# Acetylcholinesterase Inhibitors Are Neither Necessary nor Desirable for Microdialysis Studies of Brain Acetylcholine

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*Acetylcholine (ACh), one of the major neurotransmitters in brain, has an important role in various types of cognition, including attention and memory. ACh is critical to the neurocognitive dysfunction in Alzheimer's disease and possibly other psychiatric disorders such as schizophrenia. Furthermore, the development of drugs which increase the availability of brain ACh is a major research goal. Thus, direct measurement of brain ACh release under physiological conditions, using in vivo microdialysis, in laboratory animals is of great importance. Because of the low concentration of ACh, many of the microdialysis studies published to date required Acetylcholinesterase (AChE) inhibitors in the perfusion medium to increase basal ACh. However, the artificially increased concentration exerts a significant influence on the cholinergic system, thereby making interpretation of drug effects problematic. We have recently developed a highly sensitive method of ACh measurement, which requires no AChE inhibitors. Advantages of the new method are discussed.*

### *Introduction*

Acetylcholine (ACh) is important in normal brain function as well as mental illness. Specifically, ACh is of interest in cognitive dysfunction in Alzheimer's disease and schizophrenia (1), and possibly also negative and positive symptoms in schizophrenia (2). In vivo measurement of brain ACh is also important to animal studies of drugs, e.g. antipsychotic drugs (APDs) to treat Alzheimer's disease and schizophrenia (3,4,5). Because of the low sensitivity of conventional ACh measurements, most investigators have used acetylcholinesterase (AChE) inhibitors to increase basal extracellular ACh concentrations to readily detectable levels. However, there is growing evidence that the use of AChE inhibitors may interfere with the study of drug effects on ACh release. Thus, AChE inhibitors may alter muscarinic/nicotinic ACh receptor-mediated transmission because of the huge increases in extracellular ACh levels they produce. It is obviously not possible to study any additional effect of AChE inhibitors themselves on extracellular ACh, which may be of interest in some experimental disease models or drug-interaction studies. Thus, we have found that the effect of some drugs on ACh release differs in the presence and absence of neostigmine, a widely used AChE inhibitor. For example, clozapine (20 mg/kg), the prototypical atypical APD, has been reported to acutely increase ACh release in rat medial prefrontal cortex (mPFC), nucleus accumbens (NAC) and striatum (STR) in the presence of 0.3 μM neostigmine in the perfusion medium (31), whereas we have found that clozapine (20 mg/kg), in the absence of neostigmine, increases ACh release only in the mPFC, and not in the other two regions (4). Similarly, in the absence of AChE inhibitors (4), we could not replicate the report that haloperidol (1 mg/kg), a typical APD, increases striatal ACh release in the presence of 0.01 μM neostigmine (6). These results provide a strong rationale for ACh microdialysis studies without the use of AChE inhibitors. This review further discusses the importance of AChE inhibitor-free microdialysis for ACh. The review of Tsai (7) should also be consulted.

**T1**

*Microdialysis determination of the effect of AChE inhibitors on extracellular neurotransmitters in rat brain.*

** intraventricular injection*

*** given to substantia nigra, dl: dorsolateral*

| | | ACh | DA | NE | glutamate | DOPAC | region | ref |
|---|---|---|---|---|---|---|---|---|
| **Physostigmine** | | | | | | | | |
| 0.03-0.3 | mg/kg | increase | increase | increase | | | frontal cortex | 8,9 |
| 0.3 | | increase | | | increase | | striatum | 10,17 |
| 10 | µM | | | | no effect | | | 10 |
| 50 | | increase | no effect | no effect | | | frontal cortex | 8 |
| **Heptyl-physostigmine** | | | | | | | | |
| 2-5 | mg/kg | increase | increase | increase | | | frontal cortex | 8,9 |
| 50 | µM | increase | increase | no effect | | | | 8 |
| **Neostigmine** | | | | | | | | |
| 0.05* | µmol | | | | | increase | hypothalamus | 27 |
| 0.125-0.25** | | | increase | | | | striatum | 26 |
| 10 | µM | | | increase | | | hippocampus | 12 |
| 10-100 | | | increase | | | | striatum fundus | 11 |
| | | | no effect | | | | striatum dorsolateral | 11 |

### *Effects of AChE inhibitors on ACh and other neurotransmitters (T1)*

Extracellular concentrations of ACh in brain are influenced mostly by the activity of AChE and, to a lesser extent, by autoreceptor-mediated inhibition of ACh release from the neuron terminals. Inhibition of AChE activity results in a marked increase in extracellular ACh concentrations, and permits reliable measurement of ACh in dialysate samples which would otherwise not be possible due to insufficient assay detection limits. However, recent microdialysis studies suggested that AChE inhibitors may alter extracellular levels of neurotransmitters in the brain other than ACh. For example, systemic administration of physostigmine (0.03-0.3 mg/kg), another widely used AChE inhibitor for microdialysis studies, has been reported to increase extracellular dopamine (DA), norepinephrine (NE) and ACh in rat frontal cortex, whereas physostigmine (50 µM) in the perfusion medium increased only ACh in that region (8,9). Similarly, systemic administration of physostigmine (0.3 mg/kg), but not its local perfusion (10 µM), increased extracellular glutamate in the STR (10). Thus, it appears that inclusion of physostigmine in the perfusion medium has minimal effects on extracellular DA, NE and glutamate.

However, the AChE inhibitor heptyl-physostigmine increased ACh and DA in the frontal cortex after both systemic administration (2-5 mg/kg) and local perfusion (50 µM) (8,9). Furthermore, local perfusion of neostigmine has been reported to increase DA in the STR fundus (11), and NE in the hippocampus (12), respectively. These results suggest that AChE inhibitors in the perfusion medium have appreciable effects on other neurotransmitter systems as well as ACh, possibly mediated via increased ACh. In fact, ACh by itself has been reported to increase DA release in the STR in the absence of physostigmine (13); however, these increases were attenuated in the presence of 10 µM physostigmine, and completely abolished by further increases in its concentration (50 µM) (13).

Radio-receptor binding studies demonstrated that AChE inhibitors such as physostigmine displaced the agonist [$^3$H]oxotremorine-methiodide binding in rat brain (14). Neostigmine has also been reported to depress neuronal activity mediated by nicotinic ACh receptors by interacting with the ACh binding sites of the receptors (15). These results suggest that the ability of ACh receptor agonists and antagonists to affect the release of ACh, and perhaps other transmitters as well, may be altered in the presence of AChE inhibitors. The slice-superfusion studies of cat caudate putamen demonstrated that scopolamine and QNB (quinuclidinyl benzilate), non-selective muscarinic ACh receptor antagonists, dose-dependently decreased electrically-evoked [$^3$H]DA in the presence of physostigmine (1 µM). Physostigmine itself increased [$^3$H]DA, whereas both scopolamine and QNB had no effect in the absence of physostigmine (16). Taken together, these considerations provided evidence that the effect of drugs on extracellular ACh in the presence of AChE inhibitors may not reflect in vivo effects in the brain under physiological conditions.

### *Alterations of drug effects on extracellular ACh by AChE inhibitors (T2)*

The ability of AChE inhibitors to alter drug effects on extracellular ACh may depend largely upon their concentration in the perfusion medium. It has been suggested that low concentrations of AChE inhibitors in the perfusion medium only minimally modulate drug effects on extracellular ACh. For example, a low concentration of neostigmine (0.01 µM) in the perfusion medium could produce minimal perturbation of physiological conditions, permitting reliable measurement of dialysate ACh. Thus, systemic administration of physostigmine (0.3 mg/kg) has been reported to increase extracellular ACh in the STR in the presence of 0-10 µM physostigmine, whereas

it decreased ACh in the presence of 25-50 µM physostigmine (18). Under physiological conditions, or in the presence of low concentration of neostigmine (0.01 µM), high dose amphetamine (10 mg/kg) as well as the $D_2$ receptor agonist quinpirole (3 mg/kg) decreased, but low dose amphetamine (2 mg/kg) had no effect on striatal extracellular ACh, respectively (6,19). However, the results, in the presence of high concentrations of neostigmine (0.1 µM), were quite different. Thus, amphetamine, 2 mg/kg, increased striatal extracellular ACh, and the amplitude of the quinpirole-induced decrease in extracellular ACh was not increased relative to basal ACh (6,19). Local perfusion of atropine (10 µM), a non-selective muscarinic ACh receptor antagonist, has been reported to increase basal and handling-evoked extracellular ACh in rat ventral hip-

***T2***

*The effect of AChE inhibitors on the ability of drugs to affect extracellular neurotransmitters in rat brain.*

| | drug | ACh | DA | region | ref |
|---|---|---|---|---|---|
| **Neostigmine (µM)** | | | | | |
| | Oxotremorine (µM) | | | | |
| 0.1 | 100 | no effect | | striatum | 28 |
| | Atropine (µM) | | | | |
| 0.01 | 10 | no effect | | hippocampus | 20 |
| 0.1-1 | | increase | | | 20 |
| | Amphetamine (mg/kg) | | | | |
| 0-0.01 | 2 | no effect | | striatum | 6,19 |
| | 10 | decrease | | | 6,19 |
| 0.1 | 2 | increase | | | 6,19 |
| | 10 | no effect | | | 6 |
| | | increase | | | 19 |
| | L-DOPA (µM) | | | | |
| 0.01 | 2 | increase | | striatum | 30 |
| 0.05-0.1 | | attenuated increase | | | 30 |
| | Quinpirole (mg/kg) | | | | |
| 0.01 | 0.2 | increase | | striatum | 19 |
| 0-0.01 | 3 | decrease | | | 6 |
| 0.1 | 0.2 | attenuated increase | | | 19 |
| | 3 | no effect | | | 6 |
| | Haloperidol (mg/kg) | | | | |
| 0 | 0.05-0.5 | | increase | striatum | 11 |
| 10 | | | attenuated increase | | 11 |
| 0.01 | 1.0 | increase | | | 6 |
| 0-0.3 | 1.0 | no effect | | medial PFC | 4 |
| | | no effect | | striatum | 4 |
| | | no effect | | nucleus accumbens | 4 |
| **Neostigmine (µM)** | | | | | |
| | Clozapine (mg/kg) | | | | |
| 0.3 | 20 | increase | | medial PFC | 4,31 |
| | | increase | | striatum | 4,31 |
| | | increase | | nucleus accumbens | 4,31 |
| 0 | | increase | | only medial PFC | 4 |
| **Physostigmine (µM)** | | | | | |
| | ACh (mM) | | | | |
| 0 | 10 | | increase | striatum | 13 |
| 10 | | | attenuated increase | | 13 |
| 50 | | | no effect | | 13 |
| | Physostigmine (mg/kg) | | | | |
| 0-10 | 0.3 | increase | | striatum | 17 |
| 25-50 | | decrease | | | 17 |
| | 8-OH-DPAT (mg/kg) | | | | |
| 0-10 | 0.5 | increase | | hippocampus | 29 |
| 0 | 3-30 mM | no effect | | | 29 |
| 10 | | increase | | | 29 |
| **Heptyl-physostigmine (mg/kg)** | | | | | |
| | Atropine (mg/kg) | | | | |
| 0 | 2.2 | no effect | | cortex | 18 |
| | 0.2 µM | no effect | | | 18 |
| 1 | 2.2 | increase | | | 18 |
| | 0.2 µM | increase | | | 18 |

pocampus only in the presence of a higher concentration of neostigmine; 0.1 and 1 µM, but not 0.01 µM (20). Thus, it should be noted that haloperidol (1 mg/kg) had no significant effect on extracellular ACh in the STR in the absence of neostigmine (4), whereas De Boer and Abercrombie (6) reported that haloperidol (1 mg/kg) increased striatal extracellular ACh in the presence of even low concentration neostigmine (0.01 µM). These results clearly suggest that the effect of some drugs on extracellular ACh differs, even with a low concentration of AChE inhibitors, from the effects under physiological conditions or in the absence of AChE inhibitors.

The use of ACh esterase inhibitors has the major disadvantage that increased extracellular ACh causes continuous stimulation of presynaptic and postsynaptic ACh receptors, which may result in cholinergic tone significantly different from physiological conditions. These differences could bias the interpretation of the data obtained from microdialysis studies performed in the presence of AChE inhibitors. In the presence of AChE inhibitors, it is also not possible to split dialysate samples to measure DA or other substances which can provide direct evidence for their interaction with ACh. Thus, the development of methods for ACh measurement which requires no AChE inhibitors provides a necessary tool for some types of experiments.

### *AChE inhibitor-free microdialysis and low detection limit ACh measurement (T3)*

Several ACh microdialysis studies in which AChE inhibitors were not used have been reported. Determination of ACh by radioimmunoassay (RIA) was used in some of these studies. Although RIA provides sufficient sensitivity for detection of ACh at relevant concentrations (0.72-1.72 fmol/µL at basal levels), it will not be further discussed in this review. Few neuroscience laboratories are equipped for this methodology, whereas liquid chromatography (LC) is readily available.

The principle of ACh measurement with liquid chromatography/electrochemistry (LCEC) is shown in ***F1*** and ***F2***: 1.) dialysate samples are injected onto the LC system; 2.) ACh is separated on the cation exchange column (analytical column); 3.) ACh is hydrolysed by AChE to form acetate and choline in the post-IMER (immobilized enzyme reactor), and then choline is oxidized by ChO (choline oxidase) to produce betaine and hydrogen peroxide ($H_2O_2$); 4.) $H_2O_2$ is detected, oxidized on a platinum electrode with the potential set at e.g. +0.5 V (old system); and 5.) the oxidation is monitored with the detector signal indicating ACh in the chromatogram. Basal dialysate concentrations of ACh have been reported to be 0.17-10 fmol/µL, although the size and shape of dialysis probes, flow rate of perfusion medium, sampling interval and the region receiving probe implantation are different in each experiment. The detection limit based upon the signal-to-noise ratio (S/N) has not been clearly provided in most studies; however, it may range from 20 to 50 fmol per sample. While this system provides reliable measurement of ACh in dialysate samples in the presence of AChE inhibitors, it is unable to measure ACh in the absence of AChE inhibitors.

The next generation of ACh measurement has replaced the platinum electrode with a peroxidase-redox polymer coating on a glassy carbon electrode (***F2***, new system) (21,22). Peroxidase is mounted on the surface of a glassy carbon elec-

***T3***

*ACh measurement in the dialysate without AChE inhibitors.*

| | pre-IMER (peroxidase/ChO) | post-IMER (AChE/ChO) | electrode (peroxidase-redox polymer-coated glassy carbon) | basal (fmol/µL) | region | ref |
|---|---|---|---|---|---|---|
| **I. Liquid Chromatography/Electrochemistry (LCEC)** | | | | | | |
| **A.** | no | yes | no/platinum | 10.0 | hippocampus | 37 |
| | | | | 3.60 | | 32 |
| | | | | 4.10 | frontal cortex | 32 |
| | | | | 0.17 | | 34 |
| | | | | 3.50 | striatum | 32 |
| | | | | 1.26 | | 6 |
| | | | yes | 6.20 | | 21 |
| | | | | 1.6 | hippocampus | 22 |
| **B.** | no/octadecylsilane | yes | no/platinum | 5-10 | cortex | 18 |
| **C.** | yes | yes | yes | 0.60 | hippocampus | 23 |
| | | | | 1.13 | frontal cortex | 23 |
| | | | | 1.37-1.87 | | 35 |
| | | | | 0.98 | medial PFC | 4 |
| | | | | 1.39 | nucleus accumbens | 4 |
| | | | | 2.74 | striatum | 4 |
| **II. Radioimmunoassay** | | | | 1.72 | hippocampus | 33 |
| | | | | 1.00 | | 29 |
| | | | | 0.72 | | 36 |
| | | | | 0.96 | cortex | 36 |

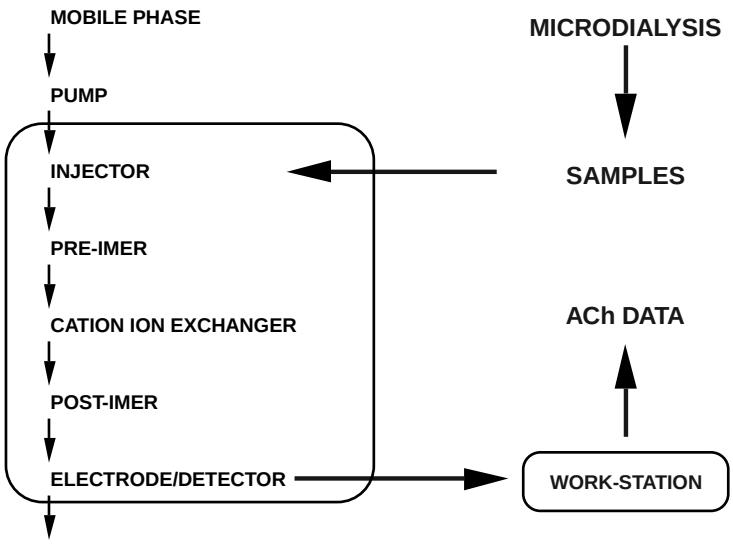
trode and coated with a redox polymer material. This type of electrode increases the signal-to-noise ratio by providing a stabilized electrode surface on which $H_2O_2$ is reduced to form $H_2O$. For example, a platinum electrode requires higher potential, e.g. +0.5 V versus the reference electrode, compared with +0.1 V for the new type of electrode. This low potential contributes, in part, to lower noise levels. However, a high concentration of choline remains in the injected samples and interferes with the peak due to ACh, which elutes prior to the choline peak in the chromatogram. The ACh peak is well separated from the choline peak in the authentic standard. In dialysate samples, the ACh peak appears on the slope of a huge choline peak. This chromatographic interference markedly increases the detection limit for the ACh measurement. Although clear separation of the ACh peak from the huge choline peak can be obtained with a reversed phase column, a microbore column, which is best fitted to the analysis of dialysate ACh, is not available at present because of technical difficulties that are being remedied.

Since it is difficult in most cases to separate the ACh peak from the huge choline peak with the cation exchange column, Kato et al. (23,24) and Tsai et al. (25) used a pre-IMER (ChO/peroxidase and ChO/catalase, respectively) set prior to the analytical column (***F2***). Choline in the dialysate samples is oxidized by the ChO to form betaine and $H_2O_2$. The $H_2O_2$ is then converted by peroxidase/catalase to $H_2O$ before entering the analytical column. The pre-IMER completely eliminates choline and $H_2O_2$ from the injected samples. This procedure eliminates overlap of choline with the ACh peak in the chromatogram (***F3***), thereby decreasing the detection limit for the ACh measurement. The extent of decomposition of $H_2O_2$ in the pre-IMER may vary based on the activity of the catalase, leaving some amount of $H_2O_2$ in the analytical column. The $H_2O_2$ left over in the injected dialysate samples increases the front peak in the chromatogram which tails and may interfere with the ACh peak. However, this interference has been shown to be minimal as long as the catalase remains active (***F3***). Thus, the use of the pre-IMER coupled with the cation exchanger microbore column is essential for the method to achieve sufficient sensitivity to permit AChE inhibitor free determination of ACh in dialysates.

We modified and improved the method of Kato et al. (24) so that dialysate ACh can be reliably and consistently measured in microdialysis experiments in the absence of AChE inhibitors (4,5). The procedure, in brief, is as follows: Three to five days following cannulation surgery, a concentric-shaped dialysis probe (2 mm length, polyacrylonitrile/sodium methalylsulfonate polymer membrane, AN69H; Hospal) is implanted into either mPFC, NAC or STR of rats under slight anesthesia with methoxyflurane (Metofane; Pitman-Moore, Mundelein, IL), which make them temporarily immobilized. The rats quickly recover from slight anesthesia and are allowed to move freely in the cage. Perfusion flow rate of the implanted probes is set at 1.5 µL/min and then dialysate samples (45 µL) are collected every 30 min. The perfusion medium is Dulbecco's phosphate buffered saline solution (Sigma, St. Louis, MO) including $Ca^{2+}$ (NaCl 138 mM, $Na_2HPO_4$ 8.1 mM, KCl 2.7 mM, $KH_2PO_4$ 1.5 mM, $MgCl_2$ 0.5 mM, $CaCl_2$ 1.2 mM, pH = 7.4). Three hours after probe implantation and its perfusion, dialysate samples are directly injected onto the LCEC system assisted by a chromatography manager (Millennium; Waters, Milford, MA), and analyzed for ACh. ACh is separated on a coiled ion exchanger ACh column (UniJet

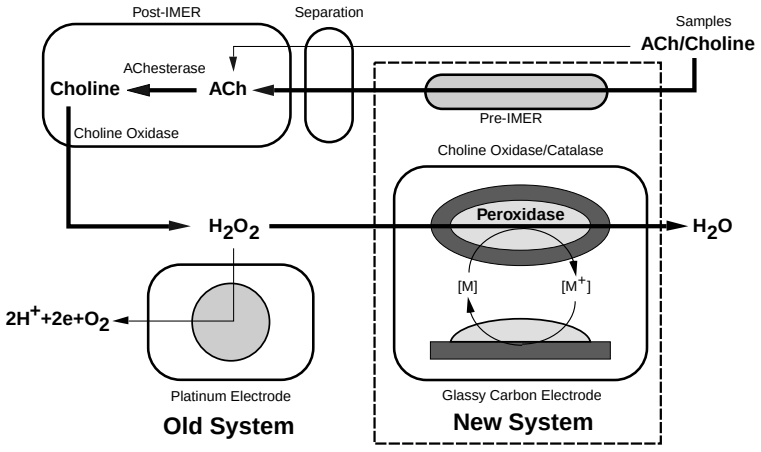
***F1***

*Scheme of bioanalysis of microdialysis dialysate samples for ACh. The arrow indicates the direction of sample analysis. IMER: immobilized enzyme reactor.*




***F2***

*Scheme comparing the new system with the old system. The arrow indicates the direction of sample analysis IMER: immobilized enzyme reactor. M: mediator of electron transfer.*



10 µm ID 530 × 1.0 mm; BAS, West Lafayette, IN). The pre-IMER (MF-8907) purchased from BAS consists of ChO/catalase, and the post-IMER (BAS) consists of ChO/AChE. The mobile phase ($Na_2HPO_4$ 50 mM, pH 8.2) including ProClin (BAS), a microbiocide, is pumped at 0.14 mL/min by a LC-10AD pump (Shimadzu, Kyoto, Japan). ACh is detected using a UniJet amperometric detector cell with the peroxidase-redox polymer-coated glassy carbon electrode (MF-2098; BAS), set at +0.1 V (LC-4C; BAS) versus AgCl-coated Ag reference electrode. The ACh peak is clearly resolved in the chromatograph (***F3***).

The ACh peak is clearly identified in all the chromatograms because there is no interference (***F3***). Areas and the heights of ACh peaks showed a linear correlation with the concentration. Due to the markedly increased S/N ratio, the detection limit is about 1-2 fmol per sample (10 µL sample loop). Thus, we can obtain stable baseline values of ACh in dialysates in all the experiments for these three regions. The basal levels of ACh are 0.6-2.74 fmol/µL for the mPFC, NAC and STR (***T3***). The basal ACh and its increase produced by clozapine (20 mg/kg) were completely eliminated by co-perfusion of the sodium channel blocker tetrodotoxin (1 µM) (4), indicating dialysate ACh is entirely of neuronal origin. With this newly developed method, it is now possible to reliably and accurately measure ACh in dialysate samples in the absence of AChE inhibitors.

In conclusion, AChE inhibitors have long been used to investigate ACh by pharmacologically increasing its basal concentrations, in push-pull, slice-superfusion, in vivo voltammetry and in vivo microdialysis studies. AChE inhibitors have significant effects on the ACh transmission and alter physiological conditions by themselves. Thus, caution is indicated in the interpretation of potentially biased data in the presence of AChE inhibitors. Highly sensitive measurement of ACh, which has been recently developed, permits measurement of ACh under physiological conditions. This new method may contribute to progress in ACh pharmacology and neuroscience, and help to develop new therapeutic drugs for Alzheimer's disease and cognitive dysfunction in schizophrenia or other mental illness.

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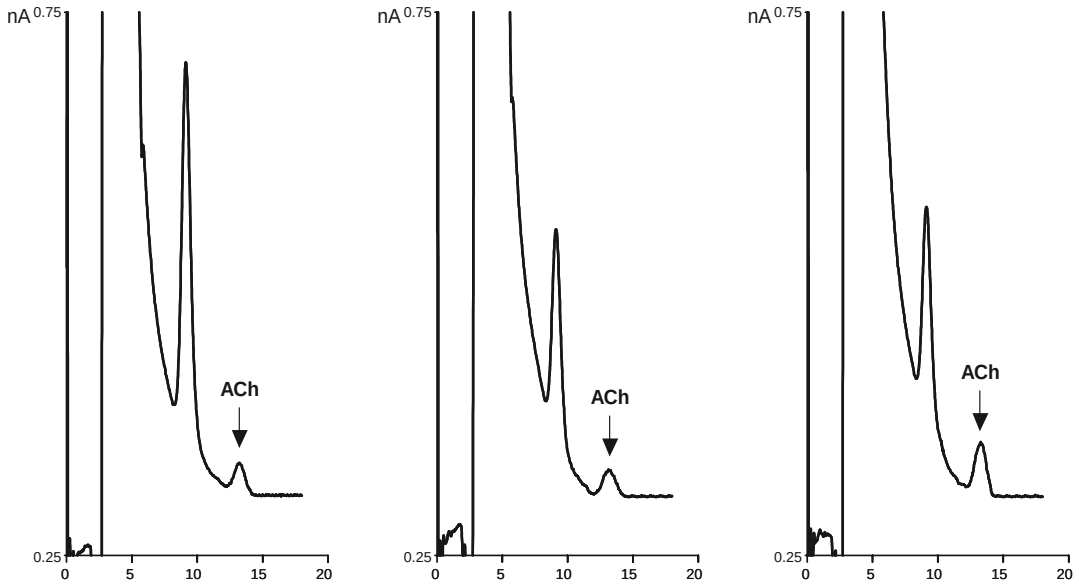
***F3***

*Separation of acetylcholine (ACh) peak in the chromatogram obtained from an authentic standard solution (10 fmol, left panel) and dialysate samples (center and right panels) in rat medial prefrontal cortex (mPFC). The ACh peak was clearly separated and reliably detected in the chromatogram. Clozapine (20 mg/kg, s.c.) significantly increased the height and area of ACh peak compared to the basal peak. Detection limit was about 1-2 fmol per sample. Vertical axis indicates nA and horizontal axis indicates time after injection (min), respectively.*



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