Improving the Temporal Resolution of *ultra*-trace Neurochemical Analysis by HPLC with Electrochemical Detection

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Overview

Purpose: The need for rapid, yet sensitive, applications for the analysis of neurochemicals is desired to help obtain enhanced temporal resolution of sampling. A simple, rapid, and accurate method has been developed for the analysis of biogenic amines and their acid metabolites. In order to provide a higher throughput assay, a rapid and sensitive method for the analysis of dopamine and serotonin was prepared. A third chemistry for the analysis of neuroactive amino acids was also developed.

Methods: Rapid resolution chromatographic techniques with sensitive electrochemical detection were employed for the various methods described.

Results: The methods enable the rapid separation of various neurochemical compounds at ultra-trace levels and without matrix interferences.

Introduction

Significant insights into the working chemistry of the brain have occurred over the past few decades due to the coupling of microdialysis perfusion sampling with HPLC analysis with sensitive electrochemical detection. These techniques are now commonly used by neuroscientists and pharmaceutical companies to study the neuropharmacological activity of neuroactive pharmaceuticals, and their ability to alter neurotransmitter levels in various brain regions. A number of different compounds act as neurotransmitters, including some amino acids and monoamines such as norepinephrine (NE), dopamine (DA), and serotonin (5HT). Methods will be presented to illustrate the analysis of different neurochemicals including biogenic amines and metabolites, as well as amino acids, using rapid separation techniques.

Newer HPLC columns and related products help improve temporal resolution by allowing shorter analysis times by means of rapid separations. These columns have a smaller particle size than traditional 3 micron columns. By employing columns with smaller particles (2.2 micron) or fused core column technologies, shorter column lengths can often be used. This results in faster analyses without sacrificing resolution performance or producing significantly higher system pressures. Some improvements in sensitivity (picomolar levels) can be realized with sharper peaks. Microdialysis experiments can now be designed with shorter collection periods due to decreased analysis times, enabling improved temporal resolution.

Figure 1A. Inert HPLC system for trace neurochemical analysis.
Figure 1B. Specialized Thermo Scientific Dionex nanoViper capillaries allow for inert high pressure connections.

A

B

Part No.	Description
5035.9200	Thermo Scientific Dionex Solvent Rack SR-3000 (without degasser). It is recommended that solvents should be degassed daily via vacuum degassing (this ensures highest possible sensitivity)
5042.0011	Thermo Scientific Dionex ISO-3100BM Pump
5827.0020	Thermo Scientific Dionex WPS-3000TBSL Analytical Autosampler
70-9143	Thermo Scientific Dionex Coulochem III Electrochemical Detector with DC Mode & Accessories
705499TA	Thermo Scientific Dionex Thermal Organizer Module
5960.0067	Thermo Scientific Dionex Chromeleon CDS software

Methods

Biogenic Amines and Acid Metabolite Analytical Conditions:

Flow:	Isocratic at 0.60 mL/min.
Temperature	45 °C
Column:	Thermo Scientific Acclaim PA2 RSLC, 2.2 μm, 3 x 100 mm, guard column (88-12305) and holder (88-12415)
Inj. volume:	10 - 20 μL partial loop
Mobile Phase:	Thermo Scientific Dionex MD-TM (part number 70-1332)
EC detector:	Thermo Scientific Dionex Model 5011A High Sensitivity Analytical cell: E1 at -150 mV: E2 at +220 mV, Thermo Scientific Dionex 5020 Guard cell: E_{GC} at +250 mV
Sample Preparation	Brain tissue samples (10-25 mg) were prepared in 0.3N perchloric acid, sonicated to disrupt the tissue and centrifuged at 13,000 RPM for 10 min.

Rapid Dopamine and Serotonin Analytical Conditions :

Flow:	Isocratic at 0.40 mL/min.
Temperature:	32 °C
Column:	Acclaim™ RSLC PA2, 2.2 µm, 2.1 x 50 mm
Inj. volume:	10 μL partial loop
Mobile Phase:	150 mM sodium dihydrogen phosphate, monohydrate, 4.76 mM citric acid, monohydrate, 3 mM sodium dodecyl sulfate (SDS), 50 μM EDTA, 15% acetonitrile, 10% methanol, adjust to pH=5.60 sodium hydroxide, 99.99%, semiconductor grade (14N solution)
EC detector:	Thermo Scientific Dionex Model 5041A High Sensitivity Analytical cell for Microdialysis with glassy carbon at 225 mV: 12 micron Mylar; Filter:5.0 s
Sample Collection:	Artificial cerebral spinal fluid (aCSF) was collected for 10 minutes at 1 μL/min from a 3 mm microdialysis probe positioned in the prefrontal cortex of the rat brain.

Amino Acids Analytical Conditions:

Flow:	Isocratic at 0.640 mL/min.
Temperature:	45 °C
Column:	Thermo Scientific Accucore PhenylHexyl, 2.6 μm, 3 x 100 mm, Accucore Ph/Hex, 2.6 μm, 3.0 x 10 mm Guard column and Uniguard Holder
Inj. volume:	5 μL partial loop
Mobile Phase:	100 mM di-sodium hydrogen phosphate anhydrous 22% methanol, 3.5% acetonitrile, adjust to pH=6.75 with H_3PO_4
EC detector:	Thermo Scientific Dionex Model 6011 ultra Analytical cell: E1 at +150 mV: E2 at +550 mV
Sample Collection:	A sample of aCSF was collected for 15 minutes at 1 μL/min from a 3 mm microdialysis probe positioned in the corpus striatum of the rat brain.

Stock OPA/βME Solution: (store at 4 °C, usable over 5-day period)
27 mg OPA is dissolved in 1 mL methanol, add 5 μL βME and 9 mL o-Phthalaldehyde (OPA) diluent.

Sample Preparation:

- Place 15 µL microdialysis samples (or standards) into glass microvials.
- Add 15 µL of working OPA/βME solution to each sample.
- Mix 3 times and wait 1 minute.
- Add 10 µL of 0.1N HCl solution to help lower the pH of the sample then inject 5 µL

Results and Discussion

An instrumental prerequisite for ultra-trace analysis is the fact that the HPLC system must be inert in order to achieve optimal sensitivity using the electrochemical detector. The system shown below in Figure 1A uses biocompatible materials in the flow path to reduce the influence of metal that can contribute to elevated background currents at the electrochemical cell. New Thermo Scientific Dionex nanoViper (Figure 1B) fingertight fittings were employed to cope with the higher pressures due to smaller column particles. These fingertight, zero dead-volume (ZDV) capillaries can operate at pressures up to 14,500 psi and are much safer to use than PEEK™ tubing which can slip when using elevated pressures. They are made of PeekSil™ tubing and are available in small internal dimensions to minimize chromatographic band spreading. Capillaries used on this system were 150 micron ID for all connections made prior to the autosampler valve and 100 micron ID for those made after the injector valve.

Analysis of Biogenic Amines and Acid Metabolites:

A common assay used for tissue and microdialysis samples is the analysis of important biogenic amines norepinephrine (NE), dopamine (DA) and serotonin (5HT) and their acid metabolites including dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole acetic acid (5HIAA), homovanillic acid (HVA) and 3-methoxytyramine (3MT). A new, rapid method is

described which allows the complete separation of these compounds in less than 10 minutes using a 2.2 micron column (Figure 2) which is approximately 25% faster than previous methods. Good linearity of response was obtained since the correlation coefficients ranged from R^2 = 0.99.1–99.4 for the seven compounds evaluated (Figure 3). The analysis of tissue samples is illustrated in Figure 4 and demonstrates that low picogram sensitivity can be obtained using this technique. Using transgenic mice that have over-expressed Parkin function contributes to the changes in dopamine levels vs. wild type animals. Figure 4 illustrates the elevated dopamine levels of 81% in tissue sample of these transgenic animals while serotonin levels only increased by 16% vs. wild type.

FIGURE 2. Rapid analysis of biogenic amines and acid metabolites (10-100 ng/mL).

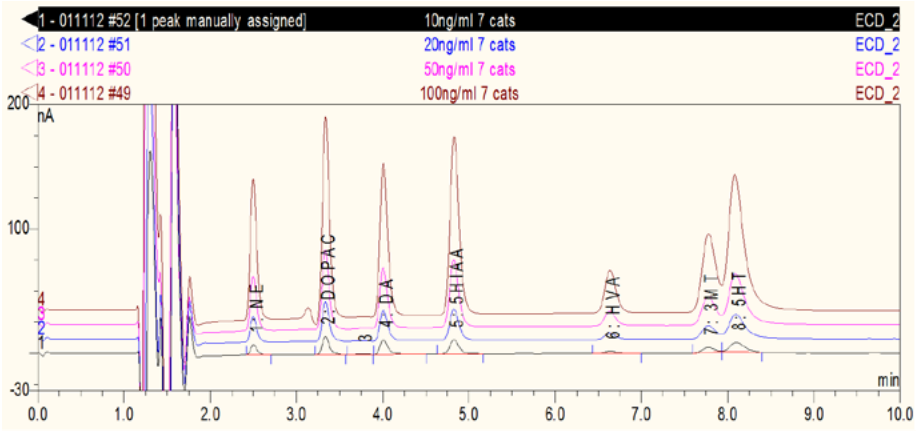


FIGURE 3. Calibration curves for biogenic amines and acid metabolites (10- 100 ng/mL).

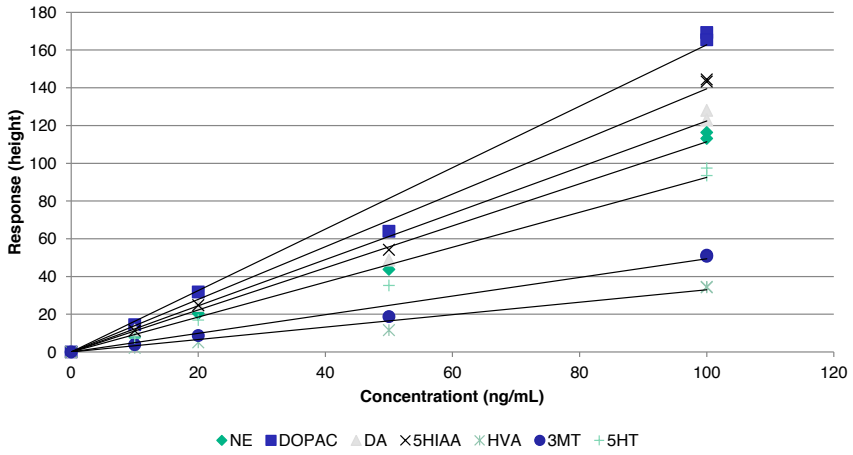
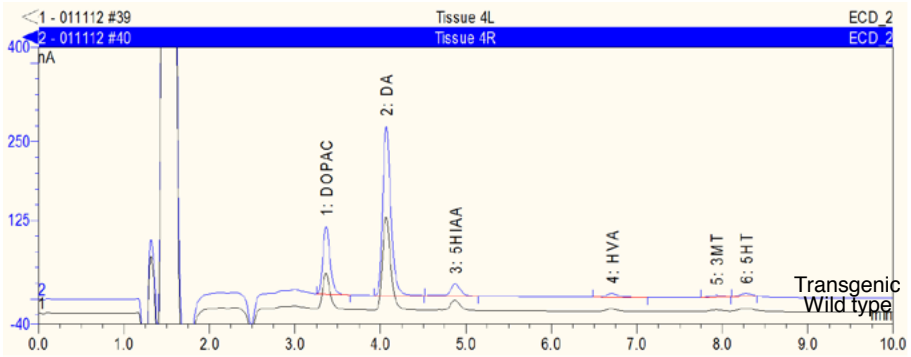


FIGURE 4. Analysis of biogenic amines and acid metabolites in tissue sample from the corpus striatum.



Rapid Analysis of Dopamine and Serotonin:

DA and 5HT were analyzed in less than five minutes as described using a short (50 mm) UHPLC column. The chemistry of the mobile phase enabled this rapid analysis by employing high concentrations of ion-pairing agent SDS (3mM) and high organic content (25% total acetonitrile and methanol) with the pH adjusted to 5.6 which facilitates the

acid metabolites to elute early from the column along with the salts from the aCSF. This method provides improved temporal resolution to access possible neurochemical changes since sampling can be adjusted to 4 minute intervals as illustrated in Figure 5. The sensitivity of this assay using an amperometric electrochemical cell with a small 2.1 mm ID column was excellent as less than 0.1 pg of dopamine and serotonin could be detected (Figure 6). This allows the analysis of trace levels of these important neurochemicals in brain regions such as the prefrontal cortex.

FIGURE 5. Rapid Analysis of dopamine and serotonin in a microdialysis sample.

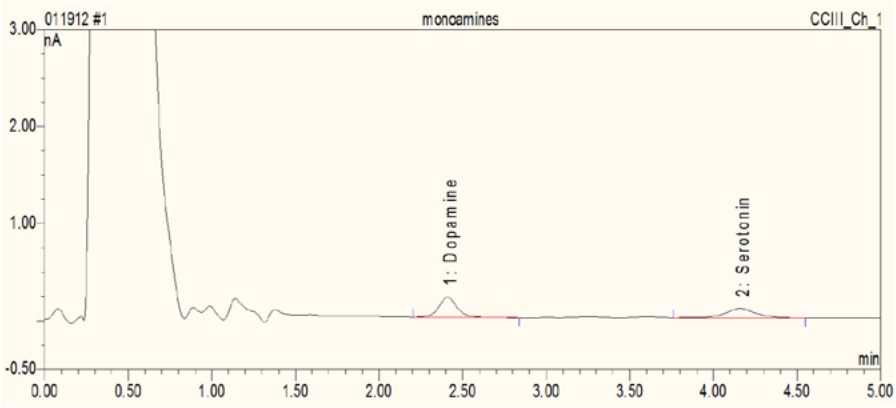
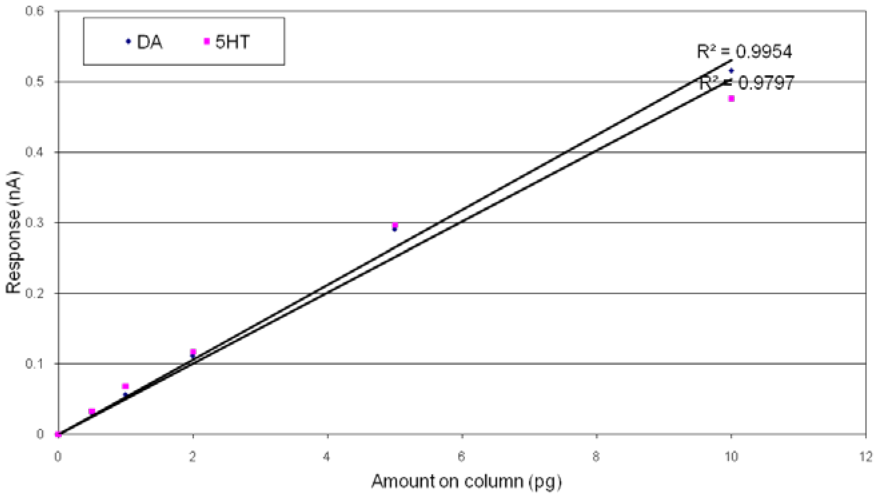


FIGURE 6. Calibration curves for trace levels of dopamine and serotonin.
Assay sensitivity is currently 0.1 pg on column with a 10 µL injection volume (10 pg/mL).



Analysis of Amino Acids:

Most amino acids cannot be analyzed directly by HPLC-ECD so the preparation of derivatives is required.[1] In order to take advantage of the sensitivity of HPLC-ECD, amino acids have to be converted to compounds that are electrochemically active. OPA/β-ME is the most common pre-column derivatization used for this approach. The isoindole derivative also confers some degree of stability, essential when measuring the ultra-low levels of neuroactive amino acids, in particular GABA. The work illustrates a fast and stable isocratic method for amino acid analysis (Figure 7). Table 1 indicates that excellent linearity can be realized for the various amino acids using this method. The electrochemical detector cell used (Thermo Scientific Dionex Model 6011 ultra analytical cell) is a coulometric, dual electrode cell. This cell has reduced volume electrodes and flow path for UHPLC resulting in enhanced peak resolution while decreasing run times when appropriate columns are employed (Figure 8). 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 over previous techniques.[2] The Model 6011 ultra analytical cell with a fused core chromatography column adds sensitivity and selectivity to this assay.

FIGURE 7. Isocratic separation of amino acids using a fused core analytical column.

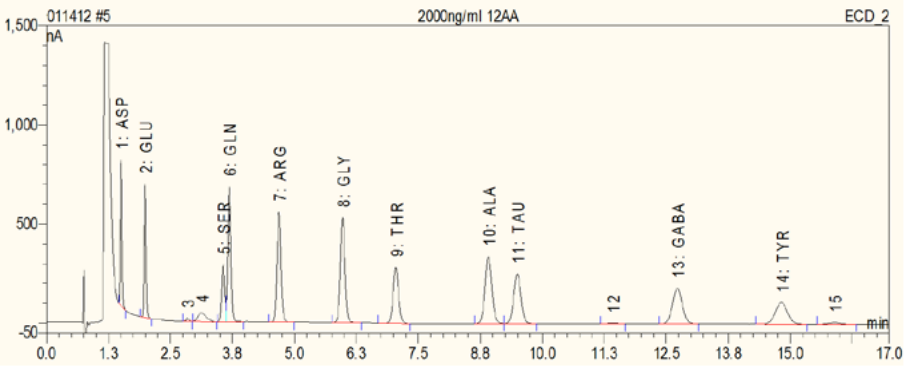
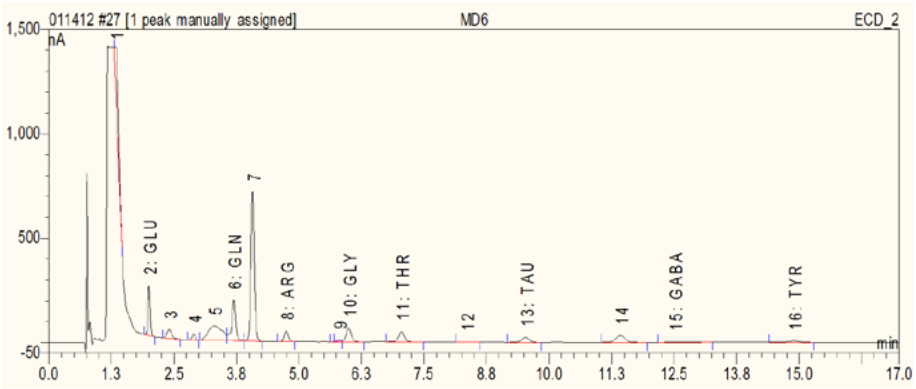


TABLE 1: Correlation data for amino acid calibration curves.

Peak Name	# Points	Rel.Std.Dev %	Corr.Coeff.%
ASP	4	4.4257	99.9607
GLU	4	9.3560	99.9532
GLN	4	11.4120	99.8425
ARG	4	4.7359	99.8828
GLY	4	5.2531	99.8611
THR	4	4.3761	99.9059
ALA	4	5.5414	99.8539
TAU	4	4.9745	99.8789
GABA	4	5.0979	99.8756
TYR	4	5.5241	99.8545

FIGURE 8. Analysis of neuroactive amino acids in a microdialysis sample from the corpus striatum.



Conclusions

- The method for biogenic amines and acid metabolites was both highly sensitive and rapid. All seven compounds were analyzed within 9 minutes and with limits of detection of about 1 picogram on-column.
- Rapid temporal changes in neurochemical levels were assessed using the method for the analysis of dopamine and serotonin. The assay was completed in just five minutes, therefore improving temporal resolution over other methods in the literature.
- The analysis of microdialysis samples for their neuroactive amino acids was completed within 17 minutes and detected levels at low ng/mL.

References

1. McGeer P. L. and McGeer E. G. Amino acid neurotransmitters, In *Basic Neurochernistry.* 4th ed. Siegel *G.,* Agranoff B., Albers R. W., and Molinoff P., Eds. Raven Press: New York. **1989**, pp. 311-332.
2. Donzanti, B. A., and Yamamoto, B. K. An improved and rapid HPLC-EC method for the isocratic separation of amino acid neurotransmitters from brain tissue and microdialysis perfusates. *Life Sci.* **1988**, 43, 913–922.

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