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Application note

Acetylcholine in Brain Microdialysates

Acetylcholine (ACh), a monoamine, is the primary neurotransmitter of cholinergic neurons. These nerve cells are located within the peripheral (e.g., autonomic ganglia, at parasympathetic postganglionic synapses and at neuromuscular junctions) and central nervous systems. ACh is involved in muscle contraction (smooth, cardiac and skeletal), hormone release, behavior and memory. ACh is also involved in a number of neurobiological diseases including myasthenia gravis, Alzheimer's disease, and Huntington's disease, and has been implicated in affective disorders and stress. Acetylcholinergic neurotransmission can be markedly affected by a number of drugs, insecticides and chemical warfare agents.

Initially studied in brain tissue homogenates, ACh and choline (Ch) are now commonly measured in microdialysis perfusate samples (Damsma and Westerink (1991); Greaney *et al.,* (1993)). This approach overcomes many of the disadvantages of using homogenates – discrete brain regions can be dialyzed in the awake, behaving animal, with minimal disruption of vital function. However, due to the destructive action of the enzyme acetylcholinesterase located within the extracellular space, the measurement of the exceedingly low levels of ACh found in microdialysis samples requires a very sensitive analytical method.

Several approaches have been used to measure ACh including bioassays, enzymatic assays, and radioimmunoassays, as well as gas chromatography- and HPLC-based techniques. HPLC-electrochemical detection (HPLC-ECD) is by far the most popular approach used to measure both tissue and microdialysis ACh levels, due to its sensitivity, selectivity and ease of use.

In order to overcome this sensitivity limitation of some HPLC-ECD systems, an acetylcholinesterase inhibitor is usually added to the medium used to perfuse the microdialysis probe. This drug diffuses from the probe and prevents the destruction of ACh by acetylcholinesterase located within the brain's extracellular space. The use of inhibitor leads to an artificial elevation in ACh levels that can now be measured by insensitive analytical equipment. However, the use of an acetylcholinesterase inhibitor, especially at higher levels, is now strongly discouraged as it can lead to altered physiology and pharmacology by altering receptor feedback mechanisms (Damsma *et al.*, (1988); de Boer *et al.*, (1990); Greaney *et al.*, (1993)).

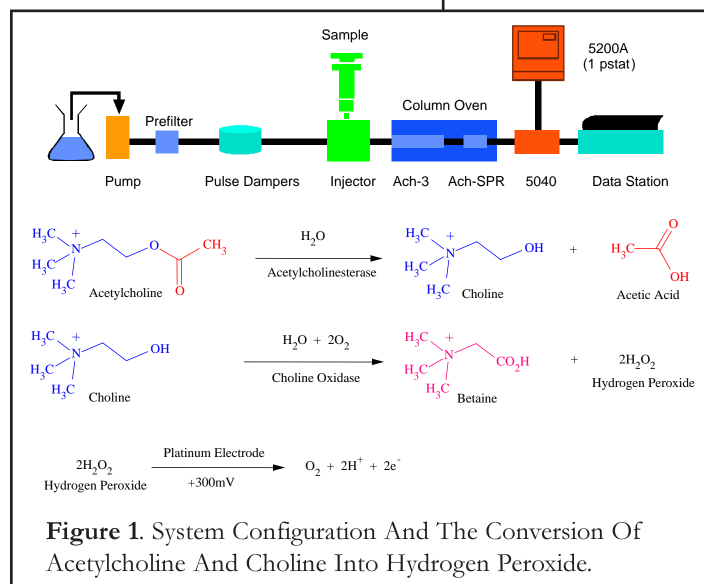

Figure 1. System Configuration And The Conversion Of Acetylcholine And Choline Into Hydrogen Peroxide.

The ESA Acetylcholine System with the Coulochem® III Electrochemical Detector, specially designed amperometric cell and enzyme reactor, delivers the sensitivity, selectivity and stability required to routinely measure low femtomole levels of ACh and Ch in brain microdialysates.

Materials and Methods

The isocratic analytical system consisted of a pump, a manual injector, a Coulochem III detector and a thermal organizer module. Other components are described below.

LC Conditions:

Column:	ACH-250
Reactor:	ACH-SPR
Mobile Phase:	100mM Disodium Orthophosphate 0.005% Reagent MB 2.0mM Octane Sulfonic Acid pH 8.0 with Phosphoric Acid. Use for up to one week.
Flow Rate:	0.35 mL/min
Temperature:	35°C
Injection Volume:	10µL

Coulochem III

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Detector Conditions:

Detector:	Model 5300, Coulochem III
Cell:	Analytical Cell, Model 5040 with Platinum Target
Cell Potential:	+300 mV

Microdialysis Conditions:

Probe design:	Concentric (3mm x 0.5mm) – CMA.
Flow Rate:	1.5µL/min.
Recovery (*in vitro*):	4-5% per membrane mm length.
Collection Period:	Every 20min. following a two hour injury period.
Animal Model:	Anesthetized (urethane) rat.
Probe Coordinates:	AP +0.5; LR 2.7; DV 7.0mm.
Artificial CSF (in mM):	Sodium – 147mM; Potassium – 3.5mM; Magnesium – 1.2mM; Calcium – 1.0mM; Chloride – 129mM; Phosphate (pH7.4) – 2.0mM.

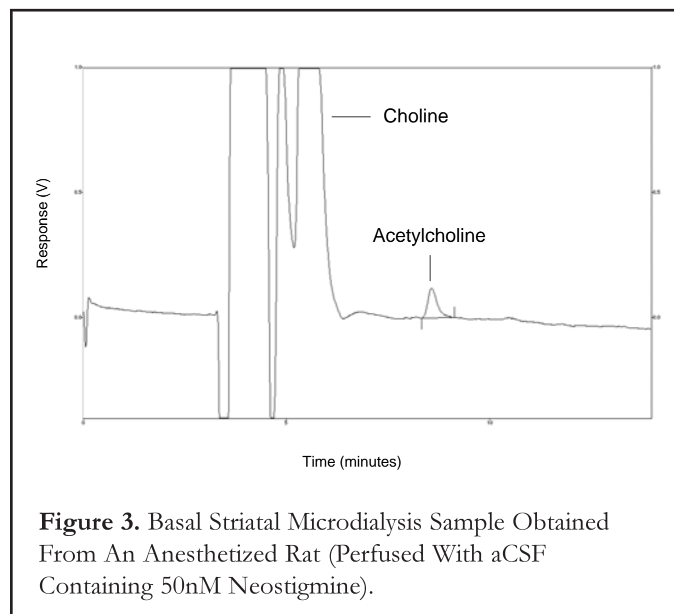

Figure 3. Basal Striatal Microdialysis Sample Obtained From An Anesthetized Rat (Perfused With aCSF Containing 50nM Neostigmine).

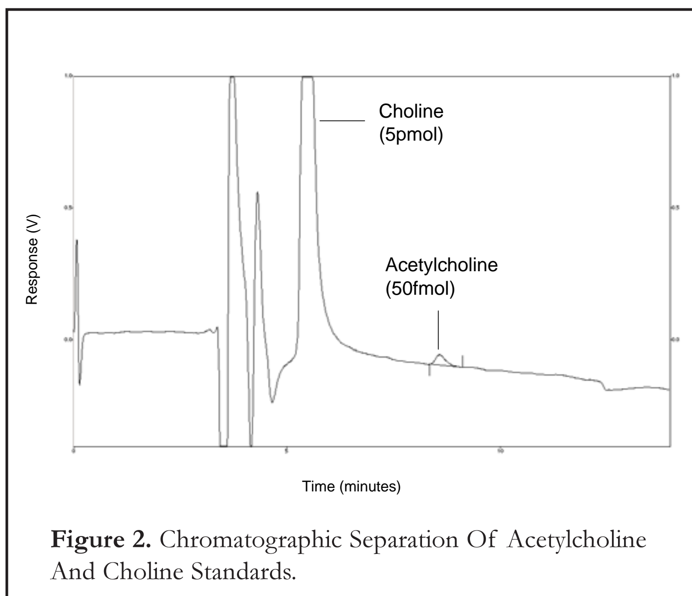

Figure 2. Chromatographic Separation Of Acetylcholine And Choline Standards.

Results and Discussion

The detection of ACh and Ch is accomplished with a post-column solid phase reactor (SPR) containing immobilized enzymes (choline oxidase and acetylcholine esterase). After separation on the analytical column, ACh and Ch are both converted into hydrogen peroxide (Figure 1) which is, in turn, quantitated using an amperometric Pt working electrode.

In order to be used for microdialysis experiments the HPLC system must provide maximum chromatographic resolution in the shortest time and with a high degree of reproducibility. Figure 2 illustrates the separation of ACh and Ch standards in under ten minutes while Figure 3 presents the measurement of ACh in striatal microdialysis perfusates (containing 50nM of the acetylcholinesterase inhibitor, neostigmine). The assay has excellent linearity even at low femtomole ACh levels (Figure 4) (Ch is linear beyond 20 pmol; data not shown)).

The ESA Acetylcholine System offers improved separation with sharper peaks, providing superior resolution and enhanced sensitivity. Due to the low noise of the Coulochem III electrochemical detector low femtomole levels of ACh can be measured routinely. Researchers now commonly use 100-1000 fold less inhibitor than with other published methods. Using this approach basal striatal ECF levels of ACh can be measured in the total absence of inhibitor and in the anesthetized animal (Greaney *et al*., (1993)) where basal ACh levels are suppressed (Bertorelli *et al*., (1990); Greaney *et al.* (1993); Kawashima *et al*., (1991)). The use of lower inhibitor concentrations creates the opportunity to study ACh release under conditions closer to the normal physiological state and even allows researchers the possibility to study decreases in ACh levels (e.g., following exposure to ACh agonists).

The composition of the perfusion medium is a critical factor in brain microdialysis. The concentration of calcium can induce profound variations in the experimental outcome. High perfusate calcium concentrations (>2.0 mM) are often used to

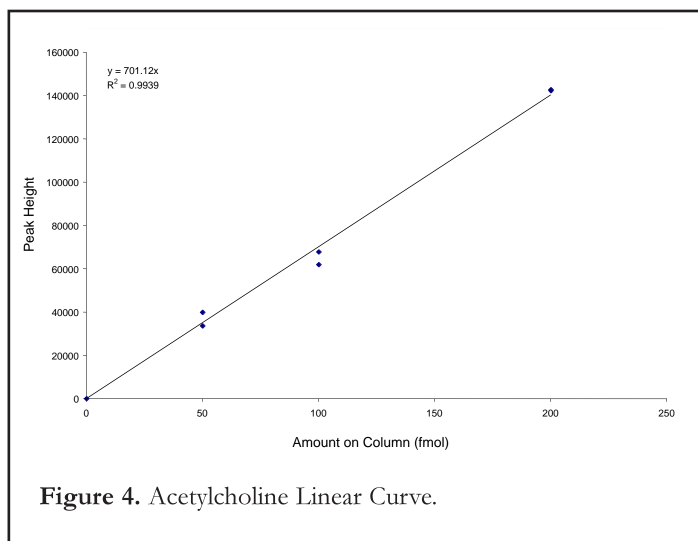

Figure 4. Acetylcholine Linear Curve.

artificially elevate brain ACh levels in order to compensate for insensitive analytical equipment. With the high sensitivity of the ESA Acetylcholine System, normal physiological levels of calcium are always used.

Conclusions

The ESA Acetylcholine System with its high sensitivity and rapid analyte separation is ideal for the analysis of microdialysis samples. The system is robust ensuring that less time is spent with system maintenance and more time spent on collecting data. Physiological calcium concentrations in the perfusion medium are used and minimal levels of cholinesterase inhibitor are required. Samples are therefore obtained from a brain region whose internal environment is as close as possible to its natural physiological state.

References

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Ordering Information

Description	**Part Number**
Coulochem III Detector, Model 5300	70-5498-120
Coulochem III Thermal Organizer Module	TBD
Analytical Cell, Model 5040 Analytical Cell with Platinum Target	70-1074
ACH-250 column with blank cartridge	70-4457
ACH-3-G Guard Cartridge	70-0639
ACH-SPR Enzyme Reactor	70-0640
Pump, Model 582	70-4049
Manual Injector, Model 9725i	70-4986
Data Station, EZChrom Elite for ESA	70-5073
Reagent MB	70-1025
Acetylcholine Start-Up Kit	70-1331

(Potentials must be optimized for each LC system.)

ESA, Inc.
22 Alpha Road
Chelmsford, MA 01824-4171 USA
(978) 250-7000
Sales (800) 959-5095
Fax (978) 250-7090
www.esainc.com

An ISO 9001 Company

ESA Analytical, Ltd.
Brook Farm, Dorton,
Aylesbury, Buckinghamshire,
HP18 9NH England
01844 239381
Fax 01844 239382

Authorized European Representative
EuroService Srl
Via Castagnevizza 11/14
I-16155 Genova, Italy
0039 010 6986021
Fax 0039 010 6986001

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