

ARTICLE

Column Chromatography Analysis of Brain Tissue: An Advanced Laboratory Exercise for Neuroscience Majors

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Neurochemical analysis of discrete brain structures in experimental animals provides important information on synthesis, release, and metabolism changes following behavioral or pharmacological experimental manipulations. Quantitation of neurotransmitters and their metabolites following unilateral drug injections can be carried out using standard chromatographic equipment typically found in most undergraduate analytical laboratories. This article describes an experiment done in a six session (four hours each) component of a neuroscience research methods course. The experiment provides advanced neuroscience students experience in brain structure dissection, sample preparation, and quantitation of catecholamines using high performance liquid chromatography (HPLC) and protein analysis using ultraviolet spectroscopic methods. The students are exposed

to useful laboratory techniques such as standard solution preparation and calibration curve construction, centrifugation, quantitative pipetting, and data evaluation and graphical presentation. Typically, only students that participate in independent neuroscience research are familiar with these types of quantitative skills. The usefulness of this type of experimental design for understanding behavioral or pharmacological effects on neurotransmitter systems is emphasized through a final report requiring a comprehensive literature search.

Key words: neurotransmitters; HPLC; brain dissection; protein assays; catecholamines; quantitation; neuroscience experiments; undergraduate neuroscience

Liquid chromatography is one of the more common analytical methods used for the isolation and quantitation of bioactive molecules. Catecholamine determination in brain tissue (Church and Rappolt, 1999; Beck and Luine, 2002; Fantegrossi et al., 2004), extracellular brain dialysate (Church et al., 1987; Young, 2004), cerebrospinal fluid (Lui et al., 1999), and blood (Ozerdem et al., 2004) is used to characterize neurochemical changes elicited by behavioral and pharmacological manipulations. High performance liquid chromatography with electrochemical detection (HPLC/ED) is used almost exclusively for this type of analysis (Kagedal and Goldstein, 1988; Bergquist et al., 2002).

Exposure to quantitative methods of neurochemical analysis tends to be limited in the typical undergraduate neuroscience major curriculum. The experiment described here is designed to provide students with an introduction to the very powerful analytical separation technique of high pressure liquid chromatography (HPLC). Liquid chromatography is applicable to over 80% of all known compounds. It is the method of choice for the separation of nonvolatile and thermally unstable compounds. The underlying mechanism by which the liquid chromatographic technique achieves the separation of different compounds is *differential migration*. This is a measure of the propensity that each compound has to move through the column at a different rate, thus separating from one another. This movement is determined by the relative partitioning of the compound into the stationary phase and can be quantitated using a *partition coefficient* (Skoog and Leary, 1992). The time required for a compound to pass completely through the column is called the *retention time* and is unique under given separation conditions (flow rate, temperature, mobile phase composition, stationary phase) for a compound.

There are several modes of liquid chromatography based on either the nature of the mobile/stationary phases or the mode of retention. The mode used in the current experiments is called reversed-phase (RP) liquid chromatography and is the most popular mode used in separation sciences. In RP liquid chromatography the stationary phase is non-polar (i.e. hydrophobic; long carbon chains bonded to silica support particle) while the mobile phase is a polar aqueous solution (typically a buffer system).

The analysis of catecholamines is most commonly achieved by reversed-phase liquid chromatography coupled to an electrochemical detector (HPLC). Catecholamines are readily oxidized at carbon-based electrodes to their respective quinones (applied potentials of +300-800 mV versus Ag/AgCl reference). The efficiency of flow-through electrochemical detector designs (amperometric and coulometric) allows for nM range detection limits (Medford, 1985; Chen and Kuo, 1995). Conveniently, the electrode material provides for detection selectivity as other common neurotransmitters (such as acetylcholine) do not undergo oxidation at carbon electrodes under mild oxidation potentials.

HPLC/ED separation conditions for catecholamines require a mobile phase that consists of an aqueous buffer, an organic modifier, an ion-pairing agent, and a metal complexing agent. The role of the buffer is two-fold: to provide an appropriate electrolyte solution for the electrochemical reactions taking place at the carbon electrode and maintain the acid/base equilibria for the various compounds in the sample. The organic modifier (typically methanol or acetonitrile) is necessary to facilitate the "solvation" of the hydrophobic stationary phase and decrease the overall separation time. The ion-pairing agent

is used to enhance the separation of the positively charged compounds (i.e. dopamine). The metal complexing agent (i.e. EDTA) is used to minimize detector noise.

Retention times can be adjusted by altering the pH, the concentration of the organic modifier, and the concentration of ion-pairing agent. The effect of various separation conditions on the retention behavior of catecholamines and their acidic metabolites has been extensively studied (Saraswat et al., 1981; Moleman and Borstrok, 1985). By characterizing changes in retention under altered separation conditions it is possible to predict optimum separation conditions.

Lesioning of specific brain structures or neuronal pathways in animals has proven to be a powerful experimental protocol for investigations into the biological substrates of behaviors. Hypothalamic and hippocampal lesions have provided insight into the neurochemical pathways involved in reproductive behavior and learning. Pharmacological ablation of the nigro-striatal dopaminergic system using 6-OHDA was used extensively up to the mid-1980s to generate an animal model of Parkinson's Disease. Recently, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone have been used to produce selective nigrostriatal dopamine depletions in animals as a means of elucidating the neurochemical pathways responsible for Parkinson's Disease neurodegeneration (Schober, 2004).

While the experiment described herein involves the analysis of specific brain structure homogenates for catecholamines and indolamines, the procedures presented are adaptable to the analysis of a wide variety of biological samples (Moleman and Borstrok, 1985). The flow chart in Fig. 1 outlines the various components of the analysis. The degree of chromatographic theory that can be investigated (mobile phase effects, column chemistry, etc.) is flexible and can be adapted to time, resources, and instructor expertise constraints.

Goals

Within the neuroscience major, we offer a course called NESC 301 – Introduction to Neuroscience Methodology which is a core course that introduces students to current methods and techniques used in neuroscience research. It

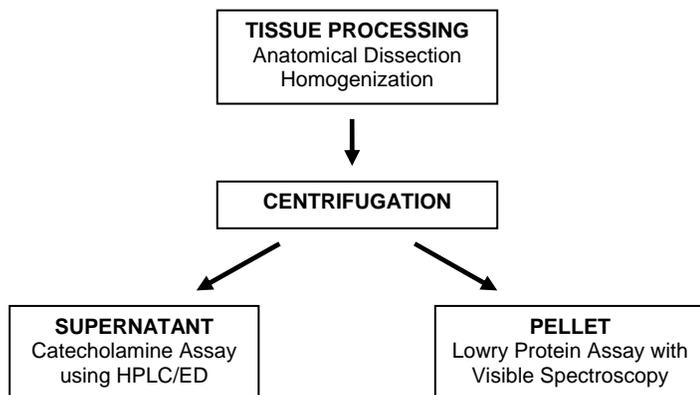


Figure 1. Flow chart diagramming the various components of the experiment. Quantitation of catecholamines is carried out using the internal standard calibration method. Quantitation of the protein content is carried out using the external standard calibration method.

consists of four three-week rotations through different research labs where students perform experiments specific to the research field of faculty assigned to that course. The experiment described here is conducted during one of the rotations and is intended to be carried out by sophomore or junior neuroscience majors who have had at least one year each of introductory chemistry and introductory biology and an introductory course in neurobiology. Goals for the experience are the introduction to and evaluation of the following:

- *Sample Preparation:* the importance of appropriate sample preparation prior to analysis such that reliable quantitation is possible.
- *Chromatographic Theory:* the mechanisms involved in the separation process are introduced. The HPLC separation is based on the distribution of an analyte between two immiscible phases.
- *Detection Methods for Biological Compounds:* the theory behind electrochemical and spectroscopic methods for the quantitation of analytes.
- *Quantitative Analysis:* the importance of quantitative methods in data reporting and the use of internal and external calibration standards for accurate reporting of experimental results.

The two instrumental methods (HPLC/ED and visible spectrophotometry) utilized in this experiment are distinctive enough in theory and operation to provide valuable operational experience without redundancy, yet are similar enough in regard to quantitation theory to reinforce the concept. Table 1 shows a typical time-line for the execution of the various experimental components of this rotation.

MATERIALS AND METHODS

Equipment

HPLC/ED System Any standard system consisting of a high pressure isocratic pump, a sample injection valve (20 μ L external loop is recommended), guard column packed with C18 packing material, 250 mm x 4.6 mm C18 reverse-phase column and an electrochemical detector will work. A strip chart recorder, integrator, or chromatography workstation can be used as an output device. The system used to develop the

HPLC Analysis of Rodent Brain Tissue Homogenates	
1 st session	Lab Orientation and Tour - Pipetting and analytical balance instruction
2 nd session	Tissue Dissection and Processing (including tissue homogenate preparation)
3 rd session	Protein and Catecholamine Assays <i>EXCEL graphing assignment due</i>
4 th session	Assays continued; Data quantification (calibration curves)
5 th session	Data Analysis and Interpretation <i>Chromatography assignment due</i>
6 th session	Student Presentations and Class Data File Generation
Final Paper due one week after presentations.	

Table 1. Typical time line for experimental procedures.

liquid chromatography lab described in this report consists of an LDC isocratic reciprocating pump, a Rheodyne 7010 injector with a 20 μL external sample loop, an Upchurch guard column, a Sigma 250 mm x 4.6 mm C18 reverse-phase column, and an EG&G PAR Model 440 electrochemical detector. The composition of the mobile phase consists of 2% citric acid, 2% K_2HPO_4 , 1 mM EDTA, 1.2% MeOH, and 70 mg/L sodium octyl sulfate. The pH of the mobile phase is adjusted to 3.0 using 6 N HCl. The mobile phase is filtered and degassed with helium prior to use. The separation is carried out using a flow rate of 0.7 mL/min. Samples are injected manually. If available, the system can be configured with an autosampler to improve the sample throughput capabilities. The electrochemical detector is configured with a glassy carbon working electrode held at a potential of +0.800 V (vs. Ag/AgCl) in the oxidation mode. The sensitivity of the detector is determined by the volume of sample injected but typically is set between 5-50 nA/V.

Visible Spectrophotometer Any standard UV/Vis or visible spectrophotometer can be used to carry out the protein analysis. In this laboratory, an HP 8453 UV-Visible spectrophotometer (Hewlett-Packard, Palo Alto, CA) is used to measure the absorption at 750 nm of individual tissue pellet samples following dissolution with 1.0 N NaOH and treatment with the modified Lowry protein assay reagents.

Methods

Isolated rat brains which have been unilaterally lesioned using 6-OHDA (and sham controls) are distributed to each member of the research team (typically two students per team depending on class enrollment; each team has at least one lesioned and one control brain). Lesioning (resulting in depletion of nigrostriatal dopamine neurons) and brain removal services are provided by Zivic Laboratories (Pittsburgh, PA). Students isolate tissue from the following structures: cortex, striatum, midbrain, and cerebellum. This is most easily accomplished using a microtome tissue slicer with a freezing stage, but it can also be accomplished manually. Students are instructed to take approximately 1 mm thick coronal slices which will isolate these structures using a rat atlas as a guide (Pellegrino et al., 1979). Manual slicing is done on a chilled glass plate using razor blades. Dry ice is available to keep the brain semi-rigid to facilitate slicing. Tissue punches (approximately 1 mm) from each hemisphere are then collected into separate centrifuge tubes. Punch devices are available commercially or can be manufactured in house.

Tissue samples are homogenized using individual plastic disposable pestles and 1.5 mL centrifuge tubes (The LabMart, South Plainfield, NJ) which contain 400 μL of tissue homogenizing solution (0.1 M perchloric acid, 1×10^{-7} M ascorbic acid; chilled on ice) containing the internal standard dihydroxybenzylamine (20 ng/mL). Following tissue disruption, the samples are centrifuged at 12000 $\times g$ for five minutes. The supernatant samples are filtered using 0.2 μm nylon disposable syringe filters and stored at -80°C until analyzed (-20°C storage is also adequate for storage times of up to three months). The centrifuge tubes containing the tissue pellet are stored at room temperature until protein

determination is carried out.

Materials

Ascorbic acid, dopamine, DOPAC, HVA, norepinephrine, 5HIAA, and 3,4-dihydroxybenzylamine (DHBA), are purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals are analytical grade and purchased from Fisher Scientific (Atlanta, GA).

RESULTS

Sample Preparation

The importance of proper collection and processing of the tissue samples is stressed during the first two sessions. The students are required to familiarize themselves with neuroanatomical landmarks using the rat brain atlas. A PowerPoint presentation (available from the author on request) of the dissection method is reviewed and brain structure identification is practiced prior to conducting the actual tissue collection.

A significant time is typically needed for the students to master pipetting and standard solution preparation techniques required for the calibration curve generation. No samples may be analyzed, neither homogenate supernatant (for neurotransmitters and their metabolites) nor protein pellet, until satisfactory calibration curves have been generated. A minimum correlation coefficient of 0.995 is typically required for sample analysis approval.

Chromatographic Theory

A general overview of chromatographic separation theory is presented to the students using the student handbook *Column Chromatography Method of Analysis in Neuroscience* (produced in-house and available from the author upon request).

The manipulation of mobile phase components is used to study the role of organic modifier, ion-pairing agent, and pH on peak shape, resolution, retention time, and selectivity of the separation. In our laboratory, we typically have each research team separate a series of standard solutions using four different mobile phases. To facilitate peak identification three different combinations of standards are run for each mobile phase investigated. Standard Mix 1 contains DHBA and dopamine; Standard Mix 2 contains DHBA, norepinephrine, and 5HIAA; Standard Mix 3 contains DHBA, DOPAC, and HVA. The combination of an early eluting analyte, the internal standard, and a late eluting analyte has proven successful in allowing accurate peak assignments and easy interpretation of changes in chromatographic behavior. These analyses are conducted outside of the regularly scheduled laboratory time and typically require two hours of lab time for each mobile phase investigated. In a typical class of 12 students, this arrangement allows for separation data from 24 different mobile phases to be generated.

The chromatographic performance variable is measured as a function of the specific mobile phase component under study using a chromatographic software package. Variables investigated are retention time, peak asymmetry, and peak resolution. Graphs of these variables are constructed by the class as a whole after combining their respective data and

discussed in relation to chromatographic theory. A typical experimental design would have mobile phases based on the composition described above for the analysis of the tissue samples. Sodium octyl sulfate concentration is varied from 20 mg/L to 120 mg/L, methanol content is varied from 0.5% to 5.0 %, and pH is varied from 2.0 to 5.0. This form of activity is useful if students have not been exposed to chromatography through their chemistry courses. It tends to be time consuming, but has the advantage of being carried out using standard solutions.

Quantification of Catecholamines Using the Internal Standard Method

Standard solutions containing mixtures of catecholamines and the internal standard, DHBA, are prepared such that the concentrations of the catecholamines range from 0.1 μM to 100 μM . The DHBA concentration remains constant at 10 μM . Each standard solution is run on the HPLC/ED and calibration curves are generated based on the internal standard ratio method using peak height measurements from the standard solution chromatograms (Skoog and Leary, 1992). Depending on the equipment available, this process can be performed using a standard chromatographic data collection/analysis software package (i.e. ChromPerfect, Justice Innovations) or developed using a commercial spreadsheet program (i.e. Excel). Figure 2 shows overlaid chromatograms of the calibration standard solutions illustrating the high resolution and short separation time of the assay.

Tissue supernatant samples are then run on the HPLC/ED system and quantified based on the calibration curve generated for each individual analyte. Figure 3 shows a typical chromatogram generated from a tissue homogenate.

If time permits, it is useful to do multiple runs of the samples to enhance the precision of the quantitation.

Protein content of the tissue samples is determined by spectroscopic analysis based on the Modified Lowry protein assay (Pierce Scientific, Rockford, IL). The standard protein solutions for the external calibration curve generation vary from 0.025 to 0.25 mg in 200 μL of 1.0 N NaOH. The catecholamine data is then normalized by expressing it as the amount (in ng) of individual compound per mg protein.

During the final lab session, research teams present their results and the class data is pooled to allow for statistical analysis of the differences in catecholamine and indolamine levels. For illustration purposes, grouped data based on the quantitation of the various analytes in the striatum are presented in Fig. 4.

DISCUSSION

The multiple aspects of this experiment make it a useful introduction to the interdisciplinary nature of neuroscience research. Dissecting out specific regions of the brain allows the students to review their neuroanatomy and provides them with a hands-on opportunity to visualize familiar structures. The use of unilateral lesions for behavioral and pharmacological studies is presented in the context of the current experiment. This type of experimental protocol provides a "within-subject" control value (the contralateral or unlesioned hemisphere) with which to carry out statistical

comparisons. During the discussion on data presentation, emphasis is placed on how tissue levels are reported as "percent of contralateral control." Additionally, the need to normalize the data by report the initial values as "ng X per mg protein" is presented. A useful "homework" assignment is to have the students obtain and present examples of this type of data reporting from the published research literature.

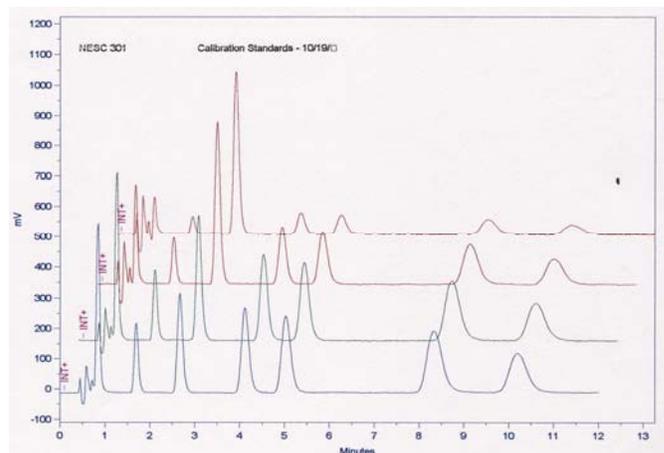


Figure 2. Overlaid chromatograms of standard solutions used for calibration curve construction. The order of elution is norepinephrine (1.7 min), DHBA (2.7 min), DA (4.2 min), DOPAC (5.1 min), 5HIAA (8.5 min), HVA (10.3 min).

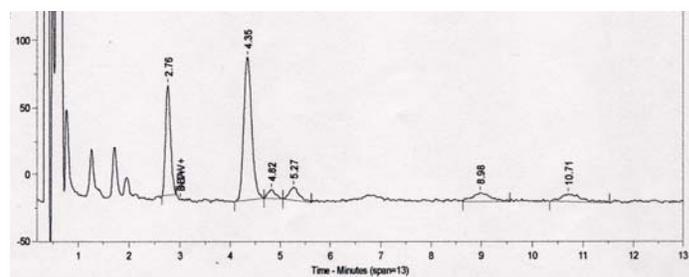


Figure 3. Representative chromatogram of rat striatum homogenate. The signal recorded (y-axis) is current in units of 10^{-9} amps.

A critical comparison of the types of neurochemical data available through various methods (tissue punch, microdialysis, metabolite turnover) is a major component of the data interpretation presentation. Neurochemical data collected in this experiment are most useful in providing a steady-state measure of neuronal synthesis capacity, whereas microdialysis generates extracellular "release" measures and metabolic turnover reflects neuronal activity indirectly reflecting "release" of neurotransmitter (Finlay and Smith, 2000). This proves to be a very powerful way of illustrating the importance of understanding the physiology of the system under study when interpreting experimental results.

The students that have not had any research experience often have a difficult time with the quantitative standard solution preparation. For this reason, it is advantageous to pair up students that have had research experience with

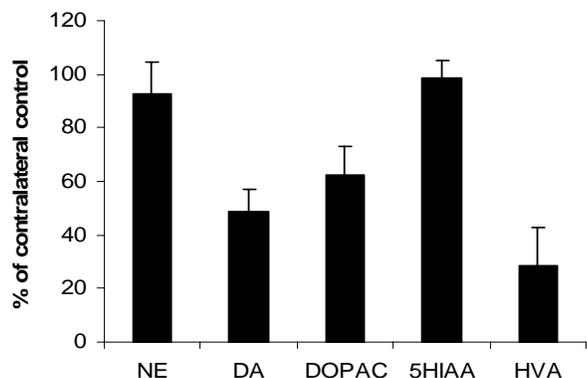


Figure 4. Effect of unilateral medial forebrain bundle 6-OHDA lesions on catecholamine and indolamine content in rat striatum. The data are reported as percent of the contralateral non-lesioned hemisphere. The data are reported as the mean \pm SEM (N=6).

those who have not. Additionally, outside lab time is often necessary for students to construct standard solutions that generate appropriate calibration curves. The chromatography and spectroscopy instrumentation should be familiar to the students from their chemistry and biology lab experiences. This lab provides an additional opportunity for exposure to analytical instrumentation and builds on previous learning experiences from introductory science labs. The graphing exercise and the presentation of the research team experimental data allow the students to explore the most effective method for conveying pertinent experimental results.

In the final class session, all the class data is combined into a “control” or “lesioned” group based on class consensus. By grouping the data, the students are obligated to investigate statistical software (Prism, GraphPad Software, Inc., San Diego, CA) for determining significant differences in analyte levels, which can be reported in the final write-up. The final session is very beneficial as the group discussion of each student’s data simulates the “real-world” research laboratory environment. Students are encouraged to speculate on the drug treatment that the animals received. This is usually readily evident from the grouped data (selective DA depletion restricted to the nigrostriatal region) and an adequate familiarity with the pertinent literature (from the literature search required for the final paper).

The students are evaluated on the following aspects of the rotation experience: lab notebook, calibration curves, lab behavior, final presentation, and final report. The group is then responsible for presenting a poster based on the overall results at the College’s annual Science Symposium.

It should be noted that the technique of tissue isolation and analysis by HPLC/ED is not strictly limited to investigations of pharmacological lesioning. In the Neuroscience Methods course students have conducted a behavioral (unilateral bar pressing, maze running, etc.) experiment coupled to pharmacological manipulations (chronic treatment with dopamine agonists or glutamate NMDA receptor antagonists) during one rotation and then have analyzed changes in brain chemistry using the technique described above during the next rotation. The

experiments that allow the students to explore the connections between behavioral and/or pharmacological manipulations and neurochemistry are numerous.

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Received January 10, 2005; revised April 27, 2005; accepted April 29, 2005.

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