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Microdialysis coupled on-line to capillary liquid chromatography with tandem mass spectrometry for monitoring acetylcholine *in vivo*

Holly M. Shackman [a], Minshan Shou [a], Nicholas A. Cellar [a], Christopher J. Watson [a], Robert T. Kennedy [a,b,*]

[a] *Department of Chemistry, University of Michigan, Ann Arbor, MI, United States*
[b] *Department of Pharmacology, University of Michigan, Ann Arbor, MI, United States*

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Abstract

Capillary liquid chromatography–mass spectrometry (cLC–MS) was coupled on-line to microdialysis sampling to monitor endogenous acetylcholine (ACh) from the rodent brain. *In vivo* microdialysate sampled at 0.6 μL/min from the striatum of ketamine or chloral hydrate anesthetized rats was loaded onto a sample loop and then injected onto a ~5 cm long strong cation exchange (SCX) capillary column. A step gradient was used to separate the analyte from ionization suppressing salts contained in dialysate in 2.4 min. Sampling coupled on-line with cLC–MS allowed for high temporal resolution (data points at 2.4 min intervals), good reproducibility (10–15% relative standard deviation, R.S.D.), and sensitive limits of detection (0.04 nM or 8 amol injected). The method successfully monitored basal and stimulated levels (induced by increased K^+ concentrations) of ACh from the anesthetized rat without necessitating perfusion of an acetylcholinesterase (AChE) inhibitor. Absolute and percent basal levels of ACh from rats receiving different anesthetics were also compared.

Keywords: Capillary liquid chromatography; Strong cation exchange; Mass spectrometry; Acetylcholine; Striatum; Microdialysis; K^+ stimulation; Ketamine; Chloral hydrate

1. Introduction

Acetylcholine (ACh) is an important neurotransmitter involved in signaling in both the peripheral and central nervous system (CNS). Within the CNS it is recognized to function in temperature control, blood pressure regulation, motor coordination, learning and memory, and in controlling stages of sleep (Sagales and Domino, 1973; Wessler et al., 1998; van der Zee and Luiten, 1999). Much recent ACh research has concentrated on its role in pathological conditions such as Huntington's disease, Tourette's syndrome, schizophrenia (White and Cummings, 1996), Parkinson's disease and Alzheimer's disease (Felician and Sandson, 1999).

Microdialysis sampling is frequently used for studying ACh function *in vivo*. A complication with this approach is that ACh is rapidly hydrolyzed by acetylcholinesterase (AChE) (Macintosh, 1959; Smith, 1984; Parsons et al., 1993) resulting in low concentrations in extracellular space and dialysate making detection difficult. As a result, AChE inhibitors are often added to the perfusing fluid to raise ACh levels and facilitate detection. While making detection simpler, this addition is less than ideal as it may alter neurotransmission and confound interpretation of results. Another limiting factor is that temporal resolution of the sampling is often limited to 10–20 min making it difficult to detect rapid changes in ACh neurotransmission such as might occur with changes in behavior or rapid pharmacological effects. Analytical methods with high sensitivity for ACh are needed to allow detection without AChE inhibitors at better temporal resolution.

ACh in dialysate is most commonly detected by high-performance liquid chromatography (HPLC) coupled to an enzyme reactor with electrochemical (EC) detection. (For review see Tsai (2000).) With mass limits of detection of 10 fmol, it is usually necessary to collect dialysate fractions off-line for up to 20 min resulting in relatively poor temporal resolution (Damsma et al., 1988; Greaney et al., 1993; Huang et al., 1995; Kato et al., 1996) with or without AChE inhibitors present.

* Corresponding author at: 930 N. University Ave., Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055 United States. Tel.: +1 734 615 4363; fax: +1 734 615 6462.
E-mail address: rtkenn@umich.edu (R.T. Kennedy).

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More recently, ACh has been detected in dialysate using HPLC coupled to mass spectrometry (MS) (Zhu et al., 2000; Hows et al., 2002; Uutela et al., 2005). The initial study used ion-pairing chromatography for separation, off-line sample collection, and tandem mass spectrometry (MS^2) on a quadrupole ion trap (QIT). Tandem MS, which allows a characteristic ion of the analyte to be collected and then fragmented for the final signal, yielded excellent selectivity by providing detection based on fragmentation, and a 1.4 fmol detection limit. While samples could be sensitively detected, throughput of the method was limited to just a few samples per day (Zhu et al., 2000). Hows et al. (2002) achieved similar detection limits and temporal resolution by coupling either a strong cation exchange (SCX) or dual-mode HPLC column (reversed-phase/SCX) to off-line MS detection. While these methods allow for routine detection of basal ACh levels without AChE inhibition, the use of off-line sampling and 1 fmol detection limits still required sampling times of 20 min thus preventing a significant improvement in temporal resolution over the HPLC-EC methods.

In this paper we report improvements in *in vivo* monitoring of ACh based on coupling microdialysis on-line to capillary liquid chromatography (cLC) with MS to detect ACh. Capillary columns require less sample volume and improve ionization efficiency via lower flow rates relative to conventional HPLC columns allowing mass limits of detection to be improved over 100-fold. The improved mass detection limits and on-line analysis allow monitoring of *in vivo* ACh levels, without the use of AChE inhibitors, at temporal resolution of 2.4 min, representing a significant improvement over previous methods.

2. Experimental

2.1. Chemicals and reagents

Benzoin methyl ether, glycidyl methacrylate, toluene, trimethylolpropane trimethacrylate, and 2,2,4-trimethylpentane were purchased from Sigma–Aldrich (St. Louis, MO). High purity water and methanol were from Burdick & Jackson (Muskegon, MI). Liquid chromatography and spectrophotometry grade anhydrous alcohol was purchased from J.T. Baker (Phillipsburg, NJ). Glacial acetic acid, ammonium formate, and HPLC grade ammonium acetate were distributed by Fisher Scientific (Fairlawn, NJ). Hydrofluoric acid (HF) and sodium chloride were from Acros Organics (Morris Plains, NJ). Calcium chloride, magnesium sulfate, and potassium chloride were purchased from ICN Biomedicals Inc. (Aurora, OH). Acetylcholine chloride and chloral hydrate were purchased from Sigma. Ketamine hydrochloride was from Fort Dodge Animal Health (Fort Dodge, IA) and medetomidine hydrochloride (Dormitor) was from Pfizer Animal Health (Exton, PA). Fused silica capillary was purchased from Polymicro Technologies (Phoenix, AZ).

2.2. Capillary liquid chromatography

Capillary chromatography columns with integrated electrospray emitters (Emmett and Caprioli, 1994) were prepared as previously described (Baseski et al., 2005; Haskins et al., 2001). Briefly, 500–800 μm long macroporous photopolymer frits were formed within 20 cm lengths of 50 μm inner diameter (i.d.) × 360 μm outer diameter (o.d.) fused silica capillary 5 cm from the terminus by photopolymerization of methacrylate solution within the capillary. Compared to the previous study that used 25 μm i.d. capillaries (Baseski et al., 2005), we found it necessary to increase the polymerization time to 45 min when using a larger i.d. capillary, as frits formed using shorter times were unstable. A 5.0–5.5 cm length of the fritted capillary was packed at 800 psi with SCX HPLC particles (5 μm, Allsphere SCX, Alltech Associates Inc., Deerfield, IL) using a dilute slurry of the particles (artificial cerebral spinal fluid, aCSF as solvent). The outlet of the capillary was then pulled to a fine tip on a pipette puller so that the i.d. at the tip was ∼3 μm.

The capillary LC system with on-line microdialysis was plumbed as illustrated in Fig. 1. A two valve (C2, Cheminert, Valco Instruments, Houston, TX) system was used with the selection valve allowing determination of the pump used to generate flow through the injection valve and cLC column. The gradient LC Pump (MicroPro, Eldex Laboratories, Napa, CA) in Fig. 1 was utilized during sample injection (0.4 min) and gradient elution (12 μL/min total flow rate and ∼800 nL/min through column after flow splitting). Pump 2 in Fig. 1 (pneumatic amplifier pump, Haskel Inc., Burbank, CA) was maintained at elevated pressure (3400 psi) and used to purge the system of bubbles or rinse the column as necessary. Dialysate was loaded on-line into a capillary sample loop (250 μm i.d. capillary with 5 μL volume) for injections. All connections were constructed using 75 μm i.d. × 360 μm o.d. capillary except for the link between the splitting-tee and the injection valve, which was 50 μm i.d. × 360 μm o.d.

A step gradient was utilized for separation. Mobile phase A was comprised of 25 mM ammonium acetate and 25 mM ammonium formate in water while mobile phase B consisted of 90% mobile phase A and 10% methanol. Both mobile phases were adjusted to pH 4 with acetic acid. The separation was initiated with 0% B and at 0.1 min stepped up to 80% B and held for 1.4 min. Mobile phases were filtered weekly with an aluminum oxide filter (0.02 μm, Anodisc 47, Whatman).

2.3. Mass spectrometry

The chromatography system was coupled to the QIT mass spectrometer (LCQ Deca, Thermo Electron Corporation, San Jose, CA) using electrospray ionization (ESI). Electrospray voltage of+1.5 kV was applied at the injection valve (see Fig. 1). The emitter tip was positioned ∼0.5 mm from the heated capillary of the mass spectrometer using a micromanipulator operated in *x*, *y*, and *z* directions as previously described (Haskins et al., 2001).

The QIT was operated with the following parameters: positive mode, automatic gain control (AGC) on, max AGC time = 300 ms, $q = 0.25$, isolation width = 3 *m/z*, activation time = 30 ms, normalized collision energy = 30, center mass = 87 *m/z*, scan width = 1 *m/z*, and the default number of microscans and target count values. Optimization of the ion optics was achieved by tuning during constant infusion of 16 μM ACh in