Application Note 1060

Comprehensive Neurochemical Profiling of Brain Tissue Samples

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Key Words
Biogenic amines, amino acid precursors, acid metabolites, UHPLC, and coulometric

Introduction

The ability to measure low levels of many different neurochemicals simultaneously is problematic due to detector sensitivity and the chromatographic challenge of resolving analytes with similar chemical structures. Most of the biogenic amines and metabolites can be oxidized electrochemically so the use of electrochemical detection is routine for the analysis of these compounds. Chromatographic techniques have advanced over the years, however, even with the use of UHPLC columns and gradient elution techniques, baseline resolution of the common neurochemicals still remains difficult due to the constraints of current electrochemical detectors. A new, modular, four-channel electrochemical array detector makes profiling biogenic amines and acid metabolites in brain tissue samples using UHPLC easier and faster.

The Thermo Scientific™ Dionex™ UltiMate™ 3000 electrochemical detector (ECD-3000RS) uses multiple coulometric electrodes in series with each electrode having a unique potential setting. This voltammetric approach provides additional resolution of analytes beyond chromatographic separation. The ECD-3000RS incorporates improved resolution with a wide dynamic range due to advanced autorange capability. This feature makes the detector compatible with gradient HPLC techniques and enables the simultaneous measurement of low and high level analytes. Qualitative information is thereby enhanced while still maintaining the ability to accurately measure low levels of analytes.

Goal

- Profiling biogenic amines, their amino acid precursors, and metabolites from brain tissue samples, in a single HPLC analysis
- Voltammetric resolution is a complementary technique for the characterization of complex sample mixtures

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 Biocompatible HPLC system, including:
  - SR-3000 Solvent Rack (without degasser)
  - ISO-3100BM Pump
  - WPS-3000TBSL Analytical Autosampler
- Thermo Scientific™ Dionex™ UltiMate™ 3000 Electrochemical Detector
- Thermo Scientific™ Dionex™ Chromeleon™ CDS software, version 6.8

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Chemical Description	Part Number
Perchloric acid (PCA)	GFS Chemicals® 67
Hydrochloric acid (HCl)	J.T. Baker® 9535-03
Test mobile phase	Thermo Scientific 70-3829
3-Methoxytyramine hydrochloride (3MT)	Sigma-Aldrich® M4251
5-Hydroxyindole-3-acetic acid (5HIAA)	Sigma-Aldrich H8876
5-hydroxytryptamine (5HT)	Sigma-Aldrich H9523
3,4-Dihydroxyphenylacetic acid (DOPAC)	Sigma-Aldrich 850217
Dopamine hydrochloride	Sigma-Aldrich H8502
Epinephrine bitartrate	Sigma-Aldrich E4375
Homovanillic acid (HVA)	Sigma-Aldrich H1252
L-Tyrosine (TYR)	Sigma-Aldrich 93829
L-Kynurenine (KYN)	Sigma-Aldrich K8625
L-Tryptophan (TRP)	Sigma-Aldrich 93659
Norepinephrine bitartrate (NE)	Sigma-Aldrich A0937
Vanillylmandelic acid (VMA)	Sigma-Aldrich H0131

Preparation of Solutions and Reagents

- ***0.3 N Perchloric Acid Solution***—A solution of perchloric acid facilitates the deproteinization of tissue samples after ultrasonic disruption. Prepare 0.3 N perchloric acid (PCA) solutions by dissolving 2.565 mL PCA (70%) in a 100 mL volumetric flask containing 95 mL water. Bring to volume with Deionized water (DI). Store at 4 °C and prepare fresh every 3–4 weeks.
- ***0.1 N Hydrochloric Acid Solution***—This acidic solution provides a suitable reducing environment to protect labile compounds. Make a 1 N hydrochloric acid solution by taking 8.25 mL of concentrated hydrochloric acid (37%) and slowly adding this to a 100 mL volumetric flask containing 95 mL water. Bring to volume with DI water. Dilute 10 mL of this 1 N HCl solution in a 100 mL volumetric flask and bring to volume with DI water to prepare a 0.1 N HCl solution. This solution is stable at room temperature for over one month.
- ***3MT Stock Solution***—To prepare a 1 mg/mL 3-methoxytyramine (3MT) (MW 167.21) stock solution, dissolve 12.2 mg of the salt into a 10.0 mL volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***5HIAA Stock Solution***—To prepare a 1 mg/mL 5-Hydroxyindoleacetic acid (5HIAA) (MW 191.18) stock solution, dissolve 10.0 mg into a 10.0 mL volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***DOPAC Stock Solution***—To prepare a 1 mg/mL 3,4-Dihydroxyphenylacetic acid (DOPAC) (MW 168.14) stock solution, dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Dopamine Stock Solution***—To prepare a 1 mg/mL dopamine (DA) (MW 656.59) stock solution, dissolve 12.4 mg of the salt into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Epinephrine Stock Solution***—To prepare a 1 mg/mL epinephrine (EPI) (MW 183.20) stock solution, dissolve 18.2 mg of the salt into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***HVA Stock Solution***—To prepare a 1 mg/mL homovanillic acid (HVA) (MW 182.17) stock solution, dissolve 10.0 mg into a 10.0 mL volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Kynurenine Stock Solution***—To prepare a 1 mg/mL kynurenine (KYN) (MW 208.21) stock solution, dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Norepinephrine Stock Solution***—To prepare a 1 mg/mL norepinephrine (NE) (MW 169.18) stock solution, dissolve 18.9 mg of the salt into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Serotonin Stock Solution***—To prepare a 1 mg/mL serotonin (5HT) (MW 176.22) stock solution, dissolve 12.1 mg of the salt into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Tryptophan Stock Solution***—To prepare a 1 mg/mL tryptophan (TRP) (MW 656.59) stock solution, dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***Tyrosine Stock Solution***—To prepare a 1 mg/mL tyrosine (TYR) (MW 181.19) stock solution, dissolve 10.0 mg into a 10.0 mL volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.
- ***VMA Stock Solution***—To prepare a 1 mg/mL vanillylmandelic acid (VMA) (MW 198.17) stock solution, dissolve 10.0 mg into a 10.0 ml volumetric flask using 0.1 N HCl. Store at 4 °C and prepare fresh every 3–4 weeks.

Table 1. Preparation of working standard solutions (prepare fresh daily).

Concentration	1st Addition	2nd Addition	Instruction
1 µg/mL	1 volume of 10 µg/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
500 ng/mL	1 volume of 1 µg/mL solution	Add 1 volumes of 0.1 N HCl	Mix well
200 ng/mL	1 volume of 1 µg/mL solution	Add 4 volumes of 0.1 N HCl	Mix well
100 ng/mL	1 volume of 1 µg/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
50 ng/mL	1 volume of 500 ng/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
20 ng/mL	1 volume of 200 ng/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
10 ng/mL	1 volume of 100 ng/mL solution	Add 9 volumes of 0.1 N HCl	Mix well
5 ng/mL	1 volume of 50 ng/mL solution	Add 9 volumes of 0.1 N HCl	Mix well

Prepare a 10 µg/mL working solution of the twelve different stock solutions shown above by placing 1.0 mL each of VMA, TYR, NE, EPI, DOPAC, DA, 5HIAA, KYN, HVA, 3MT, 5HT, TRP into a 100 mL volumetric flask, add 0.1 N HCl solution to mark, cap and mix.

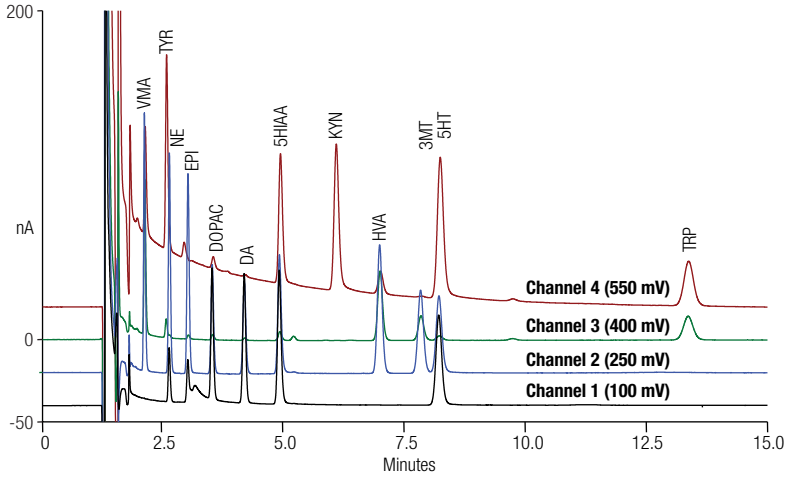

Figure 1. Neurochemical profiling with a four-channel electrochemical array detector (100 ng/mL).

A list showing the dilutions of stock standards for preparation of the calibration curves is in Table 1.

Preparation of Tissue Samples

- Administer with vehicle (saline) via i.p. injections to male Sprague Dawley® rats weighing 175–200 grams
- Wait one hour and sacrifice animal by carbon dioxide asphyxiation
- Immediately remove the brains and dissect on ice
- Freeze brain tissue at -70 °C until needed
- Sonicate brain tissue samples (10–25 mg) in in 1.0 mL perchloric acid
- Prepare sample by centrifugation at 13,000 RPM for 10 min.
- Transfer the clear supernatant into an autosampler vial and place on a thermostated autosampler set for 10 °C.
- For best results, prepare sample homogenates on the day of analysis.

HPLC Method Conditions

Column:	Thermo Scientific™ Hypersil™ BDS C18 column, 3 µm, 3 × 150 mm, including a Hypersil BDS guard column (28103-013001) and UniGuard™ guard cartridge holder (852-00)
Flow:	Isocratic at 0.50 mL/min.
Temperature:	35 °C
Injection volume (standards):	5 µL
Injection volume (tissue samples):	10 µL
Mobile phase:	Thermo Scientific™ Dionex™ Test Phase
Electrochemical cell:	Thermo Scientific™ Dionex™ 6011RS ultra Coulometric Analytical cell
Analytical cell:	E1: +100 mV: E2: +250 mV, E3: +400 mV, E4: +550 mV vs. Pd reference electrode

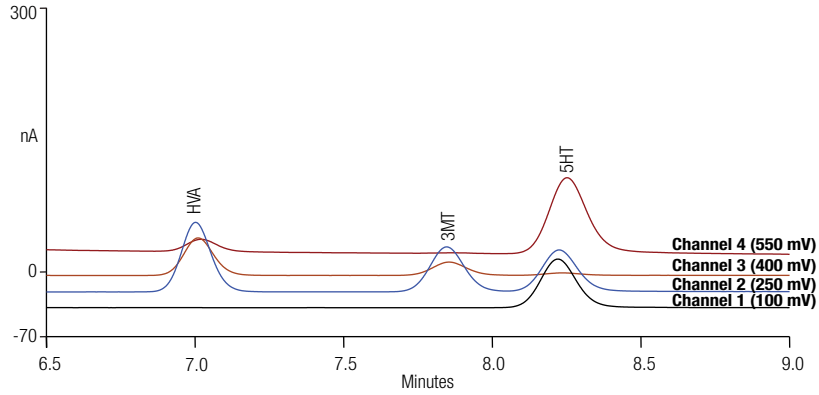

Figure 2. Chromatographic and voltammetric resolution of compounds (100 ng/mL).

Table 2. Calibration data for standards ranging from 5–500 ng/mL.

Peak	Name	RT (min)	Rel. Std. Dev %	Correlation Coeff. R^2	Dominant Channel
1	VMA	2.15	1.40	0.9999	2
2	TYR	2.60	0.94	0.9999	4
3	NE	2.65	2.08	0.9998	2
4	EPI	2.98	1.89	0.9999	2
5	DOPAC	3.60	5.48	0.9991	1
6	DA	4.25	5.22	0.9992	1
7	5HIAA	5.00	3.73	0.9996	4
8	KYN	6.10	1.15	0.9999	4
9	HVA	7.00	0.98	0.9999	3
10	3MT	7.85	3.81	0.9997	2
11	5HT	8.20	1.83	0.9999	4
12	TRP	13.3	2.88	0.9997	4

Results and Discussion

Figure 1 shows complete separation of NE, DA, and 5HT, their amino acid precursors TYR and TRP, and their metabolites including DOPAC, 5HIAA, KYN, HVA and 3MT. Good linearity of response from the dominant channel ranging from R^2 = 0.9991–0.9999 for the 12 compounds evaluated (Table 1) was obtained over a concentration range of 5–500 ng/mL. The dominant channel represents the electrode having the greatest response for each compound, repectively. The percent relative standard deviation (%RSD) for the calibration curves (seven concentrations in duplicate) is also shown in Table 2. The RSD values ranged from 0.98% to 5.48%, indicating that the coulometric electrodes provided good stability during this analysis.

In Figure 2, the section of the chromatographic trace shows both chromatographic and voltammetric resolution between the compounds 3MT and 5HT made possible through the use of coulometric electrodes. Note that serotonin shows a response at lower potentials of 100 and 250 mV, due to the oxidation of the 5-hydroxy group and one at higher potentials of 550 mV due to the oxidation of the indole ring nitrogen. Judicious choice of applied potentials to the coulometric four-electrode array enables the simultaneous measurement of co-eluting compounds with a high degree of accuracy. The array configuration is not possible using electrochemical cells with single amperometric electrodes and results in the overestimation of the analyte of choice when co-elution occurs. Voltammetric resolution of coulometric electrodes offers superior insights into the proper identification of individual compounds since each will have a unique but reproducible pattern across the four-electrode channels. Thus the multi-potential response for serotonin can be used to help authenticate the compound.

A number of different brain regions were evaluated for this study. Figures 3 and 4 are labeled with the appropriate brain region. Data from the corpus striatum and frontal cortex are presented in Figures 5 and 6, respectively. These demonstrate that picogram sensitivity can be obtained using this technique.

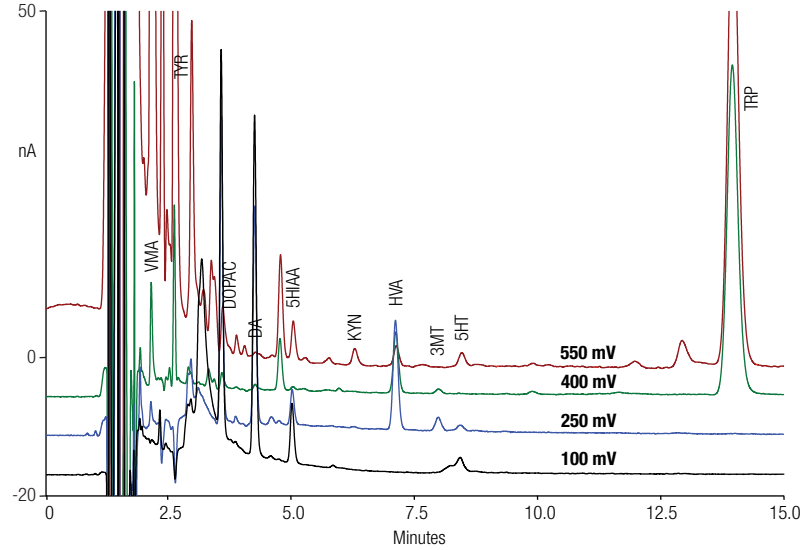

Figure 3. Neurochemical profiling of brain tissue sample (striatum).

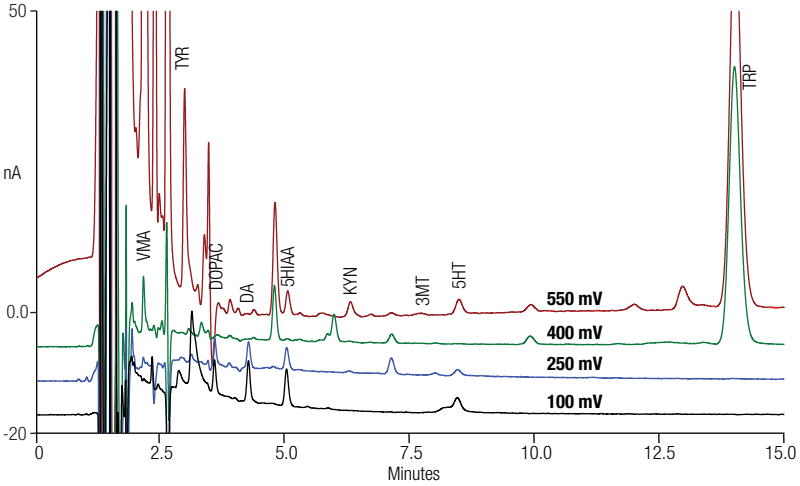

Figure 4. Neurochemical profiling of brain tissue sample (frontal cortex).

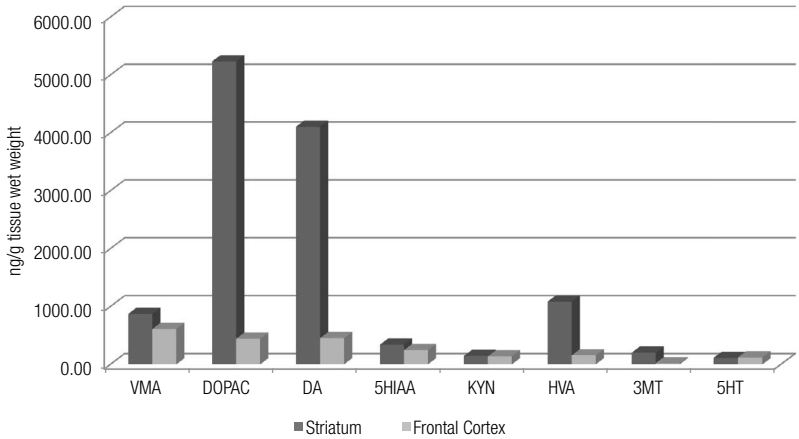

Figure 5. Neurochemical concentrations of regional brain tissue samples (striatum vs. frontal cortex regions).

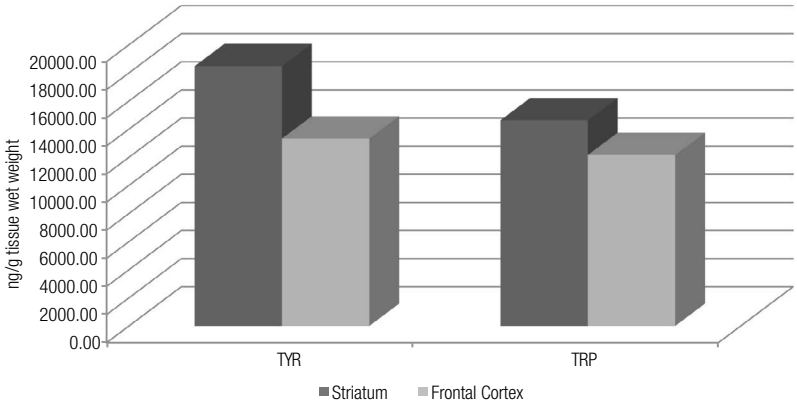

Figure 6. Amino acid precursor concentrations of brain tissue samples.

Table 3. Levels (ng/g tissue wet weight) of measured neurochemicals in regional brain tissues.

Region	VMA	DOPAC	DA	5HIAA	KYN	HVA	3MT	5HT	TYR	TRP
Striatum	873	5235	4108	342	146	1086	202	115	18472	14624
Frontal Cortex	611	443	453	246	139	156	20	122	13326	12158

The levels of neurochemicals found in regional tissue samples are presented in Table 3. These data indicate that the corpus striatum has higher levels of the majority of neurochemicals measured, except for serotonin which was slightly elevated in the frontal cortex sample, and are in agreement with other articles in literature.

Conclusion

A simple, rapid, and accurate method was developed for the analysis of biogenic amines, their metabolites and precursor amino acids using isocratic chromatography with a multichannel electrochemical detector. This enables both chromatographic and voltammetric resolution of many analytes, thereby enhancing the identification and accurate quantification of these compounds. The method enables the rapid separation of various neurochemical compounds at trace levels and without significant matrix interferences.

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