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Short communication

Improved method for the routine determination of acetylcholine and choline in brain microdialysate using a horseradish peroxidase column as the immobilized enzyme reactor

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Abstract

A modified microbore high-performance liquid chromatography-immobilized enzyme reactor-electrochemical detection system for acetylcholine (ACh) and choline (Ch) was developed. The system used the horseradish peroxidase and a solution mediator ferrocene to convert the analyte into an oxidized ferrocene species which was detected electrochemically by reduction at 0 mV. There was an excellent linear relationship between the concentration of ACh/Ch and the peak height over the range of 1–5000 nmol/l. The limit of detection for ACh was 2 fmol/5 μl (S/N=3:1). Compared with the common method recommended by Bioanalytical System Inc. (BAS), this method exhibits a 200-fold improvement in the detection limit. The ACh and Ch levels in rat brain microdialysate were examined.

Keywords: Microdialysis; Acetylcholine; Choline; Horseradish peroxidase

1. Introduction

Acetylcholine (ACh) was the earliest discovered neurotransmitter and choline (Ch) is both its precursor and metabolite. Cholinergic neurons are located throughout the entire central nervous system and are involved in such diverse behaviors as sleep, location, as well as learning and memory [1]. There is also evidence the abnormalities of central cholinergic functions are related to various neural diseases, including Alzheimer's and Parkinson's diseases [2]. These facts have made the determination of ACh and Ch an integral part of cholinergic research.

Several methods for ACh detection have been developed including bioassays, radioenzymatic assay, gas chromatography (GC) and mass spectrometry (MS), as well as techniques utilizing high-performance liquid chromatography (HPLC) [3]. Indeed, HPLC–electrochemical detection (ED) and GC–MS methods are the most frequently used methods for the analyses of ACh and Ch.

In 1983, a simple, rapid method for the determination of ACh in neuronal tissue by means of HPLC with ED was firstly described by Potter et al. [4]. Since then, several investigations have focused their attention on this method. Subsequent modifications, including column packing optimization, enzyme

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immobilization and modifications of the electrochemical detector, have improved the methodology to enable the detection of basal levels of ACh in rat brain dialysate. Damsma et al. [5] extended this method (the solution enzymes) by covalent immobilization of ACh esterase and choline oxidase in a post-column reactor. A sandwich-type enzyme reactor in which the enzymes are physically immobilized in a minimal dead space between two cellulose membranes, resulting in improved sensitivity, was developed by Flentge et al. [6]. However, research has been hampered by the inadequate sensitivity of classical HPLC techniques. A reliable microbore LC assay system is an excellent choice. In addition, commercial ACh and Ch assay kits with microbore separation column and immobilized enzyme reactor (IMER) are available from the Bioanalytical System (BAS, West Lafayette, IN, USA) and from Eicom (Kyoto, Japan).

Further modifications such as micro IMER [7], micro HPLC [8], and modified ED [9] greatly enhanced the sensitivity and selectivity. Many of these were further modifications of electrodes. Two different Pt-target working electrode designs (Kel-F/Pt and PEEK/Pt) were examined by Greaney [10] and both enabled a low fmol limit of detection. An osmium poly(vinylpyridine) redox polymer ''wired'' horseradish peroxidase (HRP) electrode has been used by Yang et al. [11]; while a peroxidase-redox polymer-modified glassy carbon electrode was used by Huang et al. [12]. With this method, a detection limit of 10 fmol (injected) for ACh ($S/N=3:1$) was obtained. Ikarashi et al. [13] used a modified glassy carbon electrode, i.e. the plastic formed carbon (PFC), which can effectively eliminate the interference of catecholamines (CAs). A horseradish peroxidase–osmium redox polymer-modified glassy carbon electrode (HRP–GCE) has been employed by Kato et al. [14] and more recently, a disposable screen-printed, film carbon electrode (PFCE), modified with osmium–poly(vinylpyrridine)-wired HRP gel polymer (Os–gel–HRP) provided a useful simplification of standard chromatographic assays. The limit of detection for ACh was 16 fmol/10 μl. In 1998, Kehr et al. [15] improved the precision of the assay by applying the HRP–Os (PVP)-coated electrode technology together with the external standardization with acetylethylhomocholine (AEHCh) or butyrylcholine (BCh). However, the preparation procedure of the electrode is very inconvenient, including several modifying steps (mixing, curing, etc.), and it was expected to be difficult to obtain reproducible results between equally prepared electrodes.

In the present study, a modified microbore HPLC–IMER–ECD system for the determination of ACh and Ch was established. The system used ferrocene (Fc) as a solution mediator. The enzyme (HRP) was immobilized on silica–NH_2 beads and packed into a short column. The linearity, sensitivity, reproducibility, recovery and the limit of detection were discussed.

2. Experiment

2.1. Subjects

ACh (A.R.), Ch (A.R.), HRP (EC.1.11.1.7), glutaraldehyde, ferrocenecarboxylic acid and neostigmine were purchased from Sigma (St. Louis, MO, USA). ProClin was purchased from BAS (Indiana, USA). Amino silica beads (YWG-NH_2, 10 μm) were purchased from Tianjin Second Chemical Reagents (Tianjin, China). All other chemicals were analytical grade. Artificial cerebrospinal fluid (aCSF) was composed of 142 m*M* NaCl, 3.0 m*M* KCl, 1.2 m*M* $CaCl_2$, 1.0 m*M* $MgCl_2$, 1.35 m*M* Na_2HPO_4, 0.3 m*M* NaH_2PO_4. All solutions were made from doubly distilled deionized water and were filtered through a 0.2-μm filter.

2.2. Microdialysis sampling

Sprague–Dawley rats (250–300 g) were anaesthetized with chloral hydrate (345 mg/kg, i.p.). And microdialysis guide cannulas (BAS/MD-2250; BAS) were implanted in the left hippocampus (AP=−4.8 mm, L=4.9 mm from bregma; V=3.5 mm from dura) [16] using standard stereotaxic procedures. At least 24 h before microdialysis, a microdialysis probe (BR-4; BAS) was inserted that projected beyond the guide cannula. The microdialysis probes were perfused with aCSF containing 0.5 μ*M* neostigmine via FEP tubing connected to a microinjection pump (CMA/100; CMA, Sweden) through a liquid swivel,

at a flow-rate of 1.0 μl/min. The outlet of the probe was connected via low dead volume FEP tubing (10 μl/m, BAS/MD; BAS) to a BAS honeycomb fraction collector. The operant conditioned reflex is used widely to study learning and memory. In this study, training was conducted in a Skinner box where foot shocks (700 μA; 8 s duration) were delivered as unconditioned stimuli (US) and frequency-modulated lights as conditioned stimuli (CS). Rats were allowed to move freely in a Skinner box at all times during microdialysis experiments using a BAS BeeKeeper. Samples were collected at intervals of 6 min and immediately frozen for off-line analysis.

2.3. HPLC–IMER–ECD system for ACh and Ch

The system consisted of a LB-1 micro LC pump (Xingda, Beijing, China), a Rheodyne HPLC valve with 5 μl injection loop, BAS microbore ACh/Ch kit, including a SepStik ACh/Ch analytical column (530×1 mm I.D., 10 μm) (MF-8904) and a SepStik ACh/Ch IMER (50×1 mm I.D.) (MF-8903), a small enzyme bed reactor (homemade, containing HRP) and a radial flow cell containing a 6-mm downstream glassy carbon electrode held at 0 mV vs. Ag/AgCl (BAS). The mobile phase was Na_2HPO_4 (50 m*M*), ferrocenecarboxylic acid (0.5 m*M*), Na_2EDTA (1 m*M*), ProClin (0.5% v/v) pH 7.0, and the flow-rate 100 μl/min. Potential was maintained using a potentiostat (EG&G model 174A; Princeton, NJ, USA) and data was stored electronically using an AC-1020 analogue to digital card and home-written software.

2.4. Preparation of the immobilized enzyme reactor

HRP was immobilized on the amino silica beads (10 μm). The preparation of HRP column was as follows. Briefly, HRP (1 mg enzyme to 10 mg beads) were linked at 4 °C to silica beads with surface amino groups using 2.5% glutaraldehyde as a cross linker. After linkage, washed beads were slurry packed at a pressure of less than 100 kg/cm^2 into PEEK tubings (20×0.75 mm I.D.) containing a frit to form the HRP enzyme microbore reactor. The buffer used for all couplings was 40 m*M* Na_2HPO_4 (pH 8) with 0.5% ProClin added as a preservative. HRP enzyme reactor was inserted between the SepStik ACh/Ch IMER and the flow cell.

3. Results and discussion

The common method is based on the separation of ACh and Ch by microbore cation-exchange HPLC followed by enzymatic conversion to betaine and hydrogen peroxide by a post-column IMER contained in a BAS microbore ACh/Ch kit. The hydrogen peroxide was then quantitated electrochemically using a glassy carbon electrode held at +500 mV vs. Ag/AgCl. The high electrode potentials required would also detect other compounds present in the dialysate and result in a complex chromatogram. Also, at the high potential, the electrode needs a longer time to equilibrate than it does at the low potential.

In the present study, we used the enzyme HRP and a solution mediator Fc to convert the hydrogen peroxide into water. The HRP is regenerated by the oxidation of the Fc species present in the buffer pumped through the short column. The Fc^+ species produced are electrochemically reversible (with an $E_{1/2}$=0.225 V) and are detected very efficiently by reduction at a downstream electrode. The low potential of electrode (0 mV vs. Ag/AgCl) prevents oxidation of other species in the sample, and a single peak is produced.

Typical chromatograms of ACh/Ch standards detection are shown in Fig. 1A–C. These chromatograms demonstrate that the separation of ACh and Ch with the present microbore HPLC–IMER–ECD system is quite satisfactory. The retention time of ACh and Ch are all less than 13.5 min. At the operated potential, the electrode equilibrates rapidly. Analysis of aCSF showed no contaminant peaks capable of co-eluting with either analyte.

3.1. Linearity and limit of detection

The calibration curves were determined based on at least 12 standard solutions prepared by sequential dilution of a stock solution with aCSF, in the concentration range 1–5000 nmol/l. The injection volume was configured with a 5-μl sample loop. There is an excellent linear relationship between the

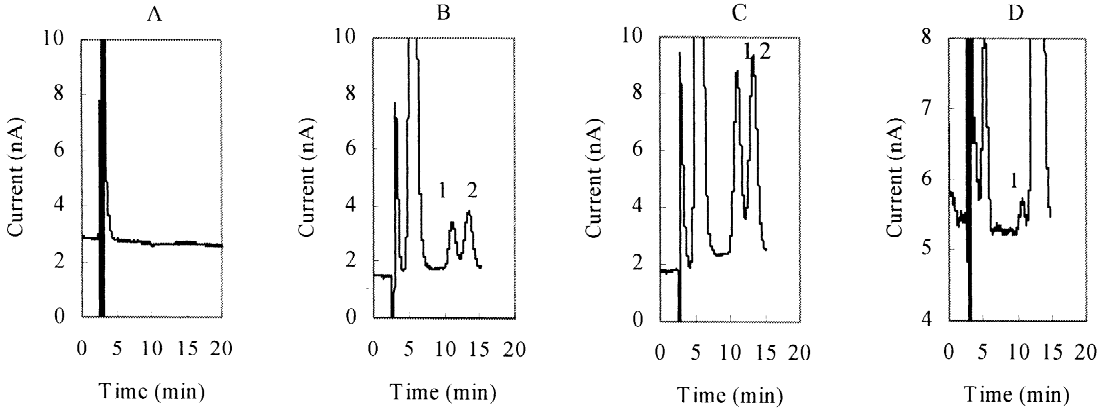

Fig. 1. Typical chromatograms of: (A) artificial cerebrospinal fluid (aCSF), (B) 0.05 μmol/l acetylcholine/choline standard, (C) 0.2 μmol/l acetylcholine/choline standard, and (D) baseline acetylcholine release from hippocampus. The microdialysis probe was perfused with aCSF containing 0.5 μ*M* neostigmine. Peak 1: acetylcholine; peak 2: choline. The injection volume was configured with a 5-μl sample loop.

concentration and the peak height. The linear equation and corresponding regression coefficients for ACh were $y = 2.72x + 0.08$ and $r^2 = 1$, respectively. Those for Ch were $y = 3.13x - 0.08$ and $r^2 = 0.9999$, respectively. The limit of detection for ACh and Ch were 2 fmol/5 μl ($S/N = 3{:}1$).

3.2. Reproducibility

The relative standards deviation for eight replicate determination of 0.2 μmol/l ACh/Ch and 0.05 μmol/l ACh/Ch solution were all <4%. The inter-day peak-height precision for 0.4 μmol/l Ch standards analyzed over three consecutive days was 12.05±0.04 nA (mean±SEM), while the figures for 0.4 and 0.05 μmol/l ACh standards were 12.47±0.04 and 1.41±0.07 nA (mean±SEM), respectively. The injection volume was configured with a 5-μl sample loop.

The HRP column is stable. No loss of activity was found within 8 months if the immobilized enzyme column was preserved carefully to eliminate the growth of bacteria (known to be efficient scavengers of H_2O_2). Addition of Na_2EDTA and the biocide ProClin help to prevent the bacteria growth.

3.3. Recovery

The recovery was determined by adding known amounts of the standard to samples of dialysate. It gives a quantitative indication of the overall influence of sample constituents on both the conversion efficiency of the IMER involved and the HRP-column reactor. Two different additions of analyte were made. The recovery of ACh and Ch were shown in Table 1.

3.4. Dialysates assay

The concentration of ACh and Ch were quantified by comparing the peak heights of the sample with the heights in the external standards. External standards were analyzed before and during the experiment to monitor the electrode's response. The chro-

Table 1
The recovery for acetylcholine (ACh) and choline (Ch)

	Added (pmol)	Mean found (pmol)	Recovery (%)	RSD (%, $n = 6$)
ACh	5.5	5.80	105.5	0.25
	8.0	7.54	94.2	0.11
Ch	105	102.9	98.0	5.5
	185	168.0	90.8	4.8

matogram of basal ACh and Ch detection in the dialysate of rat brain was shown in Fig. 1D. The concentration of Ch can be seen to be much higher and off-scale in the chromatogram. During the 2.5-h perfusion with aCSF containing 0.5 μ*M* neostigmine, the concentration of ACh in the rat dialysate samples was relatively stable. The concentration of basal ACh was 14±1 fmol/μl (mean±SEM, $n=25$). The ACh outflow observed with the method described here was similar to that reported earlier [17]. During the behavioral session, the ACh and Ch levels increased (Fig. 2).

The common detection system recommended by BAS, without HRP column and a solution mediator ferrocene (the rest is similar to the modified detection system) was tested at the same time. The linear equation and corresponding regression coefficients for ACh over a concentration range of 0.2–10 μmol/l were $y=0.51x+0.21$ and $r^2=1$, respectively. Those for Ch were $y=0.51x+0.56$ and $r^2=0.9998$, respectively. The limit of detection was 0.4 pmol/5 μl. The glassy carbon electrode has the disadvantage of a long equilibration time. Also, it is difficult to detect the ACh in microdialysates directly by this system.

The above results indicated that the present microbore HPLC–IMER–ECD system provides a sufficiently low detection limit for basal ACh determination. This assay is not only 200-fold more sensitive than the common assay for the detection of ACh and Ch, but also equilibrates significantly more rapidly. The high sensitivity is attributable to the use of the HRP column. The HRP column preparation procedure is very easy. In the present study, three HRP columns were prepared at different time and obtained reproducible results (data not shown).

4. Conclusion

In conclusion, a modified microbore HPLC–IMER–ECD system for determination of ACh and Ch was built in the present study. A 200-fold improvement in the detection limit was achieved using a HRP column reactor. Regarding its characteristic (linear range, low detection, sensitivity and reproducibility), this assay has to be considered a very attractive analytical method. The method has been used to detect ACh and Ch levels in rat brain microdialysate. The data show that during the operant conditioned reflex, the concentrations of ACh and Ch in dialysate were increased.

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References

[1] M.M. Mesulam, in: G. Adelman (Ed.), Encyclopedia of Neuroscience, Birkhauser, Boston, 1987, p. 233.
[2] A. Fisher, I. Hanin, C. Lachman, Alzheimer's and Parkinson's Deceases: Strategies For Research and Development, Plenum Press, New York, 1986.

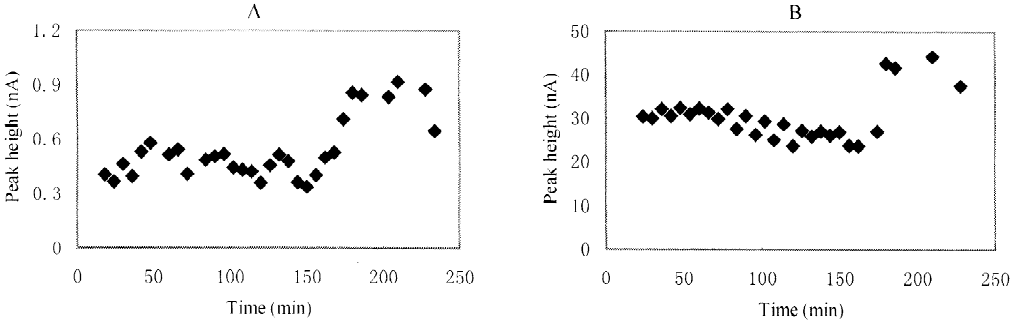

Fig. 2. The change of the microdialysis output of acetylcholine (A) and choline (B) during the operant conditioned reflex. The microdialysis probe was perfused with aCSF containing 0.5 μ*M* neostigmine.

[3] T.H. Tsai, J. Chromatogr. B 747 (2000) 111.
[4] P.E. Potter, J.L. Meek, N.H. Neff, J. Neurochem. 41 (1983) 188.
[5] G. Damsma, B.H.C. Westerink, A. Imperato, H. Rollema, J.B. De Vries, A.S. Horn, Life Sci. 41 (1987) 873.
[6] F. Flentge, K. Venema, T. Koch, J. Korf, Anal. Biochem. 204 (1992) 305.
[7] T.R. Tsai, T.M. Cham, K.C. Chen, C.F. Chen, T.H. Tsai, J. Chromatogr. B 678 (1996) 151.
[8] C. Giaroni, L. Somaini, F. Marino, M. Cosentino, O. Leoni, F.D. Ponti, S. Lecchini, G. Frigo, Neurosci. Lett. 232 (1997) 9.
[9] A.J. Carter, J. Kehr, J. Chromatogr. B 692 (1997) 207.
[10] M.D. Greaney, J. Chromatogr. 622 (1993) 125.
[11] L. Yang, E. Janle, T.H. Huang, J. Gitzen, P.T. Kissinger, Anal. Chem. 67 (1995) 1326.
[12] T.H. Huang, L. Yang, J. Gitzen, P.T. Kissinger, M. Vreeke, A. Heller, J. Chromatogr. B 670 (1995) 323.
[13] Y. Ikarashi, C.L. Blank, Y. Suda, T. Kawakubo, Y. Maruyama, J. Chromatogr. B 718 (1995) 267.
[14] T. Kato, J.K. Liu, K. Yamamoto, P.G. Osborne, O. Niwa, J. Chromatogr. B 682 (1996) 162.
[15] J. Kehr, P. Dechent, T. Kato, S.O. Ögren, J. Neurosci. Methods 83 (1998) 143.
[16] X.M. Bao, S.Y. Shu, The Stereotaxic Atlas of the Rat Brain, People's Health Publication Press, Beijing, 1991.
[17] C.M. Thiel, C.P. Muller, J.P. Huston, R.K.W. Schwarting, Brain Res. 882 (2000) 112.