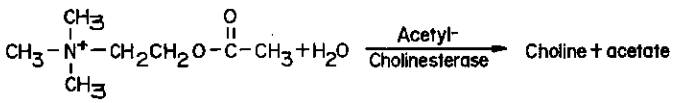
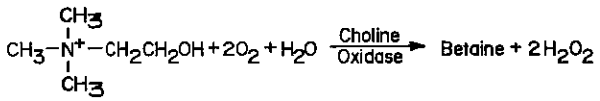
CAPSULES
preliminary notes and applications from Bioanalytical Systems, Inc.

Acetylcholine and Choline In Brain Tissue

Neither the neurotransmitter acetylcholine or its metabolite, choline, are directly detectable by liquid chromatography coupled to ultraviolet absorbance, electrochemical, or fluorescence detection. However, both substances are capable of yielding hydrogen peroxide enzymatically:





As a product of both choline and/or acetylcholine, the hydrogen peroxide may be detected electrochemically by virtue of its oxidation to O_2 on a platinum electrode at +0.5 V vs. Ag/AgCl. These reactions coupled to the effluent from the LC column form the basis for this analytical method.

This Note provides detailed technical assistance for implementing the assay, based on liquid chromatography/ electrochemistry (LCEC). The method was devised by Potter, Meek and Neff of the National Institute of Mental Health, Washington, D.C. (1). The compounds are separated from each other chromatographically (Figure 1), and the effluent is then mixed at a tee with the enzymes in phosphate buffer. After a delay period in a reaction coil, the accumulated H_2O_2 is detected in the thin-layer electrochemical detector (Pt, +0.5 V). The hardware for this assay is available as an accessory kit to various BAS chromatographs. This Note assumes that installation has already been accomplished. Further details, including tissue sample preparation, are also available from the original paper (1).

Selectivity

The main advantage of this assay is selectivity. This feature is contingent on 3 factors: chromatography, the enzymatic reactions, and the electrochemistry. Since acetylcholine and choline are located in a complex biological matrix, some components of which are electrochemically active, chromatography permits separation of the two substrates from each other and endogenous electroactive solutes. This is accomplished by reverse phase LC and ion- pairing mobile phases.

The use of enzymes as reagents in chemical analysis obviously improves selectivity. These biological catalysts can be much more specific than general organic chemistry reactions (if such were available) and also permit moderate reaction conditions, necessary for optimum detector compatibility. In this case, the reactions are accomplished at ambient temperatures. The enzymes are added post-column at a constant rate, thereby allowing calibration of the system with known amounts of ACh and Ch.

Few endogenous substrates react at platinum electrodes at +0.5 V vs. Ag/AgCl. As will be evident later, the enzyme reagent may be removed from the stream to ascertain the presence of any such interferences. Peroxide may also be detected on glassy carbon, albeit at about +900 mV vs. Ag/AgCl. The higher potential means a loss in selectivity and more noise in terms of system performance. For these reasons, Pt is mandated for this assay.

Equipment

Chromatography. The chromatography system consists of a reciprocating piston pump capable of delivering a constant flow of mobile phase at a rate of 0.8 ml/min and a 10 µm particle size BAS Acetylcholine Column (P/N MF6060, 10 cm by 4.6 mm). The use of an octadecylsilane (e.g. "ODS" or C18") column for the determination of choline and acetyl-

choline results in severe peak tailing due to hydrogen bonding between the hydroxyl groups of the packing material and the quarternary ammonium moiety. Amines, such as tetramethylammonium chloride or diethylamine, are routinely added to the mobile phase to eliminate this problem. However, in this particular application, inactivation of the enzyme results when a sufficient amount of amine is added to the mobile phase to reduce tailing. This problem is circumvented by using a polystyrene-based reverse phase column (2), as used here. After the column, a mixing tee is used to add enzyme reagent to the effluent, via a peristaltic pump. In Figure 1, you will note a 3-way valve is used to select the reagent for the peristaltic pump. Enzyme/phosphate buffer or phosphate buffer alone may be selected, to conserve the supply of enzyme. Exact details are provided below.

Mobile Phase. The mobile phase consists of 96% 0.01 M sodium acetate (pH 5.0) containing 30 mg/L sodium octyl sulfate and 4% acetonitrile. Prepare the buffer portion by dissolving 1.36 g of sodium acetate in 900 mL of distilled, deionized water (or equivalent). Adjust to pH 5.0 with 0.2 M citric acid. Add 30 mg sodium octyl sulfate and dilute to 1.00 L. Prepare the mobile phase by adding 40 mL acetonitrile to 960 mL of buffer. Filter and degas prior to use.

0.2 M. Phosphate Buffer, pH 8.5. Prepare by dissolving 28.4 g of dibasic sodium phosphate in 900 mL of distilled, deionized water (or equivalent). Adjust the pH to 8.5 with phosphoric acid (dropwise) and add water to make 1.00 Liter. Filter but do not sparge this buffer, as the presence of O_2 in the buffer is a requirement of the assay (see reaction 2). Normal membrane filtration using water aspiration is okay.

Phosphate Buffer, pH 7.0. Take a 50 mL portion of the pH 8.5 phosphate buffer (above) and titrate to pH 7.0 with phosphoric acid. Filtering is unnecessary.

Enzyme Stock Solutions. Dissolve 1000 units of acetylcholinesterase (EC 3.1.1.7, Sigma Catalog No. C-2888) in one ml of 0.2 M sodium phosphate, pH 7.0. To 500 units of choline oxidase (EC 1.1.3.17, Sigma Catalog No. C-5896) add 5 ml of 0.2 M sodium phosphate pH 7.0. When dissolving enzymes, foaming must be avoided to minimize denaturation. This can be done conveniently by capping the container and slowly rotating it in an upright and inverted position. This ensures that all of the protein gets into solution. The stock enzyme solutions should always be kept cold (+5°C) when not being used to prepare the working enzyme solution. No change in system sensitivity was noted over 3 weeks when the enzyme stock solutions were maintained under these conditions.

Working Enzyme Solution. The enzyme solution used for the post-column derivatization of acetylcholine and choline is prepared by adding 20 µL of acetylcholinesterase and 100 µL of choline oxidase stock solution per 10 ml of 0.2 M phosphate buffer, pH 8.5. We recommend adding the oxidase to the buffer followed by addition of the esterase to avoid adsorption of protein to glassware which can result in inactivation of the enzymes. Gently swirl to mix this solution. This working enzyme solution should contain 2 units/ml of acetylcholinesterase and 1 unit/ml of choline oxidase. The volume of working enzyme solution to be made will depend on how long the system will be in use. The enzyme solution is added to the column effluent at 0.5 mL/min. When calculating how much solution to prepare, it is important to provide an extra half hour (15 ml) of reagent, in order to compensate for a short equilibration period and delays between injections. A single

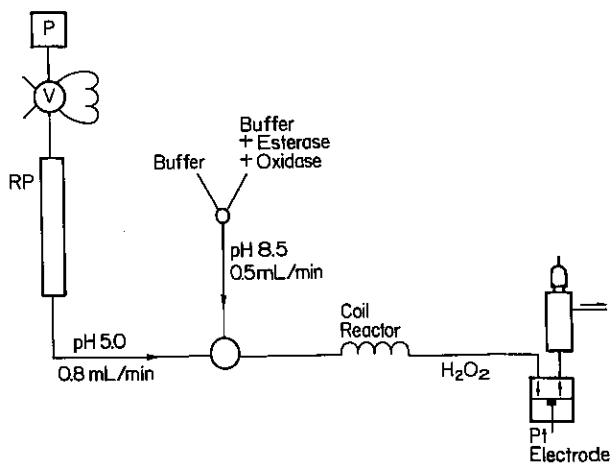


Figure 1. Schematic of instrumentation for assay.

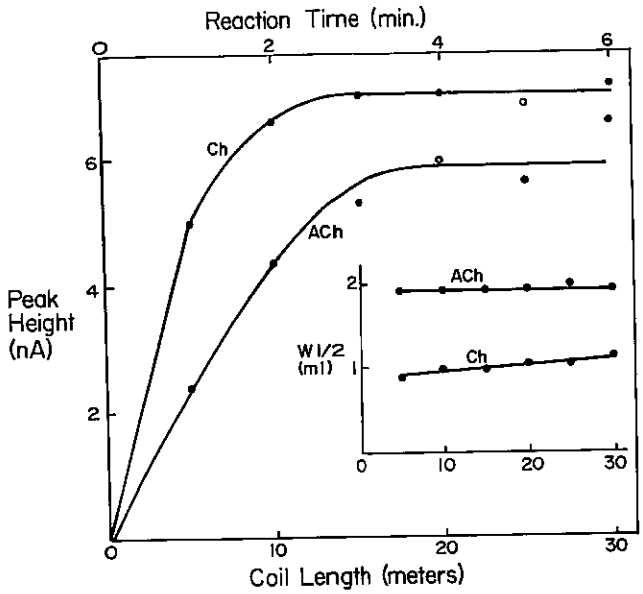


Figure 2. Formation of acetylcholine and choline as a function of reaction coil dimensions. Inset: peak width at half-height vs. reactor length.

injection under the conditions of this note requires 9.5 mL of enzyme solution. Note that the stock enzyme solutions are made up with pH 7.0 buffer and the working enzyme solution utilizes buffer at pH 8.5.

Selection of Reagent. The 3-way valve determines the reagent solution supplied by the peristaltic pump. The larger vessel contains only the 0.2 M. sodium phosphate (pH 8.5) while the smaller vessel contains the working enzyme solution, also at pH 8.5. When the system is initially turned on and is reaching equilibrium, the phosphate buffer alone is added. once the system has equilibrated, indicated by a flat baseline, the valve is switched to the working enzyme solution. A short additional equilibration period, about 15 min., is required due to the presence of the enzyme in the buffer. Samples can then be injected. This procedure reduces the volume of working enzyme solution needed and thus reduces the cost per assay. Final conditions for the assay are summarized below.

Conditions

Liquid Chromatograph: LC-304 (Bioanalytical Systems Inc.). The BAS 460 or BAS 200 replaces the discontinued LC-304 in this application.

Mobile Phase: 96% 0.01 M sodium acetate (adjusted to pH 5.0 with 0.2 M citric acid) with 30 mg/L sodium octyl sulfate/4% acetonitrile.

Flow Rate: 0.8 mL/min

Stationary Phase: BAS Acetylcholine Column (10 µm, 100 mm x 4.6 mm)

Temperature: Ambient

Post-Column Addition: 0.2 M sodium phosphate, pH 8.5, containing acetylcholinesterase (EC 3.1.1.7) 2 units/mL, choline oxidase (EC 1.1.3.17) 1 unit/mL, infused at 0.5 mL/min. The mixture of column effluent and enzyme solution reacts during passage through a Teflon coil. The delay time is about 4 min., sufficient for reaction to completion (Figure 2).

Standardizing the System. Speed is essential when preparing stock solutions of acetylcholine and choline since both compounds are extremely hygroscopic. In the solid state both materials should be kept desiccated and below 0°C. Due to the hygroscopic nature of both acetylcholine and choline, it is difficult to weigh out a predetermined amount of either solid. Thus, a standard solution is made in the following manner: tare a piece of parafilm or other nonwettable material, quickly add and note the weight of the solid analyte (about 0.075 g of choline or 0.1 g of acetylcholine is appropriate). Quantitatively transfer each solid to separate 50 mL volumetric flasks using 0.05 M perchloric acid and dilute to volume. Calculate the concentration of the solutions and then transfer 50 µL of the choline stock solution and 100 µL of the acetylcholine stock solution to a single 50 mL volumetric flask. Use 0.05 M perchloric acid to make to volume. Calculate the concentrations of each compound in this working standard solution. This solution can be serially diluted to generate a standard curve. In all cases, a 20 µL sample is injected. If the tissue sample requires that a larger aliquot be injected, then the standard curve should be generated using a similar sample size. A tissue sample homogenate can now be injected (Figure 3), and the amount of choline and acetylcholine determined from direct comparison to the standard curve.

As with all analytical procedures, it is good practice to periodically inject a standard sample to monitor

the efficiency of the system. You will find that the response of the system decreases by about 10% over a 10 hour period. This is due in part to protein adsorbing to the surface of the platinum electrode. Periodic injections of a standard solution can be used to correct for this loss in efficiency.

Care of the Platinum Electrode. Platinum is a fairly soft metal so care should be taken not to scratch the surface of the electrode. It is good practice to rinse the surface of the polishing pads with distilled water before use, and to protect them from airborne grit. The platinum electrode should be polished when needed, first with the 1 µm diamond slurry, followed by the 0.05 µm polishing alumina. A 2-5 min sonication in water is recommended, followed by a thorough rinsing under a jet of distilled water. Polishing should only be done when the electrode surface becomes passivated. In general, this procedure is necessary about every 4-6 weeks.

System Shutdown Procedure. After the working enzyme solution is exhausted, and no more samples are to be assayed, switch to the 0.2 M sodium phosphate buffer and keep the system running for about 30 minutes, with the electrode in the STANDBY position. This washes off any protein that is adsorbed to the surface of the electrode.

A faster alternative procedure is to switch to the phosphate buffer, run the system for about 5 minutes, and then turn off both pumps. Turn the electrode to STANDBY. Remove the platinum electrode (lower half of cube) and flush the electrode with distilled water.

Bacterial growth in the LC system is possible during long term shutdown. If this is the case (shutdown period > 3-4 days), flush the LC system with 50:50 methanol/H_2O (300 mL). Flush the enzyme reagent and phosphate buffer lines with water.

Miscellaneous Precautions, Recommendations

1. Conversion to fraction collection. The platinum electrode cell cube can be used for collection of fractions by a simple 90° rotation of the bottom half to allow fluid access to a threaded exit port. The addition of an RE-3 reference electrode to the top half of the cube now provides a flow cell with all 3 electrodes positioned in the thin- layer channel. Fractions collected from the cell will maintain their chromatographic integrity, thus allowing *turnover* studies to be carried out by radioisotopic measurement. The preceding description applies to the LC-17 Thin-Layer Transducers; the LC-17A Thin Layer Transducer is by design already set up for fraction collection.

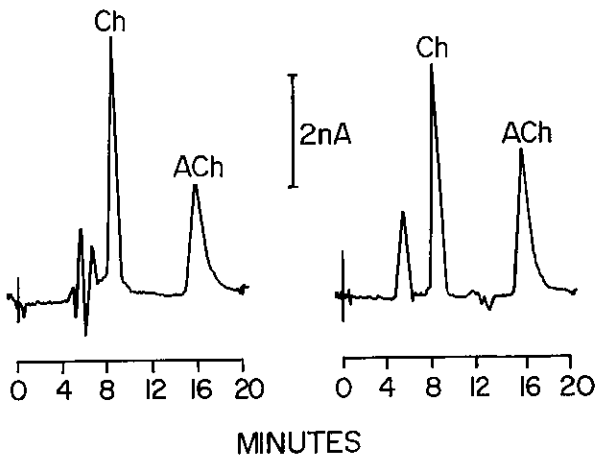


Figure 3. LCEC chromatograms for choline and acetylcholine, conditions as described in the text: (A) standard solution, 144 pmoles choline and 219 pmoles acetylcholine injected. (B) Perchloric acid extract of rat brain; choline and acetylcholine were calculated at 126 pmoles and 329 pmoles, respectively.

2. Diversion of flow. Mobile phase being pumped through the column has two directions of flow when it reaches the mixing tee. The flow must be directed to the cell by either having the phosphate buffer flowing or by shutting off this channel with a clamp or with the rollers of the peristaltic pump.

3. A Tygon tube is necessary in line between the 3-way switching valve and the mixing tee. This tube is inserted into an appropriate peristaltic pump to provide a force for the flow of either phosphate buffer or working enzyme solution. The peristaltic pump must be calibrated for a flow rate of 0.5 ml/min prior to carrying out any assays.

4. Reagent buffers. The 0.2 M phosphate buffer, pH 8.5, should be filtered but not degassed. The 0.2 M phosphate buffer, pH 7.0, used to prepare the enzyme stock does not need to be filtered since it constitutes only a very small portion of the working enzyme solution.

5. Reagent storage. The enzyme stock solutions and acetylcholine and choline standards should be refrigerated when not in use.

Summary of Assay Procedure

1. Equilibrate the analytical column with mobile phase buffer for at least 2 hours.

2. Turn on the phosphate buffer flow using the peristaltic pump.

3. Turn the cell mode switch to the CELL position, with the electrode at a potential of +0.5 V, and allow the electrode to come to equilibrium at 500 nA (range) and then 100 nA.

4. Switch over from the phosphate buffer to the working enzyme solution and allow the baseline response to come to equilibrium again.

5. Inject sample and standard solutions, measure peak heights, calculate concentrations by peak height ratios.

6. Before shutdown, flush the electrode with protein-free buffer in preparation for use the next day.

7. To begin operation on another day, steps 1, 2, and 3 can be carried out sequentially, with no lag time between step 1 and 2.

References

1. P. E. Potter, J. L. Meek, N. H. Neff, "Acetylcholine and Choline in Neuronal Tissue Measured by HPLC with Electrochemical Detection," J. Neurochem., 41 (1983) 188-194.

2. J. L. Meek, personal communication.

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