Fast UHPLC Methods for Analysis of Amino Acids

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Overview

Purpose: Methods that offer both rapid and sensitive measurement of amino acids are essential to neurochemical research. The ability to decrease analysis time enables an improvement in temporal resolution when studying amino acid neurotransmission.
Methods: Two methods using pre-column derivatization were developed: first, a fast isocratic method with electrochemical detection for the analysis of several neurochemically important amino acids, and secondly, a fast and sensitive gradient UHPLC method with fluorescence detection that measures both free D-aspartic acid and D-serine (as well as their L-enantiomers).
Results: The UHPLC methods enabled the rapid separation of various neuroactive amino acids within half of the analysis time when compared to traditional HPLC methods, while maintaining resolution and excellent sensitivity. Data from rat brain region homogenates are presented.

Introduction

Precolumn derivatization of amino acids with o-phthaldialdehyde (OPA) reagent prior to detection by electrochemical or fluorescence detection is a very sensitive and selective way to measure neuroactive amino acids at low levels in microdialysis perfusates or brain tissues samples. Applying UHPLC technology, the run time for these analyses can be greatly reduced, thereby further enhancing sensitivity and improving sample throughput. Together with the new UHPLC-compatible Thermo Scientific™ Dionex™ UltiMate™ 3000 electrochemical detector, a fast isocratic method using OPA/βME derivatization for the analysis of neuroactive amino acids in brain tissue homogenates and microdialysis samples was developed.
Although D-enatiomers of amino acids are common in lower organisms such as bacteria and were not thought to occur in mammalian tissues, it became apparent that some D-amino acids do occur in higher organisms and that they have biochemical importance. Recent publications indicating an abundance of D-amino acids in neuroendocrine tissues have prompted the development of simple analytical methods for the measurement of these amino acid enantiomers.[1]
D-Serine (D-Ser) occurs in glial cells, is particularly abundant in brain regions enriched in N-methyl-D-aspartate (NMDA) receptors and is the endogenous co-agonist of the glutamatergic NMDA receptor (not glycine).
D-Aspartic acid (D-Asp) is found in some specific neuronal pathways, but is more abundant in epinephrine-containing glandular tissue (e.g., adrenal medulla) where it appears to regulate hormone synthesis and release. We developed a rapid UHPLC method using chiral N-acetyl-L-cysteine (NAC)-OPA derivatization and fluorescence detection for the analysis of D- and L-Asp and D- and L-Ser with improvement over typical HPLC methods.[2,3]
The application of these two methods is demonstrated with the measurement of neuroactive amino acids and D- and L-Asp and Ser in different brain tissue samples.

Methods

Biological Experiments
Male Sprague Dawley rats weighing 175–200 grams were administered 2.0 mg/kg *d*-amphetamine or vehicle (saline) via i.p. injection. One hour later animals were sacrificed by carbon dioxide asphyxiation and the brains rapidly removed, dissected and frozen at -70 °C.

Sample Preparation
10 to 20 mg of brain tissue was homogenized in 1 mL 0.3N perchloric acid, then diluted either 10 or 20 times with water before analysis.

Derivatization Procedure
Stock OPA/NAC: 20 mg OPA was dissolved in 1 mL methanol, 10 mg NAC and 9 mL OPA diluent was added. Stock derivatizing reagent was stable for 5 days at 4 °C. Working reagent was prepared fresh daily by diluting 1 mL of stock reagent into 4 mL OPA diluent. Automated precolumn derivatization was performed by mixing 25 µL working reagent with 25 µL tissue sample. For enhanced column life, 8 µL 1M acetic acid was mixed into sample before injection to neutralize sample pH.

Analysis of Neuroactive Amino Acids with EC Detection

Pump:	Thermo Scientific™ Dionex™ UltiMate™ 3000 DPG-3600RS
Autosampler:	Thermo Scientific™ Dionex™ UltiMate™ 3000 WPS-3000TRS
EC Detector:	Thermo Scientific™ Dionex™ UltiMate™ 3000 ECD-3000RS 6011RS cell, E1 = 150 mV, E2 = 550 mV
Column Thermostat:	Thermo Scientific™ Dionex™ UltiMate™ 3000 TCC-3000RS
Column:	Thermo Scientific™ Accucore™ C18, 2.6 µm, 100 × 3 mm
Mobile Phase:	76.5% 75 mM Phosphate buffer, pH 6.3, 20% Methanol, 3.5% Acetonitrile
Temperature:	45 °C
Flow Rate:	0.8 mL/min
Injection Volume:	2 µL

Analysis of free D- and L-Asp and D- and L-Ser with Fluorescence Detection

Pump:	Thermo Scientific™ Dionex™ UltiMate™ 3000 HPG-3400RS
Autosampler:	Thermo Scientific™ Dionex™ UltiMate™ 3000 WPS-3000TRS
Detector:	Thermo Scientific™ Dionex™ UltiMate™ 3000 FLD-3400RS
Dual	PMT, Excitation: 340 nm, Emission: 450 nm
Column Thermostat:	Thermo Scientific™ Dionex™ UltiMate™ 3000 TCC-3000RS
Column:	Thermo Scientific™ Hypersil GOLD™, 1.9 µm, 200 × 2.1 mm
Temperature:	35 °C
Mobile Phase A:	50 mM Dibasic sodium phosphate, pH 6.5
Mobile Phase B:	Methanol
Gradient:	0–6 min: 3%B; 6.5–10 min: 20%B; 11–14 min: 80% B; then equilibrate at 3%B for 7 min
Flow Rate:	0.25 mL/min
Injection Volume:	2 µL

Data Analysis

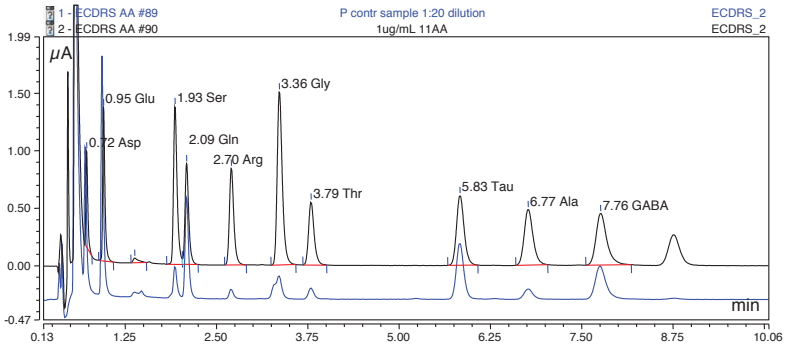
All HPLC chromatograms were obtained and compiled using Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, 6.8 SR 11.

Results and Discussion

Analysis of Neuroactive Amino Acids with EC Detection

A rapid method for neuroactive amino acids in brain tissue and microdialysis samples was developed using the new UltiMate 3000 electrochemical detector (ECD-3000RS) and ultra coulometric analytical cell (model 6011RS). With ultra-low internal volume, this new cell design minimizes dispersion to improve resolution for UHPLC separation, and still maintains near 100% efficiency for high sensitivity. Separation of 11 amino acids was completed within 10 min using an Accucore column, which reduces the analysis time in half compared to most HPLC methods that typically require >20 min. Sensitivity for these amino acids typically varied from 10 to 20 pg on column, with the exception of aspartic acid (Asp) which had a detection limit at of 40 pg on column. Although we are only presenting brain tissue homogenate data in this poster, the excellent sensitivity of this method will enable the measurement of trace amino acid levels in other biological samples such as microdialysis perfusates.

FIGURE 1: Overlay of chromatogram of prefrontal cortex control sample and standard showing the separation of 11 amino acids.

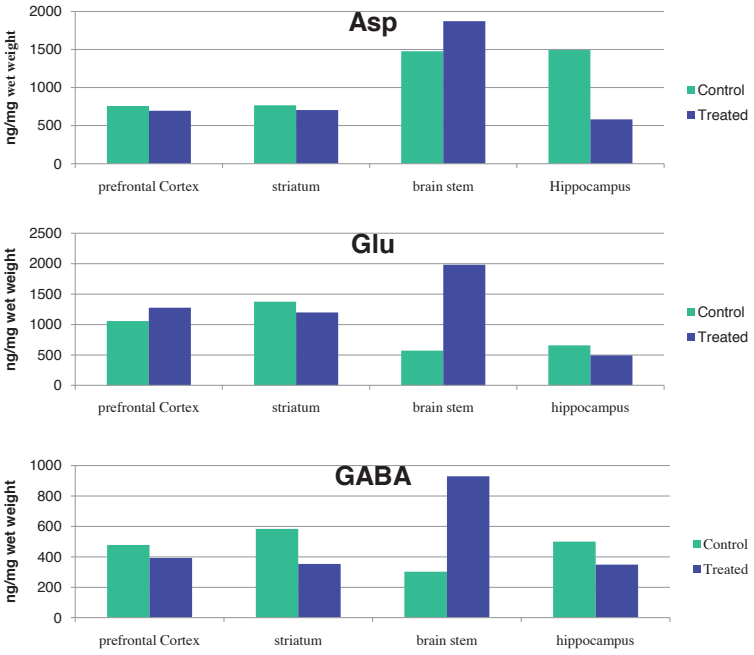


Eight brain tissue samples from four different brain regions including prefrontal cortex (P), striatum (S), brain stem (B) and hippocampus (H), from either saline control (C) or amphetamine treated (T) rats were analyzed. Figure 1 shows an overlay of chromatograms of a tissue sample and separation of 11 amino acids standards. The effect of amphetamine treatment was most notable for Asp, glutamate (Glu) and γ-aminobutyric acid (GABA) (Table 1 and Figure 2-4). Amphetamine treatment resulted in over 200% increase in Glu and GABA, and a 26% increase in Asp in brain stem, but an overall decrease in their levels in the hippocampus. No significant changes were found in the prefrontal cortex or the striatum.

TABLE 1. Levels (ng/mg tissue wet weight) of measured neuroactive amino acids in regional brain tissues

	PC	PT	% change over control	SC	ST	% change over control
Asp	758.4	694.9	-8.4	766.2	704.8	-8.0
Glu	1055.1	1275.0	20.8	1373.5	1198.5	-12.7
GABA	478.2	392.9	-17.8	583.3	652.9	11.9
	BC	BT		HC	HT	
Asp	1476.0	1873.2	26.9	1493.1	581.4	-61.1
Glu	572.1	1980.6	246.2	657.0	493.6	-24.9
GABA	302.7	928.9	206.9	500.0	348.9	-30.2

FIGURE 2: Comparison of Asp, Glu and GABA concentration levels between treated tissue sample and control samples.



Analysis of Free D- and L-Asp acid and D- and L-Ser with Fluorescence Detection

A variety of methods, including enzymatic assay, GC and HPLC methods, have been developed to separate and quantitatively determine amino acids enantiomers in biological samples. Unfortunately, most published methods either separate the enantiomers of just one amino acid e.g., D- and L-Asp, or when separating the enantiomers of more amino acids requires an analysis time of over one hour. Presented here is a UHPLC method with precolumn derivatization using OPA and NAC for simultaneous determination of D- and L-Asp and D- and L-Ser enantiomers in only 20 min, with detection limit of 200 to 400 fg on column.

Figure 3 shows the separation of D- and L-Asp, D- and L- Ser and some other amino acids, for standards and brain stem tissue homogenates. Unfortunately, an interference peak was found. Figure 4 illustrates separation of D-Asp from an unknown interference peak ”1” in the presence of large amount of L-Asp. The L/D enantiomer ratio was 360 for sample BT and 1600 for sample BC. Unknown interference peak “2” was separated from D-Asp but co-eluted with L-Asp. It has been observed during method development that its amount was insignificant relative to the endogenous amount of L-Asp, so it shouldn’t be a concern if quantitation of L-Asp is required. It was observed that the amount of unknown “2” increased significantly if the supernatant was not separated from the precipitant right after tissue extraction. Figure 5 shows the zoom-in view of the separation of D-Ser from asparagine (Asn) and L-Ser in standard and a tissue sample for a better view of separation resolution.

FIGURE 3. Overlay of chromatograms from brain stem tissue sample treated and control, overlaid with a 10 ng sample chromatogram

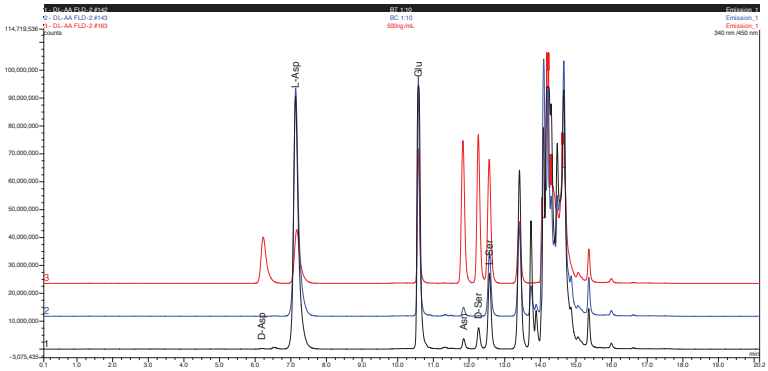


FIGURE 4. Chromatogram showing separation of D-Asp from interference peak

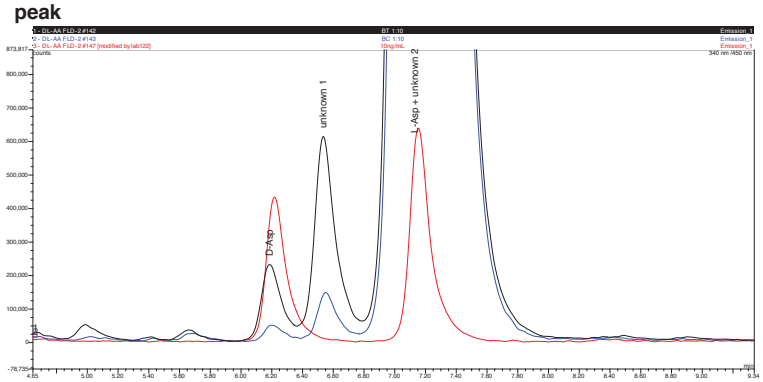


FIGURE 5. Chromatogram showing separation of D-Ser from Asn and L-Ser

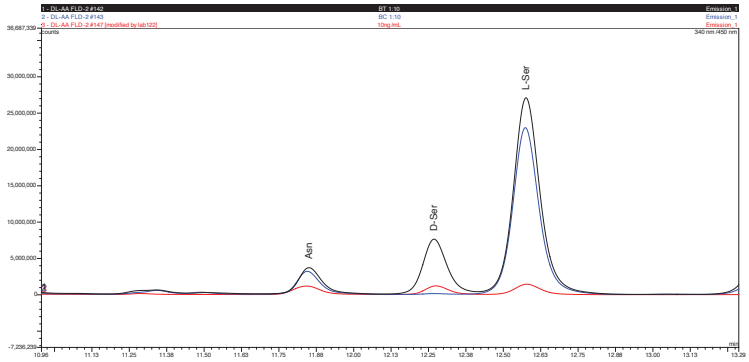
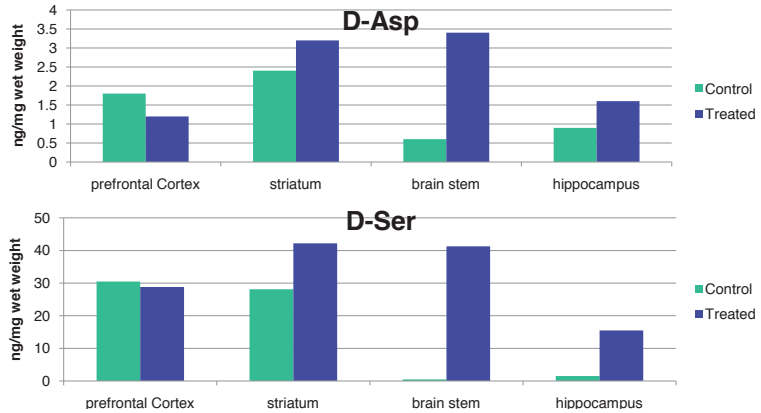


Table 2 and Figure 6 shows concentration of D- and L-Asp and D- and L-Ser in eight brain tissue samples. Amphetamine treatment was associated with region specific differences. The brain stem region was markedly stimulated with a 442% increase of D-Asp and 8498% increase in D-Ser. The hippocampus was also affected, with an increase of 875% and 958% for D-Asp and D-Ser, respectively. The frontal cortex region was least affected.

TABLE 2. D-Asp and D-Ser concentration (ng/mg tissue wet weight) in regional brain tissues.

	CC	CT	% change over control	SC	ST	% change over control
D-Asp	1.8	1.2	-32.3	2.4	3.2	31.5
D-Ser	30.5	28.8	-5.7	28.1	42.2	50.1
	BC	BT		HC	HT	
D-Asp	0.6	3.4	442.4	0.9	1.6	86.9
D-Ser	0.5	41.3	8498.8	1.5	15.5	958.7

FIGURE 6: Comparison of D-Asp and D-Ser concentration levels between treated tissue samples and control samples.



Conclusions

- A fast UHPLC method using OPA/βME and EC detection was developed for neuroactive amino acid analysis. Separation of 11 amino acids was completed within 10 min. Sensitivity was 10–40 pg on column for various amino acids.
- A UHPLC method for the simultaneous determination of D- and L-Asp and Ser enantiomers within 20 min was also developed showing improved separation of D-Asp from the interference peak. Sensitivity was 200–400 fg on column.
- Application of these two methods was demonstrated with analysis of regional brain tissue sample for the effects of amphetamine on Asp, Glu, GABA and D-Asp and D-Ser. Region specific differences was observed for the effect of amphetamine treatment, with brain stem most markedly stimulated, hippocampus was also affected, prefrontal cortex was least affected.

References

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