Feasibility of Ion-Pair Reversed-Phase Liquid Chromatography/Electrochemistry Detection for Determination of Acetylcholine in Microdialysates Collected Without Acetylcholinesterase Inhibitors

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Acetylcholine (ACh) concentration in medial prefrontal cortex (mPFC) was determined using in vivo *microdialysis and LC/EC without acetylcholinesterase (AChE) inhibitors in awake and freely moving rats. ACh and choline (Ch) in microdialysates were separated using a reversed phase column and converted to H_2O_2 by a post-column IMER where acetylcholinesterase and choline oxidase were immobilized. The H_2O_2 was detected by a polymer-modified electrode with osmium mediator and horseradish peroxidase. This method was shown to provide sufficient stability, concerning both detection limit and retention time, to apply to the determination of ACh in microdialysates without the use of AChE inhibitors. The basal ACh concentration of the diurnal phase was around 2 fmol/μL. On transition to the nocturnal phase, ACh increased to around 200% of basal level. These results illustrate that the reversed phase method is a practical alternative to ion exchange for determination of ACh in microdialysates.*

It has been suggested that cortical acetylcholine (ACh) in prefrontal cortex (mPFC) plays an important role in some domains of cognitive function such as attention (1,2), and sensory gating (3). Therefore, measurement of ACh in a living animal is important for the development of novel drugs, as well as investigation of brain function. Microdialysis has proven to be a useful sampling technique in the brain of awake freely-moving animals. However, it is difficult to determine the basal concentration of ACh *via* microdialysis due to its rapid hydrolysis by acetylcholinesterase. In order to resolve this problem, acetylcholinesterase (AChE) inhibitors, which increase the extra-cellular concentration of ACh, have been used for the *in vivo* determination of ACh. However, the AChE inhibitor-induced increase in ACh in the brain has been reported to alter the function of some drugs (4,5,6), which shows the importance of determining ACh without AChE inhibitors for *in vivo* studies, including microdialysis.

Liquid chromatography with electrochemical detection (LC/EC) has been successfully used to determine ACh brain tissue and dialysates. In this method, ACh is separated on the analytical column and converted to hydrogen peroxide on a post-column IMER in which AChE and choline oxidase (ChOx) are immobilized. The H_2O_2 is oxidized or reduced on the surface of an electrode (platinum or enzyme-modified glassy carbon, respectively) and the current is measured using an amperometric detector. Huang *et al* (7) reported the LC/EC determination of basal ACh in dialysates utilizing microbore ion-exchange chromatography and an enzyme-modified ("wired") electrode in the absence of AChE inhibitors. This combination of electrode and IMER resulted in a low background current, increasing the signal-to-noise ratio and lowering interferences. High concentrations of Ch can sometimes interfere with the quantitation of ACh. The addition of a pre-IMER (prior to the ion-exchange analytical column) containing immobilized ChOx and

catalase, which destroy the Ch prior to chromatographic separation, has enabled routine determination of basal ACh in rat brain dialysate without use of AChE inhibitors (4,5,8).

Although silica-based reversed phase columns exhibit superior separation compared to ion-exchange columns, the basic conditions required for maximum IMER activity can be detrimental to column lifetime. However, it was expected that if increased separation of ACh and Ch were obtained, the use of a pre-IMER would not be necessary. In this report, we present a method to determine basal ACh in microdialysate, without AChE inhibitors, using EC detection linked to a reversed phase column. Furthermore, in order to verify the feasibility of this method for the physiological assessment of ACh release, we applied this method to the task of assessing circadian variation of ACh in mPFC of freely moving rats.

Materials and Methods

Subjects

Male Wistar rats (Harlan Laboratory Animals), weighing 280-320g were used. Animals were housed at 25°C on a 12-hour light/dark cycle (light on from 7:00 a.m. to 7:00 p.m.) with free access to food and water. Before implantation of a guide cannula, animals were housed in pairs, in steel hanging racks. After implantation, animals were housed individually in plastic cages with shavings. All animal care and experiments were performed according to the protocol authorized by the Institutional Animal Care and Use committee of Bioanalytical Systems, Inc.

Surgery and Brain Microdialysis

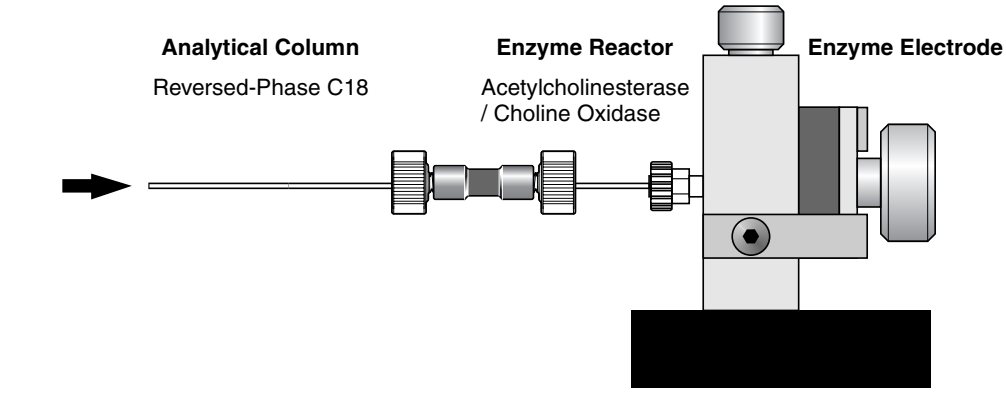
Animals were anesthetized with ketamine/xylazine (10/1) (1 mL/kg i.p.), and placed on a stereotaxic frame with the incisor bar set 5 mm above an interaural line. A guide cannula (BAS, West Lafayette, Indiana) was implanted in the medial prefrontal cortex (coordinate: 4.0 mm anterior, 0.8 mm lateral from bregma, 0.8 mm ventral from dura mater) by reference to the atlas (9). Two to three days after surgery, a microdialysis probe with 4 mm active area (BAS) was inserted through the guide cannula. Each probe was perfused at 0.3 µL/minute with Ringer solution containing the following: NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 2 mM. An acetylcholinesterase inhibitor was not used in any experiment. After 18 hours for reconstitution of the blood-brain barrier, the flow rate was increased to 0.7 µL/minute. After three hours, collection of dialysate was performed every 30 minutes.

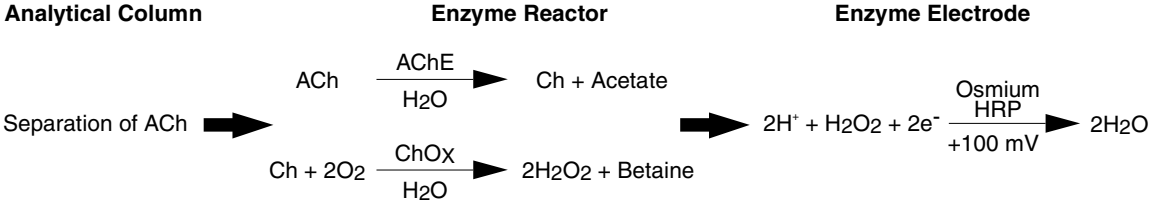
Acetylcholine and Choline Analysis

In the present study, both ACh and Ch were determined using liquid chromatography linked with an electrochemical detector (LC/EC) as shown in ***F1***. An isocratic pump (PM-92e, BAS) delivered the mobile phase containing the following: 50 mM Na_2HPO_4, pH 6.5 (adjusted with phosphoric acid), 0.5 mM EDTA, 5 mM diethylamine HCl, and 0.6 mM 1-octanesulfonic acid sodium salt, at a flow rate of 75 µL/minute. Separation occurred on an analytical column, 1 x 100 mm, protected by a guard column, 1 x 14 mm , both packed with 3 µm ODS silica, (BAS, MF-8949 and MF-8946, respectively). A post-column IMER (1 x 50 mm, BAS, MF-8903) in which AChE and ChOx were immobilized, converted ACh to hydrogen peroxide. The hydrogen peroxide was detected at a glassy carbon working electrode (diameter: 3mm) modified

F1

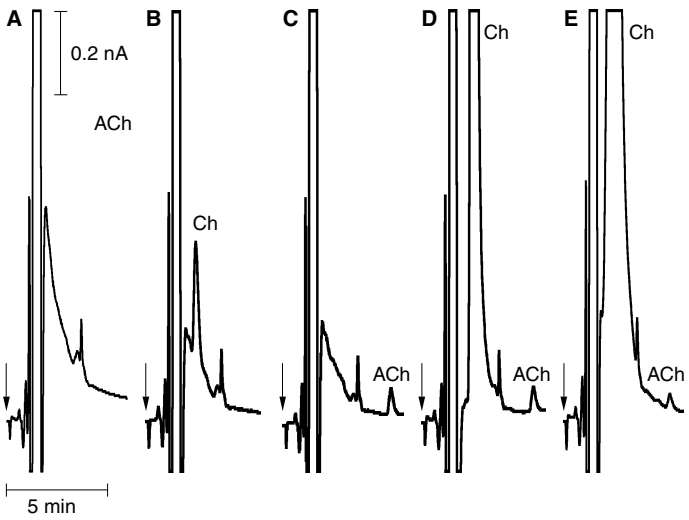
Schematic diagram of ACh determination using a reversed-phase column and electrochemical detection.





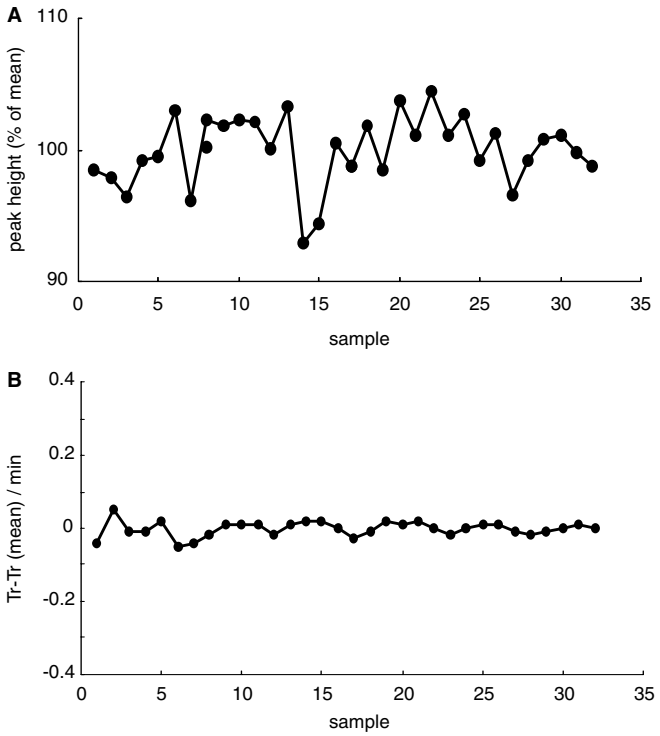
F2

Chromatograms of standards and dialysate. (A) Ringer; (B) 25 nM Ch standard; (C) 6.25 nM ACh standard; (D) 1 uM Ch and 6.25 nM ACh standard; (E) microdialysate collected from mPFC. Each injection volume was 10 µL.



F3

Consistency of response (A) and retention time (B). Amount of ACh injected on column was 60 fmol.



with osmium-horseradish peroxidase polymer (BAS). The applied potential was +100mV (vs. Ag/AgCl). The reduction current was monitored by an electrochemical detector (LC-4C and BAS ChromGraph software). Injection volume was 10 µL.

Histological Examination

After the end of each experiment, the rats were sacrificed by an overdose of sodium pentobarbital and the brains removed and fixed in 4% formaldehyde and saline mixture. The tissue was dissected into 1 mm thick slices using a Brain Matrix (BAS). Placement of the microdialysis probe in the mPFC was verified.

Data Analysis

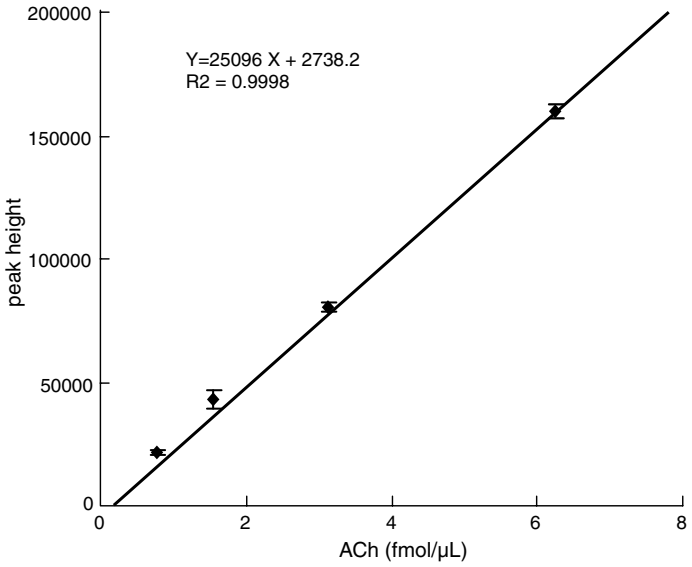
Basal concentration of ACh in the light phase was determined by averaging six samples from each rat. Each subsequent value was expressed as a percentage of basal concentration and the mean and standard error of mean (mean ± S.E.M.) in each group was calculated with respect to the time of day. Differences of ACh concentration between the light phase and dark phase were analyzed by *t*-test. Significance was attained at $p < 0.05$.

Results and Discussion

AChE inhibitors, such physostigmine or neostigmine, have been used in many studies related to the investigation of the physiological action of ACh or the pharmacological effect of drugs on ACh when microdialysis is used as the sampling technique (1,10-13). Without the added AChE inhibitors the ACh concentration is often below the detection limit of the analytical methods. However, the presence of AChE inhibitors has been reported to alter the action of some drugs: for example, effects of the non-selective muscarinic antagonist scopolamine on ACh release in rat mPFC were enhanced by physostigmine (6). Neostigmine was reported to enhance the effects of clozapine on ACh release in mPFC, striatum (STR), and nucleus accumbens (NAC) (4,15). These findings show the importance of determining ACh without the use of AChE inhibitors. In the present study, we report the reversed-phase chromatographic determination of ACh/Ch in microdialysate samples from rat mPFC without the use of AChE inhibitors. Typical results are present in ***F2***. Ch and ACh exhibited retention times of 2.5 minutes and 6 minutes (***F2B-E***), respectively. These results are different from a previous method using a cation-exchange column, which results in ACh eluting prior to Ch (7). The difference in retention time between ACh and Ch is 3.5 minutes for the present method and about 2 minutes for the previous method. Moreover, peak shape is shown to be sharper for the reversed-phase column compared to a cation-exchange column. As shown in ***F2E***, the Ch concentration in microdialysates is high compared to the ACh concentration, which has been a problem for the previous method because the ACh peak is often masked by the Ch

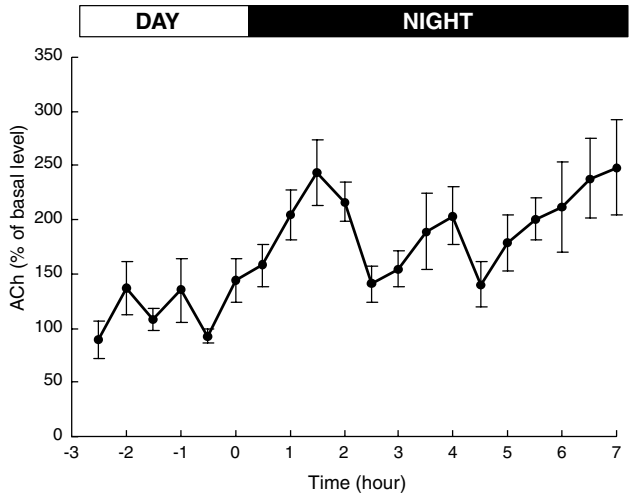
F4

Standard curve for ACh. Concentration of ACh 0.78, 1.56, 3.25, and 6.25 nM, injection volume 10 µL. Each point is the mean ± SD of five replicates.



F5

Time course of circadian variation of ACh release in mPFC. Light cycles are shown on the top. Basal concentration determined by averaging ACh concentration before lights were turned off. At time 0 (7:00 p.m.) lights were turned off. Subsequent plot shows ACh concentration as a percent of basal concentration (mean ± S.E.M.).



peak. A pre-IMER in which catalase and ChOx are immobilized was reported to be beneficial for ACh determination when using the cation-exchange column, because Ch is destroyed in pre-IMER prior to entering the analytical column (4,5,8). The present method enables determination of ACh without a pre-IMER, and allows determination of ACh and Ch simultaneously.

The chromatographic conditions for determination of ACh (by LC/EC) are rather restricted, since these are optimized more for enzyme activity than for chromatographic separation. Optimum activity is exhibited by AChE near pH 7.0. The optimum pH for ChOx, pH 8-9 (most use pH 8.5), is the most restrictive, and generally the limitation to use of silica-based packing material. Therefore, a polymer-based cation-exchange column has been used in determination of ACh because basic conditions are not suitable for silica-based reversed-phase columns. In this study, we show that the enzyme system (AChE/ChOx) maintained its activity near neutral pH during analysis (***F3A***). ***F3B*** shows the present system provides a constant retention time for ACh.

A typical calibration curve is shown in ***F4***. The square of *r* value from the linear regression is 0.9998. The detection limit of the present method was 4-8 *f*mol on column. The separation using an ion-exchange column and the same enzyme-modified electrode was reported as 2-3 and 10 *f*mol injected (4 and 7, respectively). Zhu *et al* (14) reported a detection limit of 1.4 *f*mol on column for an ion-pair reversed-phase chromatographic separation, but using mass spectrometry for detection.

We demonstrated feasibility of this method to assess ACh in microdialysate from awake rats without AChE inhibitors. ***F5*** presents the time course of variation of cortical ACh release in the circadian cycle. The mean ACh concentration collected in the diurnal phase was 2.2 ± 0.6 *f*mol/µL (n=5), which was taken as the basal level. After the lights were turned off, the ACh level increased by 243 ± 30%. The average ACh concentration in the nocturnal phase was 195 ± 27% of basal level, which is statistically significant from the diurnal level ($p = 0.00017 < 0.0005$). It has been suggested that ACh release in rat mPFC is increased in the nocturnal phase, which is the circadian active phase for rodents (11,12). These studies also reported the ACh concentration doubles in the nocturnal phase relative to the diurnal phase. Our results (***F5***) support this conclusion. It has been suggested that cortical ACh release, including from the mPFC, is involved with cognitive functions such as attention processing (1,2), and sensory gating (3). For example, a selective lesion of the cholinergic neurons in the nucleus basalis magnocellularis, which provides major projections to subcortical areas including mPFC, has been reported to cause disruption of prepulse inhibition of acoustic startle response (3) and cognitive impairment (15,16). Both are observed in psychiatric conditions such as schizophrenia. These observations lend validity to the investigation of the effects of cortical ACh in models of such diseases. Nevertheless, ACh has had less attention compared with other neurotransmitters such as dopamine (17), norepinephrine (18), 5-HT (19), or glutamate (20) viewed in studies of schizophrenia. The reasons probably include methodological problems, variable drug affects using AChE inhibitors, as well as analytical difficulties in determining the basal ACh. Here, we have re-

ported an alternative method for ACh determination in the absence of AChE inhibitors. It is expected this method will prove to be valued for the elucidation of psychiatric diseases, as well as for the development of new drugs, including antipsychotic drugs.

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