CAPSULES
notes and applications from Bioanalytical Systems, Inc.

Peak Identification using Parallel, Dual Electrode LCEC

Biogenic Amines in Brain

Purpose

It is often of interest to analyze brain parts involved in the metabolism of both dopamine and serotonin. Because there is no single extraction procedure available for these neurochemicals and their metabolites due to the diversity of their functional groups, direct injection of brain tissue homogenate supernatant is frequently employed. Since the extract is so crude, it is important to establish the validity of results obtained by the direct injection method; several checks have been employed(1,2). In our lab, peak current ratios generated simultaneously at parallel-adjacent dual electrodes were used to confirm peak purity of tissue homogenates. The method(3) described in here permits accurate quantitation of norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), dopamine (DA), 5-hydroxyindole-3- acetic acid (5-HIAA), homovanillic acid (HVA), and serotonin (5-HT).

In parallel configuration, two glassy carbon electrodes are placed side-by-side in the flow stream so the eluent passes over each electrode simultaneously. This configuration is analogous to dual wavelength ultraviolet detection. Chromatograms are generated at two potentials along the compound's hydrodynamic voltammogram. The potentials are on the diffusion-limited current plateau as shown by W1, and somewhere along the rising portion of the curve as shown by W2 (F1). The electrodes are operating simultaneously but independently. The peak current ratios are calculated by dividing the output of the lower potential by that of the higher potential (i_2/i_1).

Conditions

Liquid Chromatograph: Bioanalytical Systems LC-304 BAS 400 or BAS 200 with dual glassy carbon working electrodes. Optimized chromatographic conditions are listed below:

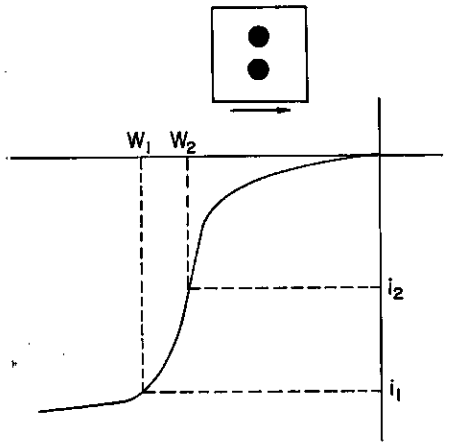

Figure 1. Hypothetical hydrodynamic voltammogram showing potentials for W1 and W2.

Mobile Phase: 3.5% acetonitrile/96.5% 0.15 M monochloroacetate buffer pH 3.0 with 0.86 mM sodium octyl sulfate, 18 mL of tetrahydrofuran(THF) per liter is added after filtering and degassing.
Flow Rate: 1.6 mL/min.
Stationary Phase: Biophase ODS 5 µm (P/N MF6017) 250 mm x 4.6 mm
Temperature: Ambient
Detector Potential: W1 at +800 mV, W2 at +650 mV (vs. Ag/AgCl/3M NaCl)
Detector Sensitivity: 5 or 10 nA fs
Volume injected: 100 µL

Results

A hydrodynamic voltammogram was generated by injecting a standard mixture at different potentials. F2 illustrates the current vs. voltage curves obtained at one of the electrodes. After evaluating the curves, applied potentials of 800 mV and 650 mV were chosen. Typical chromatograms for standards and samples are represented in F3. Samples were homogenized in perchloric acid, as described elsewhere(3). The current ratios are distinctive of a compound's redox characteristics so that they may be used to differentiate between electroactive func-

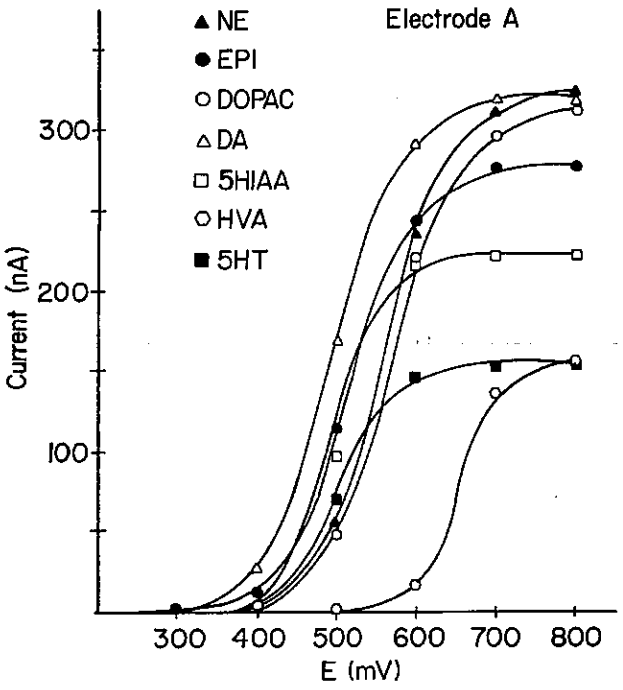

Figure 2. Hydrodynamic voltammograms for several biogenic amines and metabolites on a glassy carbon working electrode. Conditions; flow rate: 1.6 mL/min; column: Biophase ODS 5 µ, 250 mm x 4.6 mm; mobile phase: 3.5 parts CH_3CN, 96.5 parts stock buffer, and 1.9 parts THF.

tional groups. For example, ratios for NE and EPI, which are β-hydroxylated catecholamines, were different from DA and DOPAC, which are not β-hydroxylated. Shifting the potential "window" to other pairs of operating potentials (in another injection) may be used to further distinguish between various sample components if identification is still in doubt.

Peak current ratios were used to confirm peak purity. For instance, if the ratios of sample and standard agreed, the peak purity was further confirmed. A deviation from the standard in the sample's ratio would indicate an interference. The ratios for these compounds correlated well between the two types of extracts and were reproducible. Data indicate that within-day and day-to-day reproducibility was acceptable (T2). The slight decreases of ratios observed in the day-to-day results were due in part to electrode passivation. This variation presents no real problem since daily calibration, a common practice, provides accurate standard ratios. The ratio was also independent of the concentration of analyte injected.

Parallel-adjacent dual electrodes provide distinct advantages over single electrode detection in methods such as direct injection. This configuration enables the analyst to 1) confirm peak purity, 2) differentiate between electroactive functional groups, and 3) gain information on the redox characteristics of peaks containing only a few picomoles of the species to be detected. Of course, in these cases, the amount is so small as to preclude off-line characterization by other techniques.

Appendix - Standard Solutions

The standard solution should be prepared in 0.05 M $HClO_4$ with 0.1% (w:v) cysteine as an antioxidant. If the salts of these compounds are used, concentrations must be corrected for the free base. For example, if norepinephrine bitartrate (M.W. 319.39) is used instead of free base (M.W. 169.18), the actual mass of the free base is determined by multiplying the amount of the compound as the bitartrate by the factor.

$$\frac{\text{M.W. Norepinephrine free base}}{\text{M.W. Norepinephrine bitartrate}} = \frac{169.2}{319.39}$$

As an example, a stock solution (µg free base/mL) is prepared by dissolving the following amounts in 500 mL of 0.05 M $HClO_4$ with 0.1% cysteine:

45 mg	norepinephrine bitartrate	47 µg/mL
51 mg	epinephrine bitartrate	58 µg/mL
28 mg	3,4-dihydroxyphenylacetic acid	55 µg/mL
40 mg	dopamine hydrochloride	64 µg/mL
25 mg	homovanillic acid	50 µg/mL
25 mg	5-hydroxyindole-3-acetic acid	50 µg/mL
55 mg	5-hydroxytryptamine creatinine sulfate complex	50 µg/mL

The working standard is prepared by diluting 5 mL of the stock solution to 500 mL with 0.05 M $HClO_4$ with 0.1% cysteine. After mixing well, 10 mL of the dilution is further diluted to 100 mL with 0.05 M $HClO_4$ with 0.1% cysteine. The resulting concentrations (free base) will be as follows: NE, 47 ng/mL; EPI,

Table 1. Within-day and day-to-day reproducibility of peak current ratios using parallel dual electrodes at 650/800 mV. Chromatographic conditions as per F2

Day*	n†	NE	DOPAC	DA	5-HIAA	HVA	5-HT
3	2	0.62	.88	0.85	0.99	0.14	1.00
4	5	0.58 ± 0.05	0.87 + 0.02	0.83 + 0.004	0.98 + 0.005	0.19 + 0.005	0.98 + 0.004
5	9	0.46 ± 0.007	0.80 + 0.006	0.74 + 0.01	0.98 + 0.01	0.05 + 0.006	0.98 + 0.05
7	3	0.47 ± 0.02	0.81 + 0.002	0.76 + 0.02	0.98 + 0.01	0.05 + 0.006	0.96 + 0.008
8	5	0.41 ± 0.009	0.79 + 0.01	0.70 + 0.06	0.99 + 0.004	0.03 + 0.005	0.96 + 0.008
11	3	0.42 ± 0.01	0.76 + 0.04	0.71 + 0.005	0.97 + 0.01	0.05 + 0.005	0.95 + 0.01

*refers to the number of days since polishing the electrode surface.
†number of injections made on a given day to assess precision. .

58 ng/mL; DOPAC, 55 ng/mL; DA, 64 ng/mL; HVA, 50 ng/mL; 5-HIAA, 50 ng/mL; 5-HT, 50 ng/mL.

All standard solutions should be refrigerated when not in use. The shelf life of the stock solution in a tightly capped, refrigerated bottle is approximately 2 months. The working standard should be prepared every 2 weeks.

Although other antioxidants could be used, both $Na_2S_2O_5$ and ascorbate gave much larger LCEC responses than desired, occluding early peaks of interest. Cysteine is an excellent antioxidant in solution but oxidizes poorly at a glassy carbon electrode at these potentials.

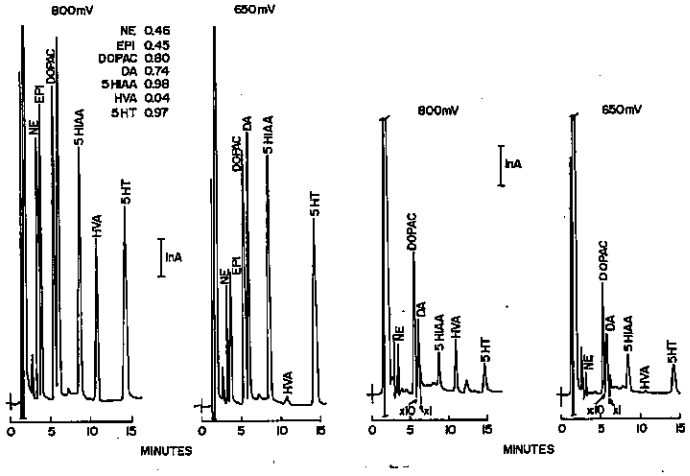

Figure 3. Simultaneous parallel-adjacent electrode chromatograms of biogenic amine standard solution and brain tissue homogenate. Conditions as described in text.

References

1. J. Wagner, P. Vitali, M.G. Palfreyman, M. Zraika, and S. Huot, J. Neurochem., 38(1982) 1241-1254.

2. W.H. Lyness, Life Sciences, 31(1982) 1435-1443.

3. G.S. Mayer and R.E. Shoup, J. Chromatogr., 255(1983), 533-544.