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Analysis of acetylcholine from extracellular fluid in brain by in vivo microdialysis and LC–ESI-MS/MS with the stable isotope-labeled internal standard

Lei Liu [a], Jianquan Huang [b,*], Kexin Li [a], Xin Hu [a], Chunhua Sun [a,**]

[a] Department of Pharmacy, Beijing Hospital, The Ministry of Health, No. 1 Dahua-Road, Dongdan, East-City District, Beijing 100730, PR China
[b] Department of Pharmacy, Aerospace Center Hospital, No. 15 Yuquan-Road, Haidian District, Beijing 100049, PR China

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ABSTRACT

Acetylcholine (ACh) associated with Alzheimer's and Parkinson's disease is the major neurotransmitter in vertebrates. In support of clinical studies on the mechanism of the illnesses and development of medicines for these diseases, the LC–ESI-MS/MS method was developed and validated for the direct quantification of ACh in dialysate samples with acetylcholine-D_9 bromide (IS) as the isotope-labeled internal standard. The analytes were separated on the Waters Hilics C_{18} Column (2.1 mm × 100 mm, 3 μm) on LC with mobile phase ultrapure water–200 mM ammonium formate (pH 3.04)–acetonitrile (30:5:65, vol/vol/vol) at a flow rate of 300 μL/min, and monitored with a fragment ion of m/z 87 formed from a molecular ion of m/z 146 for ACh and that of m/z 87 from m/z 155 for IS during multiple reaction monitoring (MRM) positive ion mode. The lower limit of quantitation (LLOQ) of ACh was lower than 0.1 nmol/L in dialysate samples, equivalent to 0.2 fmol injected on-column. The developed method could be utilized in the analysis of ACh in dialysate samples and these results were in good agreement with the gradient elution study.

1. Introduction

Acetylcholine (ACh) is a simple molecule synthesized from choline and acetyl-CoA through the action of choline acetyltransferase. ACh in vertebrates is the major neurotransmitter at neuromuscular junctions, autonomic ganglia, parasympathetic effectors' junctions, a subset of sympathetic effectors' junctions, and at many sites in the central nervous system. It is generally not used as an administered drug, due to the rapid degradation by cholinesterase, but it is useful in some ophthalmological applications. It is important to determine the concentration of ACh in the extracellular fluid of the brain, to reveal changes related to Alzheimer's and Parkinson's disease [1–6]. The concentration of ACh in the extracellular fluid is relatively reliable for the evaluation of a particular neuronal activity, and could be utilized in the research on the illness mechanisms, and development of medicines for these diseases.

Microdialysis is a technique to monitor the chemistry of the extracellular space in living tissue. In vivo microdialysis has been used extensively since the late 1960s and early 1970s. Reported methods for the measurement of ACh were based on the use of chemiluminescence [7], electrochemical (ECD) [8], fluorescence [9], MS [10,11] and UV [12] detections. A review by Tsai [13] surveyed various analytical techniques that have been adopted for the measurement of ACh. Most of these methods rely on detecting ACh indirectly and were insufficient for reliable quantification of Ach in microdialysis.

The most commonly used approach for the measurement of extracellular ACh was based on brain microdialysis coupled to an HPLC-ECD system [6,8,13–17]. The most sensitive assay that has been reported in the literature for the analysis of ACh was coupling in vivo microdialysis with liquid chromatography–tandem mass spectrum (LC/MS/MS) [18–22]. However, all the analytical methods mentioned above suffered either the poor limit of quantitation (≥1 fmol on column) or complex ion-pair or cation-exchange agents which would become the cumulative interference of analytes in mass spectrum. The ACh peak had a bad shape and width in a simultaneous determination of multiple neurotransmitters [23]. Furthermore, the researchers analyzing ACh with MS detector have been listed in a review [24]. There were various columns and mobile phases for detection of ACh and the best LLOQ was 1 fmol on column.

In support of clinical studies on ACh, a simple sample preparation coupled with LC–ESI-MS/MS analysis was developed and validated for the sensitive and reliable quantification of ACh. The developed method with the isocratic elution was certified by the gradient elution in the analysis of ACh in dialysate samples.

* Corresponding author.
** Corresponding author. Fax: +86 10 6528 3696x5.
E-mail addresses: jianquanhuang@mail.sdu.edu.cn (J. Huang), sunchunhua8888@sina.com (C. Sun).

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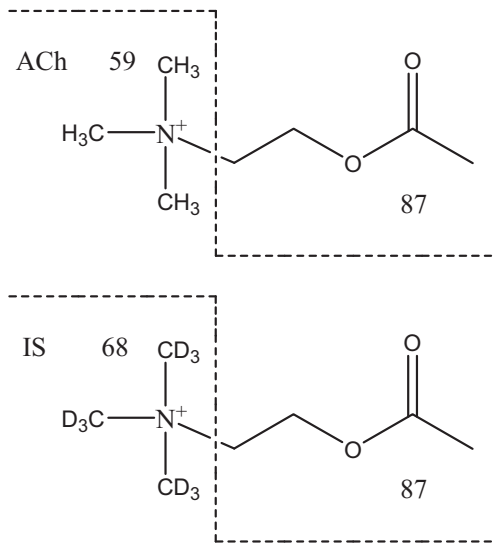

Fig. 1. The chemical structure and the fragmentation scheme of acetylcholine (ACh) and acetylcholine-D_9 (IS).

2. Experimental

2.1. Chemicals and reagents

Standards of acetylcholine chloride with the purity of >99.0% and Dulbecco's phosphate buffered saline (9.6 g/L) without calcium chloride for the development, validation and control of the method were obtained from Sigma–Aldrich (St. Louis, USA). Acetylcholine-D_9 bromide (IS) with the D atom% of 99.7% (Fig. 1) was purchased from CDN isotopes (Quebec, Canada). Formic acid, ammonium formate for mass spectroscopy and calcium chloride were all purchased from Fluka (Bush, Switzerland). Acetonitrile (Tedia, USA) and isopropanol (Sigma–Aldrich, USA) were of HPLC grade. Ultrapure water (18.2 MΩ cm, 25 °C) was prepared by passing through Milli-Q System (Millipore Corporation, Germany).

2.2. Standard sample preparation

Working Dulbecco's was prepared by dissolving a bottle of Dulbecco's (9.6 g) with 850 mL of water, then slowly mixing 0.18 g of calcium chloride in 100.0 mL, and adding pure water to 1000 mL. Stock solutions of ACh and IS were prepared in water at the concentration of 1 mmol/L. Working solutions of ACh were prepared at the concentration of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 40 and 50 nmol/L by appropriate dilution of the stock solution of ACh with working Dulbecco's, while the working solution of IS was prepared at the concentration of 5 nmol/L by appropriate dilution of the stock solution with the dilute solvent composed of isopropanol to acetonitrile of 1:2 (vol/vol). All the solutions were stored at 4 °C and were brought to room temperature (25 °C) before use.

2.3. Biological sample preparation

The dialysate samples were balancing samples collected from the medial prefrontal cortex of a rat brain. Balancing samples were working Dulbecco's solution that was pumped through the probe inserted into the brain overnight before the collection of actual dialysate samples. The animal handling protocols and study design were reviewed and approved by the Animal Ethics Committee of Beijing Hospital. All the animal experiments were carried out according to the Guide for the Care and Use of Laboratory Animals [25]. The rat was anesthetized with chloral hydrate (172 mg/kg) and pentobarbital (35.6 mg/kg), and attached in a stereotaxic device (RWD Life Science Co. Ltd., Shenzhen, China). The cannula was fastened to the skull with dental cement and three stainless steel screws. After the surgery, the rat was placed into individual test cages and allowed to recover for 5–7 days. One day before the experiment, a microdialysis probe was inserted through the guide cannula into the prefrontal cortex. The probe was infused with a working Dulbecco's solution at a flow rate of 0.3 μL/min overnight using a model 2000 syringe pump (Harvard Apparatus, Inc., Holliston, MA, USA). In the morning, the perfusion rate was raised to 1.5 μL/min for 1 h, dialysate samples (about 45 μL) were collected every 30 min and stored in a freezer (4 °C). At the end of the experiments the animals ($n = 6$) were sacrificed. The location of the dialysis probes was verified at the end of each experiment by manual brain dissection and with 100 μm brain-slices (OTS-4000, FHC, Bowdoinham, ME, USA).

The dialysate samples were transferred into an autosampler vial after diluted 5-fold with working solution of IS and a 10 μL of sample was injected into the LC–MS/MS for analysis.

2.4. Liquid chromatographic conditions

LC–MS/MS analyses were performed using a Waters 2795 HPLC system (Waters Corporation, USA) coupled to the Quattro Premier electrospray ionization tandem mass spectrometer with the software Masslynx 4.0 (Micromass, Waters Corporation, USA). Chromatographic separation was obtained using a Waters Hilics C_{18} Column (2.1 mm × 100 mm, 3 μm), fitted with a guard column of identical packing material (4.0 mm × 2.0 mm). Injection volume was 10 μL of a sample kept in an autosampler set at 4 °C. The column temperature was maintained at 25 °C. Separation of the analytes of interest from matrixes was achieved with mobile phase ultrapure water–200 mM ammonium formate (pH 3.04)–acetonitrile (30:5:65, vol/vol/vol) at a flow rate of 300 μL/min. The retention time was approximately 2.82 min for ACh and IS (Fig. 2). Meanwhile, the gradient elution was set at the same flow rate with a continuous 5% 200 mM ammonium formate and under linear gradient of 18–35% H_2O over 3 min, 35% H_2O hold for 2 min, then instantly 18% H_2O hold for 5 min.

2.5. Mass spectrometric conditions

The mass spectrometer was operated under the positive ionization mode in electrospray MS/MS conditions and data were acquired under MRM incorporated a precursor ion scanning from m/z 50 to m/z 500. The following electrospray MS/MS parameter settings were applied: electrospray capillary and cone voltage were 1.0 kV and 20 V, respectively; ultrapure nitrogen was used as nebulizer and cone gas at rate of 500 L/h and 50 L/h, respectively; source and desolvation temperature were set at 105 °C and 400 °C, respectively. With the collision energy off, ACh and IS yielded predominantly protonated molecular ion at m/z 146 and 155, respectively. Both of the precursor ions were subjected to collision-induced dissociation (CID) to determine the resulting daughter ions. The daughter ion was recorded using MRM positive ion detection mode. The monitor ion and collision energy were m/z 146 → 87 and 15 eV for ACh and m/z 155 → 87 and 15 eV for IS. Argon was used as the collision gas with a flow rate of 0.24 mL/min.

2.6. Method validation

The specificity of method was evaluated by comparing blank working Dulbecco's with those spiked with ACh standards of 0.2, 5.0, and 40 nmol/L as quality control levels (QC levels, $n = 6$). The blank working Dulbecco's was tested for interference of compounds by using the proposed operation procedure and liquid chromatographic/mass spectrometric conditions. The results were

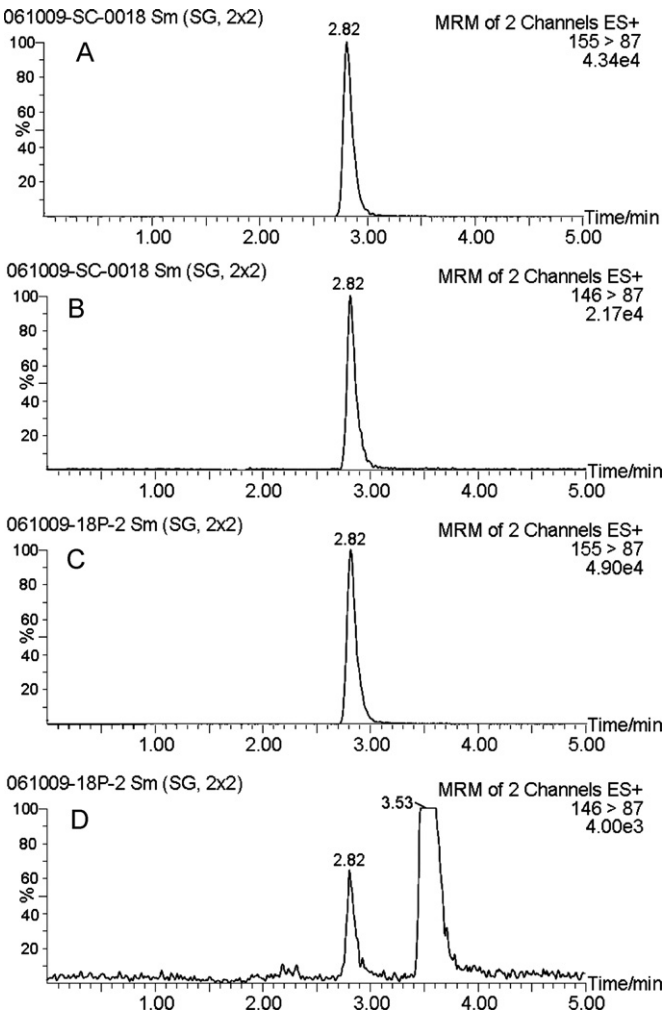

Fig. 2. The chromatograms of acetylcholine-D_9 (A) and acetylcholine (B) in Dulbecco's and acetylcholine-D_9 (C) and acetylcholine (D) in dialysate sample (Rt = 2.82 min) with isocratic elution.

compared with those containing ACh with the concentration close to the lower limit of quantification (LLOQ). Otherwise, considering the absence of blank biological sample, a gradient elution with the same mobile phase system (H_2O, 200 mM ammonium formate and acetonitrile) was set up to confirm the absence of interference of endogenous compounds in biological samples.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of IS or ACh spiking at QC levels in the mixture of blank working Dulbecco's and working solution of IS (1:4, vol/vol) with that at an equivalent concentrations of ACh and IS in pure water and dilute solvent (1:4, vol/vol).

To assess the precision and accuracy of the procedure, reproducibility for both inter-day and intra-day variations was measured. The relative recovery was calculated through the calibration curve for accuracy, while the absolute recovery was evaluated by comparing the peak area of Ach at QC levels when processing as depicted in sample preparation with that at an equivalent concentrations by adding stock solution of ACh to the mixture composed with blank working Dulbecco's and working solution of IS (1:4, vol/vol).

The stability of ACh was studied at QC levels under various conditions. Short-term stability was assessed by analyzing QC samples kept at room temperature for 6 h to cover the sample preparation. Long-term stability was tested by assaying QC samples after storage at 4 °C for 4 weeks or −70 °C for 12 weeks. Post-preparative stability was evaluated by analyzing the processed samples kept in the autosampler setting at 4 °C for 24 h. Freeze–thaw stability was estimated over three cycles.

Calibration curves were prepared by plotting the peak area ratios between ACh and IS against the concentration of ACh, and the linear regression was performed. The acceptance criterion for the correlation coefficient (r) was $r \geq 0.99$. Linearity was determined by the correlation coefficients from the calibration curves of nine concentrations of ACh which ranged 0.1–50 nmol/L. The lower limit of detection (LLOD) and the LLOQ (≤0.1 ng/mL) were determined as the concentrations with the signal-to-noise ratio of 3:1 and 10:1, respectively. Each concentration standard should meet the following acceptable criteria: no more than 20% deviation at LLOQ and no more than 15% deviation for the standards above LLOQ.

3. Results and discussion

3.1. Development of the analytical method

In the development of analytical method, the main emphasis was on the separation of ACh from the inorganic salts and endogenous compounds in dialysate samples, thus the proper retention time of these hydrophilic compounds was important for the efficient separation of each other. Therefore, the normal phase column (Waters Hilics C_{18} Column), which could provide the suitable retention for these water soluble compounds, was selected for the method development. The analytical results of dialysate sample also elucidated that ACh and the endogenous compound(s) were separated properly and the latter had a stronger retention on this normal phase column.

Based on previous chromatographic experience with these compounds [18,20,23], ammonium formate or formic acid coupled with methanol or acetonitrile systems were evaluated at various pH, concentration and compositions. Ammonium formate, which yielded satisfied chromatographic peak shapes for analytes in LC–MS/MS analysis, was chosen as the buffer (pH 3.04 adjusted with formic acid) in this mobile phase. And acetonitrile, which produced the much better baseline than methanol, was selected as the organic phase.

3.2. Results of method validation

The described method proved to be specific with respect to the interference of matrix with the same retention time as ACh, as can be seen in the ion chromatogram of the blank working Dulbecco's, though there is one peak of endogenous compound at retention time of 3.53 min with the same MRM mass transition as ACh (m/z 146 → 87) (Fig. 2). The matrix effects of ACh at 0.2, 5.0 and 40 nmol/L levels and IS were 102.03 ± 4.79%, 101.44 ± 3.57%, 99.79 ± 2.61% and 101.0 ± 3.03%.

The absolute recovery of ACh at QC levels was shown to be consistent, precise and reproducible. The mean absolute recoveries (n = 6) were satisfactory, as can be seen from Table 1. These working solutions were found to be stable for 4 weeks at 4 °C. All the results of stability validation are listed in Table 2. The results showed that there were no stability-related problems during the analysis of dialysate samples.

To assess the precision and accuracy of the procedure, reproducibility for both inter-day and intra-day variations was determined (Table 1). The accuracy was in the range 96.71–102.62%. The RSD values for intra-day precision were in the range 1.27–3.13%, while the corresponding inter-day precision was 2.32–7.16%. The mean relative recoveries of the gradient elution (n = 6) at 0.2, 5.0 and 40 nmol/L levels were 104.67%, 102.83% and 100.35%, whereas the RSDs were 7.09%, 4.73% and 2.53%, respectively. The results revealed good precision and accuracy.

Table 1
The results of recovery, precision and accuracy for acetylcholine ($n = 6$).

Conc. (nmol/L)	Abs. Rec.	RSD	Intra-day		Inter-day	
			Rel. Rec.	RSD	Rel. Rec.	RSD
0.2	100.00%	5.91%	96.71%	3.13%	98.25%	7.16%
5.0	101.17%	2.34%	100.34%	2.07%	101.01%	3.37%
40	100.22%	1.21%	102.62%	1.27%	101.97%	2.32%

Table 2
The results of stability for acetylcholine ($n = 6$).

Conc. %	0.2 nmol/L	5 nmol/L	40 nmol/L
Short-term stability	99.03 ± 5.06	103.76 ± 4.21	101.04 ± 4.95
Long-term stability (4 °C)	96.98 ± 6.96	100.63 ± 4.67	100.41 ± 3.65
Long-term stability (−70 °C)	96.29 ± 7.79	98.46 ± 3.87	97.37 ± 5.04
Post-preparative stability	98.44 ± 4.75	100.68 ± 4.02	99.04 ± 3.68
Freeze–thaw stability	99.32 ± 5.13	97.73 ± 3.99	98.32 ± 4.71

The method exhibited good linear response over the selected concentration range by linear regression analysis. Calibration curves were constructed on 3 different days. The correlation coefficient (r) and deviation for the concentration range 0.1–50 nmol/L were >0.999 and <4.2% ($n = 6$), respectively. The mean calibration curve ($n = 6$) was typically described by the least-square equation $R = 177.49 \times C + 53.79$, $r = 0.9994$, with the weight coefficient $1/C$, where R corresponds to the peak area ratios between ACh and IS and C refers to the concentration of ACh added to working Dulbecco's over a concentration range of 0.1–50 nmol/L.

The concentration corresponding to an S/N ratio of 3:1 was about 0.02 nmol/L as the LLOD and to an S/N ratio of 10:1 was about 0.07 nmol/L as the LLOQ, since the mean S/N ratio of 15.2 ($n = 6$) was determined for working Dulbecco's fortified at 0.1 nmol/L. The concentration of 0.1 nmol/L, which was selected as the lowest point of the calibration curve, covered the LLOQ and met the accepted criteria: no more than 15% deviation.

The calibration curve for the gradient elution with the weight coefficient $1/C$ was $R = 164.32 \times C + 32.58$, $r = 0.9991$, where R corresponds to the peak area ratios between ACh and IS and C refers to the concentration of ACh added to working Dulbecco's over a concentration range of 0.1–50 nmol/L. Fig. 3 shows the chromatogram of ACh in dialysate sample in gradient elution.

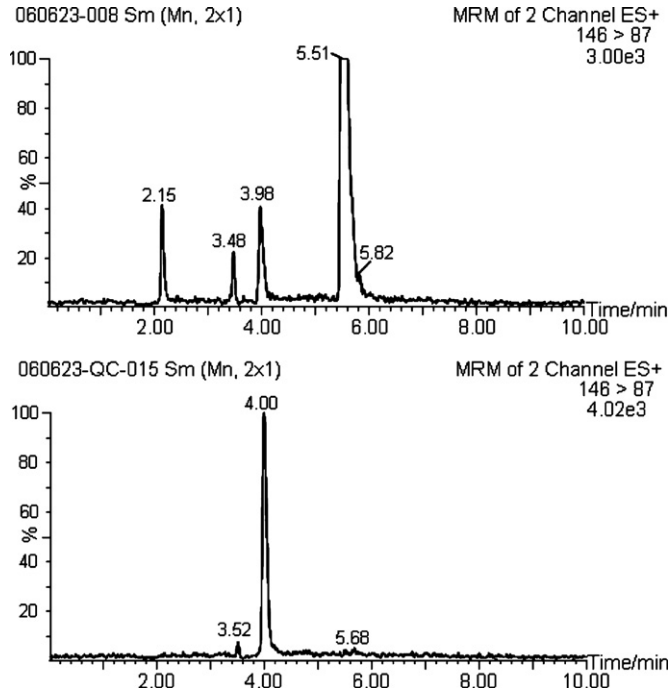

Fig. 3. The chromatograms of acetylcholine in dialysate sample (Rt = 3.98 min) and in Dulbecco's (Rt = 4.00 min) with gradient elution.

3.3. Results of application

The method was sensitive enough to apply to analyze of ACh in dialysate samples. Fig. 2 showed the chromatogram of dialysate sample. The concentration of ACh in dialysate samples from the rat brain before drug administration was 0.476 ± 0.011 nmol/L ($n = 6$) which was in good agreement with both the gradient elution study [0.502 ± 0.017 nmol/L] and the previous reports [8,19].

4. Conclusion

The described high performance liquid chromatography–electrospray ionization mass spectrometry (LC–ESI-MS/MS) method for determination of ACh in dialysate sample was proved to be rapid, reproducible, specific, sensitive and more reliable than previous reports. Compared to the gradient elution, the analysis time was curtailed with the isocratic elution. The concentration of ACh in the extracellular fluid of the brain has been reported to vary from 0.1 to 6 nmol [8,19]. With this method, not only the limit of quantitation could be sufficient enough for ACh, but also the consumption of sample was small because of the 5-fold dilution and 10 μL of injection volume. The endogenous compounds co-eluting with the Ach were absent, and the method could be applied for the analysis of ACh in dialysate samples.

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