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ScienceDirect
Journal of Pharmaceutical and Biomedical Analysis 44 (2007) 586–593
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Development of a liquid chromatography/tandem mass spectrometry method for the quantitation of acetylcholine and related neurotransmitters in brain microdialysis samples

Mei-Yi Zhang [a,*], Zoë A. Hughes [b], Edward H. Kerns [a], Qian Lin [b], Chad E. Beyer [b]

[a] *Chemical and Screening Sciences, Princeton, Wyeth Research, NJ 08543, USA*
[b] *Discovery Neuroscience, Wyeth Research, Princeton, NJ 08543, USA*

Received 1 November 2006; received in revised form 14 February 2007; accepted 15 February 2007
Available online 23 February 2007

Abstract

Monitoring concentrations of acetylcholine (ACh) in specific brain regions is important in understanding disease pathology, as well as in designing and evaluating novel disease-modifying treatments where cholinergic dysfunction is a hallmark feature. We have developed a sensitive and quantitative liquid chromatography/tandem mass spectrometry method to analyze the extracellular concentrations of ACh, choline (Ch) and (3-carboxylpropyl)-trimethylammonium (iso-ACh) in brain microdialysis samples of freely moving animals. One immediate advantage of this new method is the ability to monitor ACh in its free form without having to use a cholinesterase inhibitor in the perfusate. The separation of ACh, Ch, iso-ACh and related endogenous compounds was carried out based on cation exchange chromatography with a volatile elution buffer consisting of ammonium formate, ammonium acetate and acetonitrile. An unknown interference of ACh, which was observed in brain microdialysates from many studies, was well separated from ACh to ensure the accuracy of the measurement. Optimization of electrospray ionization conditions for these quaternary ammonium compounds achieved the limits of detection (S/N = 3) of 0.2 fmol for ACh, 2 fmol for Ch and 0.6 fmol for iso-ACh using a benchtop tandem quadrupole mass spectrometer with moderate sensitivity. The limit of quantitation (S/N = 10) was 1 fmol for ACh, 3 fmol for iso-ACh and 10 fmol for Ch. This method was selective, precise (<10% R.S.D.), and sensitive over a range of 0.05–10 nM for ACh, 0.25–50 nM for iso-ACh and 15–3000 nM for Ch. To demonstrate that the developed method can be applied to monitoring changes in ACh concentrations *in vivo*, reference agents that have previously been shown to influence ACh levels were studied in rat dorsal hippocampus. This includes the 5-HT_6 receptor antagonist, SB-271046, and the cholinesterase inhibitor, donepezil. Moreover, levels of ACh were demonstrated to be sensitive to infusion of tetrodotoxin (TTX) suggesting that the ACh being measured *in vivo* was of neuronal origin. Collectively, these biological data provided *in vivo* validation of this analytical method.

Keywords: Acetylcholine; Choline; Liquid chromatography–mass spectrometry; *In vivo* microdialysis

1. Introduction

In 1922, Otto Loewi was the first to isolate and identify the chemical messenger acetylcholine (ACh). Since these early experiments, the biological functions of ACh have been studied extensively. Although this chemical is known to regulate aspects of sleep, memory, temperature control, and blood pressure [1–4], perhaps the most widely described role for ACh is in cognitive function. In particular, deficits in central cholinergic systems are a well-known characteristic of poor cognitive performance associated with Alzheimer's disease, schizopherenia and Parkinson's disease [5–10]. Because of these purported roles, the ability to detect and monitor levels of ACh *in vivo* should not only provide valuable information for understanding disease pathology/etiology, but it may also prove useful in designing novel, disease-modifying treatments of disorders where cholinergic dysfunction is a hallmark feature.

The use of *in vivo* microdialysis to measure ACh was first pioneered by Damsma et al. [11–15], Westerink and co-workers [16–18], and Ajima and co-workers [19–21]. Analytical methods for monitoring the levels of ACh were initially based upon the use of chemiluminescence [22] and/or electrochemical,

* Corresponding author. Tel.: +1 732 274 4462; fax: +1 732 274 4505.
E-mail address: zhangm@wyeth.com (M.-Y. Zhang).

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doi:10.1016/j.jpba.2007.02.024

fluorescence and ultraviolet detection systems [23–26]. Tsai reviewed the various analytical techniques employed for the measurement of ACh [27]. However, the majority of these methods relied on detecting ACh indirectly (i.e., measuring hydrogen peroxide turnover after using an enzyme catalyzed reaction) and they were insufficient for the reliable quantification of ACh in microdialysates. Moreover, many investigators included acetylcholine-esterase (AChE) inhibitors, including physostigmine or neostigmine, in the microdialysis perfusion media to enhance levels of ACh. One fundamental problem with these methods is that the inclusion of AChE inhibitors leads to changes in the physiology of the system being investigated. For example, De Boer et al. [28] and Acquas and Fibiger [29] reported that perfusion fluid containing neostigmine quantitatively and qualitatively influenced the manner in which dopaminergic agents regulated ACh overflow in the striatum. Furthermore, AChE inhibitors contained in the perfusion fluid could mask, or indeed exaggerate, small drug-related changes in ACh levels as well as alter transmission of other systems within the perfused area [30–34].

The most sensitive assay that has been reported in the literature for the analysis of ACh is liquid chromatography/tandem mass spectrometry (LC/MS/MS) [35–40]. In these studies, the separation of ACh was based on cation-exchange [35,36], ion-pair chromatography [36,40] or hydrophilic interaction chromatography (HILIC) [38,39]. The analytes were detected by MS/MS in the positive electrospray ionization (ESI) mode. The limit of detection for ACh in the literature was between 0.03 and 0.1 nM (1–1.4 fmol) using LC/MS/MS. One of the major advantages of LC/MS/MS is that it provides direct detection of ACh. Whereas, using ECD method, ACh is first converted into Ch, and then into hydrogen peroxide using enzyme-catalyzed reactions. Furthermore, the multiple reaction monitoring (MRM) scan mode on a triple quadrupole mass spectrometer monitors specific daughter ions from dissociation of parent molecular ions. This approach provides a sensitive and selective analysis that is unique for individual analytes.

The present paper describes the development and *in vivo* validation of a liquid chromatography/tandem mass spectrometry method to monitor concentrations of ACh, iso-ACh and Ch in specific brain regions of freely moving rats. The aim of the method development was to provide a sensitive and reliable analytical approach to monitor small changes in the concentrations of ACh in brain microdialysis samples in the absence of an AChE inhibitor using a benchtop tandem quadrupole mass spectrometer with moderate sensitivity. The optimization of electrospray ionization conditions significantly increased the detection sensitivity. The chromatographic separation of ACh from iso-ACh and an unknown chemical interference in brain microdialysates ensured the accuracy of the measurements. The developed method was validated *in vivo* by monitoring ACh and iso-ACh concentrations in the dorsal hippocampus of freely moving rats after administration of the 5-HT_6 receptor antagonist SB-271046, and the AChE inhibitor donepezil. The neuronal origin of ACh measured was demonstrated using the sodium channel blocker, tetrodotoxin (TTX).

2. Experimental

2.1. Reagents and chemicals

Acetylcholine chloride (ACh), (3-carboxylpropyl)-trimethylammonium chloride (iso-ACh), choline chloride (Ch) and acetyl-β-methylcholine chloride (β-methyl-ACh) were purchased from Sigma–Aldrich (St. Louis, MO, USA). LC grade water and acetonitrile were obtained from EM Science (Gibbstown, NJ, USA). Ammonium acetate and ammonium formate were purchased from Sigma (St. Louis, MO, USA). SB-271046 and donepezil were synthesized internally. Tetrodotoxin was purchased from Sigma–Aldrich Chemicals (Milwaukee, WI, USA).

2.2. Stereotaxic surgery

Male CD rats (260–320 g; Charles River) were group housed for at least 1 week before undergoing surgery. Rats were kept on a 12:12-h light:dark cycle with free access to food and water. All experiments were conducted in accordance to the 'Guide for the Care and Use of Laboratory Animals' as adopted and promulgated by the National Institutes of Health (Pub. 85–23, 1985) and were reviewed by the Wyeth IACUC committee.

Following induction of anesthesia with 3% halothane (Fluothane; Zeneca, Cheshire, UK), animals were secured in a stereotaxic frame with ear and incisor bars. Anesthesia was maintained by continuous administration of halothane (1–2%) while a microdialysis guide cannula (CMA/12, CMA Microdialysis, Sweden) was implanted above the dorsal hippocampus (AP: −4.3 mm ML: −2.6 mm DV: −2.1 mm) [41]. The guide cannula was secured to the skull using dental acrylic and two small stainless-steel screws. Following surgery, animals were individually housed in Plexiglas® cages (45 cm × 45 cm), with free access to food and water. The following day rats were used in microdialysis experiments.

2.3. Microdialysis

Microdialysis probes (CMA12 14/02; CMA Microdialysis, Stockholm, Sweden) were equilibrated according to manufacturer's specifications. Microdialysis probes were perfused with artificial cerebrospinal fluid (aCSF: 125 mM NaCl, 3 mM KCl, 0.75 mM $MgSO_4$ and 1.2 mM $CaCl_2$, pH 7.4) prior to insertion in the guide cannula. The microdialysis probe was then inserted into the dorsal hippocampus via the guide cannula. A 3 h stabilization period was allowed following probe insertion before dialysate sampling was initiated.

In experiment 1, probes were perfused with aCSF at a flow rate of 1 μl/min and samples were collected every 20 min. After collecting 2 h of baseline samples, rats were dosed with the 5-HT_6 antagonist, SB-271046 (10 mg/kg s.c.). One hour later the perfusion fluid was switched to aCSF containing 1 μM TTX for 60 min before switching back to regular aCSF for a further 20 min.

In experiment 2, probes were perfused with aCSF at a flow rate of 0.5 μl/min and samples were collected every 40 min.

After collecting 2 h of baseline samples, rats were dosed ($t = 0$) with donepezil (0.5 or 1 mg/kg i.p.) or vehicle (water, 1 ml/kg i.p.). Following dosing, dialysis samples were collected for 3 h 20 min.

At the end of all experiments, animals were euthanized and probe placement was verified histologically. Data from animals with incorrect probe placement were discarded.

2.4. Analyzing sample preparation

Stock solutions of standard mixtures of ACh, iso-ACh and Ch were prepared by diluting a 10 mM water solution of each compound with water. A stock solution of an internal standard (IS), β-methyl-ACh, was prepared in a concentration of 10 ng/ml in water. The stock solutions were stored at 4 °C. The analysis samples were prepared by spiking 5 μl of IS and 5 μl of water into 20 μl of dialysate followed by vortexing. Standard and QC samples were prepared by spiking 5 μl of IS and 5 μl of the corresponding stock standard solutions into 20 μl of aCSF solution. Seven point calibration curves were constructed for each component in the concentration ranges of 0.05–10 nM (1–200 fmol/20 μl) for ACh, 0.25–50 nM for iso-ACh and 15–3000 nM for Ch. QC samples at two different concentration levels were used in method validation.

2.5. Liquid chromatography

An Agilent liquid chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany) was used for HPLC analysis. Chromatographic separation was carried out using a Supelco LC-SCX column (2.1 mm i.d, 150 mm length, 5 μm particle size) (Supelco, Bellefonte, PA, USA) at an oven temperature of 40 °C. The mobile phase consisted of solvent A: 15 mM ammonium acetate and 10 mM ammonium formate in water–acetonitrile, pH 4.0 (H_2O:ACN = 80:20, v/v) and B: 15 mM ammonium acetate and 10 mM ammonium formate in water–acetonitrile, pH 4.0 (H_2O:ACN = 20:80, v/v). The HPLC analysis started with a rapid gradient from 0 to 100% B in 2 min, hold at 100% B for 5 min, then ramped back to 0% B in 0.5 min, and finally hold at 0% of B for 4.5 min. The flow rate was 0.4 ml/min. The 0.4 ml/min effluent from the LC column was split before the MS and ∼0.13 ml/min effluent was directed into the electrospray interface of the mass spectrometer.

2.6. Mass spectrometry

On-line LC–MS/MS analyses were performed using a Micromass Quatro Micro tandem quadrupole mass spectrometer (Waters, Beverly, MA, USA) operated in positive electrospray ionization mode with the source temperature at 125 °C. The mass spectrometer was optimized prior to the analysis by post column infusion of 10 ng/ml of analytes with the LC flow rate set at 0.13 ml/min. The ESI ionization of analytes was optimized to a desolvation temperature of 350 °C, a spray voltage of +0.9 kV, and a cone voltage of 18 V. Nitrogen was used as both desolvation (1000 l/h) and nebuliser gas (fully open). The pressure of the argon collision gas was set at 5 psi and adjusted to an analyzer pressure of $2.0–3.0 \times 10^{-4}$ mbar. Multiple reaction monitoring conditions for each compound were then developed. The MRM analyses were performed by passing molecular ions (M^+) through the first quadrupole (Q1) and collision dissociating the molecular ions in the second quadrupole (collision cell – Q2). A selected product ion, based on intensity and structure characteristics, was isolated by the third quadrupole (Q3) and detected with the photomultiplier set at 650. The MRM transitions of m/z 146 → 87 for ACh and iso-ACh, 104 → 60 for Ch, and 160 → 101 for β-methyl-ACh were simultaneously monitored. The Dwell time for each MRM transition was set at 0.2 s. This approach provided a sensitive and selective analysis that is unique for individual analytes. β-methyl-ACh was chosen as an internal standard (IS) in the analysis. The concentrations of each neurotransmitter in microdialysates were determined by their area ratios to that of the IS using a weighting linear fit.

2.7. Data analysis

The mean of the concentration of baseline samples was calculated and denoted as 0%. All sample values were expressed as a percent change from this pre-injection mean baseline value (% change from baseline). Acetylcholine data, excluding pre-injection values, were analyzed by a two-way analysis of variance (ANOVA) with repeated measures (time). All statistical analyses were performed using SAS (v 1.03) within Excel® (Microsoft).

3. Results and discussion

3.1. Optimization of mass spectrometry

Positive electrospray ionization utilizes high voltage to ionize molecules, which have an ionisable function group, to form protonated molecular ions ($[M + H]^+$). The majority of small organic molecules analyzed in our lab showed high ionization efficiency to form $[M + H]^+$ ions at a spray voltage range of 3.0–3.5 kV and a cone voltage of 30–40 V using the Z-spray ESI ion source on a Micromass Quatro Micro tandem mass spectrometer. ACh, iso-ACh, Ch and β-methyl-ACh have a quarternary amine function group, which carries a positive charge at acidic conditions. Therefore, there is no need to ionize these molecules under the mobile phase conditions (pH 4.0) used in the present work, resulting in good sensitivity. The molecular ions (M^+) are the predominant ions in the positive ESI spectra of ACh, iso-ACh, Ch and β-methyl-ACh at acidic condition. As shown in Fig. 1a, the sensitivity of formation of molecular ions of ACh (M^+ at m/z 146) increased 2-fold when reducing the ESI capillary spray voltage from 3.5 to 0.9 kV. The optimized cone voltage for detection of M^+ of ACh was also lower than that commonly used for formation of protonated molecular ions of small organic compounds. As illustrated in Fig. 1b, the sensitivity of detection of M^+ of ACh increased 5-fold when reducing the cone voltage from 30 to 20 V. Reduced sensitivity was observed when the spray voltage and cone voltage were lower than 0.8 kV and 10 V, respectively, which was mainly because of the poor desolvation

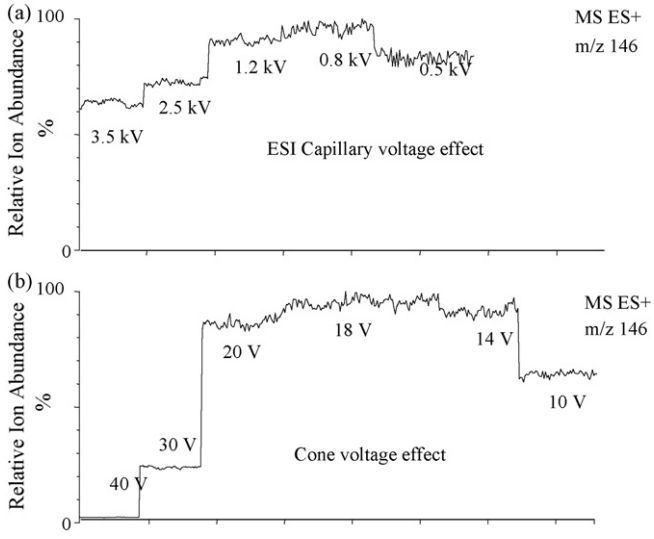
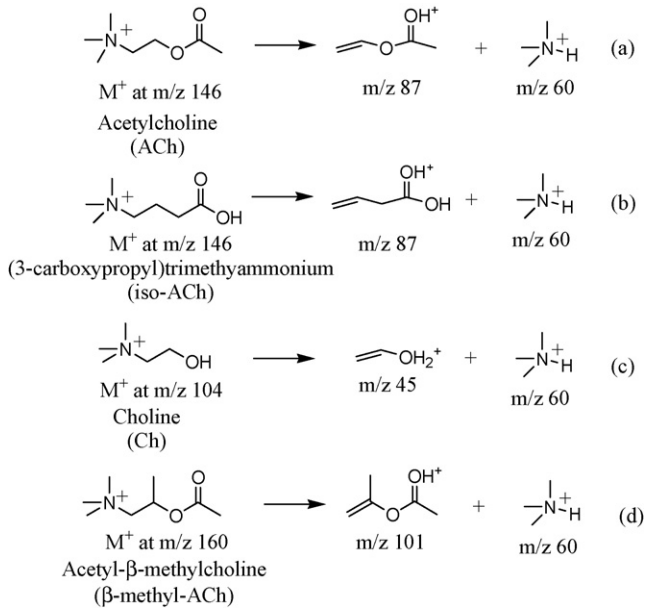


Fig. 1. The effect in formation of molecular ion of ACh (M^+ at m/z 146) by: (a) ESI spray voltage and (b) cone voltage.



Scheme 1. Dissociation pathways of: (a) ACh, (b) iso-ACh, (c) Ch and (d) β-methyl-ACh.

efficiency at such low voltages. The optimized voltages of 18 V and 0.9 kV were selected for cone and ESI capillary spray, respectively. This optimization approach achieved high sensitivity, which was necessary for detection of basal ACh, using a benchtop Quatro Micro mass spectrometer with moderate sensitivity. Another interesting observation was that the intensity of M^+ ion of ACh was insensitive to the organic composition of the mobile phase (Fig. 2). In comparison, the ionization efficiency to form protonated molecular ions was often significantly enhanced or suppressed when changing eluent composition with a LC gradient [42].

Collision-induced dissociation of molecular ions of ACh, iso-ACh, Ch and β-methyl-ACh generated only two fragment ions: a common fragment ion at m/z 60 and its corresponding counterpart ions of m/z 87 for ACh and iso-ACh, m/z 101 for β-methyl-ACh, and m/z 45 for Ch. The ion dissociation pathways of ACh, iso-ACh, Ch and β-methyl-ACh are presented in Scheme 1. The substructure of the ion at m/z 60 was assigned as trimethyl-hydro-amine. The ion at m/z 60 was a minor fragment in the product ion spectra of ACh, iso-ACh and β-methyl-ACh. ACh and iso-ACh produced identical product ion spectra. The MRM transitions of m/z 146 → 87 for ACh and iso-ACh, 104 → 60 for Ch and 160 → 101 for β-methyl-ACh were chosen for quantitation analysis.

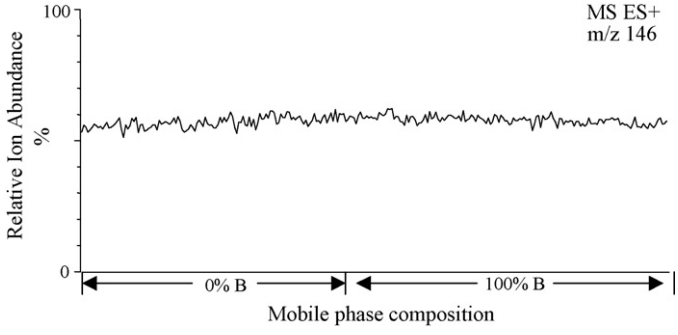


Fig. 2. The effect of mobile phase compositions on formation of molecular ion of ACh (M^+ at m/z 146).

3.2. Optimization of chromatographic conditions

The selection criteria of HPLC columns and mobile phase systems are important for detection of these neurotransmitters using LC/MS/MS techniques. ACh, Ch and iso-ACh are polar molecules with low molecular weights and their retentions on reversed phase chromatography are generally poor. Chromatographic separation techniques coupled with mass spectrometry for analysis of these small polar analytes have been achieved by ion pairing [37,43], cation-exchange [23,35,36], and HILIC [26,38,39,44,45]. Using ion pairing and cation-exchange techniques, the major concerns were the potential ion suppression of ESI process and the contamination of the ESI ion source caused by ion exchange or ion-pair reagents used in the HPLC mobile phases [40]. Using HILIC technique, the retention of polar compounds is increased when the proportion of organic solvent is increased [39]. In order to reduce the potential ion suppression of ESI process, extensive method development was often required to separate the neurotransmitters from inorganic salts in microdialysates [38]. Other concerns for analysis are the low concentrations of these extracellular neurotransmitters, the complex matrixes and the small sample volume of microdialysates.

In the present work, cation-exchange chromatography was chosen for the development of an LC/MS/MS assay to measure ACh, Ch and iso-ACh in microdialysates. Mobile phase systems containing alkanesulphonates are commonly used in cation exchange chromatography. However, these mobile phase systems are not ideal for electrospray ionization, since they could significantly reduce the ionization efficiency because of ion suppression. Hows et al. [35] developed a cation exchange

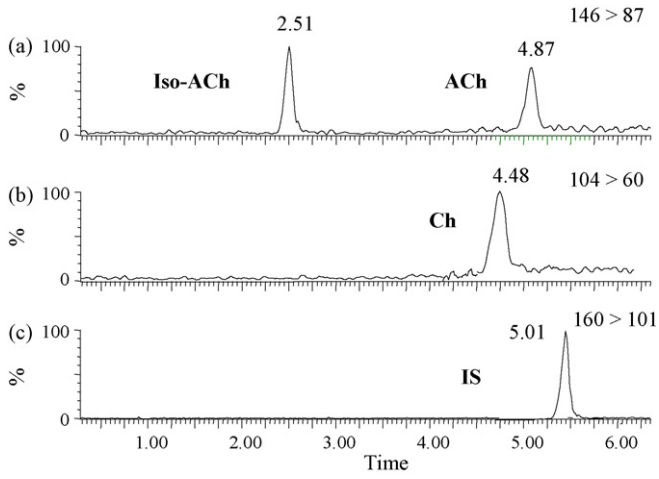


Fig. 3. The MRM ion chromatograms of: (a) Iso-ACh (1.5 nM) and ACh (0.5 nM), (b) Ch (1.5 nM) and (c) IS (13 nM) standards spiked in aCSF.

chromatography with elution buffer, consisting of a mixture of ammonium acetate, ammonium formate and acetonitrile. This system was more suitable with MS detection. A similar mobile phase system was also used in the present work, and high sensitivity detection of these neurotransmitters was achieved.

One important consideration is the potential ion suppression from the high concentrations of inorganic salts (sodium, potassium, magnesium and calcium chlorides) in dialysates. The use of a LC-SCX column (150 mm × 2.1 mm) with a rapid gradient from 0 to 100% B in 2 min resulted in early elution of the inorganic salts, which was diverted from the mass spectrometer for the first 1 min. This approach rendered minimal ion suppression typically observed during electrospray ionization, and reduced potential ion source contamination.

Fig. 3 shows the selected MRM chromatograms of an aCSF solution of ACh, iso-ACh and Ch standards spiked in aCSF solution. The selectivity of tandem mass spectrometry using MRM mode allows simultaneous determination of a number of compounds without chromatographic separation. However, iso-ACh and ACh are isobar molecules and they are both present in extracellular brain fluid [36,37]. ACh and iso-ACh produce the same MRM transition [12] (Scheme 1). Therefore, baseline separation of these two components is essential for accurate quantitation. Separation of ACh and iso-ACh was achieved using a SCX column with an eluent composition of 100% B (Fig. 3a).

It was mentioned by Uutela et al. [38] that the chloride ions of the Ringers solution (or aCSF solution) may cause some corrosion of the HPLC connection unions or the MS interface. The author suggested using the inert metal material of unions instead of stainless steel, the analysis could be carried out for weeks without clogging. We also observed a decrease in sensitivity over time that may be due to clogging of the ESI capillary, the HPLC connection unions, or the PEEK tubing. We found that the sensitivity could be easily recovered by pumping a solution containing H_2O and ACN (50:50, v/v) with 0.1% formic acid (FA) for a couple of minutes. In a good practice, our LC/MS system was cleaned-up weekly by pumping the H_2O/ACN solution with 0.1% FA for 30 min. The analysis has been carried out for several years without changing the unions, the ESI capillary or the PEEK tubing. In addition, this system clean-up procedure effectively reduced the chemical noise level in MS detection.

3.3. Method validation

The selectivity of the method was investigated in aCSF solution and microdialysis samples collected from the rat hippocampus. No chemical interference was observed for all analytes in the aCSF solution (Fig. 4). It should be mentioned that a small interference peak was observed at the same LC retention time and MRM transition as that of Ch in an old aCSF solution, which was stored at 4 °C for more than 30 days. Therefore, the aCSF solution prepared within 30 days was used for all studies. Since baseline levels of ACh in brain microdialysates are in fmol levels, there might be potential interferences for ACh detection from solutions and the instrument contamination. In practice, pure aCSF solution samples were checked daily before the analysis of microdialysates samples to avoid the potential interferences from contamination. The ion chromatograms of ACh, iso-ACh and Ch in microdialysis samples from rat hippocampus are presented in Fig. 5. Their identities were confirmed by comparing their HPLC retention times and the MRM transitions with the standard compounds. It should be noticed that an unknown chemical interference peak for ACh, which was not previously reported in the literature, was observed in many microdialysate samples from rat brains (Fig. 5b). This unknown chemical interference was more abundant than ACh in many samples. In addition, the abundance of this interference peak often increased over the sampling period. Therefore, baseline separation of ACh and this chemical interference is essential in accurately determining the small drug-related changes in ACh levels. The potential chemical interferences for the IS, β-methyl-ACh, in microdialysis samples collected from rat brain were also examined. As presented in Fig. 6a, an endogenous compound was observed with the same MRM transition as β-methyl-ACh

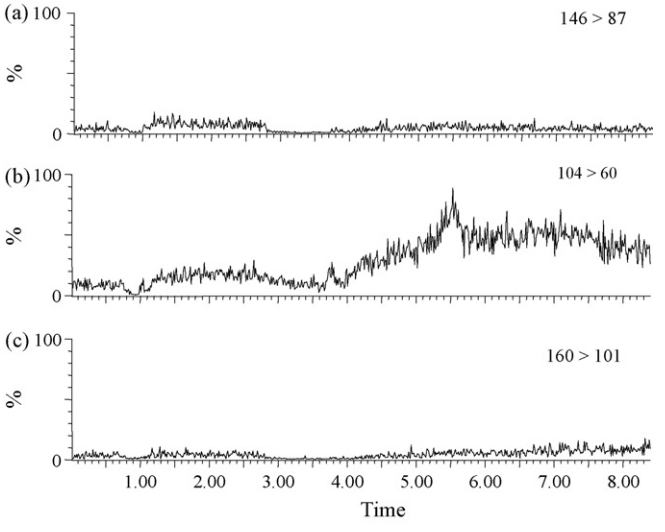


Fig. 4. The MRM ion chromatograms of: (a) Iso-ACh and ACh, (b) Ch and (c) IS in pure aCSF solution.

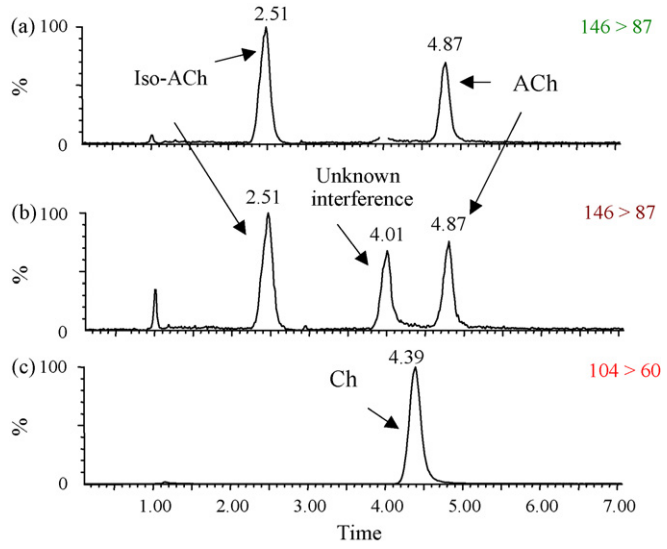


Fig. 5. The MRM chromatograms of: (a) ACh and iso-ACh, (b) an unknown interference peak of ACh and iso-ACh and (c) Ch in microdialysate from rat hippocampus.

(160 → 101) in microdialysates collected from rat brain. This endogenous compound was well separated from β-methyl-ACh (Fig. 6b) and did not interfere with the analysis.

In the method reported by Hows et al. [35], the concentrations of ACh were measured without using an internal standard. The reproducibility of the LC/MS analysis for quantitation of ACh was examined with and without using the IS. In the study, an aCSF solution contained 0.1 ng/ml of ACh and 2 ng/ml of IS was analyzed repeatedly for 88 injections in a period of 17.6 h. The eluent from the LC column was directed into the ion source of the mass spectrometer to observe the maximum effect

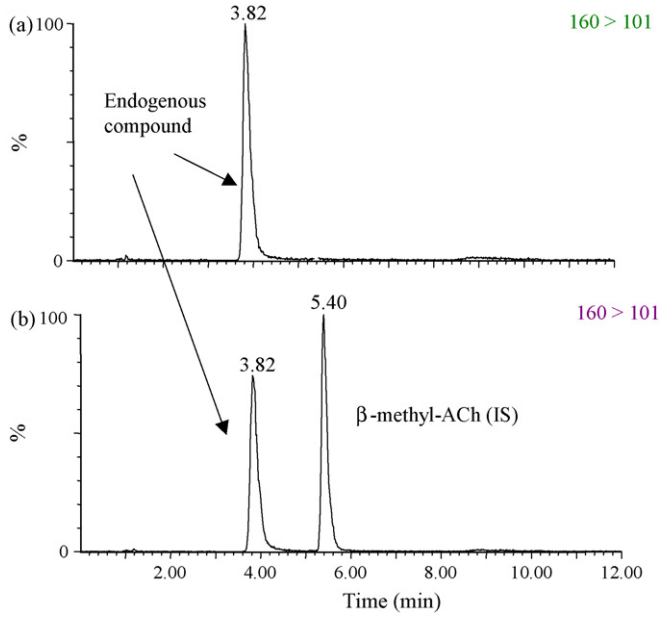


Fig. 6. The ion chromatograms for the MRM transition of 160 > 101 in: (a) microdialysis samples collected from rat hippocampus and (b) microdialysis samples from rat hippocampus spiked with IS β-methyl-ACh.

of aCSF solution on enhancement or suppression of molecular ions of ACh. A significant shift for the measured concentrations of ACh was observed when the IS was not used. In contrast, good reproducibility was achieved when the ACh concentration was determined using the peak area ratio to the internal standard. The mean concentrations of ACh ($n = 88$), measured with and without using IS, were 0.102 ± 0.006 ng/ml and 0.140 ± 0.043 ng/ml, respectively. The percent deviations of the mean values of ACh to the spiked concentration (0.1 ng/ml) measured with and without using IS were 2% and 40%, respectively. The results indicated that the IS played an important role for accurate measurement of ACh concentration in aCSF solution.

The samples collected from rat brain contained numerous endogenous compounds. The potential matrix effects on the measurements of ACh, iso-ACh and Ch in the LC/ESI/MS/MS analysis were investigated. First, the original concentrations of ACh, iso-ACh and Ch in microdialysis samples collected from rat dorsal hippocampus were measured to be 0.9, 10.6 and 1525 nM, respectively. Next, standard solutions of ACh, iso-ACh and Ch were spiked in the aliquots of the microdialysis samples to make the spiked concentrations of 0.5, 10 and 1500 nM for ACh, iso-ACh and Ch, respectively. The concentrations of these neurotransmitters in the spiked samples were then measured and the differences in the concentrations between the original samples and the spiked samples were calculated. The mean recoveries of the spiked ACh, iso-ACh and Ch were 106 ± 7, 93 ± 8 and 110 ± 10% (value ± CV%, $n = 5$), respectively. Collectively, these data indicated that there were no significant matrix effects on the measurement.

The limit of detection (LOD) in aCSF solution for ACh, iso-ACh and Ch was 0.01 nM (0.2 fmol on column), 0.03 nM (0.6 fmol on column) and 0.1 nM (2 fmol on column) with a S/N of 3:1, respectively. The limit of quantitation (LOQ) in aCSF solution for ACh, iso-ACh and Ch was 0.05 nM (1 fmol on column), 0.15 nM (3 fmol on column) and 0.5 nM (10 fmol on column) with a S/N of 10:1, respectively. The LOQ achieved in this method was 10–30 times more sensitive than the previous reported methods for ACh using ion pairing/ion trap [37], ion-exchange/tandem mass spectrometry [35], and HPLC–ECD methodologies using enzyme reactors [46]. A similar LOD and LOQ for ACh were claimed using HILIC/tandem mass spectrometry [38]. This method was three times more sensitive for Ch than the HILIC/MS method [38].

A seven-point standard curve was prepared to establish the calibration range, and linear regression analysis wit $1/x^2$ weighting was applied. Calibration curves of ACh, iso-ACh and Ch were linear in the concentration ranges of 0.05–10 nM, 0.25–50 nM and 15–3000 nM, respectively, using the peak area ratios of the analyte to that of IS β-methyl-ACh. The correlation coefficients were <0.995 for all three compounds. The intra-batch and inter-batch accuracy and precision for analysis of ACh, iso-ACh and Ch at two different concentrations in aCSF solutions are summarized in Table 1. The R.S.D. was less than 5% for ACh and iso-ACh, and less than 7% for Ch.

The stability of ACh and Ch spiked in microdialysis samples from rat hippocampus were evaluated at both 4 and 25 °C for 24 h. Our observations were in agreement with a previous report

Table 1
Accuracy and precision of the LC/MS/MS method for the analysis of ACh, iso-ACh and Ch in aCSF solution

Nominal conc (nM)	ACh		Iso-ACh		Ch	
	0.25	5.00	1.25	25.0	75.0	1500
Inter-batch ($n = 10$)						
Mean	0.249	5.01	1.24	24.7	76.2	1515
S.D.	0.0110	0.182	0.060	0.80	4.11	74.2
% R.S.D.	4.4	3.6	4.8	3.2	5.4	4.9
Intra-batch ($n = 3$)						
Mean	0.252	4.89	1.27	25.4	78.0	1491
S.D.	0.0101	0.161	0.053	0.82	4.50	92.4
% R.S.D.	4.0	3.2	4.2	3.2	5.8	6.2

S.D.: standard deviation; R.S.D.: relative standard deviation.

[38] showing that ACh was stable under these conditions. ACh was stable in the rat brain microdialysates for at least 2 months at −80 °C. In contrast to ACh, Ch decomposed totally in the microdialysis samples collected from rat brains at room temperature for 24 h. Ch was stable in aCSF solution for at least a week at room temperature suggesting that some endogenous chemicals in rat brain might cause the conversion or decomposition of Ch. When microdialysate samples were stored at −80 °C, the degradation of Ch slowed down. Ch was stable for at least a week in the freezer (−80 °C). In order to obtain reliable quantitation of Ch, the microdialysis samples should be collected under low temperature and immediately moved to a freezer after collection. The samples should be analyzed within a couple of hours after sample defrosts.

4. Application

The developed LC/MS/MS method was evaluated *in vivo* by monitoring the changes of ACh and iso-ACh concentrations in the dorsal hippocampus of freely moving rats after administration of SB-271046 and donepezil. The chemical structures of SB-271046 and donepezil are presented in Fig. 7.

As shown in Fig. 8, administration of the 5-HT_6 receptor antagonist, SB-271046 (10 mg/kg s.c.) caused nearly a 2-fold increase in the extracellular concentration of ACh related to the baseline. In a parallel experiment, tetrodotoxin (chemical structure of TTX in Fig. 7) (1 μM) was infused via the dialysis probe 60 min after administration of SB-271046. Perfusion of TTX caused a significant decrease in ACh levels to a maximum 50% below baseline ($p < 0.001$). The decrease in ACh during and after perfusion of the sodium channel blocker, TTX, indicates that ACh release was impulse dependent and from a neuronal source.

The extracellular concentrations of iso-ACh were unchanged after administration of SB-271046 or donepezil. Perfusion of TTX also had no effect on the iso-ACh levels from the rat hippocampus. The results suggested that iso-ACh could be used as an endogenous internal standard in quantitation of ACh to further increase the accuracy of the measurement.

Administration of donepezil (0.5 and 1 mg/kg i.p.) produced a dose-dependent increase in ACh levels ($F_{2,8} = 6.25$; $p < 0.0001$) (Fig. 9). The lower dose at 0.5 mg/kg produced a 50% increase whereas the higher dose at 1 mg/kg produced over 150% increase in hippocampal acetylcholine.

The increase in ACh release seen after administration of the 5-HT_6 receptor antagonist, SB-271046, was consistent with reports in the literature [47]. The increase in hippocampal ACh release seen after administration of donepezil was consistent with data in the literature for drugs that block the degradation of ACh through inhibition of the enzyme, AChE [48]. The dose dependent increase in ACh data after administration of the AChE inhibitor, donepezil, demonstrated that the developed LC/MS/MS method provided a sensitive and reliable analysis,

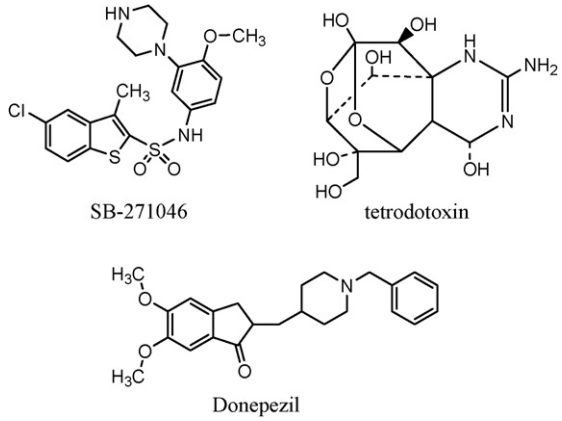


Fig. 7. The chemical structures of SB-271046, tetrodotoxin (TTX) and dosepezil.

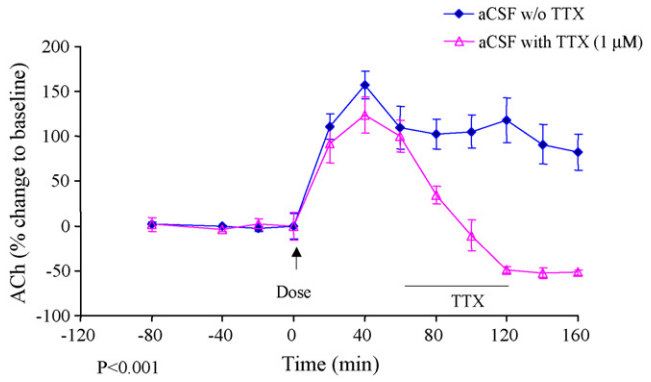


Fig. 8. Influence of the 5-HT_6 antagonist, SB-271046, on ACh release with or without including TTX in aCSF perfusion solution.

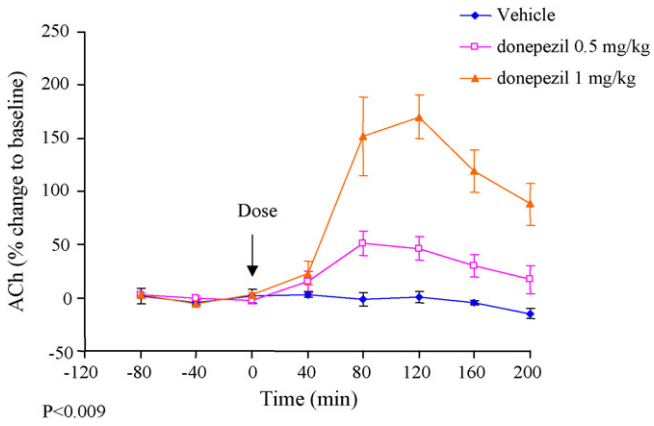


Fig. 9. Influence of donepezil (0.5–1 mg/kg i.p.) on ACh release.

which allowed the small changes in the extracellular concentrations of ACh to be monitored. Accurately measuring small changes in ACh levels in specific brain regions of freely moving animals induced by drug candidates provides important information in understanding disease pathology as well as designing and evaluating novel disease-modifying treatments. These biological data provided *in vivo* validation of the method.

5. Conclusion

A LC/MS/MS method was developed and validated to monitor the small changes in ACh concentrations of brain microdialysates using a benchtop tandem quadrupole mass spectrometer with a moderate sensitivity. This method achieved a detection limit of ACh at 0.01 nM (0.2 fmol) and a quantitation limit of 0.05 nM (1 fmol), which allowed reliable measurement of basal levels of ACh in specific regions of the rat brain. In addition, since there were no AChE inhibitors present in the aCSF, this new method enabled the measurement of ACh in its native state. The chromatographic separation of ACh from iso-ACh and an unknown chemical interference in brain microdialysates ensured the accuracy of the measurements. The developed method was validated *in vivo* by monitoring the changes of ACh concentrations in the dorsal hippocampus of freely moving rats after administration of SB-271046, donepezil and TTX. Overall, this new analytical method may demonstrate improved utility in drug discovery efforts designed to engineer compounds that interact, either directly or indirectly, with ACh and cholinergic receptor systems.

References

[1] K. Boonyapisit, H.J. Kaminski, R.L. Ruff, Am. J. Med. 106 (1999) 97–113.
[2] M. Testa, L.M. Biasucci, M. Cacciola, A. Lombardo, F. Pennestri, T.H. Lejemtel, F. Loperfido, Am. J. Cardiol. 77 (1996) 149–153.
[3] M. Sarter, J.P. Bruno, Brain Res. Rev. 27 (1998) 143–156.
[4] H. Ikawa, H.J. Yokoyama, Y. Morikawa, A. Hayashi, K. Katsumata, J. Pediatric Surg. 15 (1980) 48–52.
[5] K.E. White, J.L. Cummings, Compr. Psychiatry 37 (1996) 188–195.
[6] O. Felician, T.A. Sandson, J. Neuropsychiatry Clin. Neurosci. 11 (1999) 19–31.
[7] P. Davies, A.H. Verth, Brain Res. 138 (1977) 385–392.
[8] J.T. Coyle, D.L. Price, M.R. DeLong, Science 219 (1983) 1184–1190.
[9] W.W. Beatty, N. Butters, D.S. Janowsky, Behav. Neural Biol. 45 (1986) 196–211.
[10] M. Xu, Y. Nakamura, T. Yamamoto, K. Natori, T. Irie, H. Utsumi, T. Kato, Neurosci. Lett. 123 (1991) 179–182.
[11] G. Damsma, B.H.C. Westerink, A.S. Horn, J. Neurochem. 45 (1985) 1649–1652.
[12] G. Damsma, B.H.C. Westerink, A. Imperato, H. Rollema, J.B. De Vries, A.S. Horn, Life Sci. 41 (1987) 873–876.
[13] G. Damsma, B.H.C. Westerink, P. De Boer, J.B. De Vries, A.S. Horn, Life Sci. 43 (1988) 1161–1168.
[14] G. Damsma, P.T.M. Biessels, B.H.C. Westerink, J.B. De Vries, A.S. Horn, Eur. J. Pharmacol. 145 (1988) 15–20.
[15] G. Damsma, P. De Boer, B.H.C. Westerink, H.C. Fibiger, Naunyn-Schmiedeberg's Arch. Pharmacol. 342 (1990) 523–527.
[16] B.H.C. Westerink, G. Damsma, Trends Pharmacol. Sci. 10 (1989) 862–863.
[17] B.H.C. Westerink, P. De Boer, Neurosci. Lett. 112 (1990) 297–301.
[18] E. Moor, E. Schirm, J. Jacso, B.H.C. Westerink, Neurosci. 82 (1998) 819–825.
[19] A. Ajima, T. Kato, Neurosci. Lett. 81 (1987) 129–132.
[20] A. Ajima, T. Kato, Biogenic Amines 5 (1988) 461–464.
[21] A. Ajima, T. Nakagawa, T. Kato, J. Chromatogr. 494 (1989) 297–302.
[22] J.P. Ternaux, M.C. Chamoin, J. Bioluminesc. Chemiluminsc. 9 (1994) 65–72.
[23] T. Huang, L. Yang, J. Gitzen, P.T. Kissinger, M. Vreeke, A. Heller, J. Chromatogr. B. 670 (1995) 323–327.
[24] R.C. MacDonald, J. Neurosci. Methods 29 (1989) 73–76.
[25] B. Ulin, K. Gustavii, B.A. Persson, J. Pharm. Pharmacol. 28 (1976) 672–675.
[26] A. Guerrieri, F. Palmisano, Anal. Chem. 73 (2001) 2875–2882.
[27] T.H. Tsai, J. Chromatogr. B 747 (2000) 111–122.
[28] P. De Boer, B.H. Westerink, A.S. Horn, Neurosci. Lett. 116 (1990) 357–360.
[29] E. Acquas, H.C. Fibiger, J. Neurochem. 70 (1998) 1088–1093.
[30] G. Cuadra, K. Summers, E. Giacobini, J. Pharmacol. Exper. Therap. 270 (1994) 277–284.
[31] J.K. Liu, T. Kato, Neurochem. Intern. 24 (1994) 589–596.
[32] T.H. Tsai, C.F. Chen, Neurosci. Lett. 166 (1994) 175–177.
[33] T.R. Tsai, T.M. Cham, K.C. Chen, C.F. Chen, T.H. Tsai, J. Chromatogr. B 678 (1996) 151–155.
[34] J. Kehr, P. Dechent, T. Kato, S.O. Ogren, J. Neurosci. Methods 83 (1998) 143–150.
[35] M.E.P. Hows, A.J. Organ, S. Murray, L.A. Dawson, R. Foxton, C. Heidbreder, Z.A. Hughes, L. Lacroix, A.J. Shah, J. Neurosci. Methods 121 (2002) 33–39.
[36] M.Y. Zhang, P. Li, Q. Lin, E. Kerns, L.E. Schechter, C.E. Beyer, 52nd ASMS, Nashville, TN, May 24–27, 2004.
[37] Y. Zhu, P.S. Wong, M. Cregor, J.F. Gitzen, L.A. Coury, P.T. Kissinger, Rapid Commun. Mass Spectrom. 14 (2000) 1695–1700.
[38] P. Uutela, R. Reinila, P. Piepponen, R.A. Ketola, R. Kostiainen, Rapid Commun. Mass Spetrom. 19 (2005) 2950–2956.
[39] A.J. Alpert, J. Chromatogr. 499 (1990) 177–196.
[40] M.Y. Zhang, C.E. Beyer, J. Pharm. Biomed. Anal. 40 (2006) 492–499.
[41] G. Paxinos, C. Watson, The rat brain in stereotaxic co-ordinates, second ed., Academic Press, 1986.
[42] C. Eckers, J.C. Wolff, N.J. Haskins, A.B. Sage, K. Giles, R. Bateman, Anal. Chem. 72 (2000) 3683–3688.
[43] H. Sotoyama, Y. Zhu, J.F. Gitzen, F. Xie, P.T. Kissinger, Curr. Sep. 20 (2002) 11–15.
[44] H. Koc, M.H. Mar, A. Ranasinghe, J.A. Swenberg, S.H. Zeisel, Anal. Chem. 74 (2002) 4734–4740.
[45] E.S. Grumbach, D.M. Diehl, J.R. Mazzeo, LC–GC Eur. 19 (2004) 22–23.
[46] L. Trabace, A. Coluccia, S. Gaetani, M. Tattoli, R. Cagiano, C. Pietra, K.M. Kendrick, V. Cuomo, Brain Res. 865 (2000) 268–271.
[47] C. Riemer, E. Borroni, B. Levet-Trafit, J.R. Martin, S. Poli, R.H.P. Porter, M. Boes, J. Med. Chem. 46 (2003) 1273–1276.
[48] T. Kosasa, Y. Kuriya, K. Matsui, Y. Yamanishi, Eur. J. Pharmacol. 380 (1999) 101–107.