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Determination of acetylcholine by on-line microdialysis coupled with pre- and post-microbore column enzyme reactors with electrochemical detection

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Abstract

A sensitive procedure consisting of a pre- and post-microbore column reactor sequence of a LC–electrochemical detection system coupled with on-line microdialysis system is described in the present study to measure endogenous acetylcholine concentration in freely moving rats. The pre-column packed, with immobilized choline oxidase and catalase, was used to remove choline, whereas the post-column, packed with immobilized acetylcholine oxidase and choline oxidase, was used to measure acetylcholine selectively. The detection limit of acetylcholine was found to be 5 fmol/μl (50 fmol/10 μl). The usefulness of the described methodology was evaluated by examining the change in the striatal acetylcholine concentration of freely moving rats after physostigmine (0.5 mg/kg, s.c.) administration.

Keywords: Acetylcholine; Choline

1. Introduction

Acetylcholine (ACh) has been shown to be involved in the regulation of various neural functions. There is also evidence that abnormalities of central cholinergic functions are related to various neural diseases, including Alzheimer's disease and amnesia [1–6]. Therefore, it is important to have a sensitive method for the measurement of endogenous ACh concentration. In the past decade, numerous methods have been described for ACh and choline (Ch) measurement by using LC–electrochemical detection (ED) [7,8]. However, in order to achieve the necessary detection limit, a reliable microbore LC assay system is an excellent choice. In the previous study [9], we coupled on-line microdialysis with a microbore LC–ED system to determine biological amines, ACh and Ch in freely moving rats. Nevertheless, the peak of ACh will overlap with Ch when the performance of an analytical column is degraded. In the

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present study, we developed a sensitive on-line microdialysis/microbore LC system in conjunction with pre- and post-columns of immobilized enzyme reactor (IMER) to measure ACh selectively. This system removed Ch with the pre-column IMER and maintained selectivity for ACh determination. To validate the usefulness of the described system, the changes in striatal ACh were examined after subcutaneous administration of physostigmine in freely moving rats.

2. Experimental

2.1. Chemicals

Physostigmine sulphate (USP grade) was purchased from Research Biochemical International (Natick, MA, USA). The chromatographic reagent and solvents were obtained from Merck (Darmstadt, Germany). Triple de-ionized water (Millipore, Bedford, MA, USA) was used for all preparations.

2.2. Chromatographic conditions

The LC–ED (BAS, LC-4C, Bioanalytical System, West Lafayette, IN, USA) system consisted of a solvent delivery system (BAS PM-80), with a flow-rate of 0.1 ml/min. ACh was separated with a prepacked ACh analytical microbore column (BAS, 530 × 1 mm I.D., particle size 10 μm), using a mobile phase (pH 8.5) consisting of 28 m*M* Na_2HPO_4 and 0.5% of the antimicrobial solution Kathon (1.0%, BAS). The choline oxidase and catalase combination IMER was used as the pre-column (55 × 1 mm I.D.) to destroy choline. Further addition of the post-column IMER (50 × 1 mm I.D.) containing ACh oxidase and choline oxidase was used to determine ACh only. Hydrogen peroxide was detected by an ED using a platinum electrode set at a potential of +0.5 V vs. Ag/AgCl. The output from the ED was amplified and recorded with a Waters Chromatography interface and software (Millennium 2010, Version 2.0, Millipore, Marlborough, MA, USA) [10,11].

2.3. Microdialysis procedure

Experiments were carried out in adult, male Sprague–Dawley rats (250–320 g). The rat was anesthetized with chloral hydrate (0.4 g/kg, i.p.) and placed in a Kopf stereotaxic frame. Its body temperature was maintained at 37°C with a heating pad. A guide shaft was placed into the right striatum with its tip located at AP 0.4 mm, ML −3.0 mm, DV −4.0 mm, from bregma and dura surface, respectively [12]. One day after surgery, the dialysis probe (CMA-12, dialysing length 4 mm; diameter, 0.5 mm; CMA/Microdialysis AB, Stockholm, Sweden) was inserted through the guide shaft and then perfused with Ringer solution (147 m*M* Na^+, 4.0 m*M* K^+, 2.2 m*M* Ca^{++}) containing 1 μM neostigmine at a flow-rate of 2 μl/min, using a microinjection pump (CMA-100). The outflow of the dialysis samples was connected directly to an on-line injector (CMA-160) and the microbore LC–ED system. The injection volume was configured with a 10-μl sample loop. An injection was made every 20 min (controlled by the microinjection pump, CMA-100). The position of the probe was verified by standard histological procedure at the end of the experiment.

3. Results and discussion

3.1. Specificity

ACh and Ch are neither electroactive nor UV absorbing substances. Determination of ACh by LC is currently accomplished in conjunction with post-column enzymes (ACh esterase and Ch oxidase) to produce H_2O_2 as an electroactive product prior to electrochemical detection. The post-column chromatographic flow sequence is shown in Fig. 1.

By coupling a Ch oxidase and catalase enzyme reactor as the pre-column, the column sequence has the effect of removing Ch, due to the pre-column's Ch oxidase conversion of Ch to H_2O_2 and the subsequent catalase digestion of H_2O_2. Using a similar IMER (ACh esterase and Ch oxidase enzyme reactor) as the post-column allows for the determination of ACh specifically (Fig. 2).

The use of only the post-column IMER which

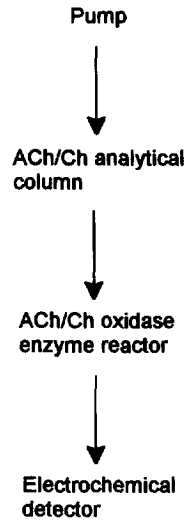

Fig. 1. Post-column enzyme reactor and analytical column sequence.

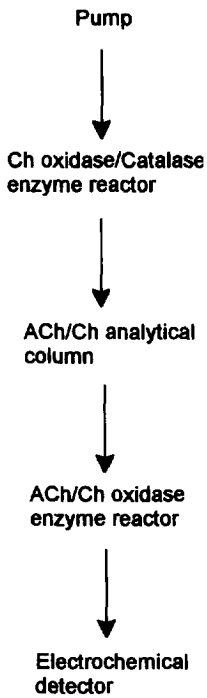

Fig. 2. Pre- and post-column enzyme reactors and analytical column sequence.

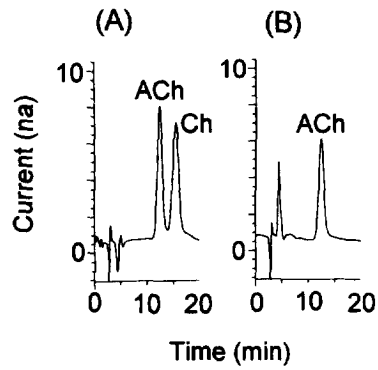

Fig. 3. (A) Chromatogram of ACh and Ch from post-column enzyme reactor sequence. (B) Chromatogram of ACh from pre- and post-column enzyme reactor sequence.

contains ACh esterase and Ch oxidase results in the ACh and Ch peaks shown in Fig. 3(A). The pre-and post-column IMER sequence results in only the ACh peak as shown in Fig. 3(B).

3.2. Linearity, limit of detection and precision

The reaction of exogenous ACh with IMER generates an electroactive product (H_2O_2). Under the chromatographic conditions used, the retention time of ACh was 12.5 min (Fig. 4). The electroactive product, H_2O_2, increased linearly in proportion to the increasing amounts of ACh, which results in a linear concentration–peak area relationship over the range 0.01–2 pmol/μl. The linear equation and corresponding regression coefficients for ACh were $y = 46.22\ x - 0.62$ and $r^2=0.999$, respectively. The detection limit for ACh, at a signal to noise ratio of three, was 5 fmol/μl. The lower practical limit of quantification was 10 fmol/μl. We found that the reproducibility of the reaction was acceptable. For both intra- and inter-assay, the coefficients of variation for determination of 1 pmol/μl ACh were 5.4% ($n = 5$) and 8.2% ($n = 5$), respectively.

3.3. Recovery

The recovery for ACh is the ratio of the ACh concentration in the dialysate, i.e. the outlet from the probe (C_{out}), to the concentration of ACh in the

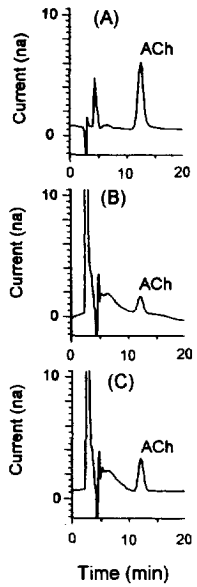

Fig. 4. Typical chromatograms of (A) standard ACh (1 pmol/10 μl), (B) baseline ACh (304 fmol/10 μl) release from striatum, (C) a dialysate sample collected 40 min after subcutaneous administration of physostigmine (0.5 mg/kg, s.c.).

medium surrounding the probe (C_{in}). $\text{Recovery}_{\text{in vitro}} = C_{out}/C_{in}$

Under the conditions described above, the in vitro recovery of the microdialysis probe, based on 1 pmol/μl ACh, was 19% (n = 4).

3.4. Microdialysis

Before the experiment, ACh standard was injected into the assay system for calibration (Fig. 4). Dialysates collected over the first 120 min were discarded to allow recovery from the acute effects of the implantation procedure. After stable baseline values (346 ± 37 fmol/10 μl, n = 5) were obtained, physostigmine (0.5 mg/kg) was injected subcutaneously and dialysates were further measured for another 120 min. Variation in ACh concentration in the dialysates was mainly due to either probe location or probe consistency. Data were, therefore, expressed as percentage of basal level within the same experiment. Administration of physostigmine caused about a 40% increase in the concentration of ACh present 20 and 40 min after administration (Fig. 5). The ACh outflow observed with the method

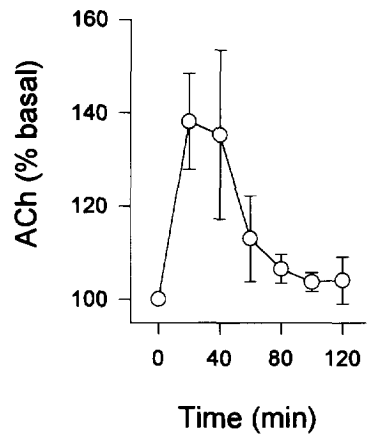

Fig. 5. The effect of physostigmine (0.5 mg/kg, s.c.) on the microdialysis output of ACh. Physostigmine was administered at time 0. Values represent the group mean ± S.E.M. (n = 5). The basal values of dialysate concentrations were based on an average of the two samples prior to physostigmine administration.

described here was similar to that reported earlier [13–16].

4. Conclusion

The present study demonstrates an on-line monitoring of ACh in the freely moving rats. The major advantage for determination of ACh using pre- and post-microbore column reactor sequence LC–ED system coupled with an on-line microdialysis system, was increasing sensitivity as well as selectivity. The method should be beneficial for further central cholinergic studies.

Acknowledgments

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