ELSEVIER

Journal of Chromatography B, 682 (1996) 162–166

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Detection of basal acetylcholine release in the microdialysis of rat frontal cortex by high-performance liquid chromatography using a horseradish peroxidase–osmium redox polymer electrode with pre-enzyme reactor

Takeshi Kato[a,*], Jian Kai Liu[a], Katsunobu Yamamoto[b], Peter G. Osborne[b], Osamu Niwa[c]

[a]*Laboratory of Molecular Recognition, Graduate School of Integrated Science, Yokohama City University, 22-2 Seto, Kanazawa-ku, Yokohama 236, Japan*

[b]*Bioelectro Analytical Science Japan Co., 1-36-4 Oshiage, Sumida-ku, Tokyo 131, Japan*

[c]*NTT Basic Research Laboratories, Morinosato, Wakamiya, Atsugi, Kanagawa 243-01, Japan*

Received 1 December 1995; revised 25 January 1996; accepted 5 February 1996

Abstract

To determine the basal acetylcholine level in the dialysate of rat frontal cortex, a horseradish peroxidase–osmium redox polymer-modified glassy carbon electrode (HRP–GCE) was employed instead of the conventional platinum electrode used in high-performance liquid chromatography–electrochemical detection (HPLC–ED). In initial experiments, an oxidizable unknown compound interfered with the detection of basal acetylcholine release on HPLC–HRP–GCE. An immobilized peroxidase–choline oxidase precolumn (pre-reactor) was included in the HPLC system, to eliminate the interference from the unknown compound. This combination could detect less than 10 fmol of standard acetylcholine and basal acetylcholine levels in the dialysate from a conventional concentric design microdialysis probe, without the use of cholinesterase inhibitor, and may facilitate physiological investigation of cholinergic neuronal activity in the central nervous system.

Keywords: Horseradish peroxidase–osmium redox polymer electrode; Acetylcholine

1. Introduction

Pathology of cholinergic function has been implicated as having a crucial role in the etiology of Alzheimer's disease [1–4]. The methods for the routine determination of basal levels of acetylcholine (ACh) in the central nervous system, without the need to use uptake inhibitors, will be an invaluable tool in the study of certain neurobiological diseases. In the last decade, brain microdialysis coupled to a high-performance liquid chromatography (HPLC) utilizing electrochemical detection (ED) with a post-column immobilized reactor (post-reactor), containing acetylcholinesterase (AChE) and choline oxidase (ChO), has been introduced as a sensitive method to measure the in vivo ACh release in the brain of freely moving rats [5–7].

Since the extracellular concentration of ACh in the brain is very low, due to its hydrolysis by AChE [8–10], many investigators have employed AChE

*Corresponding author.

0378-4347/96/$15.00
PII S0378-4347(96)00079-5

inhibitors, such as physostigmine [4–7] in the measurement of ACh. Unfortunately AChE inhibitor affects neuronal activity [10,11] and thus affects the pharmacology of the neural circuits under investigation. Recently, Vreeke et al. [12] developed a horseradish peroxidase (HRP)–osmium (Os) redox polymer-modified glassy carbon electrode (HRP–GCE) for the detection of hydrogen peroxide, and Huang et al. [13] utilized this method to determine basal ACh release in rat striatum with microdialysis. The detection limit of the method was as low as 10 fmol for ACh. However, in our laboratory we have been unable to replicate the sensitivity of these results.

This prompted us to develop an alternative method for the detection of the basal ACh level in the dialysate of rat frontal cortex using HPLC, enzymatic production and degradation of H_2O_2 and HRP–GCE.

2. Experimental

2.1. Materials

Choline oxidase, horseradish peroxidase, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and N-methyl-D-aspartate (NMDA) were purchased from Sigma. HRP–osmium redox polymer was from BAS (IN, USA). Microbore separation column (No. 51-0581, 450×1 mm) was from BASJ (Tokyo, Japan). Concentric design microdialysis probe (CMA/12 4 mm dialyzing membrane) was from CMA microdialysis (Stockholm, Sweden). Other chemicals and solvent were of analytical reagent grade. Male Wistar rats weighing 150–180 g were obtained from the Sankyo laboratory (Tokyo, Japan) and housed with ad libitum access to food and water. A 12:12 h light–dark cycle was maintained over two weeks prior to and during experimentation.

2.2. Microdialysis

Once the rat was anaesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), the animal was placed in a stereotaxic apparatus and a microdialysis cannula was slowly (1-2 min) implanted into the left frontal cortex of the rat (coordinated at 1 mm anterior to the bregma, 1.5 mm lateral to the midline and 3.5 mm ventral to the dura, with a lateral inclination of 50°) according to the atlas of Paxinos and Watson [14] and fixed with dental cement to a screw implanted into the skull. One day after the surgery, when the rat was conscious, the cannula was perfused with HEPES–Ringer's solution (147 m*M* NaCl, 4 m*M* KCl, 2.3 m*M* $CaCl_2$, 1 m*M* HEPES, pH 7.0). A flow-rate of 1 μl/min and sampling intervals of 20 min were employed. After preperfusion for 2 h, and once three or four stable cortical dialysate samples were collected as basal levels, the animal was subsequently perfused with 10 μ*M* NMDA.

2.3. HPLC

The HRP–Os-redox polymer-coated electrode was operated at 0 mV vs. Ag/AgCl. ACh was analyzed using HPLC–HRP–GCE with HRP/ChO precolumn (pre-reactor) (5×1 mm) and AChE–ChO postcolumn (post-reactor) (6×0.75 mm) (Fig. 2B). A standard sample, or 15 μl of the dialysate (perfusate), was injected into the HPLC analysis system. The mobile phase was 0.05 *M* sodium phosphate buffer (pH 8.5) containing 0.1 m*M* EDTA and the flow-rate was 60 μl/min.

3. Results and discussion

We attempted to replicate the study of Huang et al. [13] and to determine the basal level of ACh in the dialysate from the frontal cortex of a rat. However, we were continually unsuccessful. A representative chromatogram of a dialysate is Fig. 1A. We then devised an alternative method for the detection of the basal level of ACh in the dialysate of rat frontal cortex, using HPLC, enzymatic production and degradation of H_2O_2 and HRP–GCE.

To determine basal ACh release in the frontal cortex of the unanaesthetized rat, the pre-reactor, containing HRP–ChO immobilized enzymes, was connected to a HPLC–HRP–GCE unit as shown in Fig. 2A and 2B. A 20 fmol amount of ACh in standard solution showed a significant peak at 23.4 min (Fig. 1C) and had a similar detection limit to that reported by Huang et al. [13], i.e., 10 fmol

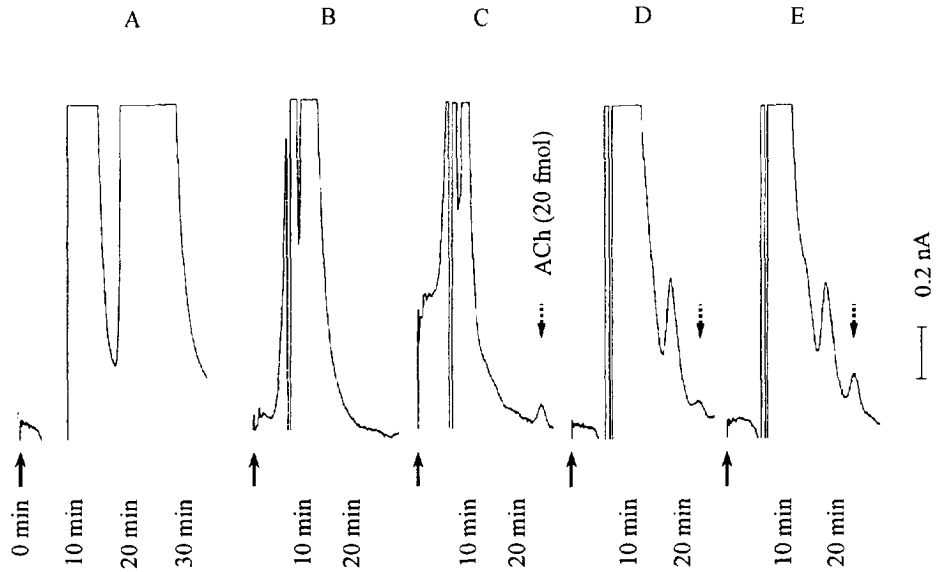

Fig. 1. Chromatograms of acetylcholine related compounds in standard solution or in the dialysate of rat frontal cortex. (A) A 15-μl volume of basal dialysate in rat frontal cortex without connecting the pre-reactor; (B) a standard solution contained 50 pmol of choline; (C) a standard solution contained 20 fmol of acetylcholine; (D) a 15-μl volume of basal dialysate; (E) a 15-μl volume of the dialysate during the perfusion of 10 μM NMDA solution. ↑=Injection; ↓=acetylcholine peak.

utilizing a signal-to-noise ratio of 3. As shown in Fig. 2 and Fig. 3, the detection limit of standard ACh was less than 10 fmol. The present method specifically detects the ACh peak. The precolumn eliminates the endogenous choline level in the dialysate of rat frontal cortex [1.3 μM (20 pmol/15 μl)] [10], because 50 pmol of standard choline does not show any peak at 25 min (Fig. 1B).

In initial attempts, when a pre-reactor was not employed, the dialysate showed two large peaks with retention times at 20 and 25 min, respectively (Fig. 1A). The slower peak at 25 min was derived from choline, because this peak is in accordance with the authentic choline peak and disappears in the presence of the HRP–ChO pre-reactor (Fig. 1B). The faster peak at 19.6 min significantly decreased in the presence of the pre-reactor (Fig. 1D) compared with the absence of the pre-reactor (Fig. 1A), while the peak did not disappear.

A typical chromatogram of basal ACh detection in the dialysate of rat frontal cortex is shown in Fig. 1D. The concentration of ACh in the dialysate was 17±4 (mean±S.D., n=7) fmol/15 μl. The basal level in the present study is slightly lower than the level in our previous experiments [10]. It may be due, in part, to the use of microdialysis probes of differing construction. In a previous study [10], we used microdialysis probes with a U-shaped dialysis membrane, thus the total length of the U-shaped probe was twice that of the I-type probe. As shown in Fig. 1E, perfusion of 10 μM NMDA significantly increased the ACh level in the dialysate, indicating that the present method is capable of determining cortical ACh release in response to a pharmacological stimulus.

The chromatograms shown in the paper by Huang et al. [13] did not show any peak around the ACh peak. Possible explanations for the discrepancy are as follows. As different neural networks between the striatum and frontal cortex have different chromatographic patterns of the dialysates in both brain regions, the peak at 20 min (Fig. 1A) may not appear in the striatum. Another possible explanation is that the animals were allowed to recover from surgery for 4 days in their experiment, whereas, in our experiment, the animals were perfused the day after surgery.

In comparison with a normal detection system using a conventional platinum electrode, HPLC–HRP–GCE is not only three to five times more

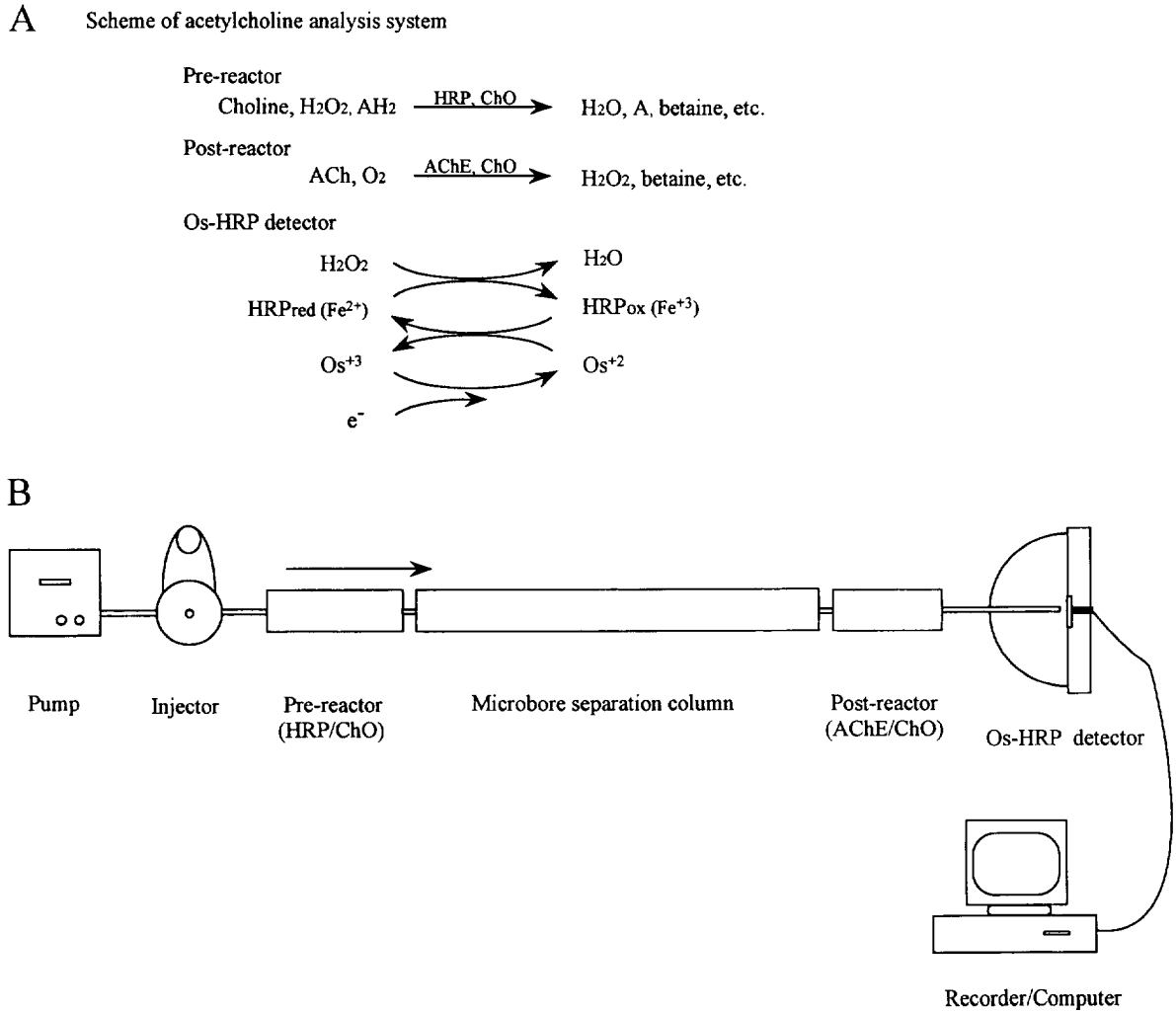

Fig. 2. Scheme of the present acetylcholine assay method by high-performance liquid chromatography–horseradish peroxidase–immobilized glassy carbon electrode with pre- and post-immobilized enzyme reactors. (A) Scheme of the principle of the acetylcholine analysis system. AChE=acetylcholinesterase; ChO=choline oxidase; HRP=horseradish peroxidase; AH_2=oxidizable compound. (B) Highly sensitive detection system for acetylcholine by HPLC–HRP–GCE with immobilized enzyme reactors. Pre-reactor, HRP and ChO-immobilized precolumn; separation column, 450×1 mm of ion-exchange microbore column; post-reactor, AChE and ChO-immobilized postcolumn; Os–HRP detector, HRP–immobilized osmium–polymer I-coated glassy carbon electrode.

sensitive for the detection of ACh, but also equilibrates significantly more rapidly, as reported by Huang et al. [13]. Furthermore, the present method regains some oxygen by the pre-reactor (Fig. 2A) and stabilizes the ChO enzyme, both in the pre- and post-reactors. It means that the present assay system is more stable than the system reported by Huang et al. [13] for the determination of the ACh level over a longer time period. Since our preliminary experiment, we have measured the basal ACh level in the microdialysate of rat hippocampus as 9±5 fmol/15 μl (n=8), further illustrating the useful application

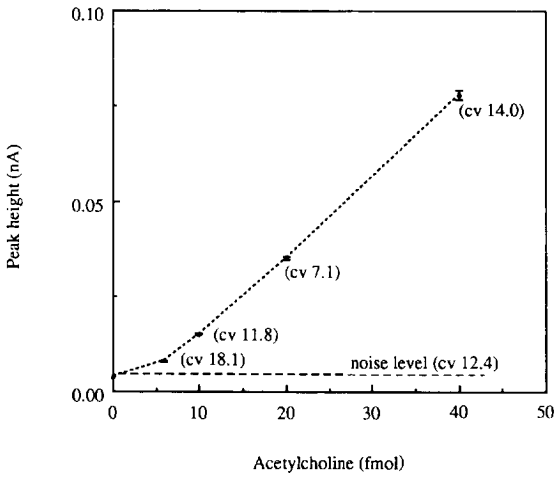

Fig. 3. Sensitivity and linearity of standard acetylcholine detection in the present HPLC system. The concentration of standard acetylcholine ranged from 6 to 40 fmol. Data are shown as mean±S.D. of three to five samples. cv=coefficient of variation; noise level=chromatogram without acetylcholine.

of this methodological refinement of ACh measurement for the study of cholinergic neuronal activity, such as memory and learning.

References

[1] P. Davies and A.H. Verth, Brain Res., 138 (1977) 385.
[2] J.T. Coyle, D.L. Price and M.R. Delong, Science, 219 (1983) 1184.
[3] W.W. Beatty, N. Butters and D.S. Janowsky, Behav. Neurol. Biol., 45 (1986) 196.
[4] M. Xu, Y. Nakamura, T. Yamamoto, K. Natori, T. Irie, H. Utsumi and T. Kato, Neurosci. Lett., 123 (1991) 179.
[5] S. Consolo, C.F. Wu, F. Fiorentini, H. Ladinsky and A. Vezzani, J. Neurochem., 48 (1987) 1459.
[6] G. Damsma, B.H.C. Westerink, J.B. de Vries, C.J. Van den Berg and A.S. Horn, J. Neurochem., 48 (1987) 1523.
[7] A. Ajima and T. Kato, Neurosci. Lett., 81 (1987) 129.
[8] G. Damsma, B.H.C. Westerink, P. de Boer, J.B. de Vries and A.S. Horn, Life Sci., 43 (1988) 1161.
[9] A. Ajima and T. Kato, Biogenic Amines, 5 (1988) 461.
[10] J.K. Liu and T. Kato, Neurochem. Int., 24 (1994) 589.
[11] P. de Boer, B.H.C. Westerink and A.S. Horn, Neurosci. Lett., 116 (1990) 357.
[12] M. Vreeke, R. Maidan and A. Heller, Anal. Chem., 64 (1992) 3084.
[13] T. Huang, L. Yang, J. Gitzen, P.T. Kissinger, M. Vreeke and A. Heller, J. Chromatogr. B, 670 (1995) 323.
[14] G. Paxinos and C. Watson (Editors), The Brain in Stereotaxic Coordinates, Academic Press, Sydney, 2nd ed., 1986.