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Development and validation of a sample stabilization strategy and a UPLC–MS/MS method for the simultaneous quantitation of acetylcholine (ACh), histamine (HA), and its metabolites in rat cerebrospinal fluid (CSF)

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ABSTRACT

A UPLC–MS/MS assay was developed and validated for simultaneous quantification of acetylcholine (ACh), histamine (HA), tele-methylhistamine (t-mHA), and tele-methylimidazolacetic acid (t-MIAA) in rat cerebrospinal fluid (CSF). The biological stability of ACh in rat CSF was investigated. Following fit-for-purpose validation, the method was applied to monitor the drug-induced changes in ACh, HA, t-mHA, and t-MIAA in rat CSF following administration of donepezil or prucalopride. The quantitative method utilizes hydrophilic interaction chromatography (HILIC) Core–Shell HPLC column technology and a UPLC system to achieve separation with detection by positive ESI LC–MS/MS. This UPLC–MS/MS method does not require extraction or derivatization, utilizes a stable isotopically labeled internal standard (IS) for each analyte, and allows for rapid throughput with a 4 min run time. Without an acetylcholinesterase (AChE) inhibitor present, ACh was found to have 1.9 ± 0.4 min *in vitro* half life in rat CSF. Stability studies and processing modification, including the use of AChE inhibitor eserine, extended this half life to more than 60 min. The UPLC–MS/MS method, including stabilization procedure, was validated over a linear concentration range of 0.025–5 ng/mL for ACh and 0.05–10 ng/mL for HA, t-mHA, and t-MIAA. The intra-run precision and accuracy for all analytes were 1.9–12.3% CV and −10.2 to 9.4% RE, respectively, while inter-run precision and accuracy were 4.0–16.0% CV and −5.3 to 13.4% RE, respectively. By using this developed and validated method, donepezil caused increases in ACh levels at 0.5, 1, 2, and 4 h post dose as compared to the corresponding vehicle group, while prucalopride produced approximately 1.6- and 3.1-fold increases in the concentrations of ACh and t-mHA at 1 h post dose, respectively, compared to the vehicle control. Overall, this methodology enables investigations into the use of CSF ACh and HA as biomarkers in the study of these neurotransmitter systems and related drug discovery efforts.

1. Introduction

Given the physiological importance of neurotransmitters as signaling molecules in the central nervous system (CNS), the ability to measure changes in neurotransmitter concentrations has become an integral part of CNS drug discovery and development. In recent years, the accurate measurement of neurotransmitter concentrations in an accessible matrix has provided the opportunity to use those concentrations as preclinical and clinical biomarkers of CNS penetration and target engagement.

Acetylcholine (ACh), one of the neurotransmitters released from cholinergic neurons in the CNS, plays an important role in sleep regulation, learning and memory, cognitive functions, and the pathology of neurological disorders such as Alzheimer's Disease (AD) [1–3]. A decrease in ACh levels in the brain is well-established as a contributor to memory dysfunction in AD [4,5]. Therefore, drugs that increase the release of ACh are being explored as potential treatments for the cognitive symptoms of AD. Histamine (HA), released from histaminergic neurons, has also been demonstrated to be an important neurotransmitter regulating many of the same brain functions as ACh [6]. Central HA is metabolized to its two major metabolites (tele-methylhistamine (t-mHA) and tele-methylimidazolacetic acid (t-MIAA)). Thus, the concentrations of HA, t-mHA, and t-MIAA in the cerebrospinal fluid (CSF) have been used as indices of brain histaminergic activity (Fig. 1B) [6–9].

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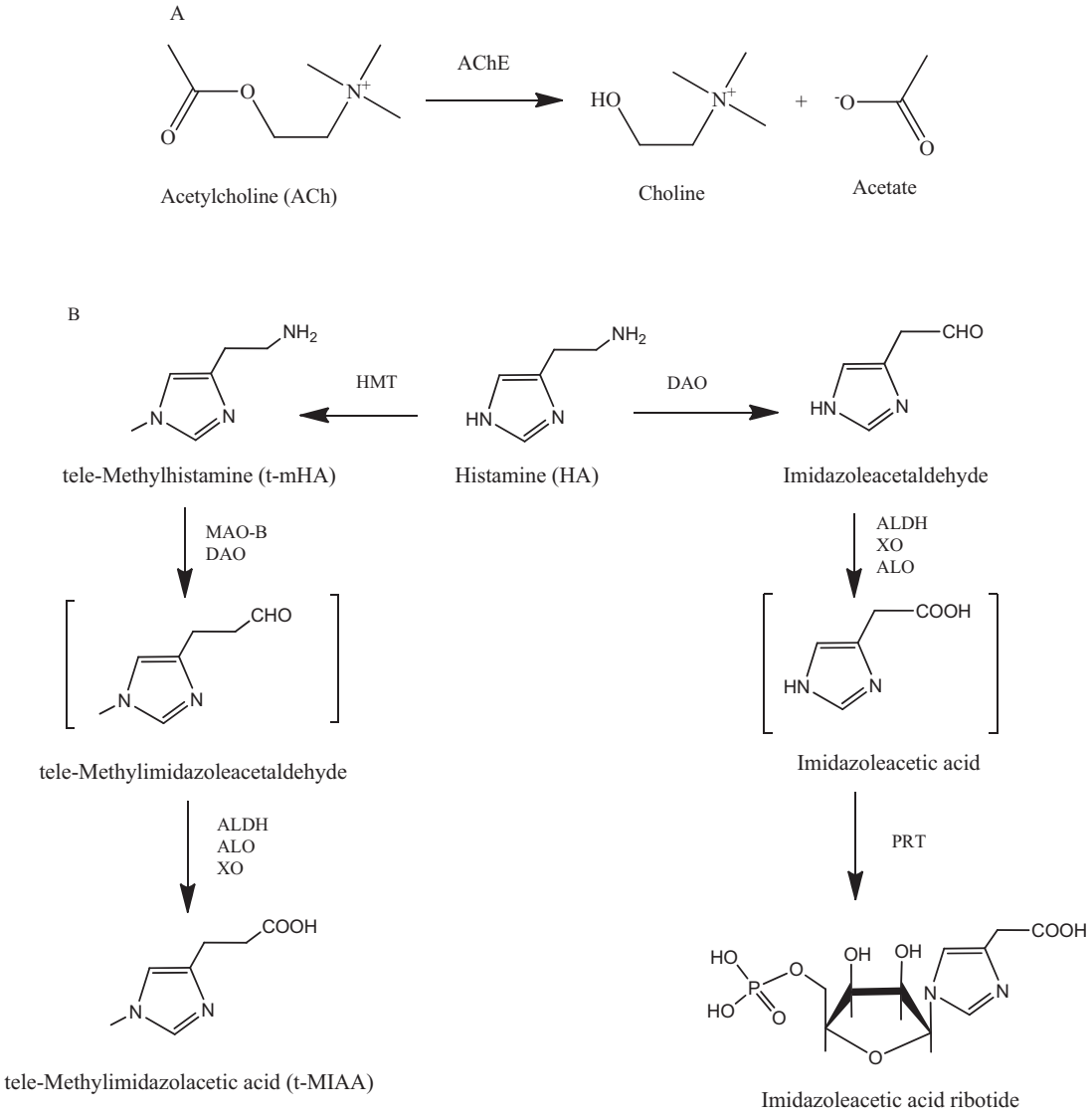

Fig. 1. Central metabolic pathways of ACh and HA. (A) for ACh and (B) for HA. AChE = acetylcholinesterase.

Dysfunction in central histaminergic activity has been linked to cognitive impairment. For instance, high levels of t-mHA were reported in CSF samples from schizophrenic patients [6], while the CSF concentrations of HA were elevated in patients with AD [10]. Hence, the central concentrations of ACh, HA and its metabolites could serve as biomarkers of cognitive impairment and as assessments of pharmacological interventions targeting such impairments.

Evidence that the cholinergic and histaminergic systems interact to regulate cognitive functions has further heightened interest in these systems for use in drug development [11,12]. Preclinical studies suggest that manipulations leading to increases in both HA and ACh can have additive or even synergistic effects on cognition [11–13]. The close functional relationship between these transmitters is further illustrated by the observation that ACh released from the CA1–CA3 region of the hippocampus of anesthetized rats can be modulated by endogenous HA [12]. Therefore, an analytical technique that provides simultaneous determinations of biomarkers of both the cholinergic and histaminergic systems in an accessible biological matrix such as CSF would be a useful research tool to better understand the underlying mechanisms and implications for therapeutic interventions.

Several challenges make the measurement of ACh, HA, t-mHA, and t-MIAA in rat CSF difficult. In particular, ACh is biologically unstable in both rat and human CSF due to its rapid conversion to acetate and choline by acetylcholinesterase (AChE) with an estimated half life of 1–2 ms at the synaptic cleft (Fig. 1A). Therefore, efforts to reduce the activity of AChE must be optimized to enable reliable ACh quantification. Additionally, ACh, HA, t-mHA, and t-MIAA are polar compounds with low molecular weights, which makes it difficult for these analytes to be retained on standard reverse-phase LC columns. It is also challenging to chromatographically separate these analytes from the inorganic salts and endogenous compounds present in enriched CSF samples to allow for the measurement of individual neurotransmitter concentrations. Furthermore, because concentrations of ACh, HA, t-mHA, and t-MIAA in rat CSF are typically very low due to their fast enzymatic metabolism in the brain, highly sensitive and selective analytical methods are required to perform these measurements.

While a number of analytical methods, including GC/MS [14], HPLC–EC [15], and mass spectrometry [16–22] have been published extensively for the measurement of ACh, only a few methodologies have been reported for the quantification of HA and its metabolites [7,23]. LC–MS/MS assay is the most commonly used quantitative

procedure to measure ACh concentrations in microdialysis samples, but no reports perform this measurement in CSF matrix. The methods utilize electrospray ionization tandem mass spectrometry (ESI-MS/MS) detection after various modes of chromatographic separation [16–22,24]. To address the chromatographic challenges of these small polar analytes, many studies have used tetrabutylammonium bromide [21,22] or heptafluorobutyric acid [18] as ion-pairing agents, which is less desirable for a mass spectrometry assay. Additionally, these methods fall short in adequately addressing the stabilization of ACh in CSF to ensure integrity through sample collection and analysis. For HA analysis, only Prell et al. reported a GC/MS method for measuring HA, t-mHA, and t-MIAA concentrations in human CSF [25]. Nevertheless, none of the methods mentioned above were capable of simultaneous, robust, and high throughput quantification for ACh, HA, t-mHA, or t-MIAA from a rat CSF matrix.

The aim of the present study was to develop and validate a rapid, simple, sensitive, selective and reproducible UPLC–MS/MS assay for simultaneous determination of ACh, HA, t-mHA, and t-MIAA in rat CSF. The biological stability of ACh in rat CSF was investigated and addressed through the choice of an appropriate AChE inhibitor. The developed method was successfully applied to monitor drug-induced changes of ACh, HA, t-mHA, and t-MIAA in rat CSF by administration of donepezil (an AChE inhibitor) and prucalopride (a 5-hydroxytryptamine-4 (5-HT_4) receptor agonist).

2. Experimental

2.1. Chemicals and reagents

HPLC-grade water, methanol, acetonitrile, formic acid, and hydrochloric acid (HCl) were obtained from Mallinckrodt Becker (Phillipsburg, NJ). ACh was purchased from Alfa Aesar (Ward Hill, MA), while (3-carboxypropyl)-trimethylammonium (iso-ACh), HA, t-mHA, t-MIAA, and diisopropyl fluorophosphates (DFP) were obtained from Sigma–Aldrich Chemical Co. (St. Louis, MO). Stable isotope-labeled ACh-1,1,2,2-d_4 (d_4-ACh) was obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada), while HA-a,a,b,b-d_4 (d_4-HA), d_3-t-mHA, and d_3-t-MIAA were from Cambridge Isotope Laboratories (Andover, MA). Eserine, also known as physostigmine, was purchased from TCI-GR (Tokyo, Japan). Artificial cerebrospinal fluid (aCSF) was supplied by CMA Microdialysis Inc. (North Chelmsford, MA). Rat, monkey, and human CSF samples for QC sample preparation and stability examination were purchased from Bioreclamation (Hicksville, NY). All other solvents were ordered from commercial sources at the highest purity grades available and used without further processing.

2.2. Chromatography

A Waters Acquity™ Ultra Performance LC (UPLC) system equipped with binary pumps, sample, and programmable column managers (Waters, Milford, MA) was used for liquid chromatography. Chromatographic separation was achieved using a 2.6 μm particle size, 2.1 × 100 mm Kinetex™ HILIC Core–Shell HPLC column (Phenomenex, Torrance, CA) at a flow rate of 0.45 mL/min. The column temperature was set at 45 °C. Mobile phase A was 0.2% formic acid and 20 mM ammonium formate in water, while mobile phase B consisted of 100% acetonitrile. The initial conditions were 90% B, and a linear gradient was performed with solvent A increasing from 10 to 70% within 1.25 min. This condition was maintained for 1.25 min to remove late-eluting substances from the column (column wash), and then the system was returned to its initial conditions with a 1.5 min equilibration period. The total analysis time including column wash and equilibration was 4 min. A Valco two-position valve was used to divert the early eluting salts to waste to prevent contamination of the LC–MS/MS interface. The samples stored in the autosampler were maintained at 4 °C.

2.3. Mass spectrometry

The chromatography system was coupled to the Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) using ESI. The source conditions were set as follows: 4.8 kV ion spray voltage, 480 °C ion source temperature, 45 psi nebulizer gas, 45 psi turbo gas, and 25 psi curtain gas. For analytes of interest, precursor-to-product ion transitions were established through direct infusion of neat standard of each compound into the ion source. The sensitivity was optimized for each compounds by manipulating entrance potential (EP), declustering potential (DP), collision energy (CE), and collision exit potential (CXP) values to achieve the best signals. The resolutions of Q1 and Q3 were set at unit. The dwell time was 50 ms for each MRM transition. Table 1 summarizes the mass spectrometry parameters for each compound and its corresponding stable isotope-labeled form.

2.4. Collection of rat CSF samples

Adult male Sprague–Dawley rats (Charles River, Raleigh, NC) weighting between 200 and 300 g were used for all *in vivo* experiments. The rats were kept in a temperature and humidity-controlled room with a 12 h light/dark cycle (lights on 6:00 am; lights off 6:00 pm for at least two weeks prior to the beginning of the study) with food and water available *ad libitum*. Rats were dosed subcutaneously with vehicle, prucalopride (5 mg/kg), or donepezil (1 mg/kg), respectively. Both prucalopride and donepezil were formulated at 2.0 mL/kg in phosphate buffered saline prior to dosing. At each sampling time point (0, 1, 2, 3, and 4 h post-dose for prucalopride, or 0, 0.5, 1, 2, 4, and 7 h post-dose for donepezil), rats were euthanized with CO_2 (slow flow of CO_2 to minimize stress to the animals) and then CSF samples were collected. CSF was collected by a puncture of the cisterna magna as described previously [26]. Briefly, after the dorsal skull and cervical region were exposed, the CSF fluid was aspirated by the introduction of a 25 gauge needle connected to PE50 polyethylene tubing and a 1 mL syringe. CSF samples were immediately transferred to a labeled collection tube coated with a stabilizing agent. Collection tubes were kept in a −80 °C freezer until 30 min prior to CSF sampling, and following collection the samples were flash frozen using a dry ice-methanol bath. Samples were stored in an −80 °C freezer until MS analysis. All animal protocols were performed according to Pfizer Animal Care Procedures and in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines for *the Care and Use of Laboratory Animals.*

2.5. Standard curve and QC preparation of ACh, HA, t-mHA, and t-MIAA

An automated liquid-handling robotic station (Hamilton Robotics, Reno, NV) was used to prepare standard curve and QC samples. The sample processing was carried out on a temperature controlled plate holder set to 4 °C to minimize the biological degradation of analytes during sample preparation process. Both standard curve and QC samples were prepared in a 96-well plate containing the stabilizing agent eserine (final concentration of 25 mM).

The stock solution of ACh, HA, t-mHA, and t-MIAA was manually prepared at 1 mg/mL for each analyte in 50/50 (v/v) acetonitrile/water. The intermediate stock solution was prepared at 125 ng/mL for ACh and 250 ng/mL for HA, t-mHA, and t-MIAA as a mixture in 2% acetic acid. The Hamilton station was used to pre-

Table 1
Mass spectrometry parameters for ACh, HA, t-mHA, and t-MIAA and their internal standards.

Compound	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	Entrance potential (V)	Declustering potential (V)	Collision energy (eV)	Collison cell exit potential (V)
ACh	146.2	87.1	10	40	22	12
HA	112.2	95.1	10	45	25	16
t-mHA	125.8	109.1	10	45	25	16
t-MIAA	141.0	95.1	10	45	25	16
d_4-ACh	150.2	91.3	10	40	22	12
d_4-HA	116.1	99.0	10	45	25	16
d_3-mHA	128.8	112.2	10	45	25	16
d_3-MIAA	144.1	98.1	10	45	25	16

pare calibration standards by serially diluting intermediate stock solution with aCSF at eight concentrations ranging from 0.025 to 5 ng/mL for ACh and from 0.05 to 10 ng/mL for HA, t-mHA, and t-MIAA (Table 3). Isotope-labeled standards (d_4-ACh, d_4-HA, d_3-t-mHA, and d_3-t-MIAA) prepared in acetonitrile were used as internal standards (IS) for the corresponding native analyte. A working solution of IS as a mixture (1 ng/mL for d_4-ACh, and 5 ng/mL for d_4-HA, d_3-t-mHA, and d_3-t-MIAA, respectively) was prepared in acetonitrile.

Rat CSF samples obtained commercially were pooled for the preparation of QC samples. The mean value of endogenous basal levels of analytes in pooled CSF samples was used in the calculations for the preparation of each QC level. When preparing QC samples, 25 mM eserine was added to the pooled CSF samples. QC target concentrations at four levels (lower limit quality control (LLQC), low quality control (LQC), medium quality control (MQC), and high quality control (HQC)), which covered the full range of the intended calibration range, were prepared by spiking this pooled matrix with a known amount of analyte to yield the desired final concentrations: (1) for ACh, 5-fold aCSF-diluted LQC (LLQC), matrix + 0.12 ng/mL (LQC), matrix + 2.5 ng/mL (MQC), and matrix + 4.5 ng/mL (HQC); and (2) for HA and its metabolites, 5-fold aCSF-diluted matrix (LLQC), matrix + 0 ng/mL (LQC), matrix + 2.5 ng/mL (MQC), and matrix + 5 ng/mL (HQC) (Table 2).

2.6. Sample preparation for the analysis of ACh, HA, t-mHA, and t-MIAA

A typical quantitative assay for ACh, HA, t-mHA, and t-MIAA consisted of an 8-point standard curve, blank (aCSF), blank spiked with IS in singlet, a QC matrix sample without IS (matrix blank), and the QC samples. All standard samples, QC samples, and unknown samples from dosed rats were transferred by a Hamilton automation system into separate wells of a 96-well polypropylene plate in 40 μL aliquots, followed by adding 80 μL of cold acetonitrile containing IS. The samples were shaken for 10 min and then centrifuged at 3200 × *g* for 15 min before analysis by UPLC–MS/MS. The sample preparation process was carried out at 4 °C.

2.7. Stability assessment

The kinetics of the *in vitro* disappearance of ACh, HA, t-mHA, and t-MIAA in the absence of any inhibitors were investigated in the fresh untreated pooled rat CSF. For comparison, this experiment was also performed in aCSF, monkey CSF, human CSF, and rat CSF pretreated with eserine. A mixture of ACh, HA, t-mHA, and t-MIAA (0.5 ng/mL for ACh, and 0.25 ng/mL for HA, t-mHA, and t-MIAA) was spiked into pooled CSF samples in duplicates at room temperature. Following spiking, at 0, 1, 3, 5, 8, 12, 15, and 60 min the enzyme reaction was terminated by quenching samples with cold acetonitrile containing IS. Samples processed at various time points were then subjected to UPLC–MS/MS analysis to measure ACh, HA, t-mHA, and t-MIAA concentrations and determine their half lives in different matrices.

To compare the effectiveness of various inhibitors, fresh CSF samples from six rats were collected and either left untreated or pretreated using three different approaches designed to stabilize ACh. Pretreated collection tubes contained the following chemical additives for stabilization assessment: (1) 100 mM HCl, (2) 0.05% DFP (AChE inhibitor), and (3) 0.05 M eserine (AChE inhibitor). Furthermore, the collection tubes were pretreated with 20 μL of eserine dissolved in isopropanol at concentrations of 0, 0.05, 0.1, 2.0 and 4.0 M. After evaporating under nitrogen at 40 °C, the coated tubes were used for CSF sample collection as described previously. The ACh levels were analyzed immediately by UPLC–MS/MS.

2.8. Measurement of prucalopride levels in rat CSF

Prucalopride measurements were performed by an in-house developed LC–MS/MS method. The LC–MS/MS system consisted of a Shimadzu HPLC system (Columbia, MD) and a Sciex API 4000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada). Chromatographic separation was achieved using a Synergy™ Polar RP column (Phenomenex, Torrance, CA), 50 × 2.1 mm, 4 μm particle size. Gradient elution with solvent A (0.1% formic acid/20 mM ammonium formate) and solvent B (100% acetonitrile) at a flow rate of 0.2 mL/min was applied. The MRM ion transitions (*m*/*z* 368 → 196) were obtained for prucalopride, and the MS parameters of EP, DP, CE, and CXP were as follows: 10 V, 70 V, 40 eV, and 15 V.

2.9. Data analysis

Analytes were detected specifically by monitoring HPLC retention times and the precursor-to-product ion mass-to-charge (*m*/*z*) values on a triple quadrupole mass spectrometer operated in posi-

Table 2
Summary of QC sample preparation.

Sample ID	ACh	HA	t-mHA	t-MIAA
LLQC	5-fold diluted LQC	5-fold diluted matrix	5-fold diluted matrix	5-fold diluted matrix
LQC	Matrix + 0.12 ng/ml	Unspiked matrix	Unspiked matrix	Unspiked matrix
MQC	Matrix + 2.5 ng/ml	Matrix + 2.5 ng/mL	Matrix + 2.5 ng/mL	Matrix + 2.5 ng/mL
HQC	Matrix + 4.5 ng/ml	Matrix + 5 ng/mL	Matrix + 5 ng/mL	Matrix + 5 ng/mL

Matrix = real rat CSF.

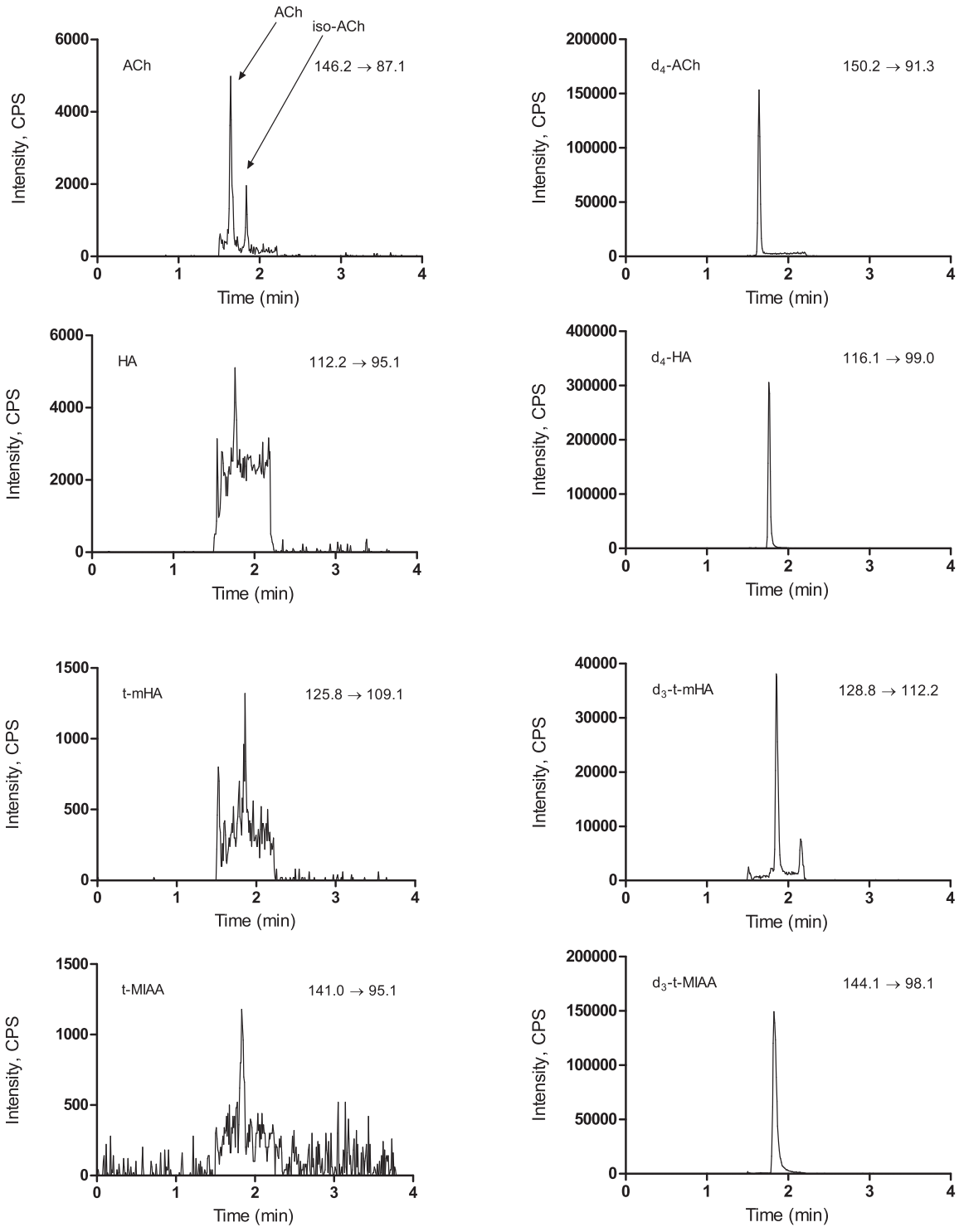

Fig. 2. Representative chromatographs of ACh, HA, t-mHA, and t-MIAA and their ISs in aCSF at concentrations of LLOQ (0.025 ng/mL for ACh and 0.05 ng/mL for HA, t-mHA, and t-MIAA).

tive ionization MRM mode. AB Sciex Analyst 1.4.1 software (Applied Biosystems, Foster City, CA) was used to acquire data and determine peak areas. Watson Laboratory Information Management System (LIMS) (Version 7.2.0.03, Thermo Fisher Scientific, Waltham, MA) was used for raw data reduction. Peak area ratios (PARs) of analytes and their IS peak areas were calculated, and all sample concentrations were then calculated from their PARs against their respective calibration lines. Samples were quantified by applying a $1/(\text{concentration})^2$ weighted linear regression analysis using Watson LIMS.

All the values are expressed as means ± SD and the number of experiments (n) is also indicated. Comparisons between two means were performed using the unpaired Student's t-test. Differences among groups were determined by one-way analysis of variance (ANOVA) or two-way ANOVA followed by PLSD or Bonferroni/Dunnett's post hoc test. For all statistical tests, $P < 0.05$ was

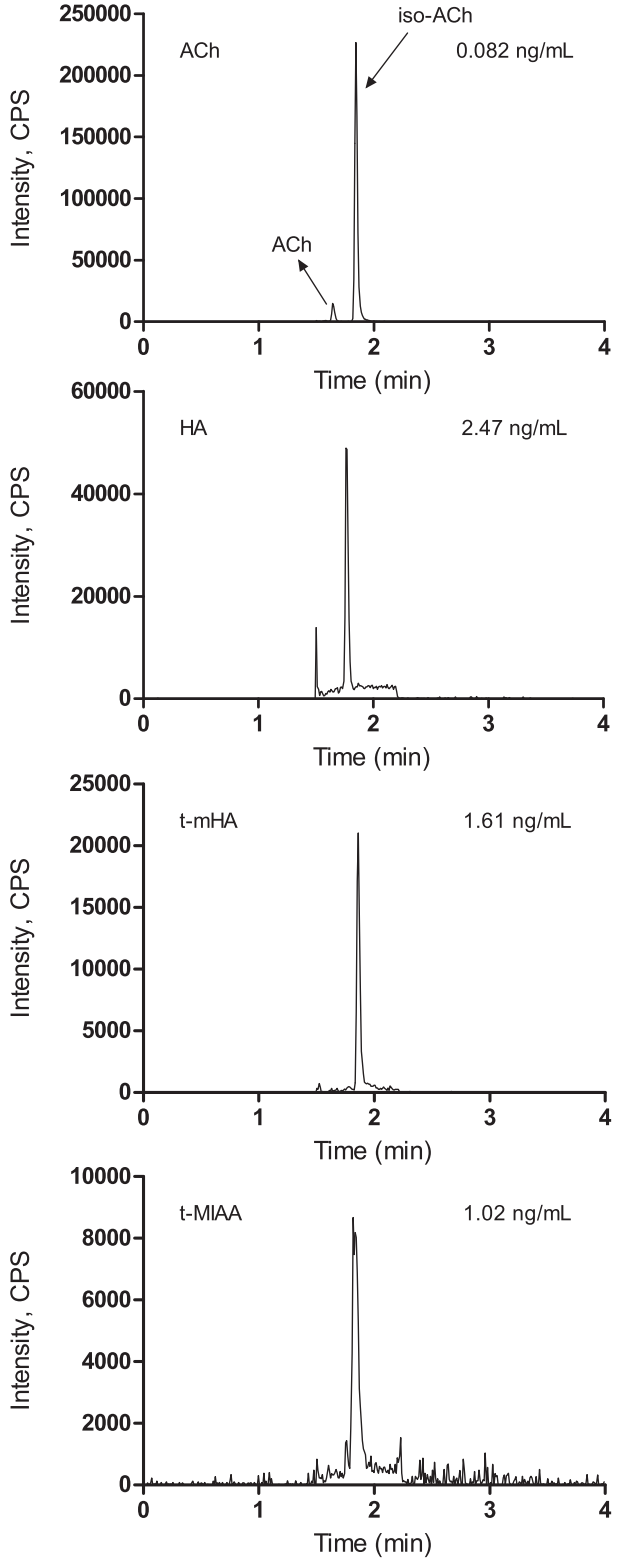

Fig. 3. Representative chromatographs of basal levels of ACh, HA, t-mHA, and t-MIAA in rat CSF at concentrations of 0.082 ng/mL, 2.47 ng/mL, 1.61 ng/mL, and 1.02 ng/mL for ACh, HA, t-mHA, and t-MIAA, respectively.

considered significant. For the purpose of clarity and biological relevance, we report significant differences *vs.* control samples. Statistical analysis was performed using GraphPad Prism (GraphPad software, Inc., San Diego, CA) or Statview (SAS Institute Inc., Cary, NC).

3. Results and discussion

3.1. UPLC–MS/MS assay development

ESI-MS/MS has been extensively used for the determination of endogenous biomarkers due to its high sensitivity and selectivity [16,27,28]. The ACh, HA, t-mHA, and t-MIAA molecules studies here all contain basic amine functionalities that allows for positive charging under acidic conditions and results in sufficient sensitivity using the ESI positive ionization mode without derivatization. The analysis was carried out using collision-induced dissociation (CID) and monitoring specific precursor-to-product ion mass-to-charge (m/z) values for individual analytes to make the assay sufficiently specific (Table 1).

While CID tandem mass spectrometry provides some selectivity, on-line HPLC separation serves as an additional purification step in an LC–MS/MS assay [28]. However, the column selection for the retention of ACh, HA, t-mHA, and t-MIAA proved to be challenging due to the highly polar and hydrophilic nature of these molecules. A number of chromatographic procedures have been previously reported for the analysis of ACh in microdialysis samples, including reversed-phase [29], ion exchange [16,30,31], or ion-pair [21,22] chromatography. However, the sensitivities of these methods were poor because of the highly aqueous mobile phases or the high concentrations of salt and mobile phase additives used. Although ion-pairing can provide improved retention, ion-pairing agents impede quantification due to the potential for ion suppression and MS source pollution. In this work, hydrophilic interaction chromatography (HILIC), a favorable alternative for the retention of polar compounds, was evaluated on several HILIC columns. First, we developed a method using a Waters Atlantis HILIC Silica (3 μM, 100 mm × 2.1 mm). This method provided efficient separation and resolution for ACh, HA, t-mHA, and t-MIAA with a run time of 7 min under optimized LC gradient conditions at a flow rate of 0.2 mL/min. This method was originally used to support discovery efforts, and has been demonstrated to be simple, robust, and reliable. Conversion of this conventional method to a UPLC method using the Acquity™ UPLC system enabled faster analysis. The Acquity™ UPLC BEH HILIC column (1.7 μM, 100 mm × 2.1 mm) separated ACh, HA, and t-mHA effectively, but yielded a broad peak shape for MIAA regardless of mobile phase composition. The Halo™ HILIC (2.6 μM, 100 mm × 2.1 mm, Mac-Mod analytical Inc.), a fused-core particle technology column, provided symmetric peak shape and retention characteristics for all analytes; but this column was unable to isolate ACh from an unknown peak present in rat CSF that was observed in many studies (data for the UPLC BEH and Halo columns not shown). Additionally the Halo™ column exhibited poor lot-to-lot reproducibility in our tests. Conversely, the Kinetex™ fused-core technology HILIC columns (2.6 μM, 100 mm × 2.1 mm) from Phenomenex achieved complete resolution of ACh, HA, t-mHA, and t-MIAA with superior peak shape symmetry (Fig. 2). While both the Atlantis HILIC and Kinetex™ HILIC columns were capable of separating analytes from the solvent front, the Kinetex™ HILIC column was chosen because it provided higher throughput (4 min run time) and a greater capacity factor (k' of 2.961, 3.251, 3.493, and 3.420 min for ACh, HA, t-mHA, and t-MIAA, respectively) with acceptable peak shape.

Quantitative analysis of endogenous biomolecules is often made difficult by matrix-induced interference and ion suppression due to co-elution of structurally related or similar molecules. The chromatographic system must resolve the analytes from each other, inorganic salts, and other existing endogenous components. For CSF sample analysis, particularly problematic are inorganic salts that exist at high concentration in rat CSF, which can cause ionization suppression and foul the ESI-MS source conditions. Adequate

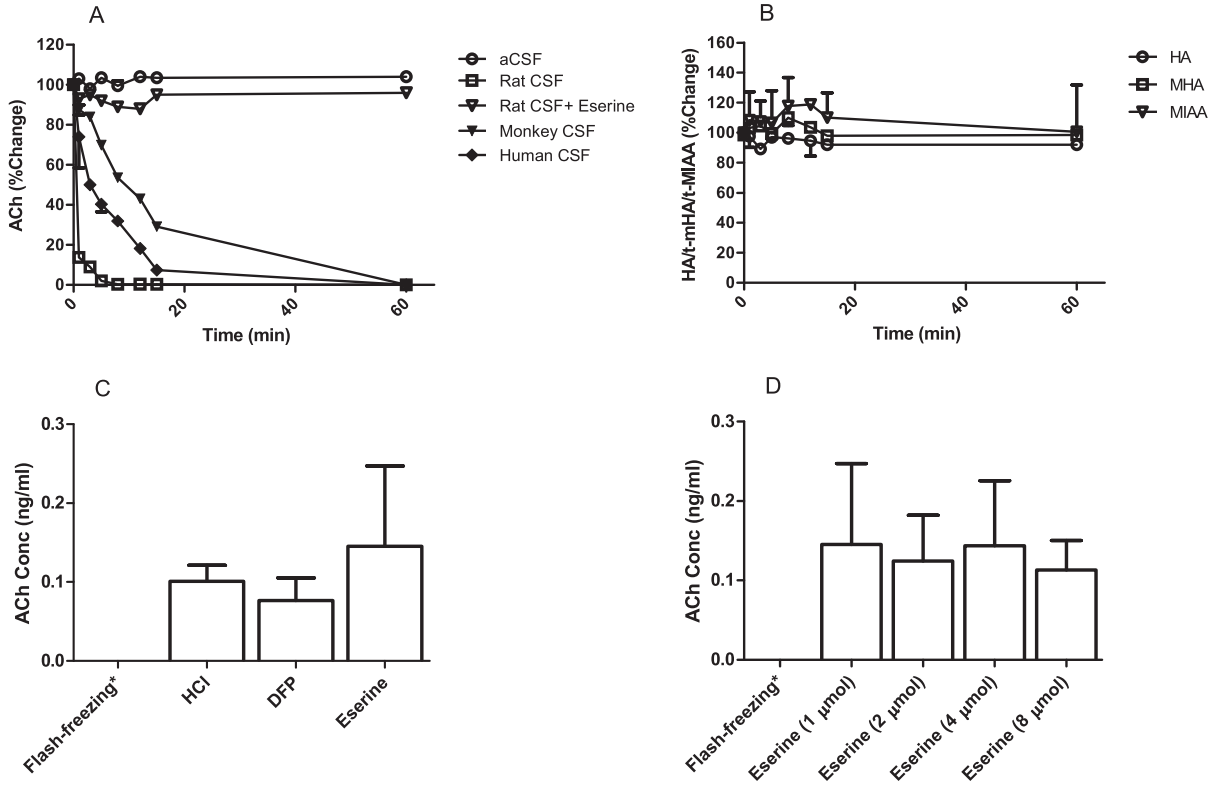

Fig. 4. Assessment of ACh biological stability in rat CSF. (A) *In vitro* half life of ACh ($n = 2$); (B) *in vitro* concentrations of HA, t-mHA, and t-MIAA ($n = 2$); (C) different stabilization methods ($n = 5/6$); and (D) different concentrations of eserine ($n = 5/6$). *The concentrations for the flash-freezing group were BLQ.

retention ($k' > 2.9$ min) of all analytes and the use of a diverter valve for the first 1.5 min addressed this problem. Additionally, isobaric interference can be a practical issue for biomarker analysis in biological samples, especially if analytes have low molecular mass. For example, the potential interfering compound iso-ACh is an isomer of ACh that is a substrate for the biosynthesis of carnitine, and it has been reported to be present at high concentrations in the rat brain [16,22]. Because ACh and iso-ACh are isobaric in the MS (i.e., they share the exact same MRM transition), it is essential to chromatographically separate iso-ACh from ACh to ensure the accurate determinations of ACh levels. The Kinetex™ HILIC column used in the present study has demonstrated effective separation of ACh from iso-ACh with retention times at 1.64 and 1.84 min, respectively (Figs. 2 and 3). Furthermore, when developing a MS-based biomarker assay, it is especially important to take ion suppression into consideration because the matrices used for standard curve preparation and unknown samples are different (see UPLC–MS/MS Assay Validation for details). In this method, stable isotope-labeled analogues, with identical chemical and physical properties as their corresponding analytes, were used as ISs, which proved to effectively compensate for ion suppression and ensure acceptable recovery and precision of the targeted analyte [32–35].

In the course of the development of the sample preparation procedure, a one-step dilution with acetonitrile was performed by an automated liquid-handling robotic station (Hamilton Robotics, Reno, NV). Because of the low protein concentration in CSF (approximately 200 times lower than that in plasma), protein precipitation was not observed in the diluted CSF sample [36]. The automated 96-well technique developed here allowed for high throughput, facilitated handling of a small sample volume, and prevented loss of analyte due to multiple liquid transfers. Additionally, carrying out the sample processing at 4 °C via the Hamilton temperature controlled plate holder minimized biological degradation of analytes during sample preparation process. In summary, monitoring the specific MRM transitions of individual analytes and judicious choice of a chromatographic system led to the development of a sensitive and selective assay for ACh, HA, t-mHA, and t-MIAA. The UPLC–MS/MS method described here used the HILIC Core–Shell column and provided acceptable separation and rapid throughput. Representative chromatograms for all analytes in extracted aCSF and rat CSF are presented in Figs. 2 and 3, respectively.

Table 3
Analytical performance of UPLC–MS/MS method for ACh, HA, t-mHA, and t-MIAA.

Compound	Dynamic range (ng/mL)	Linearity $n = 3$	Inter-assay accuracy for LLOQ (%RE) $n = 3$	Inter-assay precision for LLOQ (%CV) $n = 3$	Inter-assay accuracy for the rest of standards (%RE) $n = 3$	Inter-assay precision for the rest of standards (%CV) $n = 3$
ACh	0.025–5	0.999 ± 0.0008	−1.2	13	−5.0 to 5.0	1.4–10.2
HA	0.05–10	0.999 ± 0.0006	8.2	18.1	−6.0 to 8.2	1.2–10.2
MHA	0.05–10	0.990 ± 0.0042	−5.8	10.9	−8.4 to 13.0	5.5–14.0
MIAA	0.05–10	0.999 ± 0.0004	14.6	15.1	−10.0 to 0.8	1.5–12.7

LLOQ, lower limit of quantification; %CV, percent coefficient of variation; %RE, percent relative error.

Table 4
Precision and accuracy of UPLC–MS/MS method for the analysis of ACh, HA, t-mHA, and t-MIAA in rat CSF.

QC levels	ACh				HA				t-mHA				t-MIAA			
	LLQC (LQC/5)	LQC (EB+ 0.12 ng/mL)	MQC (EB+ 2.5 ng/mL)	HQC (EB+ 4.5 ng/mL)	LLQC (EB/5)	LQC (EB+ 0 ng/mL)	MQC (EB+ 2.5 ng/mL)	HQC (EB+ 5 ng/mL)	LLQC (EB/5)	LQC (EB+ 0 ng/mL)	MQC (EB+ 2.5 ng/mL)	HQC (EB+ 5 ng/mL)	LLQC (EB/5)	LQC (EB+ 0 ng/mL)	MQC (EB+ 2.5 ng/mL)	HQC (EB+ 5 ng/mL)
Nominal cone.	0.25	1.24	2.5	4.5	0.61	3.07	5.57	8.1	0.45	2.23	4.73	7.2	0.89	4.43	6.93	9.4
Intra-run ($n = 6$)																
Mean (ng/mL)	0.22	1.20	2.69	4.07	0.60	3.06	5.78	8.67	0.40	2.39	4.90	6.83	0.97	4.26	7.07	9.31
SD	0.01	0.04	0.08	0.08	0.04	0.19	0.18	0.49	0.05	0.28	0.32	0.51	0.06	0.19	0.18	1.05
%CV	2.3	3.2	2.9	1.9	7.2	6.2	3.1	5.6	12.3	11.5	6.6	7.5	6.0	4.3	2.5	11.3
%RE[a]	−7.1	0.0	7.6	−9.6	−2.3	−0.3	3.8	7.4	−10.2	7.2	3.6	−5.5	9.4	−3.8	2.0	−1.3
Inter-run ($n = 3$)																
Mean (ng/mL)	0.23	1.24	2.83	4.26	0.62	3.07	6.14	9.15	0.43	2.26	5.08	7.75	0.91	4.43	7.24	10.1
SD	0.01	0.07	0.14	0.18	0.04	0.19	0.39	0.51	0.07	0.28	0.43	1.24	0.13	0.33	0.29	0.89
%CV	6.4	5.5	4.8	4.1	6.1	6.2	6.4	5.5	15.9	12.2	8.5	16.0	13.9	7.5	4.0	8.8
%RE[a]	−2.5	3.3	13.2	−5.3	1.0	0.0	10.2	13.4	−5.1	1.3	7.4	7.2	2.0	0.0	4.5	7.1

EB, endogneous basel level; LLQC, lower limit quality control; LQC, low quality control; MQC, medium quality control; HQC, high quality control; SD, standard deviation.
% CV, percent coefficient of variation; % RE, percent relative error.
[a] RE calculations based on mean endogenous back-calculated concentration.

3.2. Stability assessment

While analytical methodologies on the quantitative analysis of neurotransmitters in rat brain microdialysates have been extensively reported [16,18,27,37], only a few studies have analyzed their concentrations in the CSF [8]. Although *in vivo* microdialysis is used in preclinical studies to assess the release of neurotransmitters, the translation of these findings from preclinical to clinical studies has yet to be demonstrated. To enable later clinical translatability, discovery phase researchers use rat CSF as an accessible matrix to assess central exposure and pharmacological effects. Unlike microdialysis, in addition to chemical modification these CSF neurotransmitter biomarkers are subject to further biological metabolism after sample collection due to the presence of enzymes and proteins in the CSF. Our earlier clinical data showed that when no enzyme inhibitors were added to collection vials, the ACh concentrations were below the lower limit of quantification (BLQ, 0.025 ng/mL) in human CSF, suggesting that ACh may undergo significant degradation from sample collection through analysis [38]. In this present study, we investigated the stability of ACh, HA, t-mHA, and t-MIAA in CSF and explored strategies to maintain sample integrity via enzymatic inhibitors.

The kinetics of the *in vitro* disappearance of ACh was investigated in treated and untreated rat CSF. During incubation in fresh rat CSF, the *in vitro* kinetic data indicated that exogenously spiked ACh rapidly disappeared with an *in vitro* half life of 1.9 ± 0.4 min (Fig. 4A). In contrast, spiked ACh was stable for up to 60 min at room temperature when incubated in either aCSF or rat CSF pretreated with eserine (Fig. 4A). Eserine was reported to be a potent non-competitive inhibitor of AChE when pre-incubated with the enzyme for as short a time of 6 min [39]. Therefore, the observed decreases in ACh concentration in rat CSF samples over the incubation period were linked to enzyme-related biological instability, as indicated by the fact that the enzyme AChE exists in CSF, but not in aCSF, and that the half life of ACh was prolonged to more than 60 min in the presence of AChE inhibitor. Additionally, the *in vitro* half lives of ACh in human and monkey CSF were determined as 4.6 ± 0.2 min and 8.9 ± 0.4 min, respectively (Fig. 4A), thus further confirming its instability in CSF samples. As a result, ACh cannot be measured in rat CSF under conditions commonly used for sampling due to its extremely short *in vitro* half life. Thus, identifying and incorporating a stabilizing condition that will preserve the integrity of ACh is a key factor for ACh measurement in rat CSF. Three stabilizing agents (HCl and AChE inhibitors eserine and DFP) were investigated for their ability to stabilize ACh. As seen in Fig. 4C, ACh concentrations were BLQ when samples were collected into untreated tubes, indicating that the flash-freezing alone cannot adequately prevent the ACh degradation processes. Although all three test conditions appeared to stabilize the ACh at detectable levels, ACh was best stabilized by eserine-treated tubes as indicated by an approximately 1.5-fold greater concentration when compared to HCl- or DFP-treated tubes (Fig. 4C), making it the preferred collection tube. Finally, we used an eserine concentration series to investigate the concentration-dependence of the inhibition of degradation of ACh in rat CSF. Our results demonstrated that the inhibitory effects of eserine were independent of its final concentration in the range of 25–200 mM (Fig. 4D). This is likely due to the fact that eserine concentrations at and above 25 mM far exceed the reported K_i value of 3.1 μM [39], so that these concentrations can therefore completely inhibit AChE activity, resulting in a maximal stabilization of ACh levels in rat CSF at all concentrations tested. In summary, 25 mM (i.e., 1 μmol) eserine was chosen in this study to stabilize samples to enable reliable ACh measurements.

The stabilities of ACh, HA, t-mHA, and t-MIAA in stock solution and in the final extract were also evaluated. When prepared in the stock solution at a concentration of 1 mg/mL, the neat standards

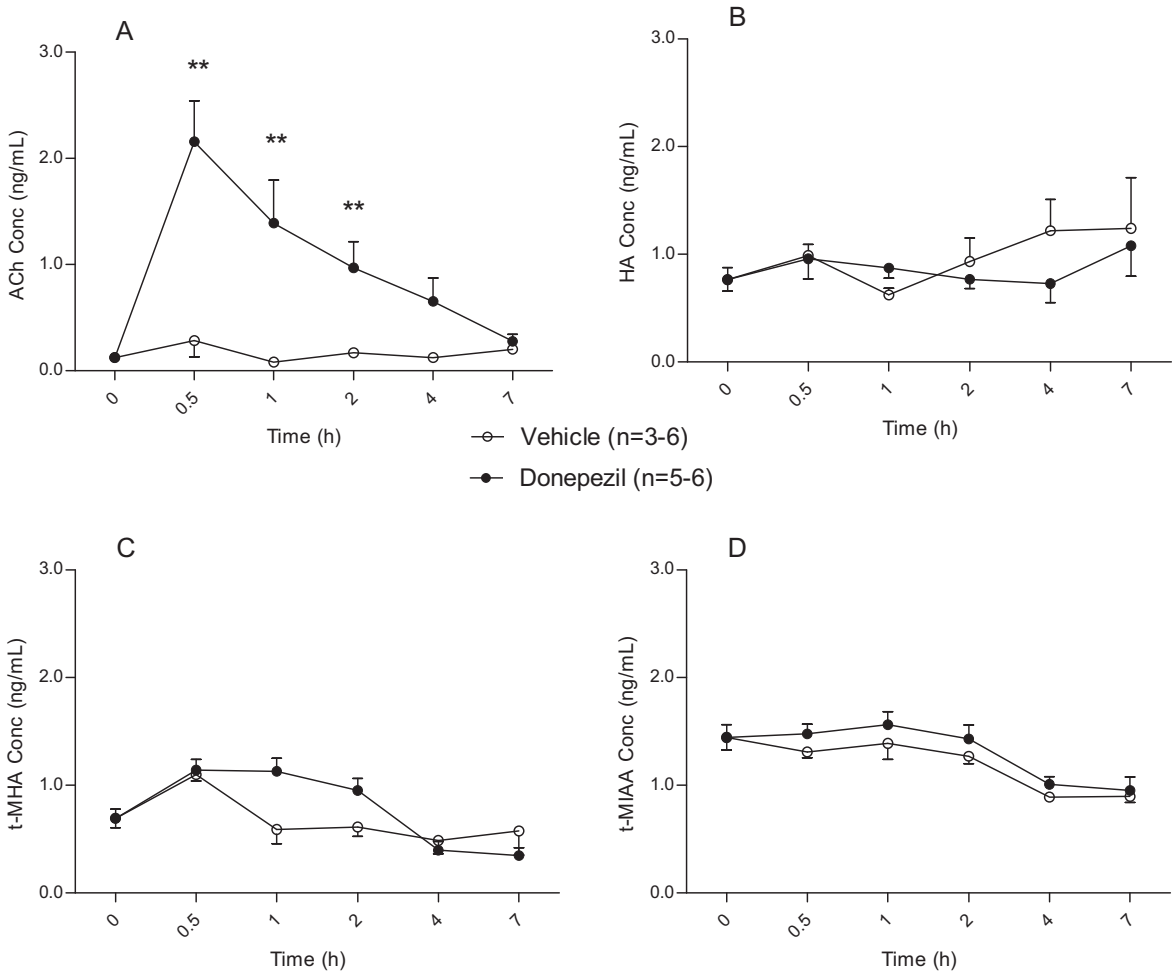

Fig. 5. Effects of donepezil on the release of ACh, HA, MHA, and MIAA in rat CSF after SC dosing 1 mg/kg of donepezil. (A) ACh; (B) HA; (C) t-mHA; and (D) t-MIAA. **A significant difference in ACh concentrations between donepezil-treated group with the corresponding vehicle group ($p < 0.05$). Vehicle, $n = 3$–6; donepezil, $n = 5$–6.

of all analytes were determined to be stable for up to 6 h at room temperature and for at least 1 month at −20 °C. Following sample processing, all analytes were verified as stable for up to 6 h in extract form while held at room temperature in a 96-well plate fitted with deactivated glass vial inserts (Waters, Milford, MA). Non-specific binding due to adherence to glass surfaces was not observed for ACh, HA, t-mHA, or t-MIAA under the conditions tested. Additionally, the biological stabilities of HA, t-mHA, and t-MIAA in rat CSF were also investigated individually. In rat CSF, without the use of any enzyme inhibitors, HA and its metabolites were confirmed to be stable at room temperature for up to 60 min (Fig. 4B).

3.3. UPLC–MS/MS assay fit-for-purpose validation

Robustness of this developed UPLC–MS/MS assay for the purpose of preclinical exploratory investigations was assessed [40]. Development and validation of quantitative biomarker assays is generally complicated by the presence of endogenous analyte in the matrix of interest. This background endogenous level elevates the analyte concentrations in spiked control samples and creates a barrier to lower levels of quantitation. Because no blank matrix sample exists that is free of endogenous analyte, it is challenging to choose standard matrix for the analysis [28]. The approach taken here was to use analyte-free surrogate matrix in combination with matrix-based QC samples to evaluate the assay performance. In the present study, commercially available aCSF, a non matrix-solution containing a similar salt content to real rat CSF, was selected as the standard curve matrix. The suitability of this surrogate matrix was demonstrated statistically in three validation runs with quantitative linearity (Table 3) and quantitative precision and accuracy compared with real rat CSF QCs (Table 4). Ultimately, this assay has been used to support several in-house preclinical programs over the past three years with consistent results. The robustness of the assay performed using aCSF as a surrogate matrix was therefore confirmed by these studies.

In an LC–MS/MS assay, the lower limit of quantification (LLOQ) is typically defined as the lowest concentration of the standard curve that be measured with acceptable accuracy and precision. Our method demonstrated that the LLOQ was 0.025 ng/mL for ACh and 0.05 ng/mL for HA, t-mHA, and t-MIAA based on an injection volume of 10 μL of spiked aCSF samples. The linearity of the assay was confirmed for the validated range of 0.025–5 ng/mL for ACh and 0.05–10 ng/mL for HA, t-mHA, and t-MIAA, with correlation coefficients (R^2) over 0.99 ($n = 3$) (Table 3). For all the analytes, the mean inter-assay accuracy and precision at the LLOQ concentration level were between −1.2 and 14.6% RE and between 10.9 and 18.1% CV, respectively ($n = 3$) (Table 3). The mean inter-assay accuracy and precision for the rest of the calibration standards were between −10.0 and 13.0% RE and between 1.2 and 14.0 CV, respectively ($n = 3$) (Table 3). The dynamic range of this assay was confirmed as appropriate for the measurement of ACh, HA, t-mHA, and t-MIAA in rat CSF collected from multiple preclinical studies.

The goal of our biomarker research is to develop an assay to generate reproducible and reliable data suitable for critical

decision-making during target and candidate selection. The analytical method must provide the ability to monitor differentiated biomarker responses between vehicle and drug-treated groups as well as to evaluate the disease progression from one stage to another. For biomarker research in neuroscience, the modulation window for these neurotransmitters could be narrow (less than 2-fold increase) depending on the target. Considering that assay variability is inversely proportional to differentiation, an analytical method with the best precision and accuracy could help to achieve the maximum differentiation power. Therefore, the assay performance was evaluated by conducting a fit-for-purpose validation via analysis of the intra- and inter-assay precision and accuracy. The intra-assay precision and accuracy were determined using CSF-based QC samples prepared at the four concentration levels with six replicates, while the inter-assay precision and accuracy were generated from three consecutive assay runs. For ACh analysis, the basal levels of ACh in rat CSF was assigned a value of zero because eserine was not initially added to these commercial rat CSF samples. QC target concentrations for ACh were then determined by adding a known amount of ACh to rat CSF samples, which were treated with eserine before QC sample preparation (Table 2). On the other hand, due to the relatively high endogenous levels of HA and its metabolites present in rat CSF, the LQC levels were assigned to their endogenous levels, which were calculated by the mean concentrations of all LQCs ($n = 18$) in three batch runs, while the LLQC levels were determined by diluting the rat CSF matrix with aCSF (Table 2). This would allow for an evaluation of assay performance near the lower end of the calibration curve if the samples were incurred in that concentration range. The analysis of the CSF-based QC samples demonstrated acceptable precision and accuracy based on pre-established validation criteria (±20% CV and RE for LLQC and ±15% CV and RE for the rest of the QCs). The intra-run precision and accuracy for all analytes were between 1.9 and 12.3% CV and −10.2 to 9.4% RE, respectively. Inter-run precision and accuracy for all analytes fell between the range of 4.0 and 16.0% CV and −5.3 to 13.4% RE, respectively. These results indicate that the assay was robust and reproducible, and they also suggest that an aCSF-based standard curve can accurately and precisely quantify the analyte in a biological matrix-based QC sample. Table 4 shows the detailed QC accuracy and precision data.

3.4. Application

The developed and validated UPLC–MS/MS method was evaluated *in vivo* by monitoring drug-induced changes of ACh, HA, t-mHA, and t-MIAA in rat CSF samples after administration of donepezil, a reversible inhibitor of AChE. In the US and many other countries, donepezil is a drug used widely for the symptomatic treatment for mild, moderate, and severe AD. The increase in ACh concentration in the brain is believed to be the major factor contributing to the therapeutic efficacy of donepezil. However, due to the instability of ACh in the CSF matrix, the degree of modulation of ACh in rat following donepezil administration is not understood. By optimizing the stabilization strategy as described above, we were able to detect basal levels of ACh in rat CSF (0.12 ± 0.04 ng/mL) without compromising the integrity of HA, t-mHA, and t-MIAA (Fig. 5). Donepezil was found to cause dramatic increases in ACh levels at 0.5 ($p < 0.05$), 1 ($p < 0.05$), 2 ($p < 0.05$), and 4 h post dose as compared to the corresponding vehicle group. The maximum increase in ACh (approximately 16.7-fold) was achieved 1 h post dose (Fig. 5A). These results are similar to increases in ACh levels observed in rat brain extracellular fluid (ECF) in response to donepezil in our earlier microdialysis work [41], although the CSF ACh response lagged approximately 30 min behind plasma exposure while ECF ACh tracked with plasma exposure. Furthermore, after a single

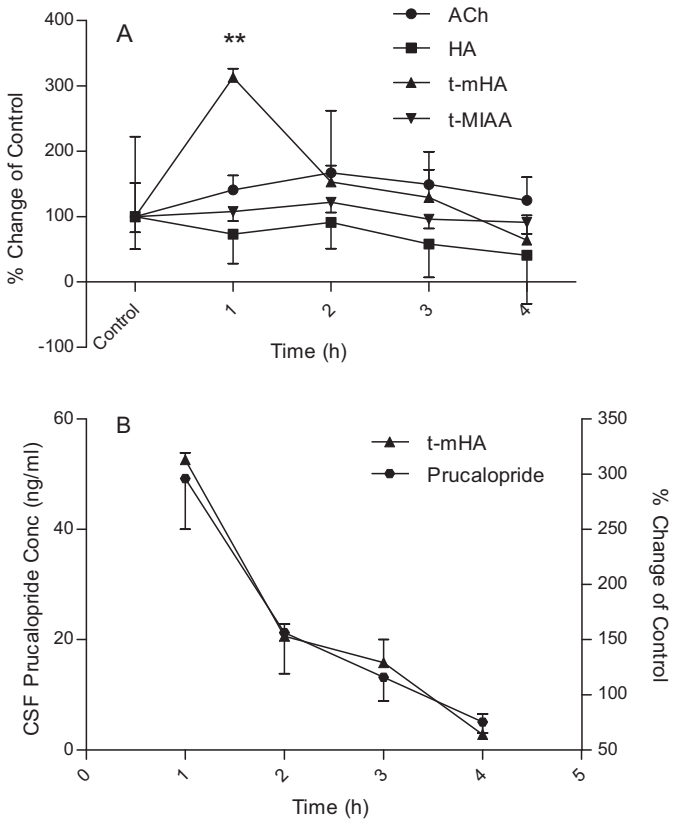

Fig. 6. Effects of prucalopride on the release of ACh, HA, t-mHA, and t-MIAA in rat CSF after dosing 5 mg/kg of prucalopride. (A) Percent changes of ACh, HA, t-mHA, and t-MIAA in rat CSF; and (B) Correlation between the concentrations of prucalopride and t-mHA in rat CSF. **A significant difference in t-mHA concentrations in rat CSF at 1 h post dose compared to controls ($p < 0.05$).

sample preparation and injection, the basal levels for HA, t-mHA, and t-MIAA in rat CSF were measured as 0.77 ± 0.27, 0.69 ± 0.21, and 1.45 ± 0.29 ng/mL, respectively, demonstrating that the levels of these species were not modulated by donepezil in the same way as ACh levels. One of the main goals of CNS drug discovery and development is to design medicines with better pharmacological efficacy and fewer undesirable side effects as compared with existing approved drugs. A drug targeting both cholinergic and histaminergic brain activities could potentially differentiate its pharmacological response with respect to donepezil in the treatment of AD.

In addition to the aforementioned example, the established method was applied to monitor changes in the concentrations of ACh, HA, t-mHA, and t-MIAA in rat CSF samples following administration of the 5-HT_4 receptor agonist prucalopride. As shown in Fig. 6A, within one hour after administration of prucalopride, approximately 1.6- and 3.1-fold ($p < 0.05$) increases in the concentrations of ACh and t-mHA were observed, respectively, as compared to the vehicle (vehicle data not shown). Maximal changes in the levels of ACh and t-mHA were attained at 2 h and 1 h post dose, respectively. Moreover, the change in t-mHA was closely correlated with CSF prucalopride concentration, with a R^2 of 0.99 (Fig. 6B). The concentrations of HA and t-MIAA were not statistically different from vehicle following administration of prucalopride. Previous reports have shown increases in ACh release triggered by other 5-HT_4 receptor agonists [42,43]. Work by Consolo et al. [42] has demonstrated a link between 5-HT_4 receptor activity and ACh release in the frontal cortex of rats [42]. Other regional activity was shown by Mohler et al. (2007), whereby a 5-HT_4 selective agonist (VRX-03011) augmented ACh efflux in the rat hippocampus for

delayed spontaneous alteration [43]. However, the current study demonstrated for the first time that the 5-HT_4 agonist prucalopride can cause an increase in ACh in rat CSF. Further interest in the 5-HT_4 receptor lies in the association of histamine in addition to its ability to potentiate ACh release. The substantial increases in t-mHA release seen after the administration of prucalopride were consistent with our in-house findings from microdialysis studies (unpublished data). To our knowledge, this is the first report of positive t-mHA response to prucalopride in rat brain, suggesting a possible linkage between brain histaminergic and cholinergic activities via the 5-HT_4 receptor. Additionally, these data were in agreement with previously published data that t-mHA might be a more sensitive CSF biomarker than HA and MIAA as a reflection of central histaminergic activities [6–9]. These data demonstrate that the UPLC–MS/MS method developed here provided a sensitive and reliable analysis that allowed small alternations in ACh levels to be monitored. Accurately measuring small changes in CSF biomarkers induced by drug candidates provided important information for understanding disease pathology, determining the pharmacokinetic/pharmacodynamic relationship, and predicting the clinical dosage. These biological data provided *in vivo* validation of the described method.

4. Conclusions

This study reports the development and validation of a UPLC–MS/MS method to simultaneously quantify ACh, HA, t-mHA, and t-MIAA concentrations in rat CSF. In this methodology, our samples were prepared by a one-step dilution with stable isotope-labeled internal standards, and then analyzed by UPLC–MS/MS. ACh was demonstrated to have extensive enzyme-related biological instability in both fresh and frozen CSF for all species tested. Thus the AChE must be reduced or blocked to enable reliable ACh measurements. Pretreatment of collection vials using the AChE inhibitor eserine was optimized to prevent ACh degradation and was a key factor ensuring ACh measurement integrity. Additionally, the method was proven suitable for *in vivo* quantification of ACh, HA, t-mHA, and t-MIAA in rat CSF over the concentration range of 0.025–5 ng/mL for ACh and 0.05–10 ng/mL for HA, t-mHA, and t-MIAA with acceptable precision and accuracy. This combination of UPLC–MS/MS and sample stabilization results in a sensitive, accurate, selective, and rapid method accommodating today's high-throughput screening requirements for drug discovery. This multiplexed assay will provide insights into the mechanism associated with cognitive dysfunction and may ultimately lead to the discovery of novel targets for pharmacological intervention.

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