Development of an Automated High-Throughput LC/MS/MS method for the Analysis of a Neurotransmitter Panel in Mouse Striata

Seth R. Bell, Joelle M. Lucarell, Changyu Quang, Mark Herberth, Liam Moran, Elizabeth Groeber
Charles River, Ashland

1 Purpose

Neurotransmitters in the brain are important biomarkers for neurodegenerative disease models. The quantitative analysis of neurotransmitters (NTs, i.e. dopamine (DA), norepinephrine (NE), serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA)) in the brain as relates to neurodegenerative disease is complicated by severe stability constraints. Bioanalytical methods for measuring neurotransmitters must be able to extract samples under conditions that don't allow for degradation during processing or analysis. In our lab, the use of hand-held homogenizers with an individual disposable polypropylene Pellet Pestle™ was able to meet these strict requirements but created a bottleneck for sample throughput with one chemist able to homogenize and process only 6 samples per 4-hour analytical session. Attempts to develop a higher-throughput automated homogenization protocol capable of meeting the strict stability requirements of the assay presented multiple challenges.

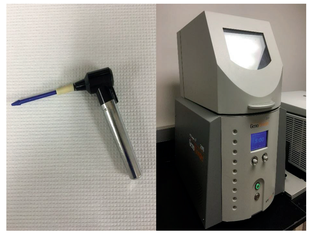


Figure 1. Comparison of the two Homogenizers. Hand-held Pellet Pestle™ (left) and Automated Geno/Grinder 2010 (right)

2 Methods

Grinding materials and parameters were investigated using simulated stability homogenate samples spiked with heavy-labeled NTs due to the difficulty in generating viable mouse striata. The results of the stability studies were vital in designing a viable protocol for these difficult to obtain one-shot tissue samples. The resulting protocol includes an LC/MS/MS method for measure of NTs in homogenized mouse striata after dilution and filtration. Using a 2010 Geno/Grinder teflon-coated bars, stainless steel, zirconium oxide, and silica grinding materials were assessed for non-specific binging, oxidation/reactivity, and adequate tissue homogenization. After identifying potential grinding media, stability studies examined the effects of grinding time, grinding speed, and grinding temperature on the assay. Teflon bars provided insufficient grinding based on visual inspection. Zirconium oxide may have resulted in analyte oxidation similar to stainless steel. Therefore silica was selected as the optimal test griding material.

Instrument Parameters

LC Conditions

Parameter:	Positive Ion Mode	Negative Ion Mode
LC System:	Waters® Acquity UPLC System	Waters® Acquity UPLC System
Column:	Restek PFPP, 50 x 2.1 mm, 1.9-mm particle size	Waters HSS T3, 50 x 2.1 mm, 1.8-mm particle size
Mobile Phase A:	0.1% Formic Acid in Milli-Q water	0.1% Glacial Acetic Acid in Milli-Q water
Mobile Phase B:	0.1% Formic Acid in Acetonitrile	0.1% Glacial Acetic Acid in Acetonitrile
Sample Temperature:	4°C	4°C
Column Temperature:	60°C	60°C

MS Conditions

MS System:	API 4000
Ionization Mode:	ESI Positive and Negative Mode
IS Voltage:	5500 and -4200
Temperature:	550°C

3 Results

Ultimately, an updated high-throughput procedure was developed using 4.0mm acid-washed silica beads grinding at 1300 rpm for one minute. The use of cryo-blocks during homogenization can extend the stability of the analytes during the homogenization process but also adds additional handling time.

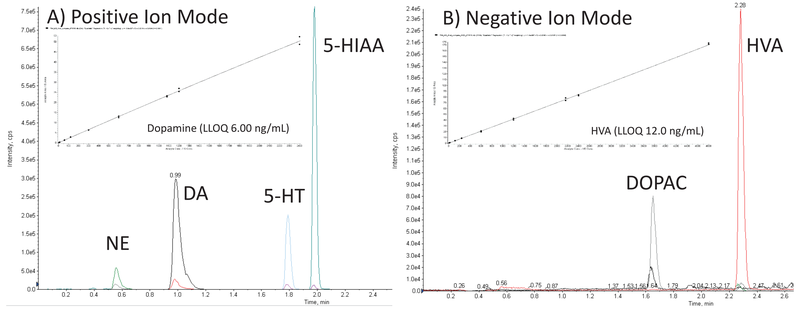


Figure 2. Representative Chromatograms A) Positive Ion Mode and B) Negative Ion Mode. Inset: Representative regression analysis results for quantitation.

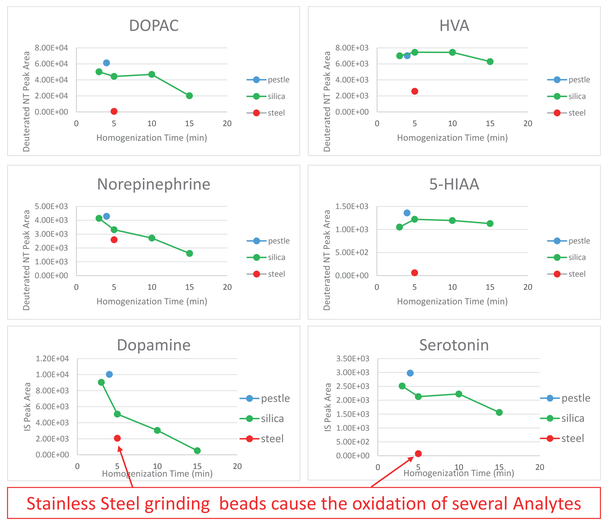
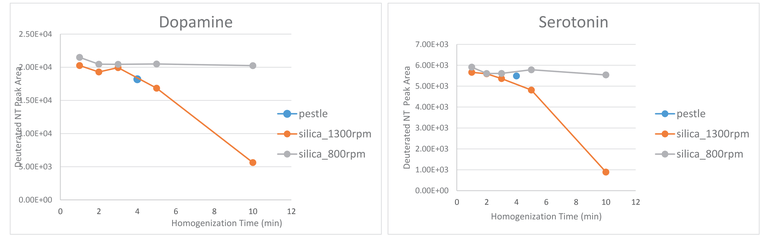
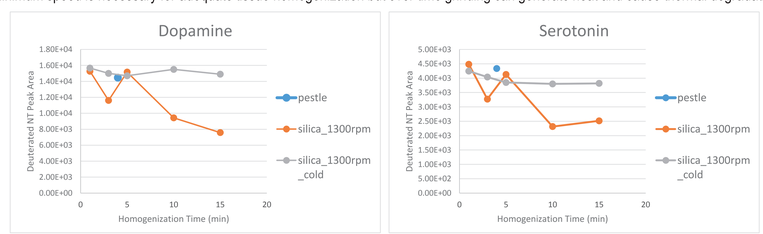


Figure 3. Investigation of different grinding materials. Representative stability plots shown comparing the effect of using polypropylene pestle, silica, and stainless steel. Homogenization time is a significant factor in determining stability.



Grinding speed is required to break up tissue but can cause thermal degradation

Figure 4. Investigation of different grinding speeds. Representative stability plots shown comparing the effect of tissue grinding speeds. A minimum speed is necessary for adequate tissue homogenization but over time grinding can generate heat and cause thermal degradation.



Cryo-block sample cooling can extend analyte stability

Figure 5. Investigation of Cyro-block Cooling. Representative stability plots shown comparing the effect of using cryo-blocks cooled to -80°C. This can extend stability but has logistical drawbacks and reduces the sample throughput from 24 to 16 per homogenization cycle with Geno/Grinder 2010.

Analyte (ng/mg)	DOPAC		HVA		Dopamine	
Replicate	Pestle	Silica	Pestle	Silica	Pestle	Silica
1	0.8440	2.40	0.996	1.67	9.83	12.10
2	1.1500	1.95	1.41	1.56	12.30	11.70
3	0.8820	2.31	1.16	1.65	9.74	12.70
Average (ng/g)	0.96	2.22	1.19	1.63	10.6	12.2
RSD	17.4	10.7	17.5	3.60	13.7	4.14

Analyte (ng/mg)	Norepinephrine		Serotonin		5-HIAA	
Replicate	Pestle	Silica	Pestle	Silica	Pestle	Silica
1	0.126	0.148	0.491	0.736	0.275	0.438
2	0.222	0.134	0.696	0.521	0.388	0.382
3	0.130	0.151	0.485	0.788	0.319	0.513
Average (ng/g)	0.159	0.144	0.557	0.682	0.327	0.444
RSD	34.1	6.29	21.6	20.8	17.4	14.8

Figure 6. Direct comparison of neurotransmitter concentrations in wild-type mouse striata determined using the historical pestle method and the new high-throughput automated Geno/Grinder 2010 method. The new method demonstrates less degradation during homogenization and less inter-subject variability.

4 Conclusion

The new homogenization protocol homogenizes 24-samples in 45 minutes for a realized 6-fold production increase. Additionally, the automated process results in lower inter-subject variability (% RSD) for a more robust and precise analysis.