Journal of Chromatography, 117 (1976) 35–46

CHROM. 8637

DUAL ELECTROCHEMICAL DETECTOR FOR LIQUID CHROMATOGRAPHY

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(First received June 16th, 1975; revised manuscript received August 15th, 1975)

SUMMARY

Selectivity, sensitivity, and wide dynamic range are all displayed by the dual electrochemical detector for use with liquid chromatography. Exhibiting a linear range of about 10^5, it is, at the same time, capable of sensing sub-picomole quantities of certain species. Moreover, only those compounds which display electroactivity (oxidation or reduction) at the chosen potential(s) are detected.

Two distinctive modes of operation are available with the dual detector. For compounds which overlap chromatographically, but have differing electrochemical formal potentials, the system offers an instrumental separation. For routine investigations, the system provides significant time reductions without causing proportionate increases in cost.

INTRODUCTION

The lack of detector sensitivity in high-performance liquid chromatography has been a major limitation in the application of the inherent capabilities of this technique[1]. Initial attempts to utilize electrochemical detection[2–4] pointed the way to the recently described thin-layer carbon paste detector[5]. This approach has provided excellent sensitivity by offering detection limits of sub-picograms for some compounds[6], while maintaining a rather large dynamic operating range. Incorporating much of the basic design philosophy of the original system, we have been able to provide capabilities for both dual series analysis and dual parallel analysis. The former application, comparable to dual wavelength monitoring in UV detection, allows the unique instrumental separation of physically overlapping components. The latter, parallel analysis, on the other hand, aids in shortening the time required for routine investigations. Both methods are, however, attainable with the same instrumental and electronic equipment.

EXPERIMENTAL

Chemicals

Dopamine hydrochloride (99%) and norepinephrine hydrochloride (99%)

were purchased from Aldrich (Milwaukee, Wisc., U.S.A.). 6-Hydroxydopamine hydrobromide was obtained from Regis (Chicago, Ill., U.S.A.). 3,4-Dihydroxybenzylamine was a gift from Dr. Don Nerland and the late Dr. Ed Smissman, Department of Medicinal Chemistry, University of Kansas.

Apparatus

The liquid chromatograph is constructed entirely from separate components. A simplified drawing of the basic set-up, for the dual series analysis, is shown in Fig. 1. To prevent oxidation of susceptible components, the solvent, 0.1 *M* $HClO_4$, is heated and degassed with oxygen-scrubbed nitrogen. The pumping system utilized is a Milton Roy reciprocating minipump (max. flow-rate, 160 ml/h, max. pressure, 5000 p.s.i.). The damping coil and other tubing connections are constructed of 0.031 in. I.D. PTFE tubing. The injection port(s) and standard connectors are obtained from the Cheminert Division of Laboratory Data Control (Riviera Beach, Fla., U.S.A.). The glass columns, also available from the same supplier, are 50 cm × 2 mm I.D., and packed with a pellicular strong cation-exchange resin, Zipax SCX, from DuPont (Wilmington, Del., U.S.A.).

Being by no means specific to this particular instrumental arrangement, the detector is attached to the column via a standard Cheminert fitting. As shown in Fig. 2 for the dual series application, the detector is simply two blocks of Lucite, appropriately machined. The details of construction are readily obtained by analogy to the previous single detector[5]. The electrode wells are packed with carbon paste and rubbed to a smooth surface on computer cards[7]. External electrical connections to the wells are provided by Pt wires.

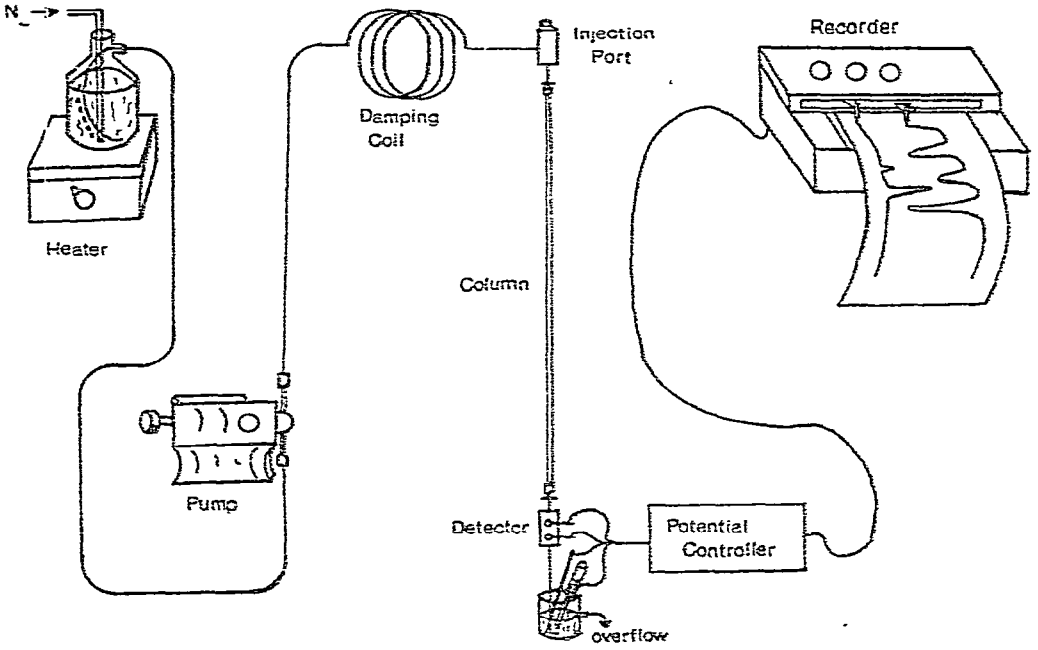

Fig. 1. Apparatus for liquid chromatography with dual series electrochemical detection.

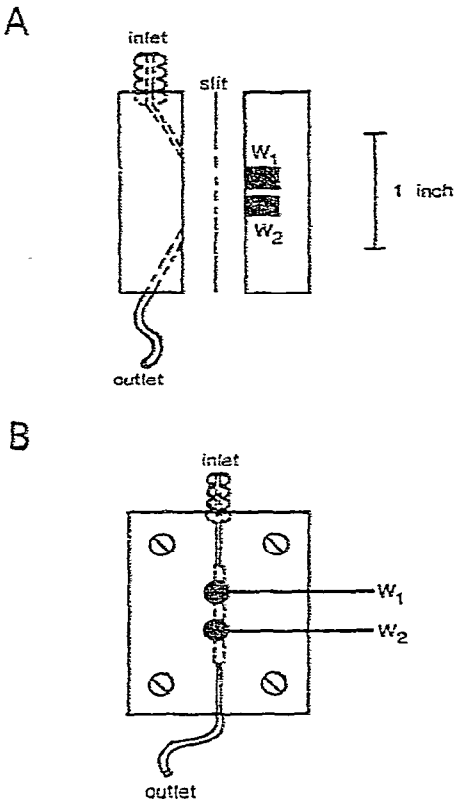

Fig. 2. Electrochemical detector for dual series analyses. The slit is a 0.005-in.-PTFE sheet with the shown rectangular opening cut out to connect the inlet stream to the outlet stream. W_1 and W_2 are working electrodes one and two, respectively: each is packed with carbon paste (see text). (A) Side view, dissembled. (B) Front view, assembled.

The outlet from the detector flows into a small suction flask, the side arm being used as an overflow. This flask contains both the reference (saturated calomel electrode, SCE) and auxiliary (Pt wire) electrodes. The electronics used to control the potentials of the electrodes and monitor the currents are described below. The outputs, directly proportional to the currents flowing in each of the carbon paste electrodes, are directed to an Omniscribe (Houston Instruments, Austin, Texas, U.S.A.) two-pen recorder.

Circuit design

The circuit for potential control and current monitoring of each of the two working (carbon paste) electrodes, W_1 and W_2, is shown in Fig. 3. The potential controlling portion, part A, and the current monitoring section, part B, for W_1 are analogous to those previously reported[8]. The potential of the first working electrode, W_1, with respect to the reference electrode is simply

$$E_1 = -E_a$$

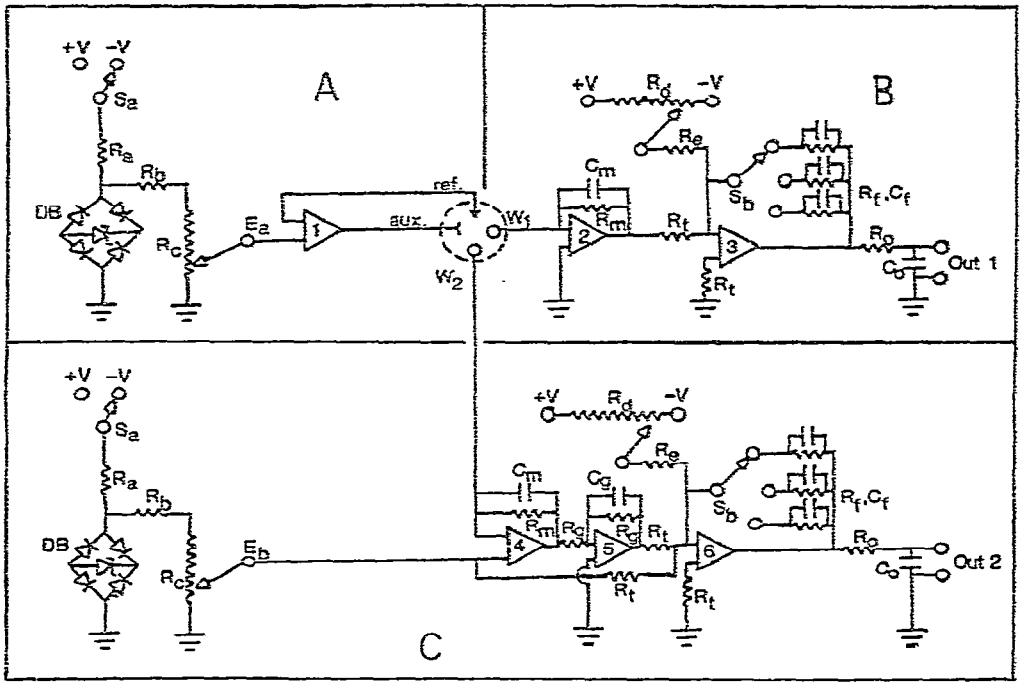

Fig. 3. Circuit diagram for dual electrochemical detector. See text for explanation.

The corresponding potential of the second working electrode is given by:

$$E_2 = E_b - E_a$$

The switches, both labelled S_a, in conjunction with the diode bridges, DB, in sections A and C help establish a stable, selectable, positive or negative potential at E_a and E_b, respectively. The arrangement shown in Fig. 3 obviously necessitates the setting of the E_1 potential prior to that of E_2. However, the very small number of times this is actually done in practice precludes the need for any further sophistication.

The final outputs, Out_1 and Out_2, are voltage representations of the currents, i_1 and i_2, flowing in W_1 and W_2, respectively:

$$Out_1 = i_1 \frac{R_m R_f}{R_t}$$

$$Out_2 = -i_2 \frac{R_m R_f}{R_t}$$

For convenience, the values of R_m (5 Mohms), R_t (2 Kohms), and R_f (50, 20, or 10 Kohms) were chosen to give output functions of 8, 20, and 40 nA/V. Since the recorder has selectable ranges from 0.01–10 V, the effective outputs extend from 80 pA to 400 nA full scale.

It should be noted that, with no current flowing in W_2, the operational am-

plifier, OA4, is really a voltage follower with an output of E_b. Using both the output from the inverting operational amplifier, OA5, $(-E_b + i_2R_m)$ and E_b itself as inputs to OA6 removes this potential offset problem.

Extension of this system to multiple (three or more), simultaneous analyses is straightforward. With the series arrangement, one would incorporate another set of electronics equivalent to part C (Fig. 3) for each additional working electrode. This same scheme could also be employed in multiple parallel analyses. However, with the parallel analyses, one normally desires $E_b = 0$ for all the added working electrodes (*vide infra*). Thus, it would be more reasonable, in this latter case, to remove the potential control (S_a, R_a, R_b, R_c, DB) of each additional part C and simply ground E_b. Likewise, OA5 and the R_t connected directly to E_b could be completely eliminated, since there would be none of the previously mentioned offset problems with OA4.

More detailed construction information for the circuit is available from the author upon request.

RESULTS AND DISCUSSIONS

Series analyses

The dual electrochemical detector is especially amenable to the separation of overlapping components of a chromatogram. A similar employment of potential

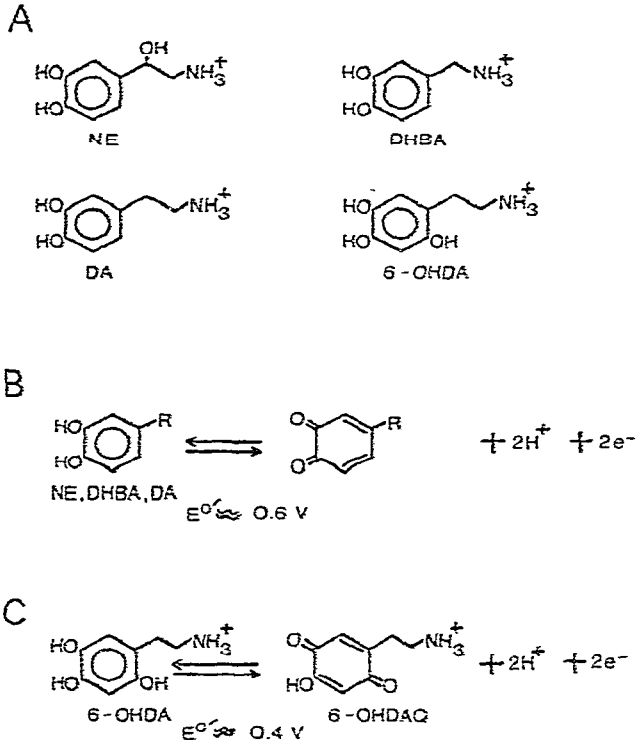

Fig. 4. A. Compounds used in this investigation. B. Oxidation mechanism for *o*-dihydroxy species. C. Oxidation mechanism for 6-hydroxydopamine.

selectivity was recently demonstrated by the single detector studies of Buchta and Papa on 1,4-naphthoquinone and benzil[9]. Complete separation and analysis of poorly resolved components by this characteristic is, moreover, conceptually quite comparable to the employment of either simultaneous UV and fluorescence (see, *e.g.*, ref. 10) or simple dual UV wavelength detection.

Our own recent interests have been concentrated upon the relationship of 6-hydroxydopamine (6-OHDA, see Fig. 4A) and catecholamines in the mammalian central nervous system[11,12]. The original idea of a dual detector was envisioned to provide an analysis for both the normal, endogenous brain components, norepinephrine (NE) and dopamine (DA) (Fig. 4A), and, additionally, any spurious 6-OHDA without lengthening the elution time presently[8] required. 3,4-Dihydroxybenzylamine (DHBA) is included as an internal standard for such samples. The oxidation mechanisms[11,13] for these molecules are shown in Figs. 4B and 4C. Due to the structural differences depicted, 6-OHDA is some 200 mV easier to oxidize than any of the other components; this potential difference is critical to the "separation".

As shown in Fig. 5 and Table I, 6-OHDA is not resolved from the other three components of concern, and is, in fact, almost completely isographic with NE. Separation and quantitation of all components can, however, be easily effected by using the arrangement shown in Figs. 1 and 2. The first, lower potential electrode (E_1 = +0.45 V) only "sees" 6-OHDA, while the second (E_2 = +0.8 V) responds to all

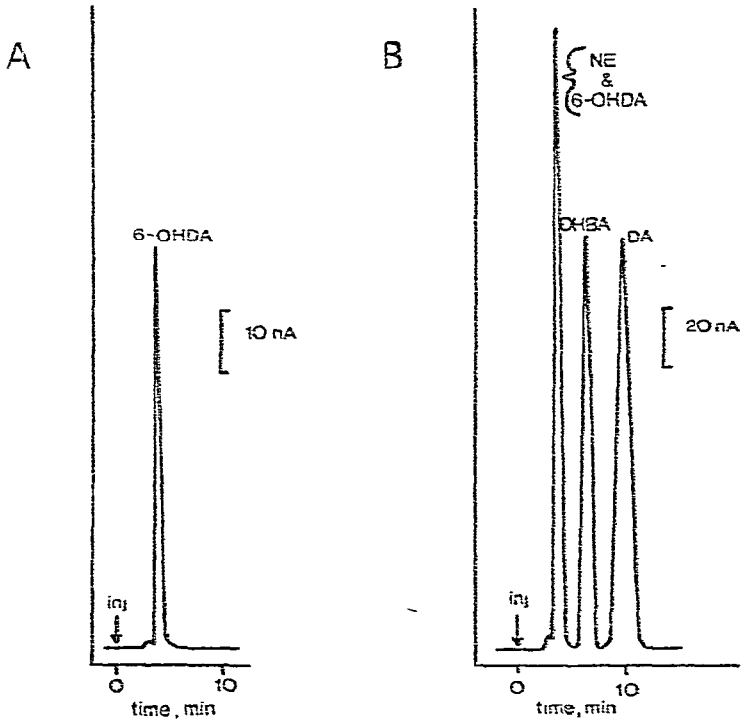

Fig. 5. Dual series chromatograms. 20-μl injection of a solution containing 7.21×10^{-5} *M* NE, 1.03×10^{-4} *M* DHBA, 2.22×10^{-6} *M* DA, and 3.7×10^{-5} *M* 6-OHDA. (A) First electrode (E_1 = +0.45 V). (B) Second electrode (E_2 = +0.80 V).

TABLE I

CHARACTERISTICS OF DUAL SERIES ELECTROCHEMICAL DETECTOR*

	Detector 1	*Detector 2*
Potential (V *vs.* SCE)	+0.450	+0.800
Retention time, t_R (min)		
NE		3.56 ± 0.03
DHBA		6.41 ± 0.03
DA		9.72 ± 0.05
6-OHDA	3.63 ± 0.04	
Response (peak height, pA/pmole)**		
NE		106 ± 3
DHBA		64 ± 1
DA		29.4 ± 0.4
6-OHDA	86 ± 2	96 ± 1
Linear range (pmole)		
NE		0.35 to 34,000
DHBA		0.67 to 92,000
DA		0.93 to 120,000
6-OHDA	0.67 to 26,000	0.46 to 30,000
Detection limit (pmole)		
NE		0.03
DHBA		0.06
DA		0.08
6-OHDA	0.07	0.07

* All injections by syringe. Uncertainties expressed as standard deviation for, at least, ten separate measurements. Flow-rate, 0.273 ± 0.008 ml/min.

** Averaged over the entire linear range.

species. In short, separation of 6-OHDA from the other three compounds has been achieved by instrumental rather than chromatographic means. Both detectors in this setup exhibit peak heights for the appropriate compounds which are directly proportional to the injected quantity (see Table I).

Appropriate corrections, in such separations, can be made for the contribution of 6-OHDA to the NE peak. But, this is only necessary if the 6-OHDA concentration is comparable to or greater than that of NE. Since such a result is not to be expected in the usual brain samples mentioned above[14], we have eliminated a discussion of these corrections.

However, 6-OHDA is so easily oxidized in 0.1 *M* $HClO_4$ that solutions of $\leqslant 10^{-7}$ *M* are readily converted to the quinone form (6-OHDAQ, Fig. 4C) upon being shaken in the presence of air. In fact, the samples of 6-OHDA mentioned previously were only maintained in the reduced state by constant coulometric (carbon cloth working electrode) reduction at +0.0 V *vs.* SCE. Quantitative transformation of a sample containing any concentration of 6-OHDA to the oxidized form can also be effected by the same coulometric technique, using a potential of +0.45 V. This potential, notably, does not oxidize NE, DHBA, or DA. But, 6-OHDAQ is much more stable than 6-OHDA, so, these solutions are much easier to handle. Thus, determination of catecholamine–6-OHDA mixtures was attempted after prior conversion of the 6-OHDA to 6-OHDAQ.

Analysis of a solution containing NE, DHBA, DA, and 6-OHDAQ is shown

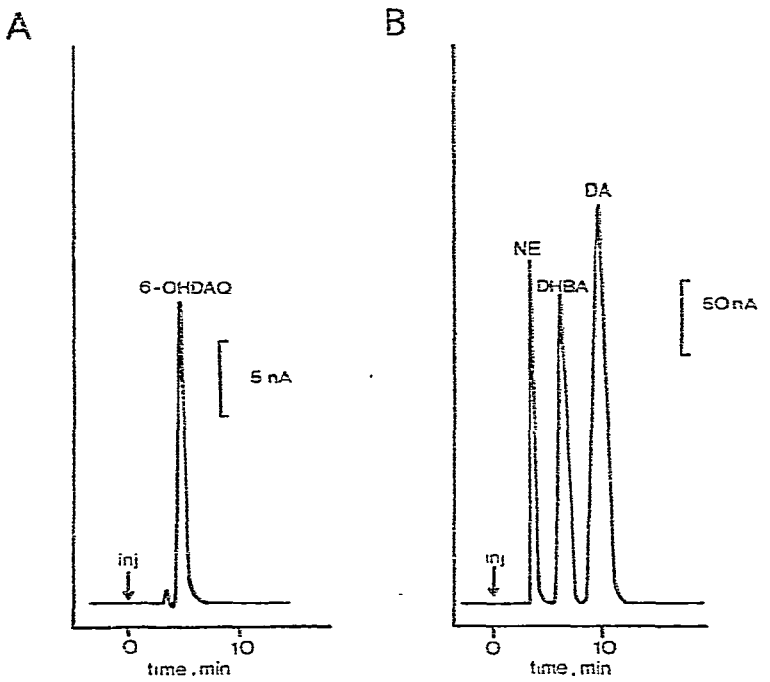

Fig. 6. Dual series chromatograms of NE, DHBA, DA, 6-OHDAQ mixture. Injection is 20 μl of a solution containing 1.09×10^{-4} *M* NE, 1.58×10^{-4} *M* DHBA, 4.43×10^{-4} *M* DA, and 9.43×10^{-6} *M* 6-OHDAQ. (A) First electrode response ($E_1 = +0.0$ V); (B) Second electrode response ($E_2 = +0.8$ V).

in Fig. 6. The potential of the first electrode is now set to reduce 6-OHDAQ ($E_1 = +0.0$ V): the second is again set to oxidize any NE, DHBA, DA, or 6-OHDA ($E_2 = +0.8$ V), the latter species being produced at the first electrode. Since the efficiency of these electrodes is only *ca.* 1%, any 6-OHDA appearing at the second electrode would only be *ca.* 1% of the injected 6-OHDAQ. Additionally, the 6-OHDAQ ($t_R = 5.02 \pm 0.04$ min under the same conditions as those in Table I) elutes directly between NE and DHBA. Significant interference by such 6-OHDA in either the NE or the DHBA peak would, thus, require very large relative injected amounts of 6-OHDAQ. Nonetheless, such interference, as for the direct 6-OHDA analysis, could be corrected if necessary.

As before, linearity, high sensitivity, and a wide dynamic range are exhibited for each of the components. NE, DHBA, and DA display results similar to those in Table I. 6-OHDAQ is linear in the range of 0.40 pmoles to 48 nmoles (20-μl injections). The lower limit of detectability for 6-OHDAQ is 0.04 pmoles, where the signal is approximately twice the noise. Thus, for the reasons given, 6-OHDA in these mixtures may be best analyzed by conversion to 6-OHDAQ prior to injection.

For those more general series applications where the arrangement outlined seems inconvenient, *e.g.*, where the product of the first electrode reaction is much more concentrated than the simultaneously eluting component detected at the second electrode, another approach is available. In these cases, the column output is split

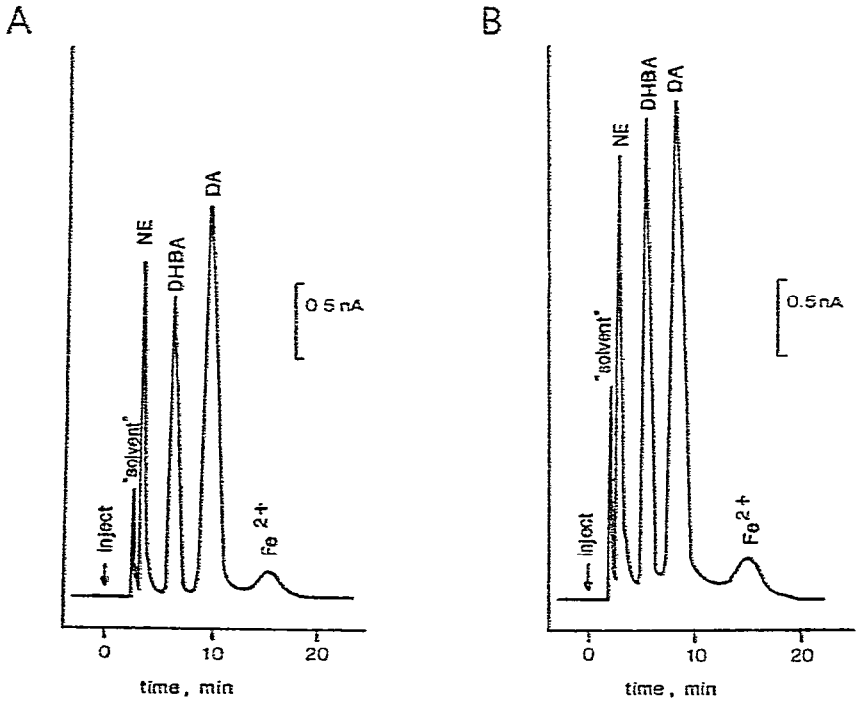

Fig. 7. Dual parallel chromatograms of NE, DHBA, and DA mixtures. Each chromatogram obtained from a 20-μl injection of a solution containing 2.36×10^{-6} M NE, 4.45×10^{-6} M DHBA, 1.04×10^{-5} M DA, and 1×10^{-3} M Na_2SO_3. (A) First column response ($E_1 = +0.8$ V); (B) Second column response ($E_2 = +0.8$ V). Note: Fe^{2+} peak frequently seen at lower sensitivities.

into two separate streams leading to two separate, single detectors. Hence, the first electrode's product is not seen at all by the second electrode.

Parallel analyses

Probably the most forthright application of this dual detector system is to routine analyses. It is obviously useful to shorten the time for such analyses since they frequently involve long elution times, many samples, or both. Yet, the cost of simply multiplying the instrumentation may be somewhat prohibitive. A typical case in which we have found this approach very useful is in the previously established determination of brain catecholamines[8]. Briefly, this technique employs the ratio of the catecholamine (either NE or DA) peak to that of the internal standard (DHBA) to establish the tissue concentrations of these compounds.

By splitting the inlet stream of the eluting solvent after the damping coil (see Fig. 1), the flow of the dual parallel apparatus is directed to two separate injection ports. These ports are, in turn, connected to two separate columns each with its own, single, electrochemical detector. The outlets from these detectors then flow into the same suction flask containing the auxiliary and reference electrodes. E_1, the potential of the detector on the first column, is set at +0.8 V *vs.* SCE ($E_a = -0.8$ V). E_2, the potential of the second column's detector, is also set at +0.8 V ($E_b = 0.0$ V).

The results of typical simultaneous injections into the dual parallel columns are given in Fig. 7 and Table II. Although it is clear from the chromatograms that the results are not reproducible from column 1 to column 2, each column is, nonetheless, independently self-consistent. Calibration curves —peak height (pA) *vs.* amount injected (pmoles)— are linear for each individual component on the separate columns. A typical dynamic range of $\geqslant 10^5$ is exhibited, covering from about 0.6 pmoles to 60 nmoles of substance injected. Quantities as low as 0.03 pmoles can be detected, but quantitation has not been attempted at this level. Careful electrical shielding and flow control of the system could certainly lead to both a wider dynamic range and a more sensitive limit of detection[5,15]. However, it was our purpose not to include unnecessary refinements, since these would not commonly be used in routine applications.

Probably the most important cause of the chromatographic differences between the two columns lies in the flow-rates, as given in Table II. The flow-rate in-

TABLE II

CHARACTERISTICS OF DUAL PARALLEL COLUMNS*

	Column 1	*Column 2*
Flow-rate (ml/min)	0.273 ± 0.007	0.308 ± 0.007
Dead volume (ml)**	0.056	0.084
Retention times, t_R (min)***		
NE	3.56 ± 0.03	2.98 ± 0.05
DHBA	6.41 ± 0.03	5.70 ± 0.04
DA	9.72 ± 0.05	8.96 ± 0.04
Peak width at half-height, $W_{\frac{1}{2}}$ (min)***		
NE	0.37 ± 0.01	0.38 ± 0.02
DHBA	0.83 ± 0.02	0.66 ± 0.01
DA	1.43 ± 0.04	1.06 ± 0.02
Peak height ratios***		
NE/DHBA	1.13 ± 0.05	0.86 ± 0.03
DA/DHBA	1.27 ± 0.03	1.14 ± 0.06
Response (peak height, pA/pmole)§		
NE	106 ± 3	134 ± 3
DHBA	64 ± 1	107 ± 2
DA	29.4 ± 0.4	43.8 ± 0.6
Linear range (pmole)		
NE	0.34 to 34,000	
DHBA	0.67 to 92,000	
DA	0.93 to 120,000	
Detection limit (pmole)		
NE	0.03	
DHBA	0.06	
DA	0.08	

* Both columns hand packed. All injections by syringe. Uncertainties expressed as standard deviation for, at least, ten separate measurements.

** Measured from the injection port to the top of the column bed and from the column outlet to the detector.

*** Chromatograms obtained from 20-μl syringe injections of a solution containing 2.36×10^{-6} *M* NE, 4.45×10^{-6} *M* DHBA, 1.04×10^{-5} *M* DA, and 1×10^{-3} *M* Na_2SO_3.

§ Averaged over entire linear range.

TABLE III

COMPARISON OF INJECTION MODES*

	Peak height (nA)	
	*Loop***	*Syringe****
NE	1.90 ± 0.03	2.21 ± 0.09
DHBA	2.13 ± 0.03	2.43 ± 0.10

* Uncertainties expressed as standard deviation.

** All loop injections were 23 μl of a solution containing 3.72×10^{-6} *M* NE and 7.42×10^{-6} *M* DHBA. The loop injector is an all-PTFE device from Laboratory Data Control.

*** All syringe injections were 25 μl of the same solution as listed in the above footnote.

congruities are not surprising, however, since both columns were hand-packed and the stream splitter exerted no control on the individual streams.

The moderately large standard deviations for peak ratios in Table II (3–5% R.S.D.) primarily arise from the mode of injection. Table III shows a brief comparison of the usual syringe injection to those performed with a loop injector (Laboratory Data Control, Cheminert Division). As can be clearly seen from the results, the loop injector is some two to three times more precise than the syringe method.

The dual detector can, thus, be readily utilized in a parallel configuration for accurate routine analyses. One must simply run separate calibrations for each column; the values for one column cannot be directly transferred to another. This technique, which virtually halves the time required for many analyses, also significantly reduces the cost compared to an entirely new setup. Considering (1) the price of another column with packing, (2) the expense of an additional detector and associated electronics, (3) that a single solvent pump could, theoretically, handle eight or more columns simultaneously, and (4) the difference in cost between a single and dual pen recorder, the second column can be presently added for only *ca.* US $ 350. This compares to about US $ 1400 for the original system.

ACKNOWLEDGEMENTS

This work was supported in part by a Cottrell grant from Research Corporation and in part by Grant No. 1 RO1 MH26866-01 PPR, awarded by the National Institutes of Mental Health, DHEW.

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