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Measurement of Acetylcholine in Rat Brain Microdialysates by LC – Isotope Dilution Tandem MS

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Abstract

An LC-MS/MS method was developed for measuring acetylcholine (ACh) in an aqueous medium using reversed-phase ion-pair chromatography, electrospray ionization on a quadrupole ion trap instrument and a tetradeuterated analogue (ACh-1,1,2,2-d₄) as an internal standard. A rapid separation was achieved on a 5-cm long octadecylsilica column (2.1 mm i.d.) by employing heptafluorobutyric acid (0.1% v/v) as an ion-pairing agent and requiring 10% v/v acetonitrile in 20 mM ammonium formate buffer under isocratic elution at 200 µl/min flow rate. The instrument's response was calibrated with samples containing known mole ratios of ACh and ACh-1,1,2,2-d₄ in an artificial cerebrospinal fluid, which afforded the conclusion that analyte concentrations could be determined by multiplying the measured analyte to internal standard ion-current ratio with the known molar concentration of the ACh-1,1,2,2-d₄ added. The rapid and simple assay was tested by measuring the basal neurotransmitter concentration in rat brain microdialysates without the use of a cholinesterase inhibitor upon sample collection.

Keywords

Acetylcholine; *In vivo* intracranial microdialysis; Liquid chromatography–tandem mass spectrometry; Electrospray ionization; Isotope dilution

Introduction

The extracellular concentration of acetylcholine (ACh), (CH₃)₃N⁺CH₂CH₂OCOCH₃, in the mammalian brain is typically very low due to its rapid hydrolysis by acetylcholinesterase (AChE), an enzyme that catalyzes the conversion of ACh to choline (Ch), (CH₃)₃N⁺CH₂CH₂OH [1]. Postmortem degradation of ACh is an additional obstacle for the determination of this neurotransmitter. Therefore, *in vivo* sampling is required for its reliable measurement in the brain. Although early *in vivo* experiments such as those using push-pull cannulae [2] also suffered from degradation of ACh in the sample prior to its detection, the introduction of intracranial microdialysis [3] has offered a solution to this problem for neurochemical studies. Microdialysis excludes proteins including AChE from the sample, because the practical “molecular-weight cut-off” for the commonly used polycarbonate membranes is 5,000 Da. Therefore, ACh hydrolysis after sampling does not occur. Determination of ACh in brain microdialysates usually employs separation of Ch and ACh by liquid chromatography with electrochemical detection after post-column enzymatic conversion of these neurochemicals to hydrogen peroxide [4]. Since the inception of this method,

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continuous efforts have been made to improve the separation by introducing narrow bore columns [5], enzyme immobilization [5,6] and improving the electrochemical (EC) detector [7]. A culmination of these improvements, along with the use of microbore ion exchange chromatography and electrode development for enhanced EC detection, afforded detection limits suitable for measuring basal ACh levels in rat brain microdialysates [8,9]. Nevertheless, determination of basal ACh levels has remained a challenge for neurochemists and routine measurements usually required the introduction of an AChE inhibitor such as neostigmine [10]. Such a practical solution may, however, affect physiology of the system and, thus, interfere with data interpretation [11].

LC–MS/MS has also been found suitable for the sensitive detection of ACh and related endogenous quaternary compounds [12]. This first report on the successful application of the technique to measure ACh in rat brain microdialysates without the use of an AChE inhibitor has prompted efforts to improve limit of detection, robustness and speed of analysis for neurochemical applications [13–17]. Most assays have relied upon the use of electrospray ionization (ESI), although a very recent study has successfully employed atmospheric pressure spray ionization (APSI) [17]. In addition to reversed-phase ion pair [12,17] and cation exchange chromatography [13,15,16], hydrophilic interaction chromatography (HILIC) has also been employed [14]. However, it was recently found that the microdialysis sample had to be diluted at least ten times with acetonitrile to obtain a reasonable peak shape by HILIC [17], which could be a disadvantage regarding assay performance using routine tandem mass spectrometers such as benchtop quadrupole ion traps [12]. Therefore, we modified the isocratic reversed-phase ion pair chromatographic conditions employed by Zhu *et al.* [12] to allow for higher throughput. Our additional improvement included the choice of internal standard (IS) for ACh quantification.

Although quantitative measurements of ACh in rat brain microdialysates have been performed without the addition of IS [13,15], the use of the latter has been found to improve assay performance [16]. Acetyl- β -methylcholine has been a commonly employed IS [16], including in the method we chose to modify [12]. However, LC–MS/MS precision improves with the use of a stable-isotope labeled internal standard [18]. One recent study has utilized nonadeuterated (N,N,N-trimethyl- d_9) ACh derived by the replacement of all the hydrogen atoms of the methyl groups of the compound with its stable isotope [17]. The use of an analogue of the analyte with such extensive deuterium labeling may carry isotope effects that could potentially impact the accuracy of quantitative LC–MS/MS analysis [19]. Therefore, we considered a deuterated ACh (1,1,2,2- d_4) with considerably less incorporation of the stable isotope than the N,N,N-trimethyl- d_9 analogue [17] as an IS. In addition, one important feature of isotope dilution quantitative MS analysis has not been considered by the previous implementation of LC–ESI-MS/MS assay for ACh in rat brain microdialysates [17]. Absolute amounts of the analyte can be determined by calibrating the instrument's response with samples containing known mole ratios of the analyte and its labeled IS based on the relationship

$$R_{\text{anal/IS}} = R_b + k \cdot (n_{\text{anal}}/n_{\text{IS}}) \quad (1)$$

where $R_{\text{anal/IS}}$ is the measured analyte to IS ion–current ratio, n_{anal} and n_{IS} are the mole amounts of the analyte and IS, respectively, and k is the MS response factor that relates the measured ion–current ratio to the mole ratio of the two species measured, and R_b is the ion–current ratio measured by the MS when the IS is measured by itself (i.e., $n_{\text{anal}}=0$) [18]. If the MS yields an identical change in the ion–current ratio upon changing $n_{\text{anal}}/n_{\text{IS}}$ ($k=1$) and the labeled IS does not produce a significant signal at the ion measured for the analyte ($R_b=0$), n_{anal} can be calculated by multiplying the $R_{\text{anal/IS}}$ with the known mole of the IS (n_{anal}) added. Our

hypothesis has been that ACh measurements by an isotope dilution-based LC–ESI-MS/MS method practically fulfill conditions for this ideal case and, thus, permits an increase in throughput compared to assays developed and validated without such considerations.

Experimental

Chemicals

ACh chloride and acetylcholine-1,1,2,2- d_4 (d_4 -ACh) chloride were purchased from Sigma-Aldrich (St. Louis, MO, USA) and C/D/N Isotopes (Pointe-Claire, Quebec, Canada), respectively. Artificial cerebrospinal fluid was supplied by Harvard Apparatus (Holliston, MA, USA). All other chemicals were of analytical grade and purchased from Thermo Fisher Scientific (Waltham, MA, USA).

In Vivo Microdialysis

Male Sprague-Dawley rats (250–300 g body weight) were used for the *in vivo* brain microdialysis experiments. Sampling of basal neurotransmitter levels was done from the ventral hippocampus according to the protocol described in our earlier study [10]; however, neostigmine (an AChE inhibitor) was not employed in the perfusion medium (artificial cerebrospinal fluid containing high concentrations of inorganic salt components). All procedures were reviewed and approved by the Institutional Animal Care and Use Committee at the University of North Texas Health Science Center before initiation of the study.

Samples and Sample Preparation

Calibration and quality control (QC) samples were prepared by dissolving ACh in artificial cerebrospinal fluid in 0.68, 1.38, 2.75, 5.5 and 13.75 nM concentrations, respectively, and by adding 2.75 nM of the internal standard d_4 -ACh. Rat hippocampal microdialysates were analyzed by taking 20 μ L from the fraction and admixing 5 μ L of deionized water containing 5.0 and 1.25 μ g/ μ L d_4 -ACh chloride, respectively. Twenty μ L of this sample solution was removed for injection.

LC–ESI-MS/MS

Analyses were performed on an LCQ ion-trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with the manufacturer's ESI source and operated in positive ion mode. Nitrogen was used as both the sheath and auxiliary gas at a pressure of 40 and 10 units, respectively. The spray voltage was set at 4.0 kV and the capillary temperature was 200 °C. Collision-induced dissociation (CID) product-ion MS/MS spectra were collected using 1.0 Th parent ion isolation width and 30% relative collision energy. The parent ions of ACh (m/z 146) and d_4 -ACh (m/z 150) were mass-selected for CID and, then, their principal product ions m/z 87 and 91, respectively, were monitored. Data acquisition and processing were performed by the manufacturer's XCalibur (version 1.4) software.

The mobile phase was delivered by a PM-80 pump (BAS, West Lafayette, IN, USA) for LC separations on a 50 $\times$ 2.1 mm i.d. Discovery HS C18 column packed with 5 μ m particles (Supelco, Bellefonte, PA, USA). The eluent was 10% v/v acetonitrile, 20 mM ammonium formate, pH 3.0, and 0.1% heptafluorobutyric acid (HFBA), and was delivered at 200 μ L/min flow rate. Samples were injected manually using a Rheodyne (Cotati, CA, USA) 7125 injection valve equipped with a 50 μ L loop. The effluent was diverted to waste for 1 min after injection to reduce the amount of salt entering the ESI source.

Assay Validation

Limit of detection (LOD) and limit of quantitation (LOQ) was defined as $3\sigma/S$ and $10\sigma/S$, respectively, where σ was the standard deviation of the response (estimated by the standard deviation of y-intercept of the regression) and S was the slope of the calibration line [21]. Intra-day reproducibility was determined by analyzing the QC sample containing 2.75 nM ACh five times in 2-h intervals on the same day with six replicates for each analysis. Inter-day reproducibility of the method was assessed by repeating the analysis of this sample (also with six replicates) on five consecutive days.

Results and Discussion

To develop a rapid and reliable LC/ESI-MS/MS assay for the quantification of ACh in rat brain microdialysate on a routine benchtop quadrupole ion trap instrument, we modified the isocratic reversed-phase ion pair chromatographic conditions employed by Zhu *et al.* [12]. Essentially, while column type and HFBA as an ion-pairing agent were adapted from the method, ammonium formate was used to match buffer capacity with mobile phase pH. With the use of HFBA instead of trifluoroacetic acid (TFA) to promote retention, the concentration of acetonitrile in the mobile phase could be raised to 10% (v/v) instead of 2% found optimal for TFA [17]. A higher proportion of organic modifier is usually advantageous for the efficiency and stability of ESI. To speed up analysis, we chose a 50-mm long column. Because microdialysates are relatively clean samples (albeit they contain high concentrations of inorganic components not detrimental to reversed-phase chromatographic columns), a guard column was not used.

No additional sample preparation was employed after mixing the calibration/QC and microdialysis samples with the solution of deuterated ACh. A stable-isotope labeled analogue is considered the most appropriate IS in quantitative bioanalytical LC-MS/MS assays [18, 20]. Recently, a deuterium isotope effect that may cause the partial resolution of analyte and its deuterated IS in reversed-phase LC has been identified as a potential source of matrix effect [19]. Although there has been no direct support to the occurrence of a deuterium isotope effect when ion pairing is believed to be responsible for the retention of the analyte, an approach to minimize such interference would be the selection of an analogue that relies on non-deuterium based (i.e., ^{13}C , ^{15}N and ^{18}O) stable-isotope labeling. However, such an approach is usually expensive and impractical for most analytes [19] including ACh. A prudent compromise may be the selection of an analogue that replaced fewer hydrogen atoms with deuterium, when alternative labeled compounds that fulfill the requirements of suitable internal standards for mass spectrometric quantification [18] are available. Therefore, we chose acetylcholine-1,1,2,2- d_4 (d_4 -ACh) instead of the alternative N,N,N-trimethyl- d_9 analogue [17] as an IS. Figure 1 shows the ESI mass spectra of ACh and d_4 -ACh, along with the CID product-ion (MS/MS) spectra of their molecular cations m/z 146 and m/z 150, respectively, on the quadrupole ion trap instrument used in our study. Accordingly, SRM chromatograms considered the principal product ions m/z 87 and 91 for ACh and d_4 -ACh, respectively.

Assay calibration correlating ACh concentrations (C_{ACh}) with SRM ion-current ratios ($R_{\text{ACh}/\text{d}_4\text{-ACh}}$) afforded the linear equation ($r^2=0.9962$) of $C_{\text{ACh}} \text{ (nM)} = 2.528 (\pm 0.090) \cdot R_{\text{ACh}/\text{d}_4\text{-ACh}} + 0.302 (\pm 0.233)$ in the presence of 2.75 nM d_4 -ACh. LOD and LOQ were 0.31 nM and 0.92 nM, respectively. We found that the labeled IS did not produce a significant signal at the ion measured for the analyte; thus, we considered that R_b was zero upon fitting data to equation (1). By plotting $R_{\text{ACh}}/n_{\text{d}_4\text{-ACh}}$ obtained from analyses of calibration/QC samples versus mole ratios of ACh and d_4 -ACh ($n_{\text{ACh}}/n_{\text{d}_4\text{-ACh}}$) in the target concentration range of 0.5 to 15 nM (Fig. 2), we also received a convincing linear relationship ($r^2=0.9944$) with slope (k , Equation 1) that could be considered $k=1$ (95% confidence interval: $0.973 < k < 1.13$). These observations have confirmed our hypothesis that ACh concentrations in brain microdialysis

samples can be reliably calculated by multiplying the measured R_{ACh/d_4-ACh} with the known molar level of the IS (C_{d_4-ACh}) added. Table 1 summarizes the results obtained from the analysis of the calibration/QC samples. Intra- and inter-day assay reproducibilities were also acceptable with 4.6% and 8.0%, respectively, as coefficients of variation (CV).

Figure 3 shows the SRM chromatograms for ACh and d_4 -ACh obtained upon the isotope dilution LC–ESI-MS/MS analysis of a representative hippocampal rat brain dialysate. In comparison with the method that employs a 150-mm long column and acetyl- β -methylcholine as an IS under identical chromatographic conditions [12,16], analysis time was reduced to 3 min (by more than 50%). Moreover, while the use of acetyl- β -methylcholine as an IS apparently requires calibration with a series of solutions containing different concentrations of ACh [12], addition of d_4 -ACh in known concentration affords a direct and reliable estimation of ACh levels in brain microdialysates samples according to our method validation. In a hippocampal rat brain microdialysis sample whose LC–ESI-MS/MS analysis is displayed in Fig. 3, 3.1 nM was calculated for its ACh concentration. Average ACh level of three consecutive 20-min fractions obtained from the same animal was 3.2 nM with standard deviation of 0.7 nM (CV of 22%), which is a typical within-subject biological variability of the *in vivo* microdialysis experiment [10]. Between-subject variability is usually considerable; hence, each animal is used as its own control, when the impact of potential pharmacological agents on extracellular ACh concentrations is tested using this experimental strategy [10]. In a separate set of brain microdialysates, we also evaluated the influence of adding ACh- d_4 at two different concentrations (4-times less ACh- d_4 was added for the second analysis) and found that the calculated neurotransmitter concentrations agreed well.

In conclusion, a rapid LC–MS/MS method (suitable for the analysis of 8 to 10 samples per hour) was developed for measuring ACh in a high-salt aqueous medium using isocratic reversed-phase ion-pair chromatography, ESI on a quadrupole ion trap instrument and a tetradeuterated analogue (ACh-1,1,2,2- d_4) as an IS. The simple assay was tested by measuring the basal neurotransmitter level in rat brain microdialysates without the use of a cholinesterase inhibitor upon sample collection. The method described here furnishes sufficient accuracy for exploratory neurochemical studies focusing on extracellular levels of ACh in the rat brain [10].

Acknowledgments

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Fig. 1. ESI mass spectra of (a) ACh and (b) d₄-ACh, along with the CID product-ion (MS/MS) spectra of their molecular cations m/z 146 and m/z 150, respectively, on a quadrupole ion trap instrument (LCQ).

Fig. 2. Selected-ion monitoring (SRM) ion-current ratios (R_{ACh}/n_{d_4-ACh}) *versus* mole ratios of ACh and the internal standard d_4 -ACh (n_{ACh}/n_{d_4-ACh}) in artificial cerebrospinal fluid samples.

Fig. 3.
LC – isotope dilution ESI-MS/MS analysis of a hippocampal rat brain microdialysate (dilution factor: 0.8; concentration of d_4 -ACh in the diluted sample: 5.4 nM).

Table 1

ACh analysis in rat brain microdialysates by reversed-phase ion-pair LC – isotope dilution ESI-MS/MS. Solutions of the analyte in artificial cerebrospinal fluid were used as quality control (QC) samples and 2.75 nM (C_{IS}) of d_4 -ACh was added as an internal standard (IS). Ratios and measured concentrations were given as averages $\pm$ standard deviations from six replicates.

C_{QC} (nM)	R_{ACh/d_4-ACh}	$C_{measured}^a$	Accuracy (%) ^b
0.69	0.30 ± 0.11	0.83 ± 0.32	20.1
1.38	0.44 ± 0.10	1.22 ± 0.28	-11.4
2.75	0.93 ± 0.12	2.55 ± 0.34	-7.1
5.5	1.86 ± 0.20	5.11 ± 0.54	-7.0
13.75	5.38 ± 0.82	14.8 ± 2.27	7.7

$$^a C_{measured} = C_{IS} \cdot R_{ACh/d_4-ACh};$$

$$^b (C_{measured} - C_{QC})/C_{QC} \cdot 100$$