Analysis of Neuroactive Amino Acids Using UHPLC and Electrochemical Detection

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ABSTRACT

Amino acids are the essential building blocks of proteins and peptides. They can also take part in intermediary metabolism and act as precursors to common biogenic amine neurotransmitters. Certain amino acids act as neurotransmitters and are the major excitatory (aspartate and glutamate) and inhibitory (GABA and taurine) commands in the central nervous system. The measurement of the profile of these neuroactive amino acids can assist with the characterization and our understanding of the factors and potential causes of various neurological conditions. Amino acids are difficult to detect directly as they lack a suitable chromophore, so derivatives are often prepared. A specific challenge of the analytical method is realized when analyses in low-volume samples are at low concentrations, as is the case with brain microdialysis samples. Isoindole derivatives of amino acids, formed through a well-established reaction with *o*-phthalaldehyde (OPA) and 2-mercaptoethanol (β-ME), can now be effectively resolved by high-resolution HPLC and detected using a new sensitive, low-dispersion, coulometrically-efficient electrochemical cell. This approach offers improved sensitivity and shorter chromatography run times for the analysis of neuroactive amino acids in microdialysis perfusate samples obtained from various brain regions of the anesthetized rat. The effects of neuropharmacological manipulation to confirm peak identification and show lack of analyte co-elution will also be presented.

INTRODUCTION

Amino acids are the most abundant neurotransmitters in the brain. There are both excitatory (aspartate, glutamate) and inhibitory (GABA, glycine) amino acids that act as neurotransmitters. γ-Aminobutyric acid (GABA) is the predominant inhibitory neurotransmitter in the brain, and its concentration in brain microdialysates is low (nM level) while levels of the major excitatory amino acid glutamate are higher (µM level).[1]

Analysis of amino acid in striatal extracellular fluid using microdialysis sampling typically requires the preparation of derivatives to achieve sufficient sensitivity for analysis due to their low concentrations. The OPA/β-ME derivative of an amino acid generates electrochemically active thio-substituted isoindole compounds that provide sufficient stability to be a suitable choice for the analysis of amino acids. The *o*-phthalaldehyde (OPA) derivatization reaction is commonly used as an automated precolumn derivatization method with electrochemical detection is shown in Figure 1.

Although the measurement of amino acid neurotransmitters by high-performance liquid chromatography (HPLC) with electrochemical detection has emerged as a reliable and sensitive method, there remain several issues related to the overall analysis time, analyte sensitivity, and stability of the precolumn derivative.

Ultrahigh-performance liquid chromatography (UHPLC) uses columns packed with sub-3 or sub-2 µm porous particles. Typically systems are operated at high linear flow velocity, thus UHPLC systems must be capable of operating at very high pressure. A major advantage of UHPLC over conventional HPLC is its capability to increase separation speed and/or efficiency. In comparison to traditional HPLC, UHPLC can achieve faster separations while maintaining or increasing peak resolution, thereby resulting in higher throughput. UHPLC enables high resolution applications resulting in significant advantages in analytical performance over traditional HPLC. Detectors used with UHPLC must be capable of high frequency data collection and low peak dispersion. Also, the production of sharper and more concentrated peaks with UHPLC can often lead to improved detection sensitivity, particularly for mass-flow dependent detectors.

The Model 6011 *ultra*™ Analytical cell is an ultrahigh performance liquid chromatography (UHPLC)-compatible, coulometric, dual-electrode cell. Importantly, coulometric ECD behaves as a mass flow-dependent device whose response (peak height) benefits from the faster peaks obtained with UHPLC and fast LC. Benefits of this cell include: reduced volume electrodes and flow path for compatibility with UHPLC; improved peak resolution; dual electrode design for flexibility in mode of operation; flow-through electrode for coulometric efficiency and a solid state, no maintenance reference electrode.

The combination of UHPLC column with an electrochemical cell specifically designed for UHPLC techniques is presented here.

METHOD

Chemicals and Standards

Amino acid standards were purchased from Sigma Chemicals Co., St. Louis, MO. The OPA and borax diluent were purchased from Pickering Laboratories, Inc. The amino acid standards were prepared at 1 mg/mL in 50% methanol/water solution. Further dilutions for calibration curves were made using water.

Surgery

Anesthetized model: The CD-1 rats were anesthetized with urethane (1.5 g/kg, i.p.) The skull was exposed and a hole was drilled for placement of microdialysis probe (CMA 11, 3 mm polycarbonate membrane) into prefrontal cortex. The coordinates, with respect to Bregma, were +3.2 mm (AP), +0.8 mm (LR) and -5.0 mm (DV). See section below for aCSF composition and flow rate.

Awake model: The CD-1 rats were anesthetized with ketamine/xylazine (60 and 8 mg/kg, i.p.). A guide cannula (CMA) was implanted, such that, when inserted, the tip of the microdialysis probe (CMA 11, 3 mm polycarbonate membrane) would be located in the prefrontal cortex at +3.2 mm (AP), +0.8 mm (LR) and -5.0 mm (DV) relative to Bregma. Animals were given 24 h for recovery from surgery. The next day, the probe was inserted through the guide cannula and animals were perfused with normal aCSF (145 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1.2 mM $CaCl_2$, 2.0 mM Na_2HPO_4) at a flow rate of 2 µL/min. A stabilization period of 2 h was allowed before collecting samples every 20 min. After collecting four baseline samples, the aCSF was switched to high potassium (60 mM). After 100 min, normal aCSF was once again perfused. The samples collected were stored on ice.

Instrumentation

The HPLC system was comprised of an ESA Model 584 pump, Model 542 Autosampler, Coulochem® III electrochemical detector and an ESA Elite chromatography data station. For the UHPLC work, the analytical cell was a Model 6011, set at E1=+150 mV and E2=+550 mV. A Model 5020 guard cell, set at +600 mV, was positioned before the autosampler to help lower the background current at the analytical cell. A reversed phase column (Shiseido Capcell Pak C18, IF, 2 µm, 2.1 × 50 mm) was employed for separation of the amino acids. The flow rate was 0.28 mL/min and the column temperature was kept at 48 °C. The mobile phase was comprised of 100 mM disodium hydrogen phosphate anhydrous, 22% methanol, 3.5% acetonitrile. The mobile phase pH was adjusted to 6.75 with phosphoric acid. A simple pretreatment program allowed for automatic preparation of the precolumn amino acid derivatives before injection.

Derivative Formation

- Stock OPA/β-ME Solution: (store at 4 °C)
- Dissolve 27 mg OPA in 1 mL CH_3OH and then add 5 µL β-ME and 9 mL OPA diluent. Note that this stock solution is usable over a 5 day period.
- Working OPA/β-ME Solution: (store at 8 °C on autosampler tray)
- Mix 1 mL OPA/β-ME stock solution with 8 mL OPA diluent and place in 10 mL glass autosampler vial. Note that this solution requires daily preparation.
- Transfer a 15 µL aliquot from each perfusate sample to a vial, then place it in the autosampler. To this vial, add 25 µL of OPA reagent and allow to react for 2 min, then inject a 10 µL amount of the derivatized sample.

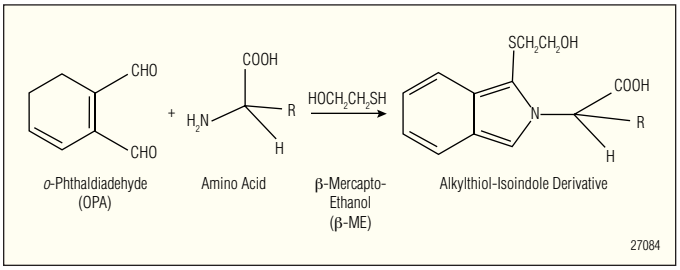

Figure 1. Derivatization of amino acids with OPA / β-ME produces electrochemically active thio-substituted isoindole.

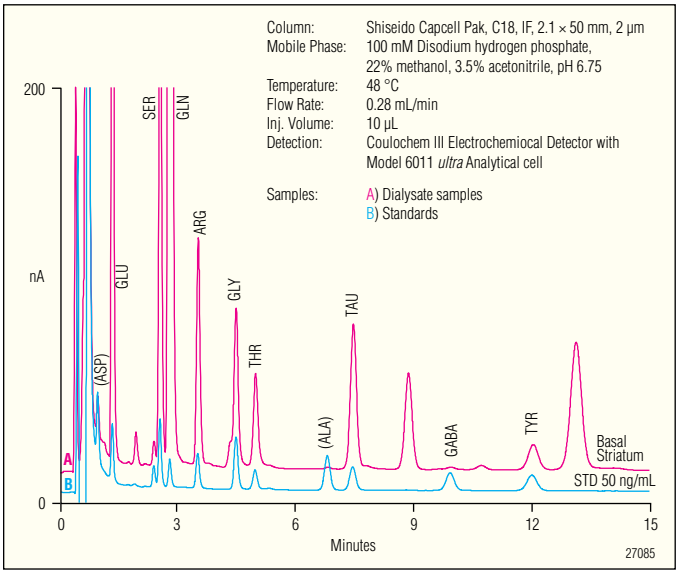

Figure 2. Overlay of amino acid analysis in samples and standard using 6011 cell.

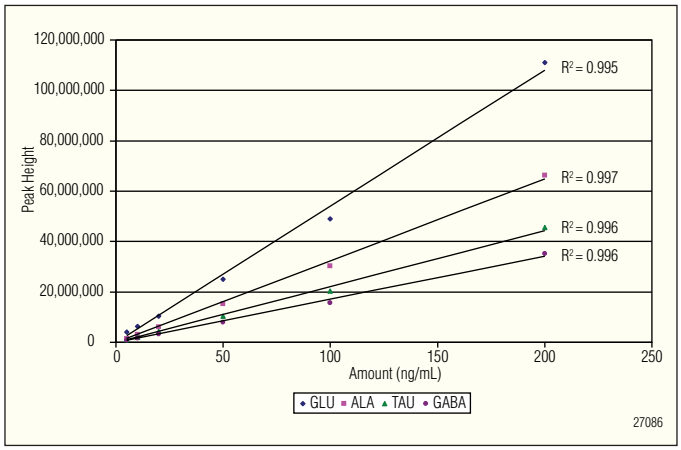

Figure 3. Calibration curves for amino acid derivatives.

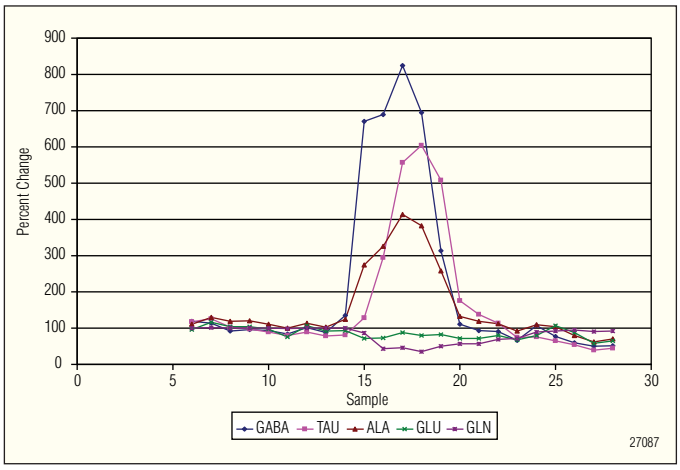

Figure 4. Effects of high potassium on amino acid levels.

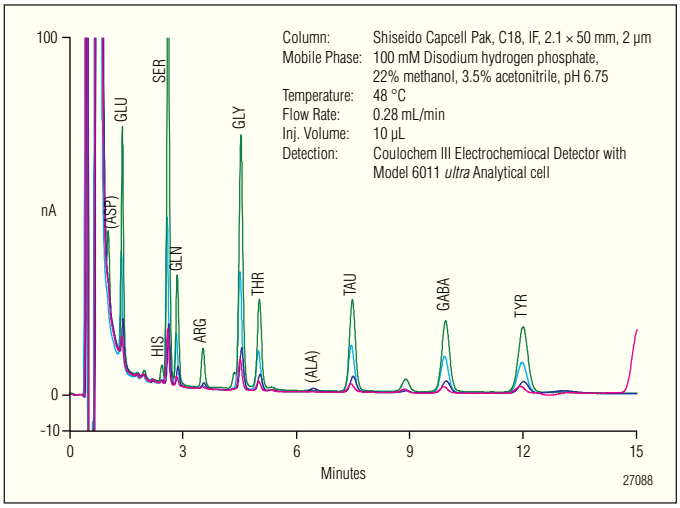

Figure 5. Overlay of four consecutive samples taken during the experimental time course.

RESULTS AND DISCUSSION

An overlay of chromatograms representing an amino acid standard and microdialysis sample are illustrated in Figure 2. Good resolution of all the neuroactive amino acids was observed due to the efficiency of the UHPLC column within a 15 min assay. Figure 3 shows that the response curves for different amino acid concentrations were linear with correlation coefficients > 0.995. The effect of aCSF with high potassium (60 mM) on amino acid levels is shown in Figure 4. Significant increases in GABA and taurine were observed but no significant increase was observed for glutamate. An overlay of four consecutive runs, shown in Figure 5, reveals that there are no major interferences from later eluting amino acids by having a 15 min cycle time.

The low peak volume of UHPLC methods puts special requirements on the cell design. The 6011 *ultra* Analytical cell has a lower swept volume for the flow-through electrodes, as well as a redesigned flow path to minimize peak dispersion. Using UHPLC with this detector cell, sharper peaks with superior resolution were observed. The UHPLC column used in this study provided better resolution and sharper peak shapes than conventional HPLC columns. The retention time for GABA using the 2 µm column was 9.9 min, which is 50% less than the retention time when using a 3 µm column packing (data not shown). The faster elution of GABA offers improved sensitivity for this compound because of several factors: 1) The peak width is smaller using the 2 µm material and this improves the limit of detection. 2) The GABA derivative is unstable with a half-life of only a few minutes.[2,3] Thus, earlier elution ensures less deterioration and a more reliable measurement. 3) The mass-flow dependent Coulometric ECD is a device whose response (peak height) increases from the faster peaks obtained with UHPLC.

Although other thiols can be used with OPA for derivatization of amino acids and many produce more stable derivatives than β-ME, the choice of β-ME actually allows for faster overall run times. Some of the later eluting amino acid derivatives such as leucine are mostly degraded by the time they reach the electrochemical cell and this prevents interferences from these compounds (data not shown). The analysis of amino acids in this work uses a simple isocratic approach that allows for a 15 min cycle time. Overlays of subsequent injections illustrate that there is no significant carryover from run to run (see Figure 5). Similar retention times of all the major peaks illustrates that good column stability was achieved.

CONCLUSIONS

Neurotransmitter amino acid analysis in brain extracellular fluid (microdialysate samples) is typically performed using HPLC-ECD after preparation of OPA derivatives. Here the authors show that a fast and stable method for amino acid analysis can be achieved using UHPLC with the new model 6011 electrochemical cell specifically designed for UHPLC techniques.

The model 6011 *ultra* Analytical cell is an ultrahigh-performance liquid chromatography (UHPLC)-compatible, coulometric, dual electrode cell. This new cell is specifically designed for UHPLC and can easily handle the improved peak resolution and throughput this approach offers. When neuroactive amino acid analysis is adapted to UHPLC, the cycle time of analysis can be reduced by as much as a factor of 2–5. Using the 6011 *ultra* Analytical cell adds sensitivity and selectivity to UHPLC. This combination brings the speed and resolution associated with UHPLC to the proven performance of coulometric electrochemical detection that provides:

- Reduced method development time
- Increased throughput
- Conservation of solvents
- High-sensitivity analysis

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