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Internal standard method for the measurement of choline and acetylcholine by capillary electrophoresis with electrochemical detection

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Abstract

An internal standard method has been developed for the determination of the neurotransmitter acetylcholine and/or its metabolic precursor choline. This approach couples the high separation efficiency of capillary electrophoresis with the sensitivity and selectivity of electrochemical detection at an enzyme-modified electrode. Indirect electrochemical detection is accomplished at a 25 μm platinum electrode modified by cross-linking the enzymes choline oxidase and acetylcholinesterase with glutaraldehyde. Although in this simple form of electrode fabrication there is a gradual loss of response from the electrochemical detector with time, accurate quantitation is achieved by the addition of butyrylcholine, which is also a substrate for acetylcholinesterase, as an internal standard. A linear response is achieved between 0 and 125 μ*M* with a limit of detection of 2 μ*M* (25 fmol). The utility of this method was demonstrated by monitoring the kinetics of choline uptake in synaptosomal preparations.

Keywords: Choline; Acetylcholine

1. Introduction

Impaired cholinergic neurotransmission has been implicated in neurodegenerative diseases such as Alzheimer's disease and in the neuromuscular disease, myasthenia gravis. Acetylcholine (ACh) and its metabolic precursor, choline (Ch), are important neurotransmitter components in a properly functioning cholinergic system. Therefore, monitoring the levels of ACh and Ch is important in characterizing these cholinergic abnormalities.

Detection of ACh and Ch is a challenging analytical problem because they are neither UV-active, fluorescent, nor electroactive. For this reason, bioassays, radiochemical methods, or derivatization methods have often been employed even with their inherently complicated procedures and difficulties in

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quantitation [1]. Detection is further complicated by the complexity of the biological fluids in which ACh and Ch are typically found. Therefore, highly selective analytical techniques that also exhibit low detection limits are necessary for investigative purposes.

Electrochemical detection methods have received significant attention for the sensitive determination of ACh and Ch. Indirect electrochemical sensing is accomplished by taking advantage of the selective reactions of ACh and Ch with the corresponding enzymes, acetylcholinesterase (AChE) and choline oxidase (ChO) to generate hydrogen peroxide, which is detected by electrochemical means. Based on these concepts, numerous approaches have been reported that describe either stand-alone micro [2–6] or macro [7–14] biosensors for ACh and Ch. Further selectivity for complex samples is gained when the enzyme reactions are coupled to a separation technique with electrochemical detection. Potter et al. [15] used a post-column reaction coil to mix the enzymes with ACh and Ch in the effluent, which had been separated by high-performance liquid chromatography (HPLC), followed by detection of hydrogen peroxide at a bare gold electrode. Subsequently, post-column reactors containing immobilized enzymes have reduced enzyme consumption and thus, have been widely used with HPLC coupled to a variety of detector electrode types and configurations [16–27]. In cases where only ACh is to be determined, a pre-column enzyme reactor has been added to remove Ch [28–30]. As an alternative to the use of a separate enzyme reactor and electrochemical detector, AChE and ChO have also been directly immobilized on the detector electrode surface [31–33].

One of the challenges of using the enzyme reactions in combination with electrochemical detection is the loss of electrode sensitivity with time [25,26,31]. Loss of enzyme, reduction in enzyme activity, or electrode fouling have been discussed as contributing factors leading to a reduction in sensitivity in various ACh and Ch detection systems. In our efforts to use an AChE/ChO-modified microelectrode as a detector for capillary electrophoresis, we have observed a similar loss of sensitivity with time. To counteract the decaying response to ACh and Ch, we have employed butyrylcholine (BuCh) as an internal standard [26] and describe the results herein.

2. Experimental

2.1. Materials

Acetylcholinesterase (EC 3.1.1.7, Type III from electric eel), choline oxidase (EC 1.1.3.17, *Alcaligenes* species), acetylcholine chloride (>99%), choline chloride (>98%), and butyrylcholine chloride (>98%) were purchased from Sigma (St. Louis, MO), stored in a desiccator at −10 °C, and weighed in a drybox when used. Choline was vacuum-dried overnight before use. *N*-Tris[hydroxymethyl]methyl-2-aminoethane-sulfonic acid (TES) (>99%), bovine albumin (>98%), Bradford reagent, and glutaraldehyde (Grade I, 25% aqueous solution) were also purchased from Sigma. Bovine albumin and Bradford reagent were refrigerated, while glutaraldehyde was stored at −10 °C. All other chemicals were of reagent grade and used as received. Hard tempered 25-μm-diameter platinum wire (99.95%) was obtained from Goodfellow (Berwyn, PA). Solutions were prepared in water distilled and deionized to a resistivity of at least 17.5 MΩ cm by a Barnstead B-pure water purification system (Dubuque, IA).

2.2. Instrumentation

Capillary electrophoresis with electrochemical detection (CEEC) was conducted on a laboratory-built instrument, which has been previously described [34]. However, in this case an on-column bare fracture decoupler was employed to isolate the electrochemical detector from the separation voltage [35]. The electrochemical detection cell was configured in a three-electrode arrangement with a Model RE-4 Ag/AgCl reference electrode (BAS, West Lafayette, IN), a platinum auxiliary electrode, and an enzyme-modified electrode as the working (detector) electrode. Potential control and current monitoring were achieved by a BAS LC-4C amperometric detector, which was modified for use with CE. Data were collected by an IBM P166 MHz computer through an A/D converter and analyzed with P/ACE MDQ Capillary Electrophoresis System software (Beckman Scientific Instruments, Fullerton, CA).

An 85-cm polyimide-coated fused-silica capillary with an I.D. of 50 μm and an O.D. of 360 μm (Polymicro Technologies, Phoenix, AZ) was used.

The distance from the bare fracture decoupler to the outlet was 2–3 cm. The enzyme-modified detector electrode was fitted with an o-ring and aligned to the end of the capillary using either a micromanipulator (Newport, Fountain Valley, CA) or a custom-made capillary electrode holder [36] (Allied Plastics, Toledo, OH). Under a microscope, ~30 μm of the electrode was carefully inserted into the capillary channel for on-column detection [37], ensuring that minimum contact of the enzyme coating with the capillary occurred.

A Varian VXRS-400 NMR spectrometer was used to acquire ^{1}H NMR data.

2.3. Methods

2.3.1. Preparation of the enzyme electrode

The detection electrode was prepared following the general procedure of Chetwyn [38]. Prior to use, 5 cm of platinum wire were cleaned by sonication in a dilute aqueous solution of Micro-90 soap (International Products, Burlington, NJ) for 10 min, rinsed in distilled water, sonicated in 1 *M* NaOH for 10 min, and finally rinsed in distilled water. A razor blade was used to cut the wire into 1-cm pieces, which were attached at one end to ~10-cm lengths of 22 gauge copper wire with colloidal silver paint (Ted Pella, Redding, CA). The wires were allowed to dry for 1 h. Once the silver paint dried, the wires were inserted into glass capillaries (1.5–1.8 by 100 mm), which had been heated and pulled to a point by hand, and then cut with a capillary cutter. The wire was threaded through the open end of the capillary until 0.5 cm of the platinum wire extended out the narrowed end. A seal was formed between the wire and the capillary by introducing UV glue (UVEXS Inc., CA) via capillary action at the narrowed end. The wire was gently pulled back until only a 300–350 μm length of platinum wire extended from the housing. The copper wire was secured to the open end of the capillary with hot glue and was allowed to dry for 1 h under a UV lamp. The entire electrode housing was hot-glued into a truncated pipette tip for ease of handling.

ChO (0.34 mg, ~4 units) was weighed in a small conical vial and dissolved in 7.25 μl of AChE (~7 units). After thorough mixing, 0.5 μl of glutaraldehyde was added to begin enzyme crosslinking. After ~2 min, the solution became cloudy and an electrode was repeatedly dipped into the solution for 45–60 s to adsorb an enzyme layer. The solution was used to coat three or four electrodes during a 6–7 min period following the addition of glutaraldehyde. The electrodes were allowed to dry for 1 h in a desiccator and then were examined under a Zeiss Stemi SV 11 optical microscope (Carl Zeiss, Thornwood, NY) to identify the most promising candidates for use. Electrodes with visible clumping of enzyme exhibited poor response characteristics. After preparation, the electrodes could be stored in a desiccator in a freezer at −10 °C for 1 week without loss of activity.

2.3.2. Capillary electrophoresis

TES (50 m*M*, pH 8) was used as the run buffer for all separations unless otherwise noted [38]. Capillaries were conditioned before use with 1 *M* HCl (10 min), H_2O (10 min), and run buffer (30 min) at an applied pressure of 25 p.s.i. for each solution. Between runs the capillary was rinsed with buffer for 5 min at 5 p.s.i. Separations were achieved with a separation potential of 27 kV, which resulted in a separation current of 15 μA. Samples were introduced by pressure injection with high purity argon for 2 s at 5 p.s.i., corresponding to an injection volume of 12.4 nl. To prevent damage to the detection electrode, the electrode was aligned after the capillary conditioning step. The capillary was rinsed and filled with water for overnight storage.

Aqueous stock solutions of ACh and BuCh were made fresh daily as they were subject to hydrolysis, but refrigerated Ch solutions were stable for at least 1 week. Calibration plots were obtained by plotting the ratio of the peak areas for ACh or Ch to the internal standard, BuCh, which was held at a concentration of either 250 or 500 μ*M*, versus analyte concentration. Each data point represents the average of triplicate measurements for each ACh and Ch concentration, unless otherwise noted.

2.3.3. Preparation of synaptosomes

A suspension of synaptosomes was prepared following the general procedure of Gray and Whittaker [39] and Patel et al. [40]. Two adult male mice (C57B16, 25–30 g, Harlan Sprague Dawley, Indianapolis, IN) were sacrificed by cervical dislocation and the forebrains removed and excised from the brainstem and olfactory bulbs, which were discarded.

The forebrains were placed in 10 volumes of a 0.32 *M* sucrose solution and homogenized for ~5 s with a PCU Kinematica GmbH Kriens-Luzern homogenizer. The homogenate was centrifuged at 1000 *g* for 10 min with an Eppendorf 5810R centrifuge and the pellet was discarded. The supernatant was centrifuged again for 20 min at 17,500 *g* in a Sorvall RC-5B Refrigerated Superspeed centrifuge. The resulting supernatant was then discarded and the pellet resuspended in ~5 volumes of 0.32 *M* sucrose. This suspension was again centrifuged for 20 min at 17,500 *g*. Both centrifuges were maintained at 4 °C. The resulting pellet was resuspended 1:1 (v/v) in Krebs-Ringer phosphate buffer (124 m*M* NaCl, 5 m*M* KCl, 1.2 m*M* Na_2HPO_4, 1.2 m*M* NaH_2PO_4, 1.3 m*M* $MgSO_4$, 0.75 m*M* $CaCl_2$, and 10 m*M* glucose, aerated with O_2, and adjusted to pH 7.4 with 1 *M* NaOH). The resulting solution consisted of mainly synaptosomes, mitochondria, and myelin fragments. The protein concentration of the synaptosome suspension was determined by a Bradford assay [41].

2.3.4. Measurement of choline uptake in synaptosomes

Incubations were carried out at 0 and 37 °C. Warm incubations were conducted in a G10 Gyrorotatory Shaker at 240 rev./min. A freshly prepared synaptosome solution (200 μl) and the stock Ch (92.5 μ*M* in Krebs-Ringer buffer) solution were equilibrated to the appropriate temperature for 20 min after which 75 μl of the Ch stock solution were added to the synaptosomes to give an initial Ch concentration of 25 μ*M*. Subsequent 75-μl aliquots were removed at specific time intervals and immediately placed on ice to halt Ch transport. The aliquots were centrifuged and filtered in centrifuge tube filters within 1.5 min of sampling for 5 min at 820 *g*. The resulting supernatant was stored at 0 °C until analysis. For analysis, an appropriate aliquot of supernatant liquid and BuCh were mixed in a 1:1 (v/v) ratio to give a final internal standard concentration of 500 μ*M*. Samples were then analyzed by capillary electrophoresis in triplicate.

3. Results and discussion

To utilize electrochemistry for detection of ACh and Ch, the enzymes AChE and ChO were used as biocatalysts to selectively recognize the substrates and generate hydrogen peroxide, which is easily detected electrochemically at +0.600 V vs. Ag/AgCl. The basis for this indirect approach to detect ACh and Ch is described in Fig. 1 and has been previously demonstrated to be both selective and sensitive [2–33].

Fabrication of a miniature enzyme electrode was easily accomplished by cross-linking AChE and ChO with glutaraldehyde onto the tip of a 25 μm diameter platinum wire electrode to form a biocatalytic layer. The AChE/ChO modified electrode was then used as a selective detector in capillary electrophoresis in either an on-column or end-column configuration. A problem in using an enzyme electrode as a detector for CE is the stability of the biocatalytic layer under hydrodynamic conditions. This may be especially important given the small amounts of enzyme deposited on the miniature platinum surface. To examine electrode stability, the response for repetitive injections of ACh was monitored for 5 h and was found to gradually decay with time with the most significant drop occurring in the first hour (10% loss of response) and reaching 22% after 5 h. Although the rate of decay varied from electrode to electrode, even a slight rate of decay limits the utility of the detector system for accurate quantitative analysis in the absence of an internal standard. Presumably, loss of enzyme, enzyme denaturation, or electrode fouling could all contribute to the signal decay observed in our experiments similar to previous observations from liquid chromatography methods [25,26,31].

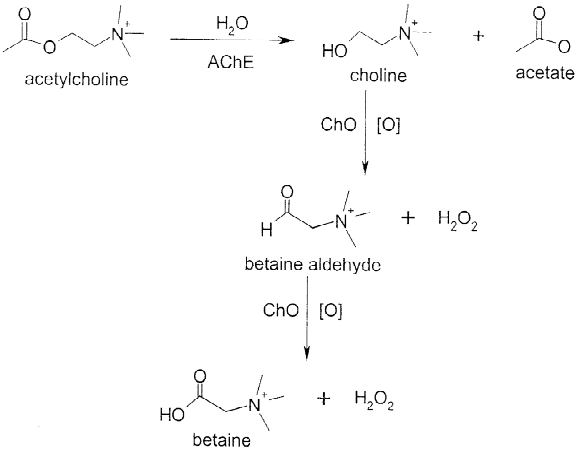

Fig. 1. Enzyme reactions for the detection of ACh and Ch.

In order to take advantage of the separating power of CE and the sensitivity and selectivity of the biocatalytic AChE/ChO electrode detector, butyrylcholine was employed as an internal standard. Butyrylcholine is an excellent choice as an internal standard for studies focusing on ACh and Ch since BuCh is recognized by AChE, although at a lower affinity. Furthermore, BuCh is not native to mature brain. It should be noted that BuCh is endogenous to the brain of developing embryos or neonates. However, these systems are not the focus of our studies and thus, endogenous BuCh is not present.

Fig. 2 shows a typical electropherogram for the injection of a mixture of 75 μ*M* Ch (7.3 min) and ACh (7.9 min) with 500 μ*M* BuCh (8.4 min) as the internal standard. All three species were separated with baseline resolution in less than 9 min and an average efficiency of 10^4 depicting the ease with which CE and the AChE/ChO electrode can respectively separate and detect Ch, ACh, and BuCh. As before, the individual responses for all three components steadily decayed with time. In contrast, when the ratios of peak areas for Ch/BuCh and ACh/BuCh were monitored as a function of time and averaged, the ratios were determined to be 0.253±0.006 and 0.213±0.006, respectively. This is well within the precision necessary for calibration of the electrodes and their use as detectors for accurate CEEC measurements of ACh and/or Ch. These results clearly indicate that the decaying response can be essentially eliminated with the BuCh internal standard.

A freshly prepared AChE/ChO electrode was examined over a range of ACh and Ch concentrations from 0 to 2000 μ*M* using the BuCh internal standard (Fig. 3). A biphasic response was observed with linear portions between 0 and 125 μ*M* and 500 to 2000 μ*M* with a transition region between 125 and 500 μ*M*. Based on numerous measurements for biological tissue, the expected concentrations for ACh and Ch are in the n*M* to μ*M* range [1]. Therefore, for most biological studies the linear region between 0 and 125 μ*M* is clearly the most valuable for analysis. At higher concentrations the response is typical for a modified enzyme microelectrode where the electrode becomes saturated with substrate [42]. Subsequently, the relative response of a series of three electrodes was examined between 0 and 125 μ*M*. A representative calibration plot for ACh and Ch is shown in Fig. 4 at a freshly prepared electrode. For each electrode the response for ACh and Ch was linear (R^2: 0.98 to 0.995) with limits of detection of ~2 μ*M* (25 fmol) for ACh and Ch at a *S*/*N* of 3. Electrode sensitivity, as measured by the slope of the calibration plot (ratio of peak area versus concentration), varied from 0.004 to 0.015 μM^{-1}, which is attributed to the variability in enzyme loading during the cross-linking process. A further observation from this data is the presence of a reproducible non-zero intercept in the Ch analysis.

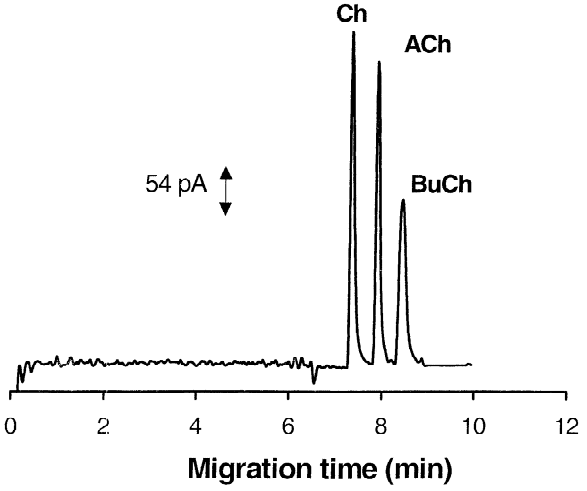

Fig. 2. Electropherogram of a mixture of Ch (75 μ*M*), ACh (75 μ*M*), and BuCh (500 μ*M*). Run buffer, 50 m*M* TES, pH 8; separation voltage, 27 kV; detection potential, +600 mV vs. Ag/AgCl.

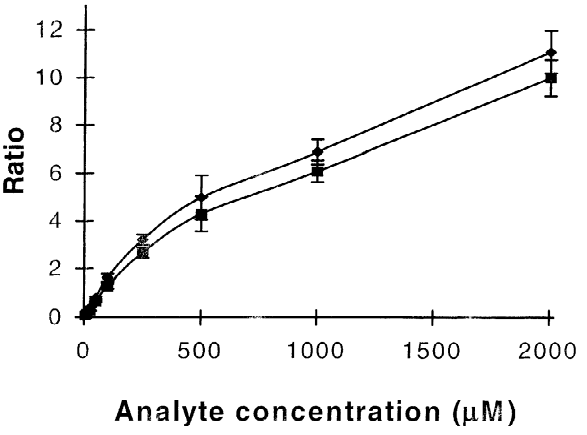

Fig. 3. Calibration plot for Ch (♦) and ACh (■) from 0 to 2000 μ*M*.

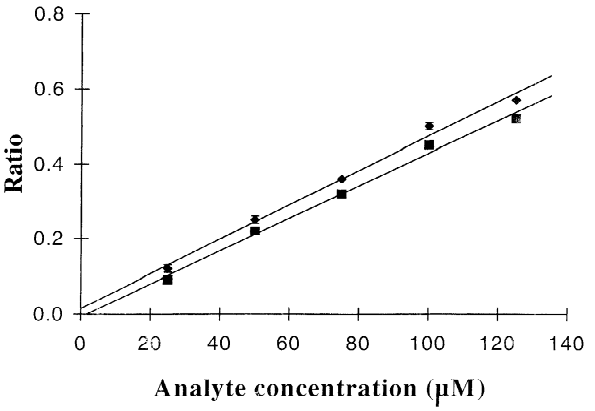

Fig. 4. Calibration plot for Ch (♦) and ACh (■) from 0 to 125 μ*M*.

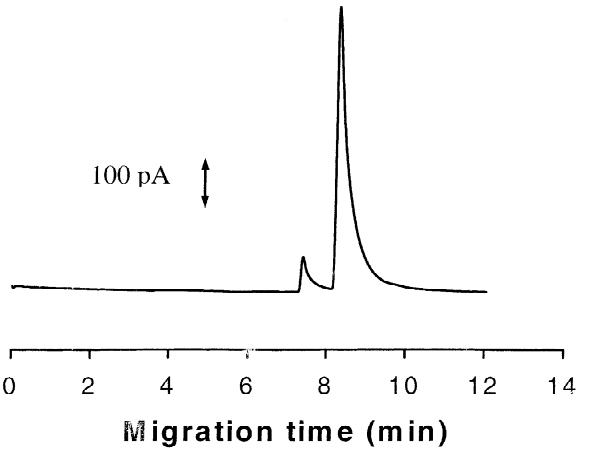

Fig. 5. Electropherogram at $t=0$ during the incubation of Ch with a synaptosome suspension at 37 °C. Ch (7.3 min), BuCh (8.4 min).

Injection of a fresh BuCh solution reveals an additional small peak (~2–3% of the BuCh peak) in the electropherogram at a migration time suggesting the presence of Ch. ^{1}H-NMR confirms the presence of choline in the BuCh purchased from the manufacturer. Repetitive injections of BuCh yield a stable response as a function of time and rules out hydrolysis of BuCh during analysis as a potential problem for electrode calibration. Variations of enzyme loading and the presence of a small amount of Ch are readily accounted for by calibration of each new electrode. Finally, it is important to note that the migration times for Ch, ACh, and BuCh were very reproducible. For example, 15 repetitive injections were made during the generation of the calibration plot of Fig. 4 and the migration times were found to be 7.34±0.06, 7.89±0.08, and 8.44±0.09 min, respectively.

3.1. Biological application

The goal of developing a sensitive and selective detection method for monitoring ACh and/or Ch was to utilize this approach for quantitative measurements in biological assays. In particular, we are interested in evaluating Ch transport through the high affinity choline uptake transport channel under conditions of selective inactivation by a well-established group of cholinotoxic agents [40,43–47]. The feasibility of the CEEC method described here was demonstrated using freshly prepared synaptosomes as a model system for Ch transport and monitoring the decrease in Ch concentration in the supernatant solution. The Ch concentration was assayed by CEEC as a function of time (Fig. 5), normalized to the initial concentration of Ch at time zero, and plotted versus time. Fig. 6 depicts the kinetics of Ch uptake by synaptosome suspensions at 0 and 37 °C after the addition of Ch to the synaptosome solution. In these control experiments it is clearly observed that different rates of Ch uptake can be readily distinguished with this approach. The relative Ch concentration after 20 min at 0 and 37 °C are

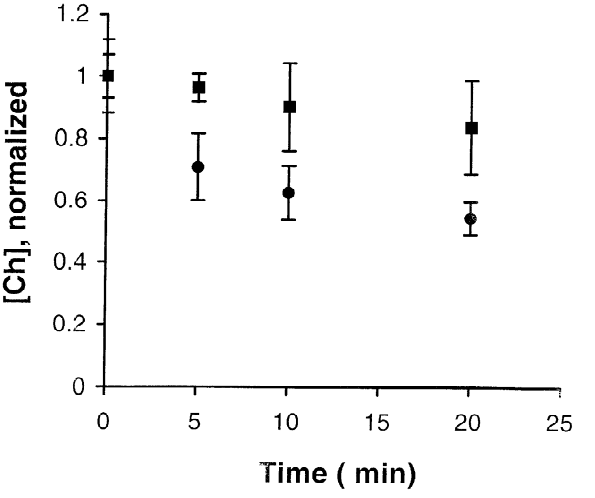

Fig. 6. Choline transport assay at 0 °C (■) and 37 °C (●).

0.84±0.15 and 0.59±0.05, respectively, indicating significant transport of Ch into the synaptosomes at 37 °C. At the physiological temperature of 37 °C it is assumed that normal Ch transport occurs, whereas at 0 °C this transport mechanism is shut down and any apparent decrease in Ch concentration in the supernatant is likely due to other mechanisms such as diffusion. The results presented in Fig. 6 are consistent with the previous results from Patel et al. [40] using a radiochemical assay.

4. Conclusions

Capillary electrophoresis with electrochemical detection at an enzyme-modified electrode has been used for the separation and determination of the neurotransmitter ACh and its metabolic precursor, Ch. The electrochemical detector was prepared by chemically crosslinking ChO and AChE on a 25-μm platinum wire with glutaraldehyde. Under the hydrodynamic conditions of CE, the adsorbed enzyme layer is gradually lost and results in a decrease in electrode response with time. To counteract this instability, BuCh was employed as an internal standard. This approach combines the simplicity of electrode modification with stability of the quantitative measurement by the internal standard in order to accurately determine Ch and/or ACh. Limits of detection of 2 μ*M* (25 fmol) were achieved, which rival detection limits reported for HPLC separations with amperometric detection schemes utilizing post-column reactors [18,19,23,24,27–29] or an immobilized enzyme electrode [31]. The feasibility of this method was demonstrated by monitoring Ch uptake through the high affinity choline uptake channel in synaptosomal preparations. Approaches to stabilize the biocatalytic layer and lengthen the lifetime of the enzyme electrode are currently being investigated and will be the subject of a future report.

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