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Short communication

Microbore high-performance liquid chromatographic method for measuring acetylcholine in microdialysis samples: optimizing performance of platinum electrodes

Adrian J. Carter[a,*], Jan Kehr[b]

[a]*Department of Biological Research, Boehringer Ingelheim KG, D-55216 Ingelheim, Germany*
[b]*Department of Neuroscience, Karolinska Institute, S-17177 Stockholm, Sweden*

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Abstract

A microbore high-performance liquid chromatographic method with electrochemical detection was applied to the measurement of acetylcholine in microdialysis samples. There was an excellent linear relationship ($r=0.99998$) between the concentration of acetylcholine injected onto the column and the peak height (0.05–10 pmol/5 μl). During the validation of this method, we noticed that the peak height for acetylcholine decreased over time, coupled with the appearance of a brown coating on the surface of the platinum electrode. Repeated measurement of acetylcholine standards which had been stored at 4°C and −20°C before and after cleaning the platinum electrode with ethanol or methanol indicated that the decrease in the peak height of acetylcholine is caused by a decrease in sensitivity of the electrode itself. Results with a second microbore high-performance liquid chromatographic system confirmed these findings. On the basis of these results, we recommend that the platinum electrode is cleaned periodically with ethanol or methanol, and that quantitation is regularly calibrated with external acetylcholine.

Keywords: Platinum electrodes; Acetylcholine

1. Introduction

Acetylcholine (ACh) was the very first ever neurotransmitter to be isolated and characterized. It is synthesized from choline by means of acetyl coenzyme A and choline acetyltransferase (EC 2.3.1.6) [1]. The actions of ACh in the central nervous system are mediated by two different classes of receptor, namely nicotinic and muscarinic. With the revolution in molecular biology over the past decade, the primary structures of several subtypes of both classes of receptor have been elucidated, as have the structures of the enzymes which synthesize, transport and eliminate this important neurotransmitter [1]. Similarly, modern techniques of liquid chromatography and microdialysis have also revolutionized the measurement of ACh in neuronal tissue over the same period.

In 1983, Potter et al. [2] described a simple, rapid method for the determination of ACh in neuronal tissue by means of high-performance liquid chromatography (HPLC) with electrochemical detection. The method was based on the separation of ACh from choline by ion-paired, reversed-phase HPLC

*Corresponding author.

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followed by enzymatic conversion to betaine and hydrogen peroxide by mixing the effluent with a solution containing choline oxidase (ChO) and acetylcholinesterase (AChE) enzymes. The hydrogen peroxide was then quantitated electrochemically. Microdialysis is a technique which allows continual perfusion of discrete areas of the brain with minimal exposure of the brain tissue to the perfusion medium by means of a special probe [3]. The techniques of HPLC and microdialysis have been used together in the past to determine the effects of various centrally acting drugs on ACh release in the brains of rats [4,5]. However, research has been hampered by the inadequate sensitivity of classical HPLC techniques. The advent of microbore HPLC is now changing this. Comparison of microbore HPLC methods with normal-bore techniques emphasizes three inherent advantages of microbore systems: Firstly, there is a greater mass sensitivity; secondly, the analysis time is shorter; and thirdly, the use of mobile phase is considerably reduced [6,7]. It has only been a matter of time before microbore techniques have been developed for the measurement of ACh in microdialysis samples [8,9].

During the validation of such a method we observed that the apparent concentration of ACh in a given tube, irrespective of whether it was a standard or actual sample, decreased over a period of several hours. Therefore, the aim of this study was to investigate the cause of this apparent decrease with two different HPLC systems, and to define the conditions for optimal sensitivity of the platinum electrode of the electrochemical detector.

2. Experimental

2.1. Apparatus

The first HPLC system consisted of a Merck–Hitachi (Darmstadt, Germany) L-6200 intelligent pump, a refrigerated Gilson 231-401 autosampler (Abimed, Düsseldorf, Germany) with a Rheodyne 7021 injection valve (5 μl loop) and an LC-4C amperometric electrochemical detector (BAS, West Lafayette, IN, USA) purchased from Axel Semrau (Sprockhövel, Germany). The electrochemical detector was equipped with a platinum working electrode and a Ag/AgCl reference electrode. The potential was set to +500 mV, the range to 5 nA, the filter to 0.02 Hz and the offset to 1–100 nA. An online DG-1200 degasser from VDS Optilab (Berlin, Germany) was also used to ensure that the mobile phase was free of air. A SepStik® (BAS) microbore polymeric column (530×1 mm I.D.) of particle size 10 μm was used in association with an ChO/AChE immobilized enzyme reactor and guard column (BAS).

The second narrow-bore HPLC system for separation of choline and ACh consisted of an LC100 intelligent micro liquid chromatographic pump (BASJ, Tokyo, Japan), a 3-channel on-line degasser (BASJ, Tokyo, Japan), a CMA/200 refrigerated microsampler (CMA/Microdialysis, Stockholm, Sweden) and a BAS LC-4B amperometric detector (BAS). Choline and ACh were separated on a 120× 2 mm I.D. column packed with polymeric reversed-phase particles and coupled to a 2×5 mm immobilised enzyme reactor (both purchased from BASJ). This column was replaced with a microbore (530×1 mm I.D.) column (BAS) for simultaneous determination of physostigmine, choline and ACh.

2.2. Reagents

Sodium acetate, Na_2EDTA, sodium-1-octanesulfonate, Na_2HPO_4 and NaH_2PO_4 were purchased from E. Merck (Darmstadt, Germany), sodium hydroxide from Fluka (Neu-Ulm, Germany), ACh and choline from Sigma Chemie (Deisenhofen, Germany) and Kathon® reagent from BAS. All other reagents were at least of reagent grade and purchased from reputable sources. Purified water was prepared by means of a Millipore Q-System.

2.3. HPLC procedure

The mobile phase for the first HPLC system was prepared freshly each week and consisted of 29 mmol/l NaH_2PO_4, 22 mmol/l sodium acetate and 5 ml/l Kathon reagent. Kathon reagent is a 1% (v/v) dilution in 0.01 mol/l sodium acetate buffer adjusted to a pH of 5.0 of a mixture of 1.5% (w/v) 5-chloro-2-methyl-4-isothiazolin-3-one and 2-methyl-4-isothiazolin-3-one. The mobile phase was adjusted to a pH of 8.5 with NaOH (8 mol/l), filtered through a

0.2-μm membrane filter (Schleicher and Schüll, Dassel, Germany), and pumped at a flow-rate of 120–130 μl/min. A pulsation-free chromatogram was achieved without the need for a splitting system or microheads with the Merck–Hitachi intelligent pump. Peak identification was performed with external standards and concentrations were calculated on the basis of peak heights. The injection volume was 5 μl.

The mobile phase for the second narrow-bore system consisted of 50 mmol/l Na_2HPO_4, 0.3 mmol/l sodium 1-octanesulfonate and 1 mmol/l disodium EDTA. The mobile phase was adjusted to a pH of 8.5 and pumped at a flow-rate of 300 μl/min. Physostigmine, ACh and choline were separated with a mobile phase consisting of 50 mmol/l Na_2HPO_4, 0.5 mmol/l disodium EDTA and 0.05% Kathon CG purchased from Rohm and Haas (PA, USA). The mobile phase was adjusted to a pH of 8.5 and pumped at a flow-rate of 100 μl/min.

2.4. Reproducibility studies

During the course of attempts to determine the reproducibility of the ACh measurements we noticed that the peak height for ACh in standard mixtures decreased over time. A brown coating also appeared on the surface of the working platinum electrode. Therefore, we determined whether this apparent loss was caused by an actual decay in the standard itself or whether it was because of a loss of sensitivity of the working electrode caused by the brown coating. A standard solution of ACh was prepared containing 1 pmol/5 μl. The working platinum electrode was dismantled before the beginning of the experiment and cleaned by wiping with a tissue moistened in methanol. Thereafter, the electrode was reassembled and the baseline of the electrochemical detector was allowed to return to normal. A standard containing 1 pmol/5 μl was initially injected five times onto the column followed by the first of 24 hourly injections of a 1 pmol/5 μl standard which had been previously split into two parts. The first part was used for the stability measurements, whereas the second part was immediately frozen at −20°C. At the end of 24 h, the sample which had been stored at −20°C was re-measured. The electrode was then disassembled, cleaned with a tissue as before and re-assembled. The baseline was allowed to return to previous levels and the initial standard which had been stored at 4°C was injected onto the column five times. The entire procedure was performed a total of four different times and all results are expressed as mean height of peak ± S.D.

2.5. Data calculation

HPLC data from the first HPLC system were collected with a Nelson analytical series 900 interface from ESWE Analytik (Sinsheim, Germany). Peak integration was carried out with Turbochrom® software from Axel Semrau (Sprockhövel, Germany) on a personal computer. Data from the second HPLC system were calculated with an SP 4400 integrator (Spectra-Physics, San José, CA, USA). All figures and descriptive statistics were prepared with SigmaPlot 5.01 from Jandel Scientific (Erkrath, Germany).

3. Results and discussion

The first HPLC method described in this paper provides an excellent separation of ACh from choline in standard mixtures and microdialysis samples (Fig. 1). There was an excellent linear relationship between the concentration of ACh and the peak height over the range 0.05–10 pmol/5 μl (r= 0.99998). The limit of quantitation was 0.05 pmol/5 μl or 10 nmol/l which is comparable with a previously published microbore system which contained a similar column [8]. Both methods used commercially available microbore columns and HPLC pumps. Nevertheless, careful attention must be given in both systems to degassing. A better baseline, and therefore sensitivity, was always obtained when a degasser was connected on-line.

The mean height of the standard injected five times onto the column directly after initially cleaning the electrode and waiting for a stable baseline was 30.70±2.44 mV. Fig. 2 shows the subsequent decrease in the peak heights measured hourly for 24 h. The mean height was 23.26±2.12 mV for the 24 injections. This represents a coefficient of variation of 9.1% and an apparent loss of 24% when the first standard is compared with the twenty-fourth. The loss was not steady over the entire 24 h but seemed

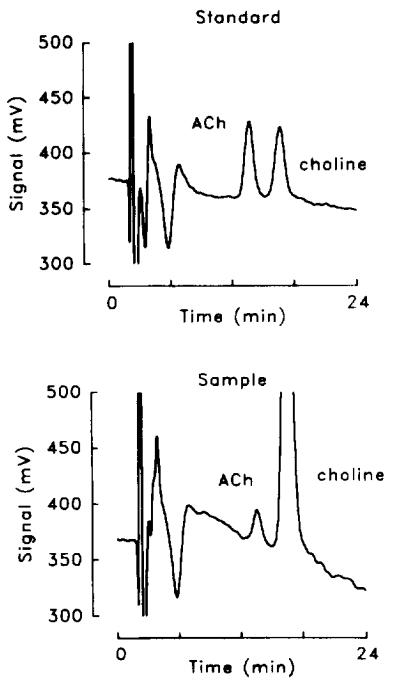

Fig. 1. Microbore HPLC separation of a standard mixture of ACh and choline (1 pmol/5 μl) and a typical baseline microdialysis sample from an awake, freely moving rat. The microdialysis probe (CMA/12) with a membrane length of 3 mm was perfused with 10 μmol/l neostigmine and found to contain 0.48 pmol ACh/5 μl.

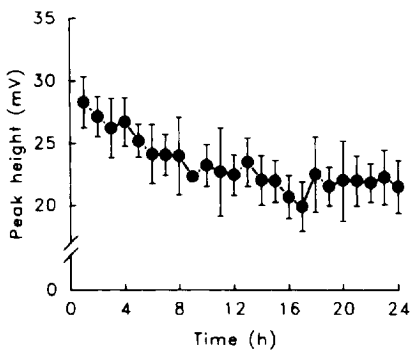

Fig. 2. The effect of time on the stability of peak height measurements of a standard containing 1 pmol ACh/5 μl injected hourly for 24 h. The coefficient of variation for the measurements is 9.1%. This represents an apparent loss of 24% when the first standard is compared with the twenty-fourth. Results are the mean ± S.D. of four different experiments.

to occur mostly over the first 8 h. This experiment confirmed our original observation that there was an apparent loss of ACh in the sample over several hours. Injection of part of the remaining half of the sample which had been stored at −20°C produced a peak height of 20.88±1.64 mV. This was considerably less than the standard injected onto the column at the beginning of the experiment. Hence, the apparent loss occurred not only if the sample was stored at 4°C but also if the sample was stored frozen at −20°C. Finally, when the standard injected onto the column at the beginning of the experiment was re-injected after the electrode had been cleaned of brown material by wiping with a tissue moistened in methanol, the peak height was 28.44±4.38 mV. This value is not significantly different from the initial value even though this standard had been stored at 4°C for more than 24 h. Hence, the apparent loss of ACh in the standard is caused by a decrease in sensitivity of the platinum electrode itself. This may be related to the brown coating present on the electrode. An accumulation of peroxide oxidation products on the surface of the electrode may cause degradation of hydrogen peroxide before it reaches the electrode itself. We are not the only group to have reported problems in the measurement of ACh. Recently, an unknown oxidizable compound was shown to interfere with the measurement of ACh by a polymer-modified glassy carbon electrode [10]. During routine use of the HPLC system for measuring microdialysis the monthly between-assay coefficients of variation for the retention time of ACh ranged from 0.59% to 1.66%, and for the peak height of a 1 pmol/5μl standard from 17.1% to 29.8%.

Results with the second HPLC system indicated that at higher flow-rates (300 μl/min) the loss of the sensitivity of the working platinum electrode was even more pronounced. Here, the ACh peak heights were reduced by more than 60% after 4–5 days of continuous use and analysis of about 300 microdialysis samples. As with the previous microbore column, the largest drop in the sensitivity of ACh occurred within the first 6–8 h after starting the injections with a stable baseline obtained with a newly polished electrode, as also reported earlier for a laboratory-made electrode cell [11]. We noticed that, apart from a gradual loss of activity of the immobilized enzymes in the postcolumn reactor, the

major contribution to the sensitivity loss was a coating on the platinum electrode of oxidation products (probably betaine) and other directly oxidizable substances. The chromatograms depicted in Fig. 3 show the effect of cleaning the platinum electrode surface with ethanol on the peak height of ACh. Rapid cleaning of the electrode surface with a paper tissue moistened with ethanol and a subsequent 3-h stabilization of the baseline current caused restoration of the initial sensitivity to that obtained with an electrode polished with alumina. However, the latter polishing procedure usually requires at least 2 days before a stable baseline is achieved. Earlier studies with amperometric oxidation of hydrogen peroxide on a platinum electrode led to the suggestion that the reaction is mediated by the formation of PtO or a PtOH layer on the electrode surface [12]. At the same time, the electrochemical reaction is controlled by the kinetics of a catalytic cleavage of H_2O_2 to H_2O and O_2 [13]. This could also explain why the baseline requires longer to become stable after mechanically polishing the electrode. Apart from the build-up of tiny scratches and alumina particles on the electrode surface, a re-equilibration time of the Pt(II) oxide electrical double-layer may also contribute to the high initial background currents. After the peak had dropped to about 60% of its initial levels, the peak heights remained relatively stable. The coefficient of variation for the mean peak height of 12 injections of 0.2 pmol/μl over 2–3 days was 2.3%.

Neostigmine and physostigmine are the most commonly used inhibitors of acetylcholinesterase (AChE) which are added to microdialysis perfusates to enhance the extracellular concentration of ACh to detectable levels. We have previously shown that the concentration of AChE inhibitor in the perfusate can influence the effects of pharmacological agents such as caffeine on the extracellular concentration of ACh [9]. In contrast to neostigmine, previous work has shown that physostigmine can be detected electrochemically using a dual-electrode cell in a redox mode and normal-phase chromatography [14]. The present mode, based on ion-exchange microbore column and a single oxidation step on a platinum electrode, allows simultaneous determination of ACh, choline and physostigmine in microdialysis samples. The chromatogram depicted in Fig. 4 shows separation of a typical microdialysis perfusate from

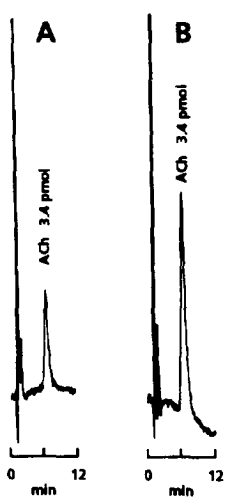

Fig. 3. Chromatograms showing the effect of cleaning the platinum electrode with ethanol on the peak height of ACh. A volume of 17 μl of a standard solution containing 0.2 pmol ACh/μl was injected either onto (A) a deteriorated platinum electrode or onto (B) the same electrode 3 h after cleaning with ethanol. The second narrow-bore HPLC system was used as described in Section 2.

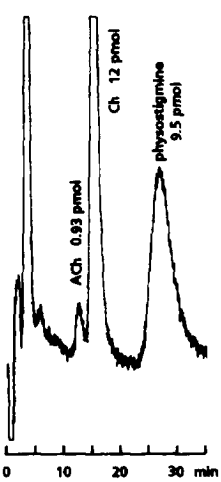

Fig. 4. A chromatogram of a typical baseline microdialysis sample containing physostigmine from the lateral striatum of an awake, freely moving rat. The microdialysis probe (CMA/12) with a membrane length of 2 mm was perfused with an artificial physiological solution containing 10 μmol/l physostigmine at a flow-rate of 2 μl/min. The perfusate was found to contain 0.93 pmol ACh, 12 pmol choline and 9.5 pmol physostigmine in 5 μl of injected sample.

the rat striatum and illustrates the application of microdialysis as a technique for simultaneously recovering and delivering small molecules from and into the extracellular fluid. Here, endogenous ACh and choline diffuse from the extracellular space into the perfusion fluid, while exogenous physostigmine is infused locally into the brain tissue. The original physostigmine concentration in the perfusate before entering the striatum was 10 μmol/l. The concentration of physostigmine found in the perfusate afterwards was only 1.9 μmol/l, a fact which indicates a high transport of physostigmine into the brain. This gradient is probably caused by the activity of AChE in the vicinity of the microdialysis membrane which in turn produces a rapid flux of physostigmine into the extracellular space. AChE is one of the most efficient enzymes known and has the capacity to hydrolyze 600 000 ACh molecules per molecule of enzyme per minute, a fact which implies a turnover time of 150 μs [15]. Because neostigmine and physostigmine have a carbamyl ester linkage, they serve as alternate substrates with a similar binding orientation to ACh for the active site of AChE. In contrast to ACh however, they are hydrolyzed by AChE to form carbomyl derivatives which are much more stable than the acetyl derivative with ACh [15]. This interaction provides the driving force for the steep gradient of physostigmine concentration.

In summary, we have applied two different microbore HPLC methods for the measurement of ACh in microdialysis samples. We observed a gradual loss of sensitivity with both methods over time which was linked to a build-up of oxidized products on the surface of the working electrode. This loss of sensitivity has important implications for microdialysis experiments. A typical experiment usually contains 10 to 20 consecutive samples for measurement which take approximately 25 min per sample to measure according to the chromatograms described here. This represents a total analysis time between 4 and 8 h. Based on our experiments, we strongly recommend that the platinum electrode is regularly cleaned with a tissue moistened in methanol or ethanol, and that the quantitation is calibrated with standards at the beginning and end of a series of measurements.

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