



Fast Quantitation of Biomarkers N-Acetylaspartate and N-Acetylaspartylglutamate in Mouse Brain Homogenates Using HILIC and Tandem Mass

Authors

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Overview

PURPOSE

Develop a fast, accurate, and precise LC/MS/MS method for the quantitative determination of N-Acetylaspartate (NAA) and N-Acetylaspartylglutamate (NAAG) from mouse brain with minimal sample preparation.

METHODS

- Parallel homogenization of mouse brain samples using a bead-beater type homogenizer
- Hydrophilic interaction chromatography using an amino column
- Detection using a triple quadrupole mass spectrometer operated in positive ion electrospray mode

RESULTS

- Less than four minute LC/MS/MS method
- NAA accuracy and precision $\pm 5\%$ and $\leq 5\%$
- NAAG accuracy and precision $\pm 10\%$ and $\leq 10\%$

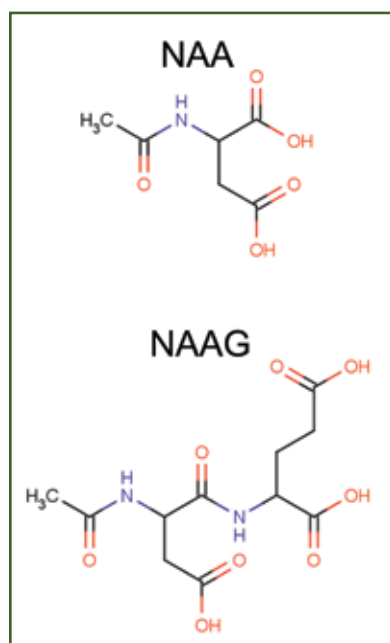


Introduction

Altered N-Acetylaspartate (NAA) and N-Acetylaspartylglutamate (NAAG) levels in the brain have been linked to multiple neurological conditions, including autism, schizophrenia, Parkinson's disease, Alzheimer's disease, and Huntington's disease.¹⁻³ These biomarkers have also been shown to have altered concentrations following administration of antipsychotic drugs in vivo and in vitro.^{4,5} With the recent availability of rodent models for many neurological disease states, the direct, quantitative measurement of NAA and NAAG from brain tissue may be an attractive way to monitor the effectiveness of potential treatments.

NAA and NAAG are hydrophilic and respond poorly using UV detection, so some methods for their quantitative measurement using chromatographic techniques involve laborious derivatization procedures that introduce either hydrophobic moieties or chromophores.^{6,7} Recently, some LC/MS/MS methods have been reported that eliminate the need for derivatization.^{8,9} However, these LC/MS/MS methods still suffer from relatively long analysis times (greater than 10 minutes) or poor chromatographic retention.

In this work an LC/MS/MS method was developed that couples a hydrophilic interaction chromatographic (HILIC) column with a triple quadrupole mass spectrometer (API 4000). The resulting method is able to rapidly analyze (less than four minutes) minimally prepared mouse brain samples with excellent analyte retention and separation. The accuracy, precision, recovery, and matrix effects associated with the LC/MS/MS method were evaluated and results are presented here. NAA and NAAG were also found to be unstable under certain conditions,¹⁰ so critical sample preparation parameters are identified below.





Methods

SAMPLE PREPARATION

- Frozen mouse brain samples were weighed into 2 mL FisherBrand centrifuge tubes and held on dry ice
- Five stainless steel ball bearings (3/16" diameter) were added to each tube
- A four-fold volume of Homogenization Solvent (0.1% formic acid in 95/5 methanol/water) was added to each tube (volume added in mL = brain mass in g x 4)
- Following solvent addition, samples were immediately homogenized using an MP Biomedical, LLC FastPrep™ 24 (4.0 m/s for 60 seconds) and then centrifuged (16,000 relative centrifugal force for 10 min)
- A 25 uL aliquot of the resulting supernatant was added to 475 uL of an internal standard (ISTD) solution (NAA-d3 at 15.0 ug/mL in Homogenization Solvent) in a polypropylene autosampler vial, the vial capped, and vortex mixed
- Standards and quality control (QC) samples were prepared in matrix-free Homogenization Solvent, then diluted with ISTD solution in the same manner as brain homogenates

CHROMATOGRAPHY

- Agilent Polaris NH2 2.0 x 50 mm, 3um column; ambient temperature
- Shimadzu LC-10ADvp pumps; LEAP HTS-PAL autosampler
- Mobile phase A: 100 mM ammonium formate with 2% formic acid
- Mobile phase B: Acetonitrile with 0.1% formic acid
- Flow rate: 0.3 mL/min
- Gradient program: 0.0 – 0.5 min = 50%B; 0.5 – 3.0 min = 50% - 10% B; 3.0 – 3.3 min = 10% B; 3.3 – 3.4 min = 10% - 50% B; 3.4 – 3.6 min = 50% B

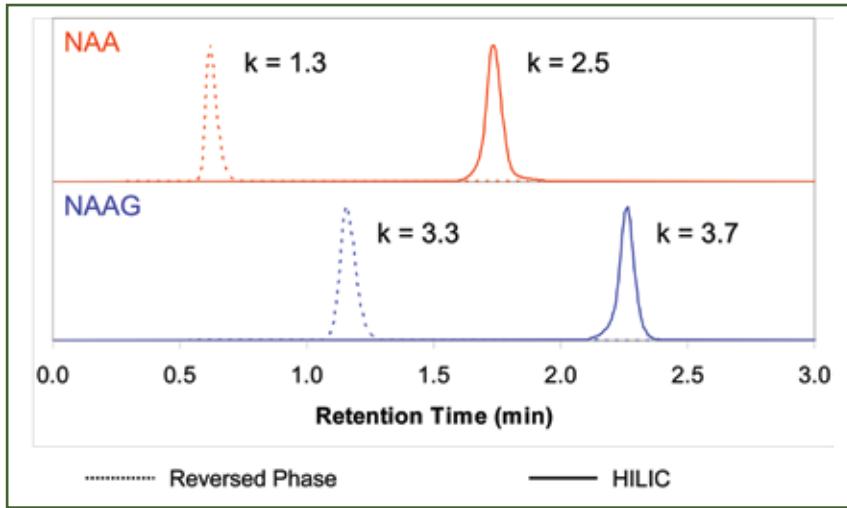
MASS SPECTROMETRY

- AB Sciex API 4000™ triple quadrupole, TurboV source, positive ion mode "Turboionspray"
- NAA and NAAG Q1/Q3 ions: 176.0/133.8 and 305.1/148.1

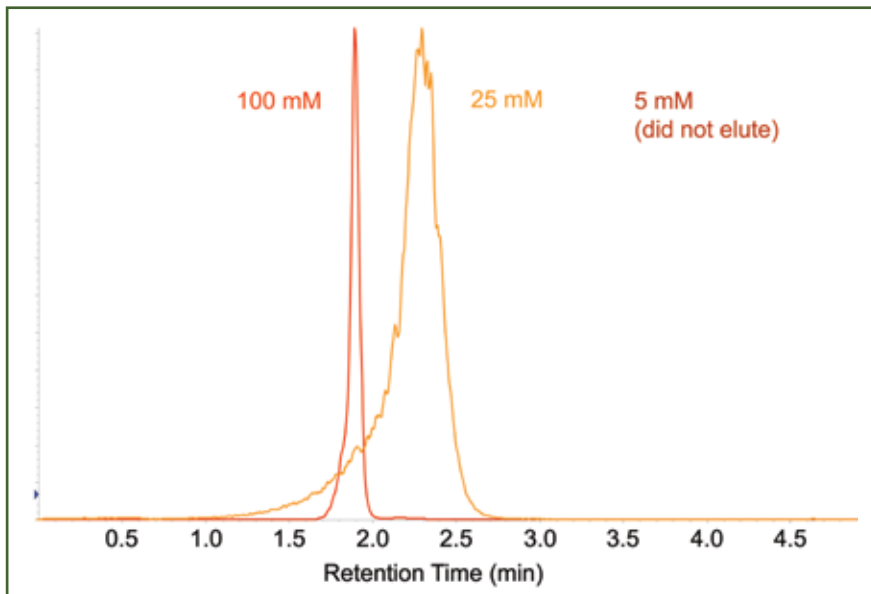


Results – Chromatography

Retention factors (k): Reversed phase versus HILIC separations (generally, $k > 2$ is desirable)



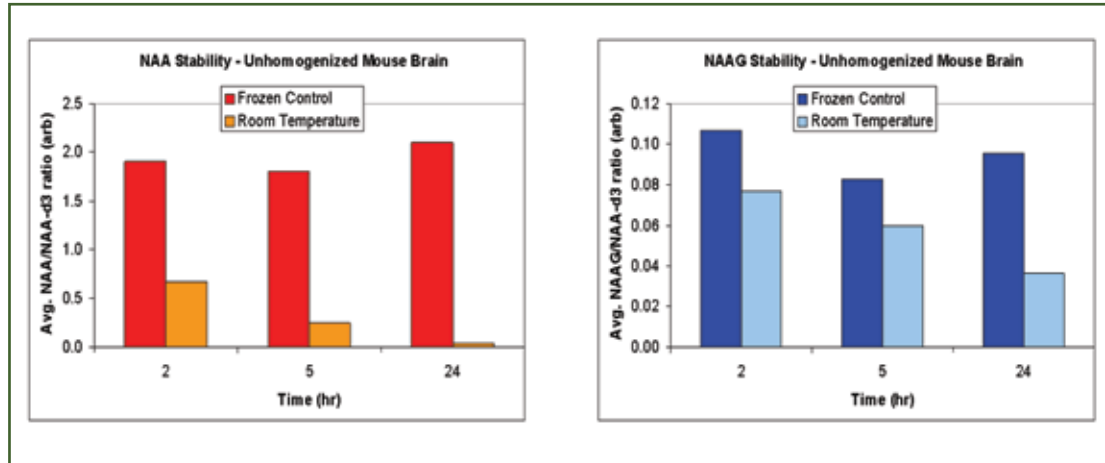
Mobile phase A ammonium formate concentration (NAA):



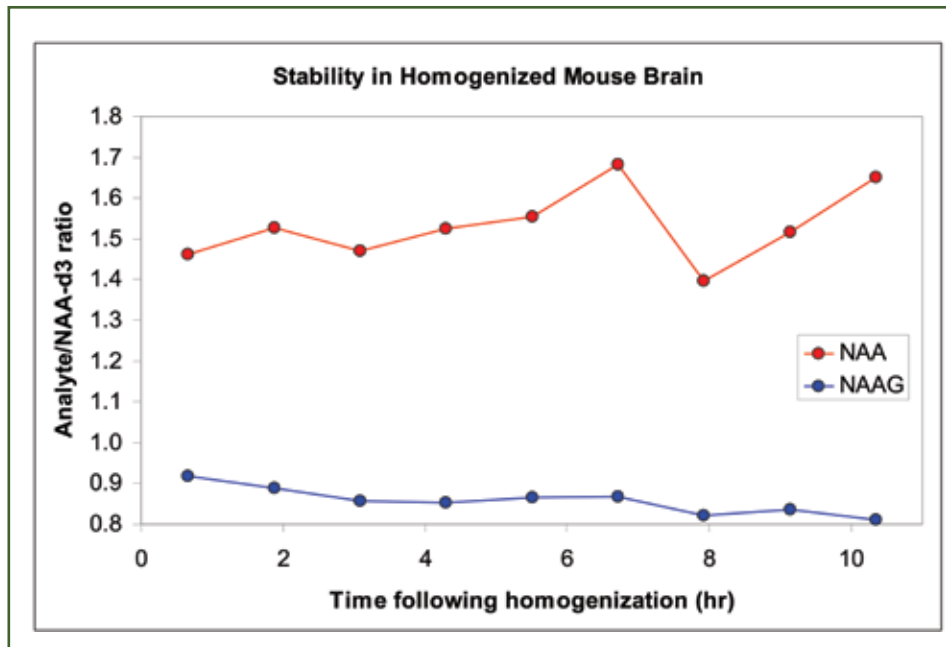


Results – Stability

- Stock solutions were stable ($\pm 10\%$) for at least 91 days (0.1 M HCl at 4°C)
- Working solutions were stable for ≤ 3 days (Homogenization Solvent at 4°C). New solvent conditions to be investigated.
- Both analytes degraded rapidly in unhomogenized mouse brain samples at room temperature



- Both analytes show improved stability following homogenization and storage at 4°C





Results – Method Qualification

QUALITY CONTROL SAMPLE ACCURACY AND PRECISION						
	NAA			NAAG		
	Low QC 67.5 ug/mL	Mid QC 158 ug/mL	High QC 338 ug/mL	Low QC 7.50 ug/mL	Mid QC 17.5 ug/mL	High QC 37.5 ug/mL
	67.9	165	349	8.05	18.7	38.0
	68.1	155	341	8.33	18.6	41.3
	67.8	162	333	7.76	18.4	38.2
	68.8	160	337	8.01	17.4	36.0
Mean	68.2	160	340	8.04	18.3	38.4
SD	0.458	4.07	6.69	0.234	0.631	2.20
%CV	0.7	2.5	2.0	2.9	3.5	5.7
Accuracy	101.0	101.5	100.5	107.2	104.4	102.4
n	4	4	4	4	4	4



Repeatability

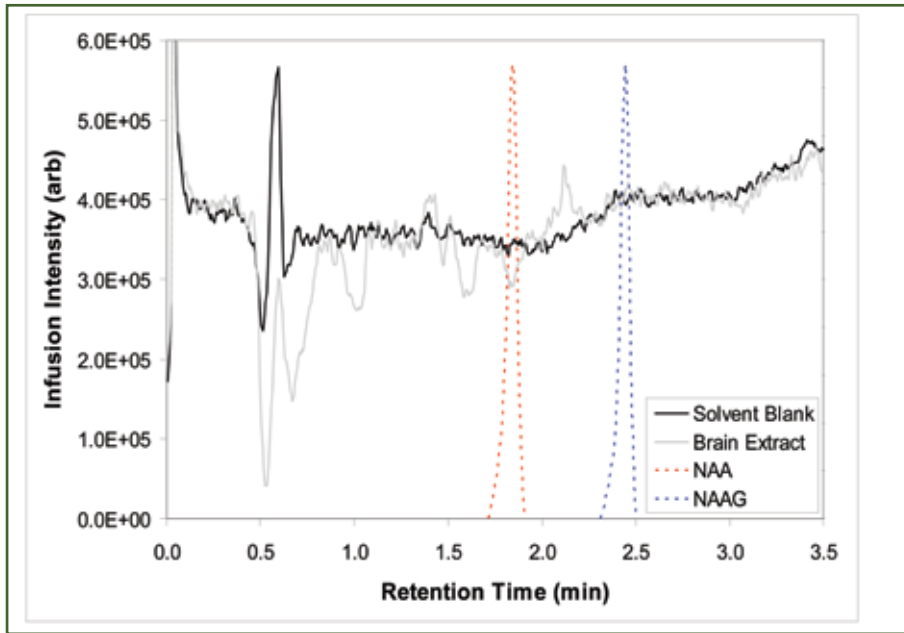
NAA REPEATABILITY						
Brain #	Brain Hemisphere	Average Concentration (ug/mL)	Injection-to-injection		Extraction	
			%CV	Average %CV	[% difference] left/right	Average [% difference]
1	Left	583	0.3%	1.0%	12.5%	14.1%
	Right	661	1.3%		14.5%	
2	Left	547	0.7%		9.7%	
	Right	473	0.7%		19.7%	
3	Left	482	1.5%			
	Right	531	1.8%			
4	Left	646	1.6%			
	Right	530	0.4%			

NAAG REPEATABILITY						
Brain #	Brain Hemisphere	Average Concentration (ug/mL)	Injection-to-injection		Extraction	
			%CV	Average %CV	[% difference] left/right	Average [% difference]
1	Left	90	4.9%	5.1%	20.1%	17.8%
	Right	73	5.3%		12.3%	
2	Left	91	4.8%		2.8%	
	Right	103	5.5%		35.9%	
3	Left	85	6.9%			
	Right	87	6.0%			
4	Left	70	5.1%			
	Right	101	2.7%			

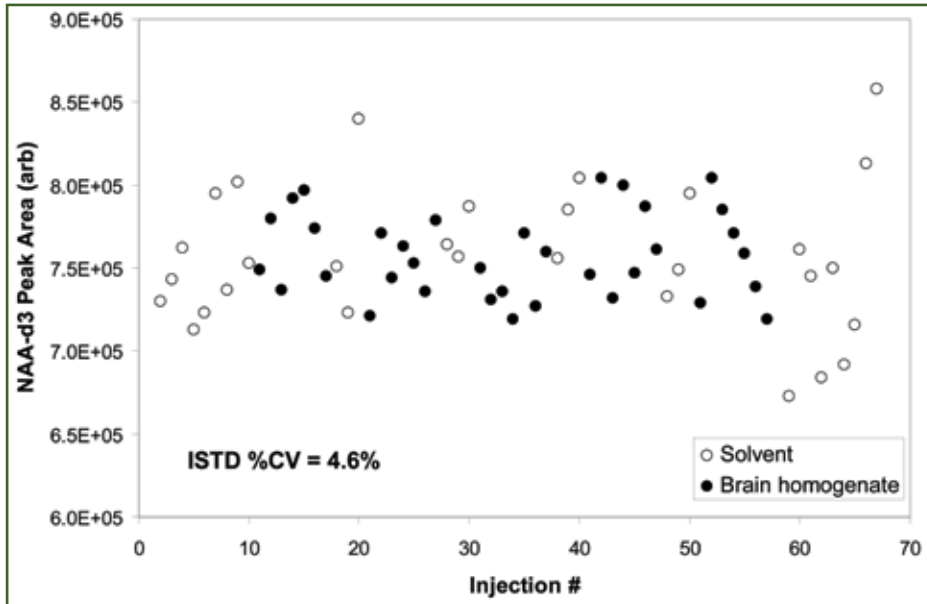


Matrix Effects

Matrix Effect (Infusion Test)



Matrix Effect (Internal Standard Response)





Recovery

Pre-spike: Spiked brain prior to homogenization

Post-Spike: Spiked brain following homogenization

Relative recovery: Pre-spike result / Post-Spike result

Absolute recovery: Pre-spike result / known concentration spiked (900 µg/mL for NAA and 100 µg/mL for NAAG)

NAA RECOVERY							
Brain #	Spike	Raw Concentration (ug/mL)	Corrected Concentration (ug/mL)	Relative % recovery	Average relative % recovery	Absolute % recovery	Average absolute % recovery
5	Pre	1760	901	96.8%	102.3%	100.1%	106.2%
	Post	1790	931				
	None	859	---				
6	Pre	1570	1067	109.2%			
	Post	1480	977				
	None	503	---				
7	Pre	1440	926	102.2%			
	Post	1420	906				
	None	514	---				
8	Pre	1540	929	101.1%			
	Post	1530	919				
	None	611	---				

NAAG RECOVERY							
Brain #	Spike	Raw Concentration (ug/mL)	Corrected Concentration (ug/mL)	Relative % recovery	Average relative % recovery	Absolute % recovery	Average absolute % recovery
5	Pre	197	80	63.0%	69.9%	80.0%	86.5%
	Post	244	127				
	None	117	---				
6	Pre	269	155	102.6% ¹			
	Post	265	151				
	None	114	---				
7	Pre	163	84.4	69.0%			
	Post	201	122				
	None	78.6	---				
8	Pre	201	95	77.9%			
	Post	228	122				
	None	106	---				

¹ Apparent outlier; not included in average % recovery calculations



Conclusions

- An amino column operated in HILIC mode allows for good retention of NAA and NAAG ($k > 2$).
- Analytes were unstable in unhomogenized, thawed matrix and under some solvent conditions.
- The developed LC/MS/MS method had good accuracy, precision, repeatability, and recovery. NAA generally outperformed NAAG in these categories, likely due to use of the heavy isotope version of NAA (NAA-d3) as the internal standard
- There was no significant matrix effect.

Future Directions

- Re-evaluate working solution solvent conditions to increase stability time-frame.
- Increase speed of method further (< two minutes) through faster flow rate and/or use of UPLC columns.
- Increase performance of NAAG through use of more suitable internal standard.

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