A Rapid UHPLC Method for the Analysis of Biogenic Amines and Metabolites in Microdialysis Samples

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Overview

Purpose: To develop an Ultra High Performance Liquid Chromatography (UHPLC) method to resolve the major biogenic amine and their acid metabolites in a microdialysis sample collected from the rat brain with improved throughput using the Thermo Scientific™ Dionex™ UltiMate™ 3000 HPLC platform.

Methods: A sample of extracellular fluid from the prefrontal cortex was collected for a 20 minute duration using a 2 mm microdialysis probe. Subsequent analysis of the sample for biogenic amines and acid metabolite content was performed via the UltiMate 3000 HPLC system using a 2.4 μm, 2.1 × 100 mm C18 column.

Results: A UHPLC method for the measurement of neurochemicals in microdialysis samples was developed. The major biogenic amines and acid metabolites were detected from the extracellular fluid sample using a combination of electrochemical oxidation and reduction techniques.

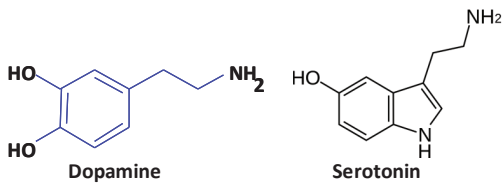
Introduction

Evaluating the efficacy of psychotropic pharmaceuticals often involves *in-vivo* animal experiments with repeated sampling of brain extracellular fluids for their neurochemical content. By using a microdialysis probe, any changes in the extracellular levels of biogenic amines and major metabolites as shown in Figure 1 can be monitored over time. This offers insights into the psychoactive nature of the drug candidate. Therefore, experimental samples must be repeatedly and accurately analyzed so that any possible changes of major neuroactive chemicals can be observed with reasonable temporal resolution. Rapid analytical techniques such as UHPLC with sensitive electrochemical detection provide advantages over traditional HPLC assays since the sampling rates can be increased due to shorter run times involved with analysis.

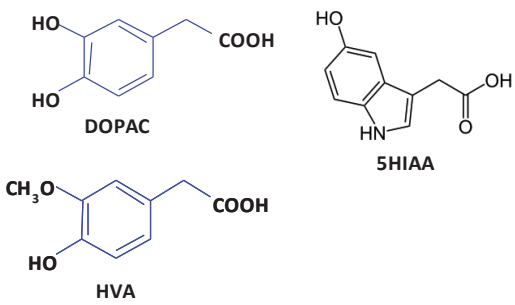
The analysis of biogenic amines and major metabolites in these samples is challenging since these compounds are labile and the levels of monoamines in extracellular fluids from various brain regions are typically quite low due to active reuptake systems and catabolism. Transport of these compounds by diffusion processes across the microdialysis probe is limited, so only partial recovery of these chemicals from the extracellular fluid actually occurs. These issues present significant assay sensitivity challenges. Combining low microliter sample volumes with short collection periods means the assay must be capable of detecting picomolar levels of analytes. By using a specialized Liquid Chromatography / Electrochemical Detection (LCEC) system, capable of handling the elevated pressures related to UHPLC operation while maintaining the full sensitivity requirements of the electrochemical detection to femtogram levels of detection, assay requirements can be fully satisfied. This work describes the analytical conditions for the successful measurement of biogenic amines and major metabolites in microdialysis samples.

FIGURE 1. Structures of A) Biogenic Amines and B) Major Acid Metabolites.

A) Biogenic Amines



B) Acid Metabolites



Methods

Liquid Chromatography using an UltiMate 3000 HPLC system:

- ISO-3100BM pump
- WPS-3000TBRS autosampler
- ECD-3000RS electrochemical detector

Flow rate:	Isocratic at 0.50 mL/min.
Column:	Thermo Scientific™ Hypersil™ BDS, 2.4 μm, 2.1 x 100 mm
Column Temp.:	40 °C via internal ECD-3000RS column oven
Mobile Phase:	75 mM sodium dihydrogen phosphate, monohydrate, 1.7 mM 1-octane sulfonic acid, sodium salt (OSA), 25 μM EDTA, 100 μL/L triethylamine (TEA), 10% acetonitrile, adjust to pH=3.0 with phosphoric acid
EC Detector:	Thermo Scientific Dionex Model 6011RS cell with porous graphite; E1 set at -150 mV, E2 at +250 mV and Model 6041RS cell with glassy carbon set at -325 mV: 12 micron Mylar; Filter:5.0 s, data collection rate 20 Hz
Inj. Volume:	20 μL partial loop
Sample Tray Temperature	12 °C
Sample Collection:	Artificial cerebral spinal fluid (aCSF) was collected for 20 minutes at 1 μL/min from a 2 mm microdialysis probe positioned in the prefrontal cortex of the rat brain.

Data Analysis

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, 7.2

Results

Relevant neurochemicals include the biogenic amines, dopamine (DA) and serotonin (5HT), and their acid metabolites dihydroxyphenyl acetic acid (DOPAC), 5-hydroxyindole acetic acid (5HIAA), and homovanillic acid (HVA). The neurochemical output from specific brain regions can be monitored using the technique of *in-vivo* microdialysis.[1] In this work, a 2 mm microdialysis probe was surgically implanted into the region of the prefrontal cortex of the rat. The extracellular fluid from this brain region was sampled by pumping an artificial cerebral spinal fluid (aCSF) through the probe at 1 μL/min. This aCSF was collected for 20 minute periods and then samples were analyzed for neurochemical content using HPLC with sensitive electrochemical detection. One of the challenges of this analytical technique is due to the high salt content of the aCSF which can influence the stability of the electrochemical cell. In order to improve temporal resolution, researchers have attempted to shorten the analysis time of the chromatographic run. However, these attempts have been largely hindered due to issues related to metal capillaries required in UHPLC systems and their promotion of auto-oxidation of labile compounds such as dopamine.

Newer UHPLC columns have been available for some time and can increase sample throughput due to the shorter chromatographic run times they provide. A major requirement to make use of these UHPLC columns is related to the upper pressure limits of the HPLC system. However, in order to obtain extreme sensitivity, most HPLC systems combined with ECD use low pressure PEEK capillaries to prevent any significant issues related to metal auto-oxidation of these labile compounds. The HPLC system described in this work is capable of reaching 620 bar and makes use of specialized Thermo Scientific™ Dionex™ BioViper™ (MP35N) capillaries that are biocompatible with labile compounds and electrochemical detection. Thus a UHPLC column packed with 2.4 μm particles could be installed. These alterations to the HPLC system contributed to the rapid separation of all analytes within 4 minutes as illustrated in Figure 2.

The ECD-3000RS electrochemical detector provides unique capabilities of sensitivity and selectivity by utilizing a multiple cell configuration for the analysis of neurochemicals and acid metabolites from extracellular fluids. The electrochemical cell configuration shown in Figure 3 provides a sensitive arrangement to measure both biogenic amines and acid metabolites present in a microdialysis sample obtained from the prefrontal cortex. Using the redox configuration shown it was possible to first oxidize all compounds on the oxidative coulometric channel set at +250 mV and then selectively reduce dopamine on the reductive amperometric channel set at -350 mV. It should be noted that in Figure 2 the high salt concentration of the aCSF did contribute to a baseline disturbance of the coulometric cell at the beginning section of the chromatogram. Concentrations of the acid metabolites were relatively high and could be easily measured at this electrode. Fortunately, the subsequent amperometric electrode using reductive potentials was quite stable and provided good sensitivity (femtogram levels of detection for Dopamine could be obtained as presented in Figure 4). This allows the analysis of trace levels of these important neurochemicals in brain regions such as the prefrontal cortex.

FIGURE 2. Overlay of standard chromatogram of oxidative and subsequent reduction electrodes at +250 and -325 mV, respectively.

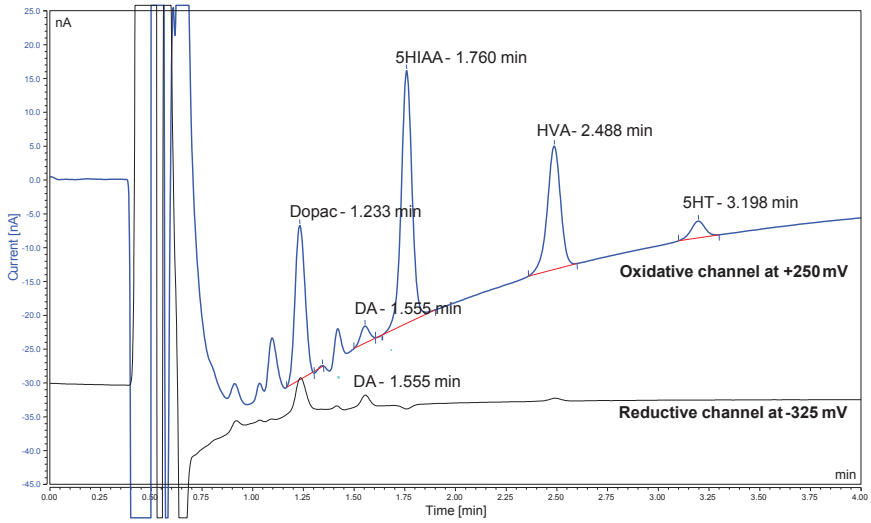
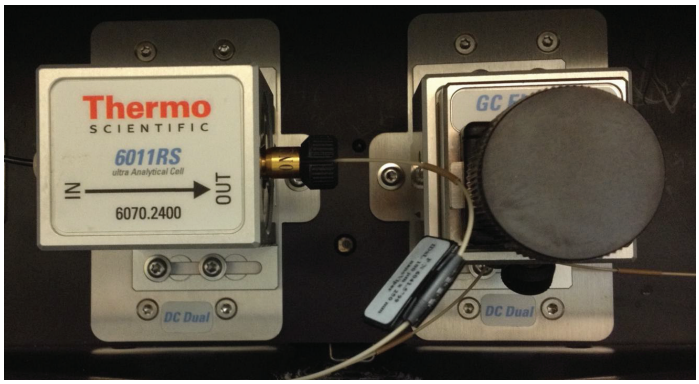


FIGURE 3. Electrode Arrangement for Redox Cycling of Catechol Compounds.



Dual Coulometric cell Amperometric cell / GC Electrode

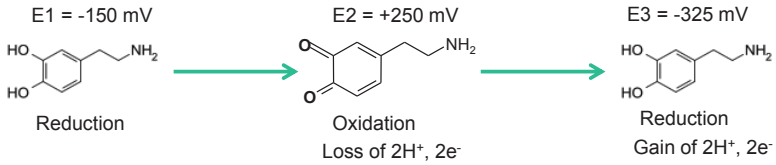
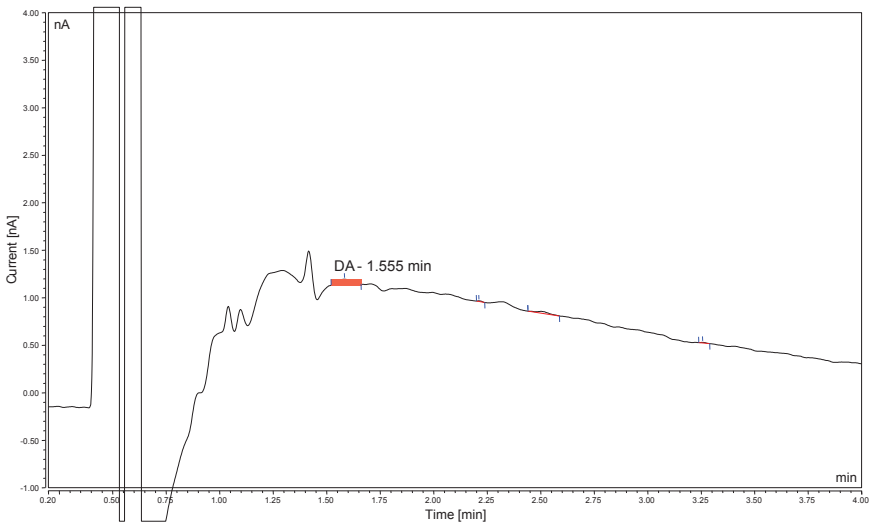


FIGURE 4. Analysis of Dopamine (0.2 pg) on the Amperometric cell using redox cycle conditions.



Calibration data for biogenic amines is shown in Figure 5 and for the acid metabolites as shown in Figure 6. Good response linearity was obtained with correlation coefficients ranging from R^2 = 0.997 – 0.999 for the biogenic amines and R^2 = 0.992 – 1.0 for the acid metabolites as shown in Table 1. Baseline stability was a real concern for the measurement of biogenic amines at low levels since dopamine has a short retention time and is present at very low levels in microdialysis samples. Dopamine will undergo redox cycling and by taking advantage of this technique femtogram sensitivity was achieved on the amperometric cell using the redox cycling configuration (Figure 3). Serotonin does not redox cycle so was measured oxidatively on the second coulometric electrode (LOD 1.5 pg). Since the three acid metabolites present in the extracellular fluid are much more abundant than the biogenic amines these calibration standards were prepared at a 10X concentration over the biogenic amine levels. These higher levels are reflected in the data shown in Figure 6.

FIGURE 5. Calibration curves of Biogenic Amine Standards ranging from 0.2 – 10 pg amounts on-column.

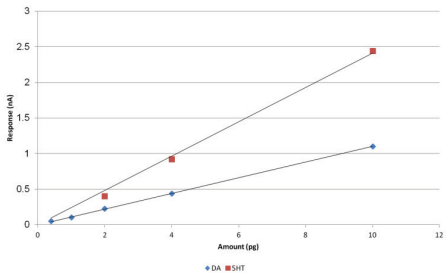


FIGURE 6. Calibration curves of Acid Metabolite Standards ranging from 2 – 100 pg amounts on-column.

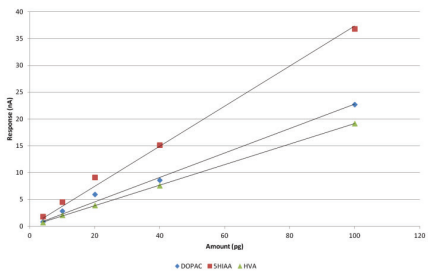


TABLE 1. Correlation data for biogenic amines and acid metabolites calibration curves.

Compound	Selected Channel	Number of Points	Correlation Coefficient, R^2	Linear Equation	fmol / fraction
Dopamine (DA)	Channel 3, Reductive	3	R^2 = 0.9997	y = 0.1102x	137
Serotonin (5HT)	Channel 2 Oxidative	3	R^2 = 0.9958	y = 0.241x	23
DOPAC	Channel 2 Oxidative	3	R^2 = 0.9918	y = 0.2281x	168
5HIAA	Channel 2 Oxidative	3	R^2 = 0.9952	y = 0.3736x	34
HVA	Channel 2 Oxidative	3	R^2 = 0.9999	y = 0.1916x	11

All five compounds were separated and detected in the microdialysis samples from the prefrontal cortex. Chromatographic data illustrating the detection of dopamine from the microdialysis sample using the reduction channel at -325 mV is shown in Figure 7. Chromatographic data illustrating the measurement of serotonin and acid metabolites from the microdialysis sample using the oxidation channel at +250 mV is shown in Figure 8. The levels indicated in Table 1 are similar to the levels indicated in literature publications.[2]

Conclusions

- The described UHPLC method with subsequent electrochemical detection provided a suitable technique to monitor rapid temporal changes in neurochemical levels in microdialysis samples obtained from the prefrontal cortex.
- Excellent sensitivity and improved baseline stability were obtained using the electrochemical redox configuration described in this work. Redox cycling provided better stability for the analysis of low levels of dopamine from microdialysis samples when using UHPLC separation methods.

FIGURE 7. Redox Detection of Dopamine in a Microdialysis Sample (137 fmol) and Standard.

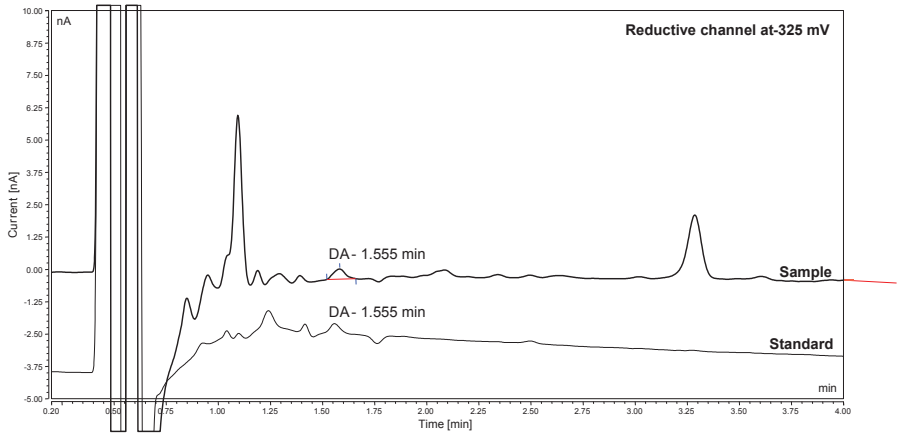
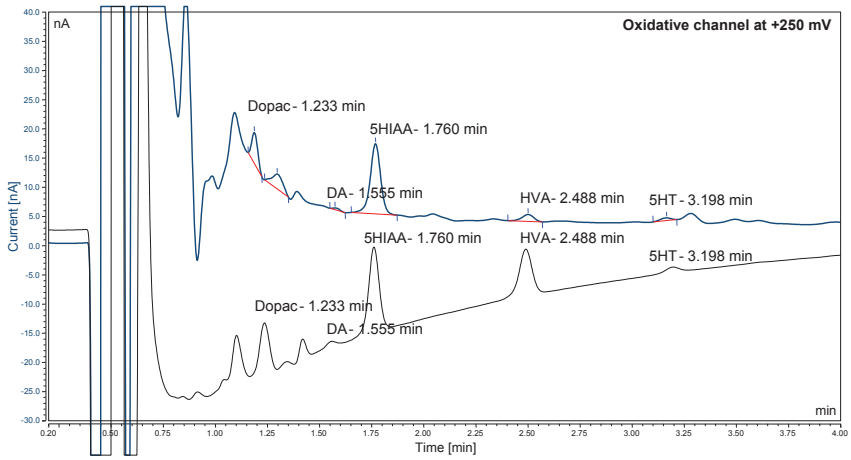


FIGURE 8. Coulometric Detection of Serotonin (23 fmol) and Acid Metabolites in a Microdialysis Sample and Standards.



References

1. Acworth, I.N. and Cunningham, M.L. The Measurement of Monoamine Neurotransmitters in Microdialysis Perfusates Using HPLC-ECD. Methods Mol Med. **1999**, *22, 219-236.*
2. Amargos-Bosch, M., Artigas, F. and Adel, A. Effects of acute olanzapine after sustained fluoxetine on extracellular monoamine levels in the rat medial prefrontal cortex. Eur. J. Pharmacol. **2005**, *516*, 235 – 238.

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