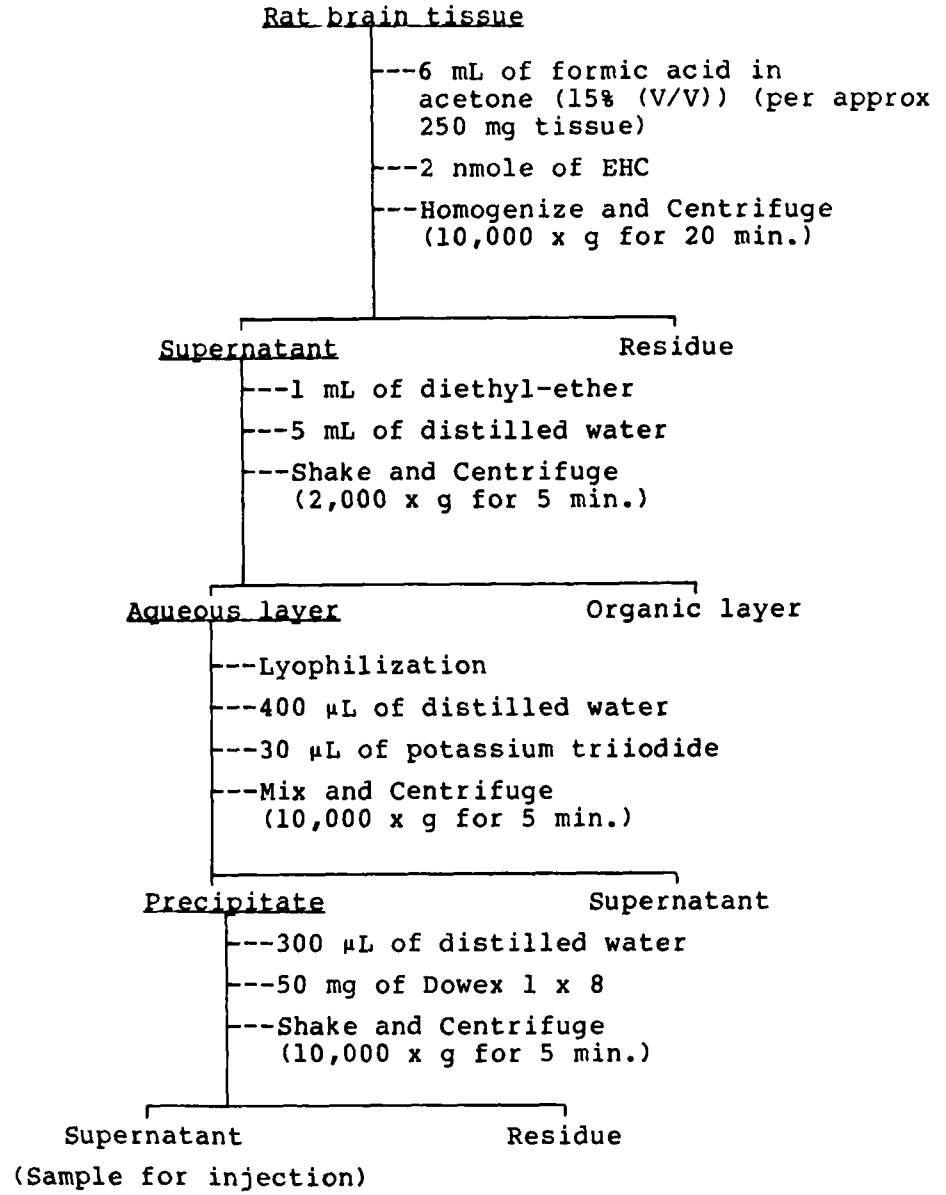

Fig. 3. Flow diagram of ACh, Ch extraction procedures from rat brain tissues.

Na_2EDTA. This solution was then added to 500 mg of glutaraldehyde treated porous glass beads (9, 10). The glass beads, with immobilized enzymes, were used to slurry pack a 4x10 mm column.

Sample Preparation

Wistar strain rats, weighing about 120 g, were killed by head focussed microwave irradiation (0.8 s, 9 kw, 2.45 GHz, New Japan Radio Co., Tokyo, Japan) (11, 12). The brains were quickly removed and dissected into regions consisting of typically 250 mg diencephalon, 200 mg of medulla oblongate, 200 mg of cerebellum, and 700 mg of cerebrum. The tissue parts were homogenized with 6 ml of 1 N formic acid in acetone to which was added 10 nmole of EHC.

The basic extraction procedure for the homogenate was carried out as follows (11): the homogenate was sonicated for 5 min., and centrifuged at 10,000 x g at $4^{o}C$ for 20 min. The supernatant was transferred to a tube containing an equivalent volume of diethylether and 5 ml of distilled water. After shaking, the sample was centrifuged at 2,000 x g for 5 min. The organic layer was aspirated, and the aqueous portion was lyophilized. The residue was dissolved in 400 µL of distilled water and filtered through a 0.45 µm membrane filter (Millipore, Bedford, MA USA). A potassium triiodide solution (30 µL) which consisted of 20% KI and 18% I_2 dissolved in distilled water was added. After mixing, the mixture was centrifuged at 10,000 x g for 5 min. The precipitate was dissolved in 300 µL of distilled water and approximately 50 mg of Dowex 1 x 8 was added. After shaking, the mixture was centrifuged at 10,000 x g for 5 min. A 10 µL sample of the supernatant was then injected into the LCEC system.

RESULTS AND DISCUSSION

Properties of immobilized AChE and COD Column

1) pH optimum

We studied the influence of pH on the reaction with ACh and Ch using the immobilized AChE and COD column. For this experiment, the guard and analytical columns were omitted. Each standard (200 pmoles) was injected and mixed with reaction buffer solutions of varied pH before passage through the immobilized enzyme column. As shown in Figure 4, the optimum pH was about 8.5.

2) Linearity and reproducibility

The oxidation current response to various concentrations of standards was studied. The observed linearity for ACh and Ch was from 1 to 200 pmoles (see Fig.5). The lowest ACh and Ch concentration which could be detected with this column was 100 fmoles using a signal to noise ratio of 2. The relative standard deviation for 10 injections of ACh and Ch was 1%, for 100 pmoles injected.

3) Selectivity and temperature effects

The selectivity was dependent on LCEC and the immobilized enzyme column. To interfere, a compound would have to co-elute with ACh or Ch and either possess native electrochemical activity on Pt at +0.5 V or be converted enzymatically to similar products. The tissue cleanup outlined in Figure 3 provides additional selectivity against other easily oxidized tissue components such as ascorbate or catechols. AChE is specific to ACh; COD is specific to Ch (11, 13, 14, 15). Addition of EHC to the brain tissue samples compensates for possible minor changes of the platinum electrode's sensitivity during continuous measurements.

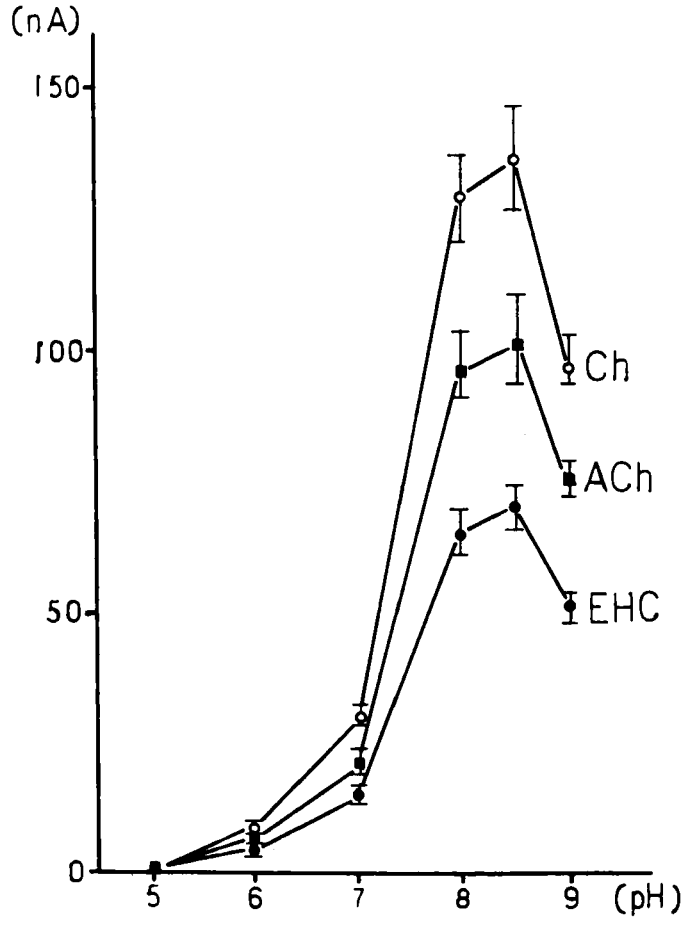

Fig. 4. pH profile for the immobilized AChE and COD column. 200 pmoles of each compound were injected. Column Temperature: 37°C. Chromatographic conditions are described in Fig. 7. Data are presented as the mean ± SEM, n=3.

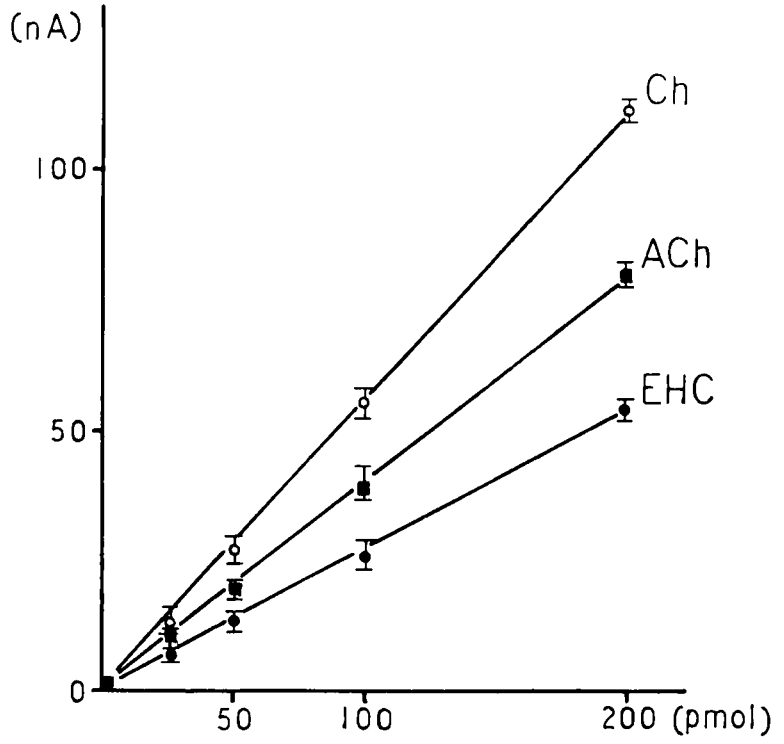

Fig. 5. Calibration curve for ACh, Ch and EHC. Standards of ACh, Ch and EHC were injected using LCEC with the immobilized enzyme column. Column temperature: 37°C Chromatographic conditions are described in Fig.7. Data is presented as the mean ± SEM, n=10.

The response of the immobilized enzyme column to ACh and Ch was investigated at different temperatures. Oxidation current increase was proportional to temperature increase, thereby indicating better enzymatic conversion efficiencies. The oxidation current reached a plateau for temperatures over 37°C (see Fig. 6).

4) Stability of the immobilized enzyme column

The immobilized enzyme column was stored at 4° C in 0.05 M phosphate buffer, pH 7-8, when not in use. Long-term stability

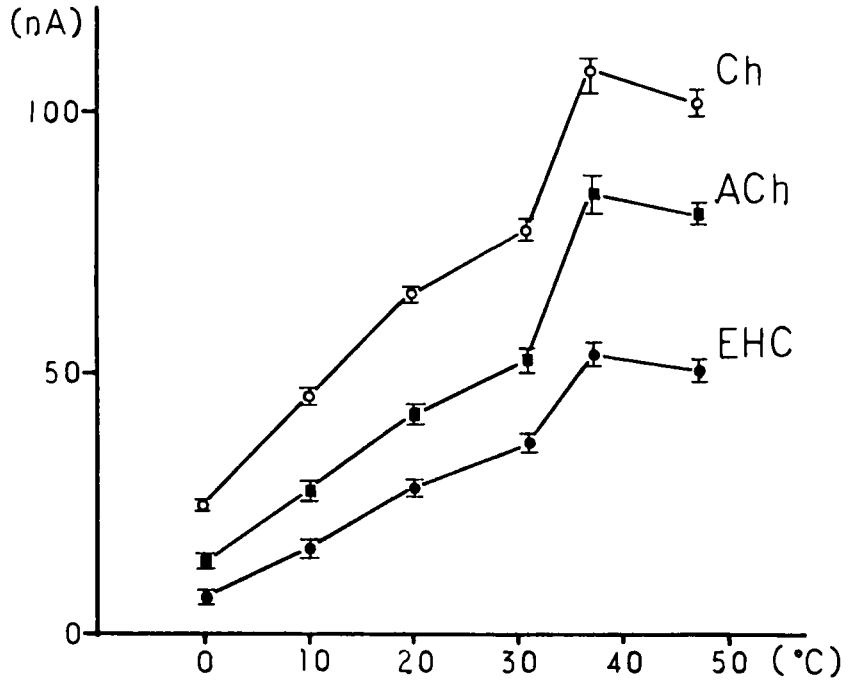

Fig. 6. Effect of temperature on the ACh, Ch and EHC response. 200 pmoles each of ACh, Ch and EHC were injected. The immobilized enzyme column temperature was varied as indicated. Data is presented as the mean ± SEM, n=3.

was verified by periodically injecting 100 pmoles each of ACh and Ch into the system. A slight increase in response was observed during the first 2 or 3 days (16) which then stabilized. This phenomenon has been attributed to the establishment of permeable channels in the porous glass beads and changes in the fraction of enzyme immobilized in an active conformation (17). The immobilized enzyme column retained most of its original activity for approximately 60 days.

5) Condition of the chromatographic column

ACh had a tendency to strongly adsorb to the reverse phase support and yielded a broad tailing peak. To prevent this

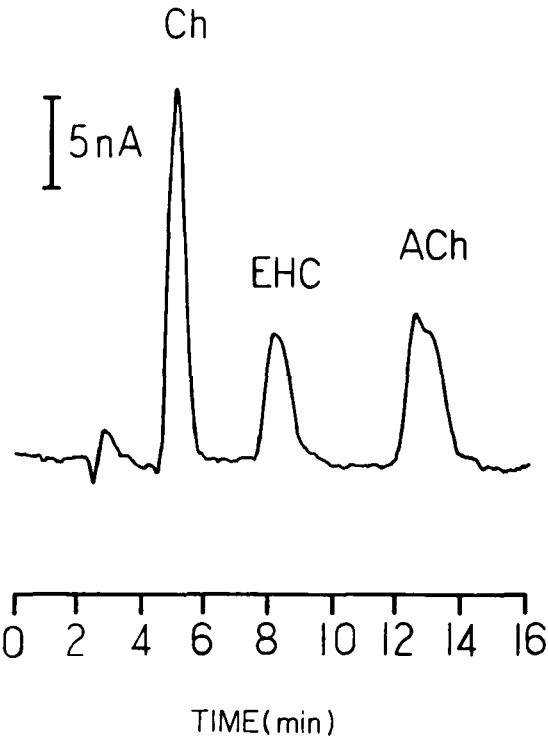

Fig. 7. Effect of ACh adsorption to C18 support.
Injection volume: 200 pmoles of ACh, Ch and EHC.
Chromatographic conditions;
Column: Chemco 5-ODS 150(L) X 4.6 mm (I.D.)
Mobile phase: 0.091 M sodium acetate, pH 5.0, 25 mg/L of SOS, 1.2 mM TMA, 10 mM Na_2EDTA.
Column temperature: 37° C.
Detector: Electrochemical detector set at +500 mV vs. Ag/AgCl.

effect, 1.2 mM TMA was added to the mobile phase, but the TMA concentration increasingly inhibited COD activity, and detector response to both ACh and Ch was reduced (7).

With 1.2 mM TMA, we tested the effect of ACh adsorption on a C18 support (Chemco 5-ODS, carbon content: 18%). The resulting peak shape gradually broadened and then divided into 2 peaks (see Fig.7).

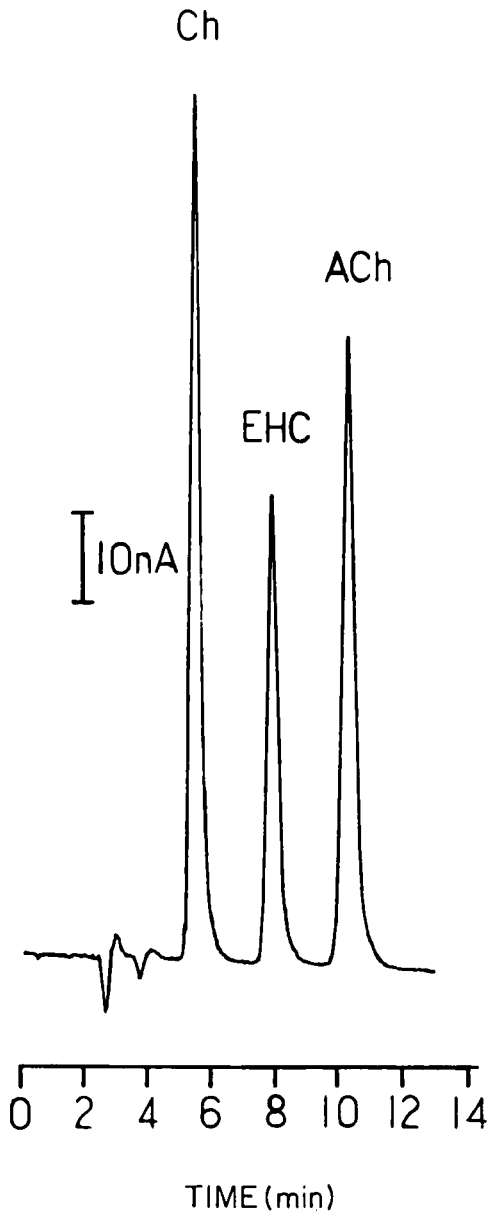

Fig. 8. Chromatogram for standard mixture.
Chromatographic conditions are described in Fig.7.
Column (Nucleosil C-18, 150 (L) X 4.6 mm (I.D.).

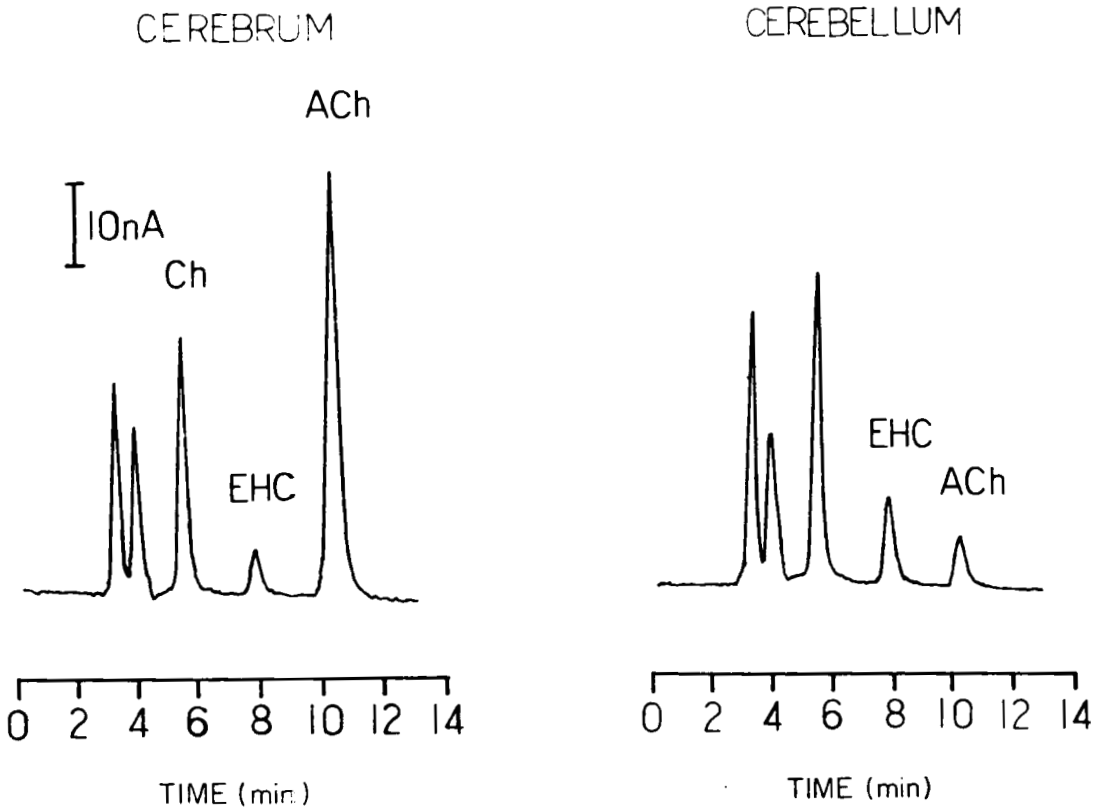

Fig. 9. Typical chromatograms for rat cerebrum and cerebellum. Chromatographic conditions are described in Fig. 8. Cerebrum and cerebellum contained 200 pmoles of EHC as internal standard. Injection volume: 20 µL.

Table 1 Regional ACh and Ch content in the rat brain. Data is presented as the mean ± SEM, n=3-5.

Brain region	(pmole/mg) Ch	ACh
Cerebrum	99.0 ± 0.7	9.2 ± 1.1
Cerebrullum	14.9 ± 1.5	3.8 ± 1.4
Medulla Oblongate	68.5 ± 13.8	14.1 ± 1.9
Diencephalon	24.2 ± 3.3	38.8 ± 3.1

An alternate C18 silica support (Macherey-Nagel) with low carbon content was used for determination of ACh and Ch. The elution pattern for ACh, Ch and EHC improved as shown in Fig.8, because of the reduction of silanol groups contained on this particular support.

6) ACh and Ch analysis in rat brain tissue

Chromatograms of brain tissue extracts using LCEC with the immobilized enzyme column are shown in Fig. 7 and 8. Table 1 shows regional ACh and Ch concentrations in rat brain.

In conclusion, LCEC using the immobilized enzyme column proved to be a simple, reliable, and inexpensive method to determine ACh and Ch in a biological sample, since immobilization is more conservative of precious enzyme than the continuous addition approach (7). Also, in the case of continuous enzyme flow, the surface of the platinum electrode can become coated with protein resulting in a gradual reduction in sensitivity. Utilization of the immobilized enzyme column provided good

sensitivity and increased electrode life. The method proved to be simple to operate and possessed lower operating costs without sacrificing accuracy.

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