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SIMULTANEOUS MULTIPLE ELECTRODE LIQUID CHROMATOGRAPHIC–ELECTROCHEMICAL ASSAY FOR CATECHOLAMINES, INDOLEAMINES AND METABOLITES IN BRAIN TISSUE

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SUMMARY

   To enhance the selectivity of liquid chromatographic–electrochemical assays for biogenic amines and metabolites in brain tissue, multiple electrode transducers were investigated. Two configurations of dual working electrodes were examined: parallel–adjacent and series arrangements. Using the raw detector currents with each configuration, peak-height ratios from simultaneously generated chromatograms were calculated to assess the selectivity of the instrument for direct injections of brain tissue supernatant. Ratios were consistent with injections of standards. Nearly coeluting peaks such as norepinephrine and 3-methoxy-4-hydroxyphenylglycol were resolved by using dual detector electrodes in series; only the catecholamines were detected at the downstream electrode owing to their electrochemical reversibility. The scheme was applicable to the assay of norepinephrine, 3,4-dihydroxyphenylacetic acid, dopamine, homovanillic acid, serotonin, 5-hydroxytryptophan and 5-hydroxyindole-3-acetic acid in brain tissue in less than 15 min.

INTRODUCTION

   Over the last several years, it has been popular to employ liquid chromatography–electrochemical (LC–ElCD) techniques for the analysis of biogenic amines in biological samples. The procedure involves separation of sample constituents by liquid chromatography prior to their direct oxidation at a carbon electrode in a thin-layer electrochemical cell. The technique is reasonably selective since two requirements, retention time and redox activity at the selected potential, must be met simultaneously. The sensitivity of the instrument permits detection limits of *ca.* 0.2 pmoles.
   In regions of the brain where both dopamine and serotonin metabolism are involved, it is desirable to measure both of these compounds as well as their metabolites and precursors. Since the diversity of functional groups encountered does not permit selective extractions such as the aluminium oxide adsorption step used with catechols, the "brute force" approach of injecting brain tissue homogenate directly is required. The selectivity of the instrumentation becomes paramount, particularly for solutes with capacity factors less than 2.
   We have recently explored the use of multiple-electrode transducers as another
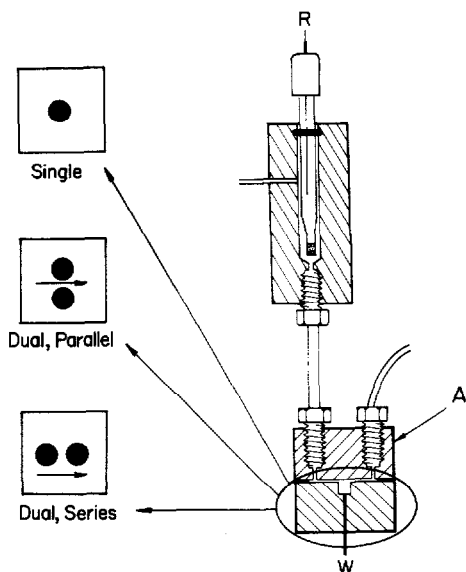
Fig. 1. Options for a dual electrode, thin-layer electrochemical detector. The auxiliary electrode is the entire top half of the cube and is constructed from type 316 stainless steel.

dimension in selectivity. These devices place at least two working electrodes in the thin-layer flow stream; the electrodes are usually held at different applied potentials (Fig. 1). The *parallel* mode is analogous to dual-wavelength UV-absorbance detection; two chromatograms are obtained simultaneously by operating at two different potentials. With *series* dual electrodes, one electrode is upstream of the other; the products of its reactions may be monitored downstream or excluded, depending on the electrochemistry. Blank[1] described circuitry for monitoring dual columns using separate transducers, and more recent publications[2,3] have described series dual electrode configurations for urinary catecholamines[2] and phenolic acids[3]. Here we report on the use of both series and parallel dual electrode detectors for the assay of seven neurochemically important species in brain tissue.

MATERIALS AND METHODS

Instrumentation

A Bioanalytical Systems LC-304B liquid chromatograph was used for all experiments. The detector consisted of 2 LC-4B electronic controllers operating in tandem using the standard interconnect cable, and a modified LC-17 glassy carbon transducer (Fig. 1). The top half of the working electrode cube was replaced by a stainless-steel block of identical dimensions; it served as the auxiliary electrode. In the bottom half, two glassy carbon electrodes 3 mm in diameter were symmetrically spaced along the center line 0.020 in. apart. Each LC-4B controller provided the operating potential, sensitivity, time constants, and output signal for one of these electrodes. A 250 × 4.6 mm Biophase ODS 5-μm column (Bioanalytical Systems) provided the separations. The flow-rate was 1.6 ml/min.

Mobile phases

A stock buffer was prepared as follows: 28.3 g monochloroacetic acid, 9.35 g sodium hydroxide, 400 mg of sodium octyl sulfate, and 0.5 g of Na_2EDTA were dissolved in 2 l of deionized, distilled water. To prepare mobile phase, sufficient stock buffer was added to 70 ml of acetonitrile to make 2 l of solution, which was then filtered through a 0.20-μm membrane filter (Rainin). After filtering and removing vacuum, 36.0 ml of UV-grade tetrahydrofuran (THF) (Burdick and Jackson, Muskegon, MI, U.S.A.) were mixed gently with the solution. This mobile phase was capped at all times to prevent THF evaporation. In some instances, a mobile phase of 160 ml of acetonitrile and stock buffer to make 2 l was used. This mobile phase was filtered and degassed after mixing.

Reagents

Norepinephrine bitartrate (NE), epinephrine bitartrate (EPI or E), dopamine (DA), homovanillic acid (HVA), serotonin (5HT), 5-hydroxyindole-3-acetic acid (5HIAA), 3,4-dihydroxyphenylacetic acid (DOPAC), and 5-hydroxytryptophan (5HTP) were obtained from Sigma, Aldrich or Calbiochem and used as received. Standard solutions containing 50 μg/ml and 50 ng/ml of each solute (as a mixture) were made up in 0.05 M perchloric acid containing 0.1 % cysteine as an antioxidant. Without cysteine, a loss in DOPAC was obvious upon standing at room temperature. The cysteine is not retained chromatographically.

Sample preparation

Adult Sprague Dawley rats weighing 350–400 g were decapitated, and their brains were removed rapidly and placed on chilled glass plates over ice. The brain stem (which includes the medulla oblongata and pons in this case), hypothalamus, and striatum were dissected as described by Glowinsky and Iversen[4]. The mean weights of excised tissue were 160 mg of stem, 40 mg of hypothalmus, and 60 mg of striatum. Tissue parts were placed in 2 ml of ice cold 0.05 M $HClO_4$ and sonicated for 3 min at 0°C, then centrifuged at 15,000 g for 20 min at 4°C. The supernatant was removed and stored in Eppendorf polypropylene tubes at -70°C.

LC–EICD analysis

Typically 100 μl of the sample were injected; the sensitivity was 5, 10 or 20 nA f.s. For the parallel–adjacent arrangement, the potentials were 800/650 or 800/700 mV *vs.* Ag/AgCl–3 M NaCl. The current ratio was expressed as the peak current at the low-potential electrode divided by that at the higher potential. The higher-potential electrode was used for quantitative calculations. Calculations of supernatant concentrations were performed by direct comparison of sample peak heights to those of a 100-μl injection of an external standard containing all seven neurochemicals, using the higher-potential electrode.

For the series arrangment, the upstream electrode was held at $+850$ mV while the downstream electrode was at -150 mV. The collection efficiency was defined as the downstream current divided by the upstream current. Quantitation using both electrodes was used by comparing peak heights of unknowns to standards. In cases where interferences were present, the more selective downstream electrode was used for quantitation.
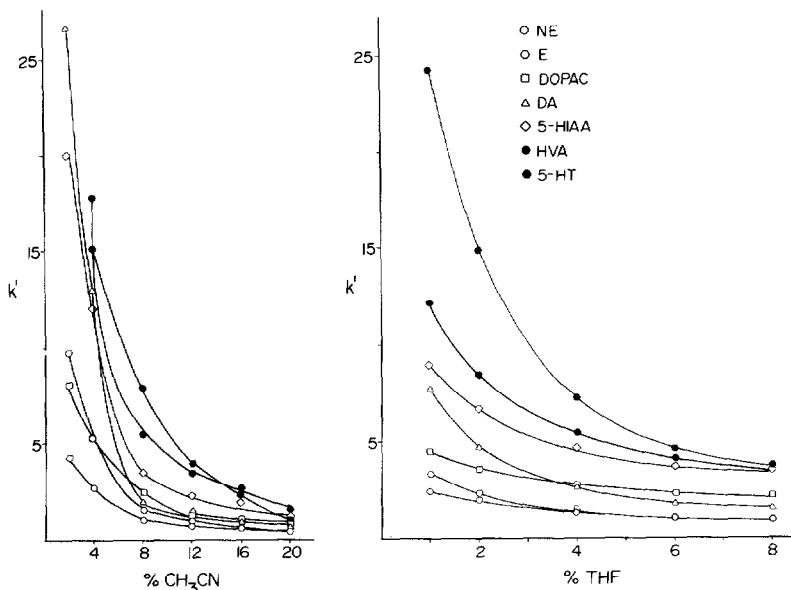
Fig. 2. (A) k' vs. % acetonitrile, and (B) k' vs. % THF using the stock buffer containing $8.6 \cdot 10^{-4}$ M sodium octyl sulfate. Flow-rate: 1.6 ml/min; column: 250 × 4.6 mm Biophase ODS, 5-μm.

RESULTS AND DISCUSSION

Chromatography

In order to compress the separation into the smallest possible time span, the use of multiple solvents and ion-pairing agents was investigated. It is common practice to use ion pair agents with concentrations between 0.1 and 5 mM with 0% to 15% methanol[5,6]. The ion pairing provides sufficient retention for poorly retained solutes such as norepinephrine and epinephrine while the added solvent reduces excessive retention for the indoles and HVA.

Using multiple solvents, resolution of all seven neurochemicals in tissue extracts could be achieved with capacity factors (k') of less than 10 (retention time approx. 14 min), using isocratic elution. The behavior of using only acetonitrile as modifier (Fig. 2A) allowed unexpected selectivity differences. For example, the elution order for 5HT and HVA changed twice in going from 4 to 15% (v/v) acetonitrile; DA and 5-HIAA and EPI and DOPAC interchanged once. However, the requirement that $k' < 10$ for all solutes prevented adequate resolution of DA and DOPAC. Using only 2% THF and no acetonitrile as modifier permitted separation of all but 5HT in the desired time span (Fig. 2B). EPI and NE coeluted at THF concentrations greater than 4%. Only DA and DOPAC inverted when using THF, at approximately 3.9%.

The role of THF at 3.5% (v/v) acetonitrile is shown in Fig. 3. Satisfactory resolution of all seven compounds is achieved at 1.8% THF–3.5% acetonitrile, with a maximum k' of 8.9. The effect of the two solvents together is dramatic; an error of only 0.2% (v/v) THF at 1.8% THF can move 5HT from 14 to 20 min, without appreciably altering the capacity factors of NE, EPI, 5-HIAA and HVA. Glajch *et*
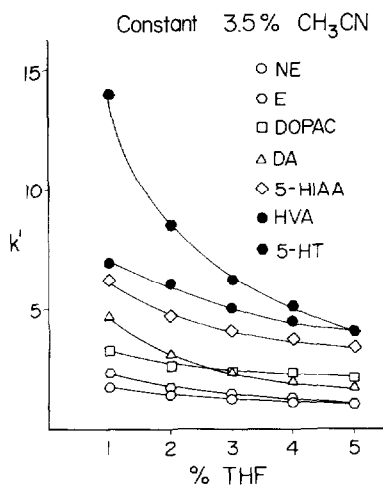
Constant 3.5% CH₃CN

○ NE
○ E
□ DOPAC
△ DA
◇ 5-HIAA
● HVA
● 5-HT

% THF

▲ NE   Electrode A
● EPI
○ DOPAC
△ DA
□ 5HIAA
○ HVA
■ 5HT

Current (nA)

300 400 500 600 700 800
E

Fig. 3. Capacity factor *vs.* % THF using 96.5% stock buffer and 3.5% acetonitrile. Other conditions as in Fig. 2.
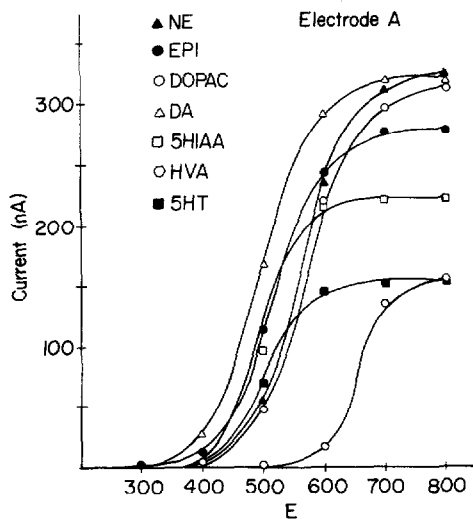
Fig. 4. Hydrodynamic voltammograms for several biogenic amines and metabolites using a glassy carbon electrode and a mobile phase containing 3.5 parts acetonitrile, 96.5 parts stock buffer, and 1.8 parts THF. Other conditions as in Fig. 2. Potentials in mV *vs.* Ag/AgCl–3 *M* NaCl.

al.[7] have described symbiotic effects with multiple solvents which cannot be obtained by any solvent operating singly; it is apparent that similar circumstances may be operating here. 3,4-Dihydroxyphenylglycol and ascorbic acid eluted in the void volume, while 3-methoxy-4-hydroxyphenylglycol (MHPG) was resolved slightly before norepinephrine. Since THF is volatile, the use of a capped mobile phase reservoir bottle is essential; otherwise, the mobile phase becomes more polar as evaporation occurs and the retention time precision is unsatisfactory. EDTA slowly precipitates at concentrations of 0.75 g/l when the mobile phase contains THF; the concentration was correspondingly reduced to 0.25 g/l. Without EDTA, baseline oscillation with a period of 10–20 min occurs, particularly using dual series electrodes.

Depending on the age of the column, an alternative mobile phase containing 92% stock buffer and 8% acetonitrile provided a useable separation (see Fig. 2A). However, MHPG and NE were not resolved, except through the use of series multiple electrodes (see below).

Multiple electrode detection

Parallel–adjacent configuration. The parallel–adjacent arrangement can provide qualitative estimates of peak purity through the generation of current ratios. This mode is analogous to dual-wavelength UV-absorbance detection, where simultaneous chromatograms at the two wavelengths as well as the absorbance ratios at any point in time are provided. Here the detector electrodes are operated at two different potentials along the hydrodynamic curve. For multiple electrode detection, it is therefore wise to establish current–voltage curves for the solutes of interest; a
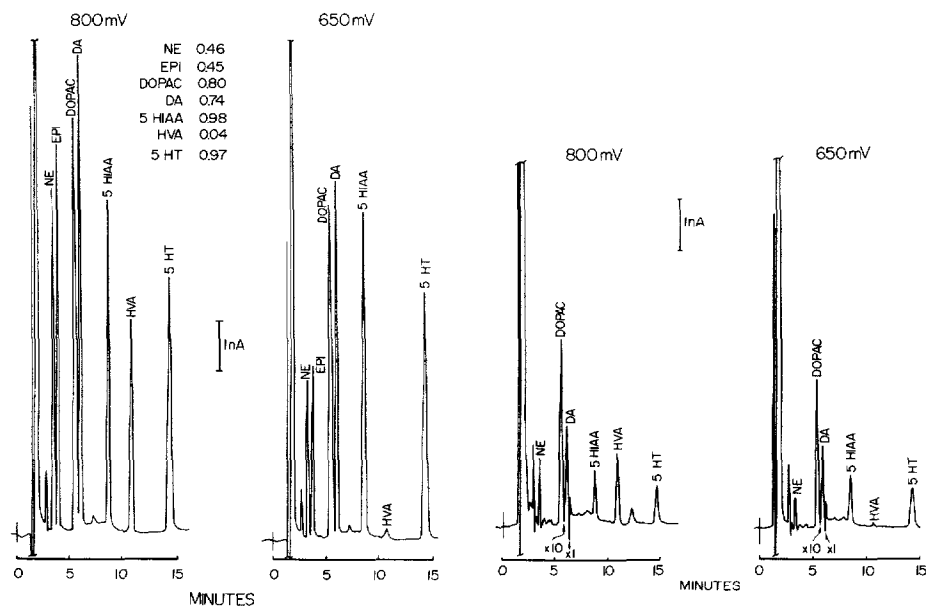
Fig. 5. Simultaneous parallel, dual-electrode chromatograms of a standard mixture (left) and brain tissue homogenate (right). The peak-height ratios (i_{650}/i_{800}) are listed between the dual chromatograms of standards. Conditions: mobile phase: 3.5 parts acetonitrile, 96.5 parts stock buffer and 1.8 parts THF. Other conditions as in Fig. 2.

standard mixture was chromatographed several times with each run at a different operating potential. The curves for a dual electrode cell are shown in Fig. 4. All of these compounds except HVA oxidize under diffusion-limiting conditions at a potential of +800 mV. Depending on the past history of the electrode surface, the other electrode may or may not have identical response curves. Our experience has indicated that freshly polished electrodes agree to within 10%; but when one electrode is operating under much higher potential, the half-wave potential for the latter may eventually be skewed more positive. In our experiment one electrode was operated in the diffusion-limiting region for catecholamines, while the other electrode was poised at 150 mV less (Fig. 5). These settings provide peak-current ratios ranging from 0.1 for HVA to near 1.0 for 5-HT and 5-HIAA.

TABLE I

PEAK CURRENT RATIOS FOR PARALLEL, DUAL ELECTRODES AT VARIOUS OPERATING POTENTIALS USING A FIXED 150 mV "WINDOW"

Operating conditions as in Fig. 4. nd: ratio is approximately 0.0.

Potentials	NE	E	DOPAC	DA	5-HIAA	HVA	5HT
650/800 mV	0.95	0.96	1.02	1.02	1.05	0.10	1.02
550/700 mV	0.29	0.29	0.64	0.66	0.65	nd	0.70
450/600 mV	0.05	0.05	0.06	0.10	nd	nd	nd

TABLE II

WITHIN-DAY AND DAY-TO-DAY REPRODUCIBILITY OF PEAK CURRENT RATIOS USING PARALLEL DUAL ELECTRODES AT 650/800 mV

Chromatographic conditions as in Fig. 4.

Day*	n**	NE	DOPAC	DA	5-HIAA	HVA	5HT
3	2	0.62	0.88	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 ± 0.05	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.

TABLE III

AGREEMENT BETWEEN SAMPLES AND STANDARDS OF CURRENT RATIOS AT 650/800 mV, USING PARALLEL DUAL ELECTRODES

Values in parenthesis refer to the concentration range examined for ten different rat brain homogenates.

	Sample	Standard
NE	0.48 ± 0.03 (4.2–49 ng/ml)	0.46 ± 0.007 (47 ng/ml)
DOPAC	0.80 ± 0.03 (1.1–36 ng/ml)	0.80 ± 0.006 (55 ng/ml)
DA	0.75 ± 0.03 (3.9–260 mg/ml)	0.74 ± 0.01 (64 ng/ml)
5-HIAA	0.98 ± 0.08 (1.7–30 ng/ml)	0.98 ± 0.01 (50 ng/ml)
HVA	* (1.0–31 ng/ml)	0.05 ± 0.01 (50 ng/ml)
5-HT	0.98 ± 0.02 (1.5–87 ng/ml)	0.96 ± 0.007 (50 ng/ml)

* Peak height at 650 mV fell below 1 % of 10 nA f.s.; ratio was not calculated.

For various pairs of potentials along each compound's hydrodynamic voltammogram, the peak-current ratio approaches zero as the "window" is shifted negative (Table I). For freshly polished electrodes operated at 650/800 mV, the ratios for all peaks are nearly unity, since the electrodes are placed on the diffusion-limiting plateau. However, at 550/700 mV, differentiation of the ratios according to electroactive functional groups occurs. NE and EPI, both β-hydroxylated catecholamines, gave ratios of 0.30, whereas DA and DOPAC gave ratios of 0.65. The latter compounds are not β-hydroxylated. Such differences were unexpected from minor side-chain substituent changes. The 5-hydroxyindoles gave similar ratios here but further differentiation was possible at potentials between 650/800 mV and 550/700 mV. Homovanillic acid was easily distinguished at 650/800 mV due to its 3-methoxy-4-hydroxyphenyl group.

Table II provides day-to-day reproducibility data for a dual working electrode cell for several days after polishing. The ratio values eventually diminish; this is due partly to (1) drift in the half-cell potential of the reference electrode due to diffusion of chloride ions into the mobile phase, and (2) the changing intrinsic reactivity of the electrode surface. Since it is common to calibrate the chromatograph daily, recalculation of response ratios at this frequency is a simple matter.

An electrode which had been in continuous use for several days (e.g., not freshly polished) was used to test the agreement of ratios between standards and brain homogenates (Table III). Standards were injected between every three brain samples to check ratio reproducibility. The ten brain part homogenates used to provide the mean sample data were of varying concentrations since parts of different size and origin were injected. Note that the ratios are easily distinguishable in terms of the electroactive functional group. Over a series of twenty injections the ratios were sufficiently precise for qualitative purposes. Interspersing the samples between standards had no significant effect on the value or precision of the calibration ratios.

Precision of the ratio against amount injected is described in Fig. 6. The ratio of any analyte at any pair of potentials should be constant, subject only to the problems of adequate determination of the peak height at high gain and the linearity of the detector in its upper dynamic range. Both electrodes gave correlation coefficients greater than 0.9984, and the slope of the ratio vs. amount injected for NE was $1.02 \cdot 10^{-4}$/ng with a y-intercept (zero amount injected) of 0.347. This is very compa-
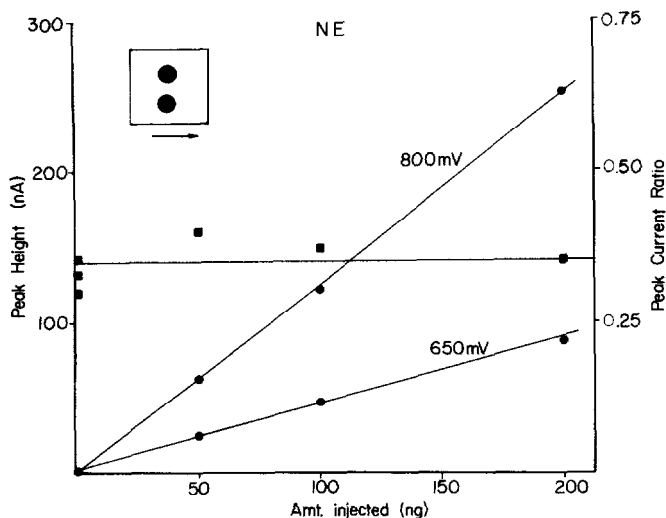
Fig. 6. Linearity of peak height (nA) *vs.* amount injected for each of two parallel glassy carbon electrodes. The peak-current ratio ($i_{p,650}/i_{p,800}$) *vs.* amount injected is the horizontal line. Conditions as in Fig. 4.

rable to the ratio obtained as the ratio of the slopes of the two curves (0.352).

In summary, we have found that parallel dual electrodes can: (1) provide reproducible peak-height ratios for both brain samples as well as standards, (2) discriminate between closely-related redox functional groups by appropriate selection of the operating potentials, and (3) offer qualitative information on the redox identity of unknown eluting peaks.
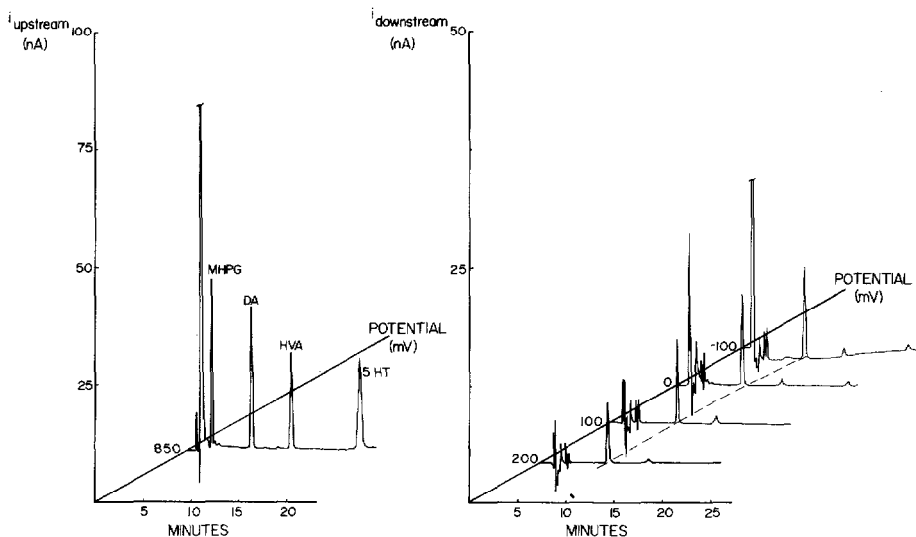

Fig. 7. Simultaneous series dual electrode chromatograms. Left side: upstream glassy carbon electrode, +850 mV, 20 ng of each solute were injected. Right side: downstream glassy carbon electrode operated at the indicated potentials. Only dopamine demonstrates an appreciable collection efficiency (0.32). Mobile phase: acetonitrile–stock buffer (8:92). Other conditions as in Fig. 2.

TABLE IV

COLLECTION EFFICIENCY VALUES FOR BIOGENIC AMINES AND METABOLITES

Conditions as in Fig. 7. i_d = downstream current; i_u = upstream current.

Compound	Functional group	Collection (i_d/i_u)
NE	Catechol	0.30
DOPAC	Catechol	0.31
DA	Catechol	0.31
5-HIAA	Hydroxyindole	0.05
HVA	Vanillyl	0.05
5HT	Hydroxyindole	0.02
MHPG	Vanillyl	0.05

Series configuration

The primary benefit of series electrodes in this assay is improved selectivity. The flow is arranged so that the reaction products from an upstream electrode pass over a second electrode. This two-stage process is analogous to fluorescence detection; here, the additional specificity accrues by the selective detection of only quasi-reversible species. However, both the "absorbance" and "fluorescence" chromatograms are available simultaneously. For example, series electrode chromatograms for MHPG, DA, HVA, and 5HT are shown in Fig. 7. The upstream electrode was operated at + 850 mV and the potential of the downstream electrode was varied from + 200 mV to − 100 mV in steps, after successive injections. The maximum downstream signal was obtained at − 150 mV vs. Ag/AgCl. Only the catechol (dopamine) gives an appreciable peak downstream, due to its electrochemical reversibility on this very short time scale. Since the transit time between the electrodes is 110 msec at 1.6 ml/min, substances which undergo fast homogeneous reactions to electroinactive products following the first electrochemical step (an EC mechanism) may still yield an appreciable reverse peak. A case in point is uric acid, which is irreversible on the time scale of a 3-sec cyclic voltammetry experiment but quasi-reversible here[8]. The ratio of the downstream peak current divided by the upstream current is termed the "collection efficiency"; values for these compounds are listed in Table IV. This ratio is an important measure of peak purity and also provides the analyst with some hint as to the types of functional groups being oxidized or reduced in unknown peaks. Due to the dimensions of the flow-cell and the electrodes, the collection efficiency may theoretically approach only 0.4 (refs. 9 and 10).

The series electrodes resolved a potential interference problem in the brain tissue samples. Using the 8 % acetonitrile mobile phase, NE and an unknown tissue constituent were not resolved (Fig. 8, bottom left). In homogenate injections, the retention times upstream and downstream did not agree exactly (although they differed by less than 0.1 min), and the collection efficiency in samples varied from 0.20 to 0.32, whereas the standard always gave values of 0.30 for norepinephrine. Quantitation using either the upstream or downstream electrode was consistent for DOPAC and DA but inconsistent for NE (Table V). The occasional positive error is consistent with the discrepancy in collection efficiency and indicates the presence of an interference upstream. Based on the retention time and the poor collection efficiency,
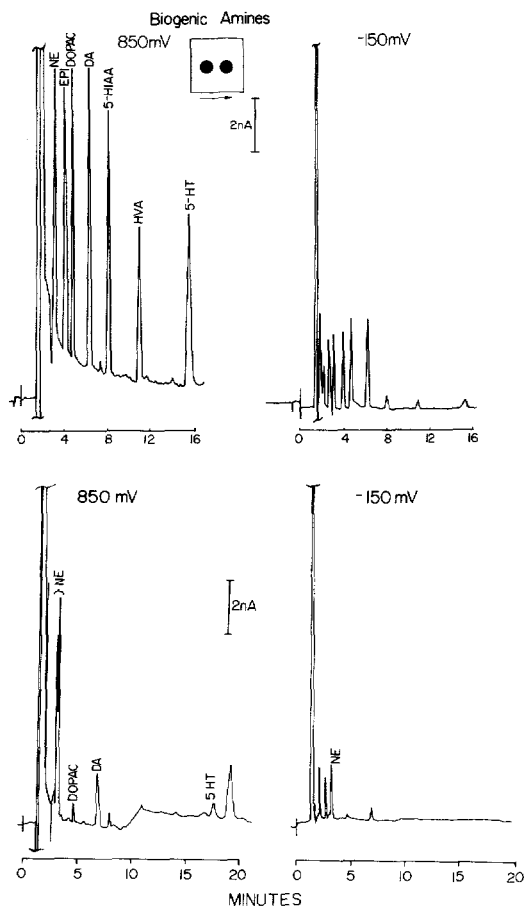
Fig. 8. Simultaneous series dual electrode chromatograms of a standard mixture (top) and brain tissue homogenate (bottom). The peak in rat brain immediately preceding NE at the 850 mV electrode is MHPG, which is subsequently filtered out at the downstream electrode at −150 mV. Conditions as in Fig. 7.

MHPG was suspected and later demonstrated using these same procedures to be the interference.

Since the downstream electrode was more selective, quantitation of each catecholamine was carried out there. The initial void volume response downstream is much less disturbing than at the upstream sensor and the peaks are well resolved. In essence the analyst has accomplished instrumentally what would otherwise demand a separate, wet chemical liquid–solid adsorption step onto alumina[11]. The alumina step does sometimes offer an important preconcentration factor: reducing the original sample volume. However, if the initial sample volume is already small, an alumina cleanup —due to its 60–70 % recovery— only loses sample, and the dual series approach becomes very attractive in both speed and simplicity. If only catecholamines are to be measured, the current upstream need not even be monitored, since the electrode is simply considered an on-line reactor.

Since the noise at either electrode is approximately equal, the minimum de-

TABLE V

BRAIN HOMOGENATE NE, DOPAC AND DA CONCENTRATIONS AS DETERMINED BY EACH OF TWO SERIES GLASSY CARBON ELECTRODES

Conditions as in Fig. 8.

Sample	NE		DOPAC		DA	
	850 mV	−150 mV	850 mV	−150 mV	850 mV	−150 mV
1	42	7.7	47	46	308	302
2	27	★	38	38	252	249
3	39	★	34	34	221	224
4	39	38	★	★	12	★
5	10	★	★	★	★	★
6	30	★	34	32	180	176
7	27	28.	★	★	★	★
8	31	33	★	★	7.2	★
9	42	37	★	★	6.8	★
10	34	★	28.9	27	213	202

★ The peak height fell below 1 % of full scale (20 nA).

tectable quantity injected (MDQI) is no better than using a single working electrode. For an MDQI downstream equal to the upstream electrode, the noise downstream must diminish by a factor equal to the inverse of the collection efficiency. In actual tests, an upstream electrode operated at +850 mV yielded an MDQI of 100 pg for norepinephrine while the downstream electrode at −150 mV gave MDQI of 360 pg. Hence the series dual detector is more advantageous for its selectivity than sensitivity.

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