Fast UHPLC Method for the Simultaneous Determination of Free D-Aspartic Acid and D-Serine in Brain Tissue Extracts

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Key Words

D-Aspartic Acid, D-Serine, Fluorescence Detection, Precolumn Derivatization

Goal

To develop a rapid and sensitive UHPLC method for the measurement of both D-Asp and D-Ser in brain tissue extract

Introduction

Although D-enatiomers of amino acids are common in lower organisms such as bacteria and not thought to occur in mammalian tissues, it has became apparent that some D-amino acids do occur in higher organisms and have biochemical importance.[1] Recent publications indicating an abundance of D-amino acids in neuro-endocrine tissues have prompted the development of simple analytical methods for the measurement of these amino acid enantiomers.[2] D-serine (D-Ser) occurs in glial cells, is particularly abundant in brain regions enriched in NMDA receptors, and is the endogenous coagonist of the NMDA receptor (not glycine).[3] 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.[4]

A variety of methods including enzymatic assays, gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been developed to separate and quantitatively determine amino acid enantiomers in biological samples. The most widely used is a conventional reversed-phase HPLC–fluorescence method with precolumn derivatization. With this method the amino acid enantiomers are converted into the diastereomeric isoindole derivatives formed by reaction with o-phthaldialdehyde (OPA) and a chiral thiol (e.g., *N*-acetyl-L-cysteine (NAC),

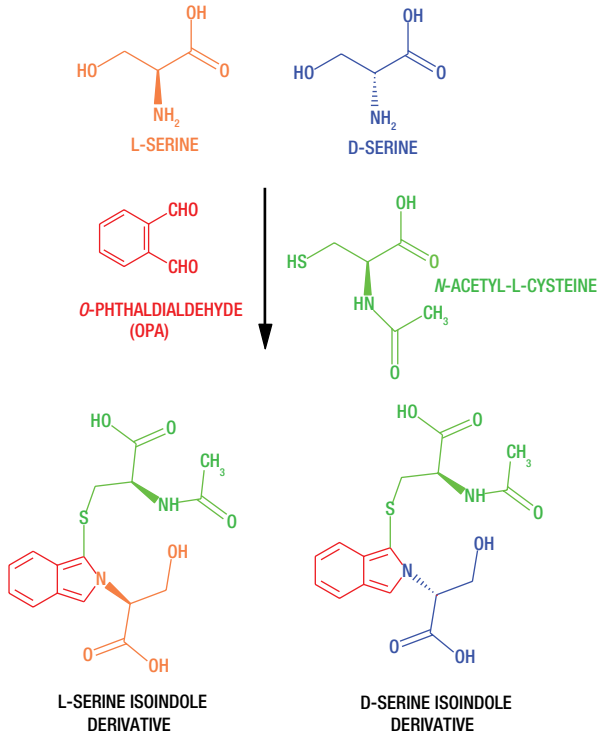

Figure 1. The reaction of amino acids with OPA/NAC to form chiral derivatives.

N-t-butyloxycarbonyl-L-cysteine or *N*-isobutyryl-L-cysteine).[5-9] Unfortunately, most published methods either separate the enantiomers of just one amino acid D-Asp/L-Asp or D-Ser/L-Ser or when separating the enatiomers of other amino acids require an analysis time of over one hour. The reaction chemistry used to form the chiral derivatives is shown in Figure 1. Described here is a UHPLC method with precolumn derivatization using OPA-NAC and fluorescence detection for the analysis of both D-Asp and D-Ser which is faster and provides enhanced resolution over typical HPLC methods.

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Experimental

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLC System including:
  - HPG-3400RS Binary Rapid Separation Pump with Solvent Selector Valves (1034 bar)
  - WPS-3000TRS Rapid Separation Wellplate Sampler, Thermostatted
  - FLD-3400RS Fluorescence Detector with Dual-PMT
  - TCC-3000RS Rapid Separation Thermostatted Column Compartment
- Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 6.80 (SR11)

Conditions

Column:	Thermo Scientific™ Hypersil GOLD™ 1.9 µm, 200 × 2.1 mm, P/N 25002-202130
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; equilibrate at 3% B for 7 min
Flow Rate:	0.25 mL/min
Inj. Volume:	2 µL
Temperature:	35 °C
Detector:	Excitation 340 nm, emission 450 nm, sensitivity 4
Backpressure:	Maximum 620 bar

Consumables

Deionized Water, 18.2 MΩ-cm resistivity	
o-Phthaldialdehyde (OPA)	Pickering® Laboratories, P/N 0120
OPA Diluent	Pickering Laboratories, P/N OD104
N-Acetyl-L-Cysteine (NAC)	Fluka® Analytical/Sigma-Aldrich® P/N 01039
Perchloric Acid	GFS Chemicals, P/N 67
Acetic Acid, Glacial	Fisher Scientific™, P/N A35-500
D-Serine	Fisher Scientific, P/N 113506
L-Serine	Fisher Scientific, P/N AC13266
D-Aspartic Acid	Fisher Scientific, P/N AC20426
L-Aspartic Acid	Fisher Scientific, P/N ICN19463380
L-Glutamate	Fisher Scientific, P/N ICN19467780
L-Asparagine	Fisher Scientific, P/N ICN100736800
L-Glutamine	Fisher Scientific, P/N AC11995
Glycine	Fisher Scientific, P/N AC38590
DL-Threonine	Fisher Scientific, P/N 13894
Taurine	Fisher Scientific, P/N 16654
DL-Alanine	Fisher Scientific, P/N15909
DL-γ-Amino-n-Butyric Acid	Fisher Scientific, P/N 207531
L-Tyrosine	Fisher Scientific, P/N 14064
Reagent Vials and Seals (for OPA/NAC Reagent and Acid Diluent)	Thermo Scientific, P/N 6820.0023
Sample Vial	Thermo Scientific, P/N 6000.0077
Sample Vial Cap	Thermo Scientific, P/N 6000.0076

Mobile Phase Preparation

Prepare 1 L of 50 mM phosphate buffer by dissolving 7.1 g of dibasic sodium phosphate into 800 mL 18.2 Ω deionized (DI) water, adjust pH 6.5 with phosphoric acid, and adjust volume to 1 L. Filter the buffer solution with a 0.20 µm membrane filter.

Standard and Sample Preparation

0.3 N and 0.03 N Perchloric Acid Solution: Prepare a 0.3 N perchloric acid solution (PCA) by dissolving 2.5 mL 70% PCA into 100 mL 18.2 Ω DI water; prepare 0.03 N PCA solution by dilution of 0.3 N PCA with 18.2 MΩ-cm DI water.

Standard Solution of Amino Acids: Prepare a stock solution of amino acid standard at 1 mg/mL with 0.03 N (PCA, including D- and L-Asp, D- and L-Ser, glutamate (Glu), glutamine (Gln), asparagine (Asn), arginine (Arg), glycine (Gly), threonine (Thr), taurine (Tau), alanine (Ala), γ-aminobutyric acid (GABA) and tyrosine (Tyr). Prepare a working amino acids standard solution by diluting the stock solution with 0.03 N PCA to the respective concentrations.

Brain Tissue Sample: Weigh about 10–20 mg rat brain (prefrontal cortex or other regions) and have the sample homogenized in 1 mL 0.3 N PCA. Centrifuge the homogenates at 13,000 rpm for 15–20 min at 4 °C. Dilute the supernatant by a factor of 10 with 18.2 MΩ-cm DI water before analysis.

Derivatization Reagent Preparation

OPA/NAC Reagent: Prepare an OPA/NAC derivatization reagent stock solution by dissolving 20 mg OPA in 1 mL methanol, and then adding 10 mg NAC and 9 mL OPA diluent. Stock reagent is stable for 5 days at 4 °C. Prepare working reagent fresh daily by diluting 1 mL of stock reagent into 4 mL OPA diluents.

1 M Acetic Acid: Prepare 1 M acetic acid solution by dissolving 5.8 mL glacial acetic acid into 100 mL 18.2 MΩ-cm DI water.

Perform automated precolumn derivatization by mixing 20 µL working reagent with 20 µL standard or tissue sample and allowing the reaction to occur for 2 min.

Note: It is important to neutralize the sample's pH with acid before the injection for enhanced column life. In this work, after the derivative formation was completed, a volume of 8 µL of 1 M acetic acid was mixed into the sample before injecting. The successful neutralization of the base derivatization mixture by adding the acid solution can be checked in preliminary experiments by using standards.

User-Defined Autosampler Program

The Thermo Scientific™ Dionex™ UltiMate™ 3000 Well Plate Autosampler series provides sample preparation commands that help define automated sample derivatization procedures. The user-defined program (UDP) wizard in Chromeleon CDS software assists in creating a UDP, making it easy to specify the single steps for sample preparation. Table 1 shows a step-by-step description of how to create a UDP for an automated precolumn derivatization.

Table 1. Step-by-step description of commands applied in the UDP for automated derivatization.

Action	UDP Command	UDP Parameter/Comment
Activate the UDP mode of the autosampler. This command activates the UDP mode of the autosampler. In this mode, every single movement of the autosampler has to be programmed. Chromeleon CDS software ignores the injection volumes of the sequence table and uses the value provided in the UDP.	InjectMode=UserProg	
Define positions for derivatization reagents. The WPS-3000 Rapid Separation Wellplate Sampler allows definition of up to four reagent vial positions and an additional Prep Vial position. The work described here uses two reagent vials.	Reagent AVial=R1 ReagentBVial=R2	Position of derivatization reagent Position of acetic acid
Draw air plug (avoid mixing with wash solvent).	UdpDraw	From=air, Volume=1 µL, Syringe Speed=GlobalSpeed
Draw derivatization reagent.	UdpDraw	From=ReagentAVial, Volume=20 µL, SyringeSpeed=5.000
Dispense derivatization reagent into sample vial.	UdpDispense	To=SampleVial, Volume=20 µL, SyringeSpeed=30.000
Wait 5 s to allow equilibration of liquid in sample vial.	UdpMixWait	Duration=5
Mix reagent and sample four times.	UdpDraw UdpDispense UdpDraw UdpDispense UdpDraw UdpDispense UdpDraw UdpDispense	From=SampleVial, Volume=20 µL, SyringeSpeed=5.000 To=SampleVial, Volume=20 µL, SyringeSpeed=30.000 From=SampleVial, Volume=20 µL, SyringeSpeed=5.000 To=SampleVial, Volume=20 µL, SyringeSpeed=30.000 From=SampleVial, Volume=20 µL, SyringeSpeed=5.000 To=SampleVial, Volume=20 µL, SyringeSpeed=30.000 From=SampleVial, Volume=20 µL, SyringeSpeed=5.000 To=SampleVial, Volume=20 µL, SyringeSpeed=30.000
Allow reaction of reagent mixture for 60 s.	UdpMixWait	Duration=60
Wash the outer surface of the needle with a 100 µL needle wash solution.	UdpMixNeedleWash	Volume=100.000
Draw acetic acid for pH decrease.	UdpDraw	From=ReagentBVial, Volume=8 µL, SyringeSpeed=5.000
Dispense acetic acid into sample vial.	UdpDispense	To=SampleVial, Volume=8 µL, SyringeSpeed=30.000
Wait 5 s to allow equilibration of liquid in sample vial.	UdpMixWait	Duration=5
Mix reagent and sample four times.	UdpDraw UdpDispense UdpDraw UdpDispense UdpDraw UdpDispense UdpDraw UdpDispense	From=SampleVial, Volume=25 µL, SyringeSpeed=5.000 To=SampleVial, Volume=25 µL, SyringeSpeed=30.000 From=SampleVial, Volume=25 µL, SyringeSpeed=5.000 To=SampleVial, Volume=25 µL, SyringeSpeed=30.000 From=SampleVial, Volume=25 µL, SyringeSpeed=5.000 To=SampleVial, Volume=25 µL, SyringeSpeed=30.000 From=SampleVial, Volume=25 µL, SyringeSpeed=5.000 To=SampleVial, Volume=25 µL, SyringeSpeed=30.000

Action	UDP Command	UDP Parameter/Comment
Perform a buffer and sample loop wash with three sample loop volumes of wash solution.	UdpSyringeValve UdpMoveSyringeHome UdpDraw UdpDispense UdpDraw UdpDispense UdpDraw UdpDispense	Position=Waste SyringeSpeed=GlobalSpeed From=Wash, Volume=100µL, SyringeSpeed=GlobalSpeed To=Drain, Volume=100µL, SyringeSpeed=GlobalSpeed From=Wash, Volume=100µL, SyringeSpeed=GlobalSpeed To=Drain, Volume=100µL, SyringeSpeed=GlobalSpeed From=Wash, Volume=100µL, SyringeSpeed=GlobalSpeed To=Drain , Volume=100µL, SyringeSpeed=GlobalSpeed
Draw sample for injection.	UdpMoveSyringe	Load=2.000, SyringeSpeed=1.000
Wait for stabilization before injection.	UdpMixWait	Duration=5
Inject sample.	UdpInjectValve	Position=Inject Unload=2.000, SyringeSpeed=5.000
Generate an inject maker pulse. This command is required in UDPs. The injection can be performed only after this pulse.	UdpInjectMarker	
Reset the syringe after injection. After the injection valve switches to inject, the syringe plunger has to be moved into the home position.	UdpSyringeValve UdpMoveSyringeHome	Position=Waste SyringeSpeed=GlobalSpeed
Perform a buffer and sample loop wash.	UdpDraw UdpDispense UdpDraw UdpDispense	From=Wash, Volume=100µL, SyringeSpeed=GlobalSpeed To=Drain, Volume=100µL, SyringeSpeed=GlobalSpeed From=Wash, Volume=100µL, SyringeSpeed=GlobalSpeed To=Drain, Volume=100µL, SyringeSpeed=GlobalSpeed

Results and Discussion

This method could readily measure both D-Asp and D-Ser in only 20 min, with a detection limit of 200 fg on column for D-Ser and 400 fg for D-Asp.

Figure 2 shows the separation of D- and L-Asp, D- and L-Ser and 10 other amino acids in a 500 ng/mL standard. This UHPLC method not only reduced total run time compared to the HPLC method, but also provided enhanced resolution between D- and L-Asp which allowed more accurate determination of D-Asp in biological samples. Figure 3 shows examples of this method for the separation of amino acids in rat brain stem tissue homogenates. A magnified view of the D- and L-Asp region shown in Figure 4 illustrates near baseline separation of D-Asp from an unknown interference (Peak 1) in the presence of a large amount of L-Asp (L/D ratio 360 for sample BT and 1600 for sample BC). The enhanced resolution in the separation of D-Asp from interference peaks and L-Asp compared to our previous HPLC method allows more accurate quantitative determination of D-Asp. Unknown interference (Peak 2) was separated from D-Asp and interference Peak 1, but coeluted with L-Asp. It was observed during method development that its amount was insignificant relative to the endogenous amount of L-Asp, so the unknown interference peak shouldn't be a concern if the quantitation of L-Asp is required. It was observed that the amount of unknown Peak 2 increased significantly if the supernatant was not separated from the precipitant right after tissue extraction.

The linearity test was in the concentration range of 1–200 ng/mL with a 2 µL injection. Excellent linearity was observed, with coefficient of determination of $R^2 > 0.99$ for both D-Asp and D-Ser. Calibration curves are shown in Figure 5.

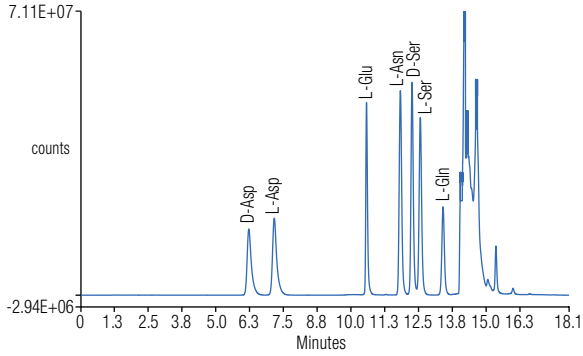

Figure 2. Separation of D- and L-Asp and D- and L-Ser and 10 additional amino acids (L-Glu, L-Asn, L-Gln, Gly, DL-Thr, Tau, DL-Ala, DL-GABA, L-Tyr) at 500 ng/mL.

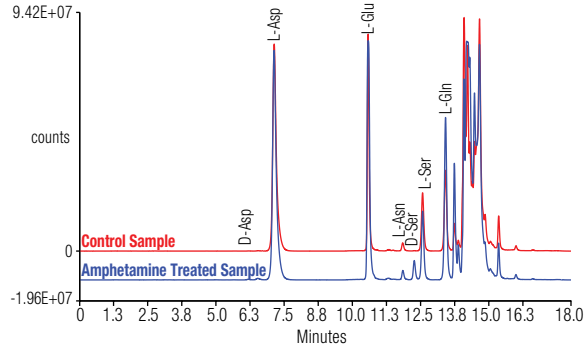

Figure 3. The separation of amino acids in a rat brain stem sample: upper red trace - control sample; lower blue trace - amphetamine treated sample.

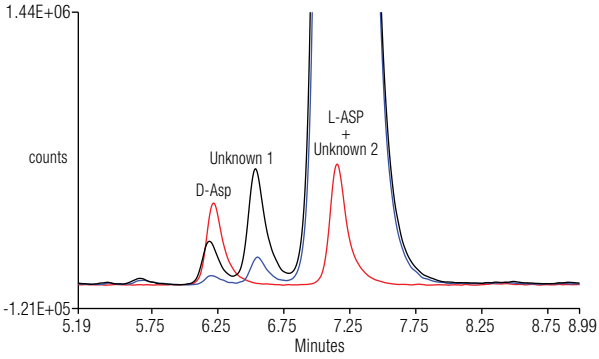

Figure 4. A chromatogram showing the separation of D-Asp from the interference peak.

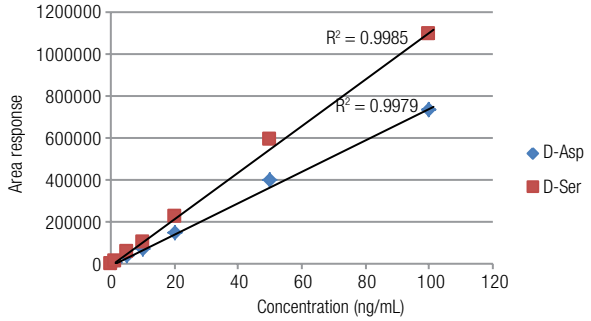

Figure 5. Calibration curves of D-Asp and D-Ser.

Table 2 shows concentrations of D- and L-Asp and D- and L-Ser in brain tissue samples. 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), were analyzed. The effect of the amphetamine treatment on D-Asp and D-Ser levels in brain tissue were 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.

	PC	PT	% 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

Conclusion

A fast UHPLC method using OPA/NAC precolumn derivatization and fluorescence detection was developed for D-Asp and D-Ser measurement in brain tisue extracts. UHPLC technology greatly reduced the run time to only 20 min versus the typical 30–60 min HPLC method, with enhanced resolution for the seperation of D-Asp from interferences and L-Asp. This method involves simple biological sample extraction and offers excellent sensitivty for the detection of D-Asp and D-Ser in the presence of a large amount of their corresponding L-enantiomers, with detection limits of 200 fg for D-Ser and 400 fg for D-Asp.

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