Journal of Chromatography, 281 (1983) 59–71
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 16,187

NOVEL COULOMETRIC DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received February 2nd, 1983; revised manuscript received August 1st, 1983)

SUMMARY

A sensitive coulometric detector was developed for determining electrochemically active compounds in high-performance liquid chromatography. An extremely thin flow layer was formed between a wide glassy carbon electrode and a pore-glass plate, and thus the volume of the detection passageway was sufficiently small. A reference and an auxiliary electrode were placed in an electrolyte chamber on the other side of the pore-glass plate, and thus a potential drop across the extremely thin flow layer on the wide working electrode was completely avoided. The detection limit was 10 fmole for catecholamines in chromatography and 1 fmole in injection to constant flow.

INTRODUCTION

In addition to the conventional UV absorption method, a few sensitive detection methods are presently available for high-performance liquid chromatography (HPLC) analysis of biogenic amines. Until a few years ago, the most widely used micro-analysis for HPLC was fluorometric measurement. More recently, the application of electrochemical detection (ED) for HPLC analysis of catecholamines (CA) was introduced by Kissinger[1] and the use of this type of detection has rapidly increased not only for the assays of CAs[2–23] and other hydroxy compounds[10,24–46] but also for many other electroactive substances[47–54].

ED for HPLC may be divided into two groups: amperometry[1–59,67] and coulometry[60–66]. Amperometric detectors electrolyse only a small fraction of the electroactive species, whereas coulometric detectors exhibit an electrolytic efficiency (coulometric yield) of 100%; amperometric detectors have a less complex construction and higher resolution. In spite of the considerably lower electrolysis yield, even the sensitivity of amperometric detectors is said to be better than that of coulometric detectors because the ratio of the sample current to the background current is higher with the former. It was found in the present investigation, however, that both the sample and background currents could be stabilized, and an extremely small detec-

0021-9673/83/$03.00

tion limit was obtained with coulometric detector of the right construction.

For application to HPLC, Johnson and Larochelle[60] used a platinum tube packed with platinum chips as the working electrode of a coulometric detector. In a coulometric detector proposed by Takata and Muto[61], and electrode made of carbon cloth, platinum gauze or silver wire net was interposed between two auxiliary electrodes separated by diaphragms made of ion-exchange membranes. Lankelma and Poppe[64] used two closely facing, wide carbon plates as the working electrode and the auxiliary electrode. Hashimoto and Maruyama[66] made a similar detector using a carbon plate and platinum plate. These two detectors, having a thin, flat, small-volume detection passageway, are thought to be more useful than other types for HPLC. However, such an arrangement of the auxiliary electrode may induce complicated errors when the test sample contains various kinds of electrochemically active impurity, because the auxiliary electrode, which has potential significantly different from that of the working electrode, may reduce (or oxidize) some of the impurities in the test sample and then the working electrode may oxidize (or reduce) them again.

We have constructed a new coulometric detector which (1) stabilizes every junction potential in the detector, (2) reduces noise in the electrolysis currents due to both the sample and background substances, (3) avoids the potential drop along the thin flow layer on a large working electrode, and (4) makes it possible to adopt both a "three-electrode system" and a "two-electrode system". The latter system was found to be suitable for detecting an extremely small amount of electroactive species using a large carbon electrode with large electric capacity (see Discussion). In this detector, the eluate from an HPLC column passes through a very thin (30 μm) layer situated between the carbon electrode and a controlled pore-glass (CPG) plate. The latter does not allow passage of the eluate, but has high electrochemical conductivity due to the solution contained in the pores. The reference and auxiliary electrodes were placed in an electrolyte chamber on the other side of the pore-glass plate. Because the same solution as used for HPLC was contained in the chamber and in the CPG plate, the junction potentials among the flowing eluate, the CPG plate, and the electrode chamber solution were very small and stable. Because of the high electrochemical stabilities and the large and stable electrolysis current, this detector exhibits a very low detection limit of 10 fmole of CA in HPLC system and 1 fmole in a constant-flow injection system.

EXPERIMENTAL

Construction of the detector

The construction of the detection cell is shown in Fig. 1. The glassy carbon used for the working electrode is of the hardest grade with the fewest gas pores (Grade GC-10, Code SB-2, Tokai Carbon, Tokyo, Japan). A carbon plate, 15 × 70 × 2 mm, was fixed into the centre part of the side of a 60 × 90 × 20 mm plastic block using epoxy (Epikote 828, Yuka Shell Epoxy, Tokyo, Japan, and Fujikyua 5300 as hardener, Fuji Kaseikogyo, Tokyo, Japan). The block was made of acrylic resin when aqueous solution was used for HPLC, or of Diflon resin when the eluate contained an organic solvent. Three holes of 1.5 mm diameter were drilled as shown in Fig. 1, and a PTFE tube (1.5 mm O.D., 0.2 mm I.D.) was inserted into each hole

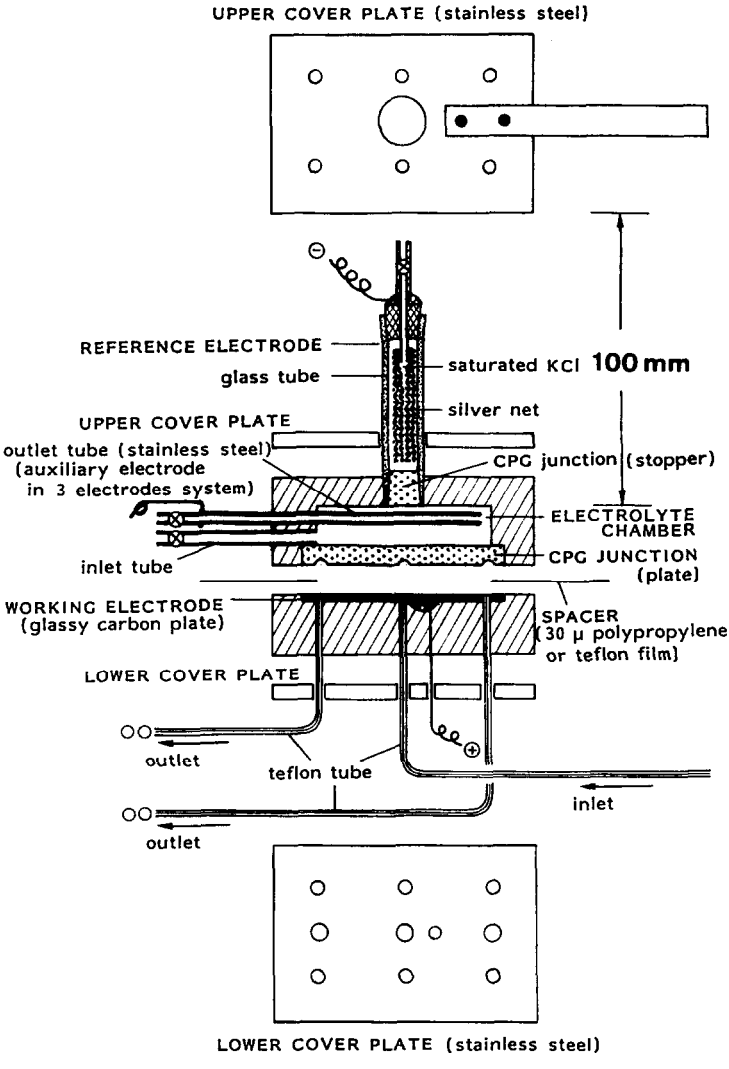

Fig. 1. Construction of the coulometric detector. Upper and lower drawings, top view of suporting plates; middle drawing, section view of detector. (Six screws for attaching the plates are not shown.)

after the outside of the tube had been etched using a metal sodium reagent (Tetraech, Junkosha, Tokyo, Japan) and coated with epoxy. The central tube was used as an inlet, and the other two as outlets. A 13 × 65 × 6 mm CPG plate with pores of 4 nm diameter (Vycor No. 7930, Corning Glass Works, Corning, NY, U.S.A.) was set with epoxy into another plastic block of 60 × 90 × 30 mm. An electrolyte solution chamber of 10 × 60 × 14 mm was positioned in the block on the other side of the CPG plate. This electrolyte chamber was filled with the elution solution used for the HPLC column, and the solution was electrolytically connected to the reference electrode through a pore-glass junction serving as a stopper at the bottom of the glass tube of the electrode. In order to obtain a large electrochemical capacity, the reference

electrode contained a silver-wire net wound into a spiral and then roughly plated with silver in order to increase the surface area. The surfaces of both the carbon electrode and the CPG plate were ground using carborundum papers and fine emery papers on a flat glass plate, and finally polished with an alumina powder of 0.5 μm on a buff cloth. The surface of the carbon electrode was washed thoroughly with a detergent solution using ultrasonic treatment. The two blocks were pressed together using a 30-μm polypropylene film, with a 10 × 50 mm removed section, as a spacer. To keep the detector structure stable fastened, the plastic blocks were held together by two 5-mm thick stainless steel plates with six 4-mm screws.

Electrical parts

The detector can be used as a two-electrode or a three-electrode system. Schematic diagrams of these circuitries are shown in Fig. 2. The "two-electrode system" was used for the analysis of extremely small amounts of CA (less than 1 pmole). In

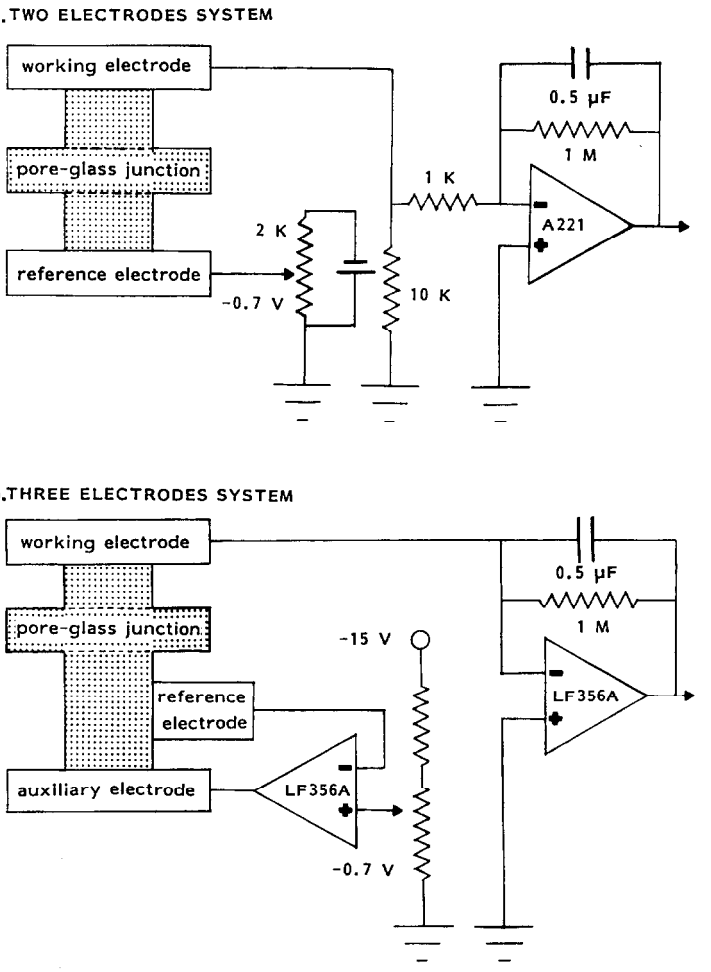

Fig. 2. Main circuit elements.

this system a mercury battery was used as the source of voltage, and the electrolysis current was amplified using a low-noise operational amplifier (A-221) followed by an active first-order filter of *ca.* 50 msec RC time (Fig. 2A). For determination of a higher level of CA (more than 1 pmole), a "three-electrode system" could be used (Fig. 2B). In this case, a stainless steel tube in the electrolyte chamber acts as the auxiliary electrode.

A two-pen recorder (Model D3R2 Minonoi, Okhura Electric, Osaka, Japan) was used for simultaneous recordings of the chromatograms obtained by this new detector and by optical measurement.

Flow injection test

In a test of the detector in a constant-flow system, sodium formate (0.1 *M*, pH 4.0 or 3.6, containing 0.2 *M* sodium chloride and 10 μM EDTA) was passed through the detector at a flow-rate of 0.3 ml/min applying a hydrostatic pressure of 800–1300 mm of water. For the injection of the CA solution, a micro-injector (Microfeeder, Furue Science, Tokyo, Japan) was used with a 1-ml glass syringe. The injection rate was instantaneously adjustable from 0.01 to 10 μl/sec and any injection time from 0.1 to 10 sec could be applied, so a very wide range of injection volumes was possible. A 2-m PTFE tube of 1 mm I.D. (2 mm O.D.) was used from the buffer bottle to the injection point and that of 0.1 mm I.D. (1.5 mm O.D.) was used from the injection point. The buffer bottle and the injector were positioned at the same height. From the injection point to the detector, and after the detector, *ca.* 20 cm of PTFE tube of 0.2 mm I.D. (1.5 mm O.D.) and that of 0.3 mm I.D. (1.5 mm O.D.) were used, respectively. The injection samples were 1, 10 or 100 μM of each CA or a mixture of three CAs and DOPA.

Chromatographic equipment and conditions

An HPLC assembly (HCL-803A, Toyo Soda, Tokyo, Japan) comprising a pump, a manometer and a sample injector with a 25-μl pipe-loop was used, together with a stainless steel column (225 × 4 mm I.D.) packed with a strongly acidic ion-exchanger of silica-gel base (IEX-510, particle size 6 μm, Toyo Soda). For the comparison of optical detection with ED, a UV absorption detector of 10-mm optical path (Spectroflow Monitor SF 770, Toyo Soda) was connected to the outlet of the chromatography column, and the CD was connected to the outlet of the optical cell. For the development of CAs injected on the column, 0.1 *M* sodium formate buffer (pH 4.0) containing 0.2 *M* sodium chloride and 10 μM EDTA (formate–NaCl–EDTA) was passed at a flow-rate of 1 ml/min.

Chemicals

Analytical grade potassium chloride, sodium chloride, formic acid, phosphoric acid and sodium hydroxide were used. Double-distilled water was used as a solvent. DOPA (D), epinephrine (E), norepinephrine (NE), and dopamine (DA) were obtained from Wako, Tokyo, Japan. They were dissolved in 0.01 *M* hydrochloric acid to a concentration of 1 m*M*, after which they were diluted with the buffer solutions used for the continuous flow system or HPLC system. The solutions were kept at −20°C.

RESULTS

Change in sensitivity and noise

When the formate–NaCl–EDTA solution was passed through our detector with a freshly polished glassy carbon electrode at a potential of +0.7 V (*vs.* SSE), the amount of the output current (background current) and noise level of this current rapidly decreased at the beginning and the rate of decrement gradually lowered as time passed. After the solution had been passed for 24 h at a flow-rate of 0.1–0.3 ml/min, the background current and noise had both decreased to a few percent of the original level. Thus, the signal-to-noise ratio was much improved after this ageing. Various characteristics of the detector were tested after ageing for at least 24 h.

Coulometric yield

As shown in Table I, the coulometric yield of this detector was practically 100% for three CAs, *i.e.*, E, NE, and DA, at the applied voltage from 0.7 to 0.9 V when measured in the flow-injection test at 0.3 ml/min. A slightly lower yield was obtained at 0.7 V for DOPA and dihydroxybenzylamine (DHBA). Even at the same potentials of the working electrode, the yield decreased as the flow-rate increased, and in the analysis of HPLC system at 1 ml/min, the detector showed yields from 80 tot 90% for four CAs at 0.7 V and this percentage was only slightly increased at higher voltages (see Table I).

TABLE I

COULOMETRIC YIELD OF THE PRESENT DETECTOR FOR FIVE CATECHOLAMINES INJECTED AT CONSTANT FLOW-RATE AND SEPARATED BY HPLC

See Experimental for abbreviations and conditions. Coulometric yields were calculated from peak areas, *i.e.* time integral of currents.

Method	*Potential (V)*	*Coulometric yield (%)*					*Residual current (nA)*
		DOPA	*NE*	*E*	*DA*	*DHBA*	
Injection at constant flow-rate (0.3 ml/min)*	0.3	0	4	4	15	0	−1
	0.4	5	8	15	35	6	−1
	0.5	35	58	65	95	52	3
	0.6	78	92	96	100	86	15
	0.7	95	100	100	100	97	40
	0.8	100	100	100	100	100	140
	0.9	94	95	98	98	97	440
	1.0	92	93	96	97	90	1500
HPLC (1 ml/min)**	0.4	10	12	13	44	–	0.2
	0.5	56	55	57	78	–	4
	0.6	83	83	84	86	–	22
	0.7	89	86	84	89	–	52
	0.8	90	84	83	87	–	180
	0.9	84	82	81	84	–	600

* 100 pmole of each derivative were injected.

** 20 μl of sample containing 10 pmole of each derivative were applied.

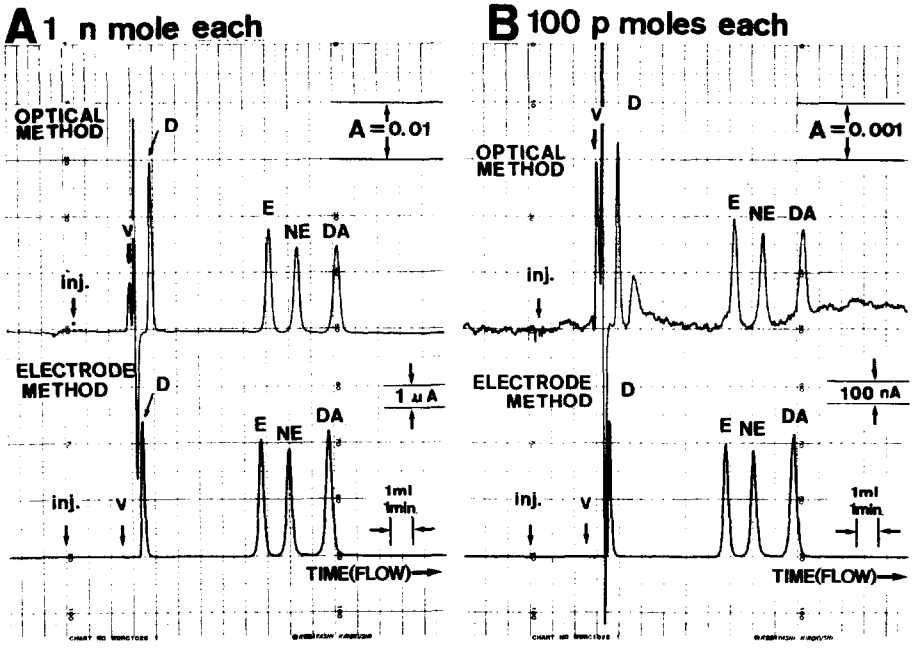

Fig. 3. HPLC chromatograms of catecholamines obtained simultaneously using optical and coulometric detectors. Sample abbreviations, see Experimental; flow-rate, 1.0 ml/min; electrode potential, +0.7 V, two-electrode system; optical detector, UV (280 nm) absorption.

Background current

The background current of the detector was negligibly small at 0.4 V and lower, and then steeply increased as the potential increased (see Table I). The amount of current was largely affected by flow-rate. The noise level of the background current at a completely constant flow-rate at constant temperature was extremely small (less than 0.001% of the amplitude of this current) when a "two-electrode system" was applied. When a "three-electrode system" was applied, the above noise level became more than 20 times as high as that obtained by a "two-electrode system" (see Discussion).

Linearity

An almost linear relationship was obtained between peak height of the electrolysis current and the amount of CAs over a very wide range (from 0.01 pmole to 1 nmole), both in the flow-injection test and the HPLC system (see Figs. 3 and 4). Even greater linearity was obtained when the current-time integral (peak area) was used instead of the peak height (see Fig. 5).

Detection limit

When a sample was injected into a constantly flowing solution, much better detection was achieved than in the case of HPLC, because the background current was more stable with the constant flow-rate than with the HPLC flow-rate which fluctuated because of the high-pressure pump. In the experiment shown in Fig. 6, 1

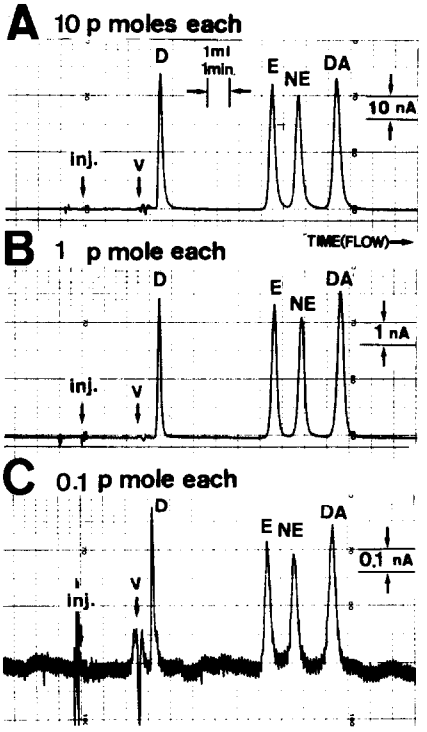

Fig. 4. HPLC chromatograms of catecholamines obtained by coulometric detector. Same conditions as Fig. 3.

μl (10 fmole, *ca.* 2 pg) and 2 μl (20 fmole, *ca.* 4 pg) of 10 n*M* E was injected four times and twice, respectively, into the formate–NaCl–EDTA at a flow-rate of 0.3 ml/min. As seen from the figure, the shape and height of the peaks are quite reproducible.

The noise level of the background current is *ca.* 3 pA and the peak heights in the case of 10 fmole (2 pg) E are shown to be 66 pA (Fig. 6). Therefore, the detection limit as expressed by the signal-to-noise ratio of 2.0, was estimated to be 1 fmole (0.2 pg). This detection limit increased as the flow-rate increased.

The detection limit of CAs by our detector increased remarkably in HPLC analysis owing to the background noises caused by the HPLC pump. Using the HPLC assembly described in Experimental, this limit was *ca.* 10 fmole (2 pg) at a flow-rate of 1 ml/min (see Fig. 4C).

Comparison of CD and optical method

Fig. 3 shows HPLC elution patterns of four CA derivatives obtained simultaneously by an optical detector and our coulometric detector. In these experiments, 20 μl of a sample containing 1.0 nmole (Fig. 3A) or 0.1 nmole (Fig. 3B) each of four CAs was injected onto the ion-exchange column, and the eluate was passed first through the UV detector and then through the coulometric detector. No significant difference was observed between the optically and the electrochemically obtained

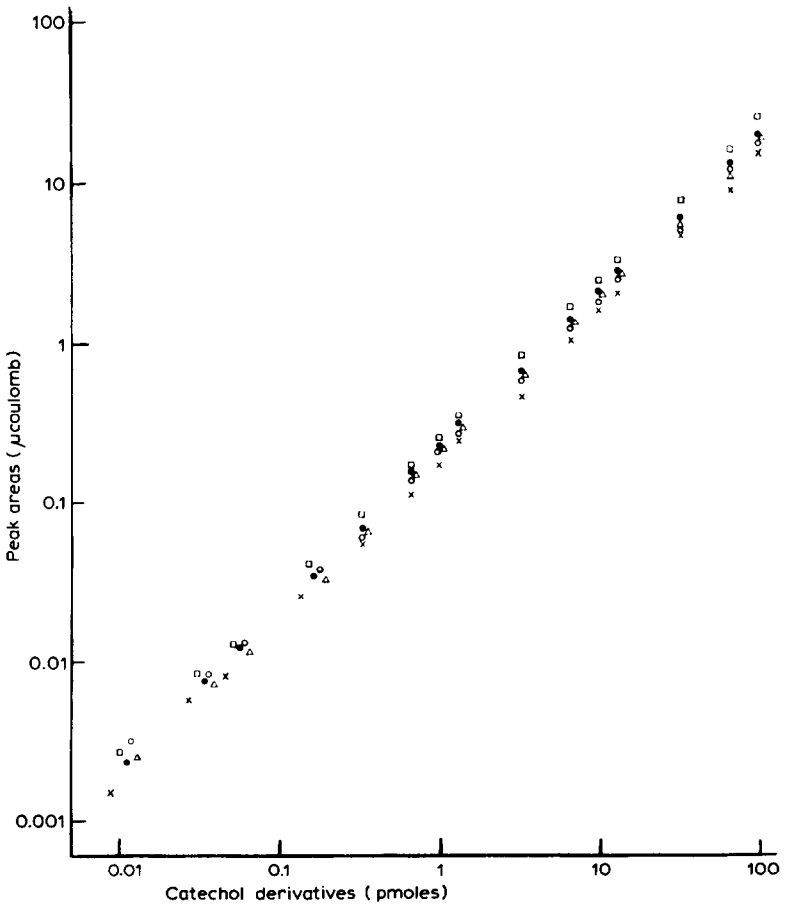

Fig. 5. Relationship between peak area and amount of catecholamines injected at constant flow-rate of 0.3 ml/min. Key: □. DOPA; ○, NE; ●, E; △, DA; ×, DHBA.

chromatograms of 1 nmole of each CA (Fig. 3A). However, in the case of 0.1 nmole (Fig. 3B), the background current of the optically obtained chromatogram contained much larger noise and drift than that of the coulometric detector.

Fig. 4 shows three chromatograms obtained using our detector when samples containing 10, 1, and 0.1 pmole each of the four CAs were analysed by HPLC. Although the optically detected chromatograms were recorded simultaneously, these recordings are not shown in this figure because no CA peak was observed. In the CD chromatogram obtained with 10 pmole CA (Fig. 4A) and even in that with 1 pmole CA (Fig. 4B), peaks of the four CAs were clearly obtained on a straight baseline without noise or drift. In the case of 0.1 pmole each (Fig. 4C), much noise was observed in the background current. However, the drift was not large and the noise pattern was regular. Therefore, the detection limit (signal-to-noise ratio = 2) was estimated to be less than 0.01 pmole. Consequently our detector can be said to be more than 1000 times as sensitive as the conventional UV detector (see Fig. 3B).

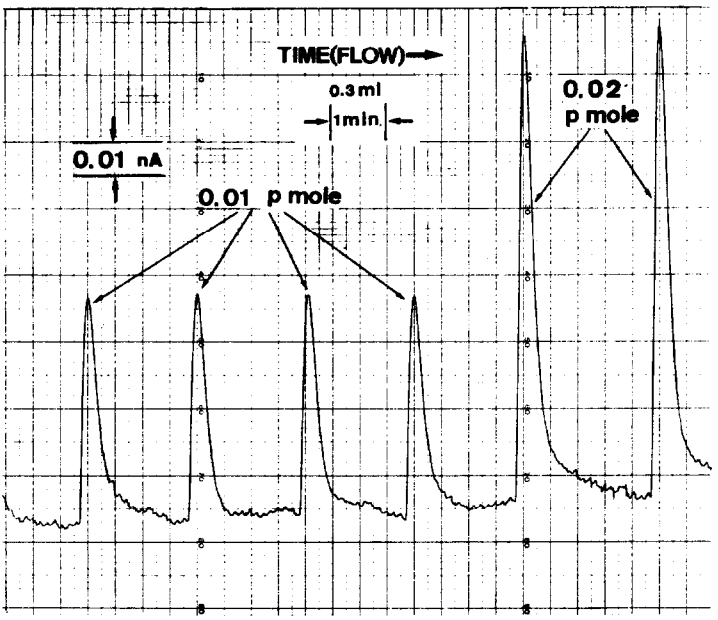

Fig. 6. Response to injection of trace amount of epinephrine at constant flow-rate of 0.3 ml/min. Electrode potential, 0.65 V.

DISCUSSION

For the HPLC analysis of CAs and other electroactive compounds, amperometric detectors have been more widely used than coulometric detectors. However, the present investigation suggests that CD is more advantageous for the analysis of extremely small amounts of CAs if a proper construction is adopted.

The basic requirements for ED in HPLC analysis are (1) high sensitivity, (2) high stability (high signal-to-noise ratio), (3) low and stable background (residual) current, (4) large range of linearity, and (5) small volume of detection space. Comparison of the advantages and disadvantages between amperometric and coulometric detectors is very complicated, because the characteristics of the detector greatly differ according to the construction and materials used.

Coulometric detectors exhibits much larger (usually 20–50 times) output current for the same amount of test compound. Generally speaking, a large output current is not necessarily an advantage, because even a small current can be amplified. However, in the case of HPLC analysis of a picogram level of a CA, the output (peak height) was *ca.* 0.02 nA even using a detector with 100% coulometric yield, and such a small current was very difficult to amplify without a very elaborate amplifier. In this respect, coulometric detectors are more useful than amperometric detectors.

The stability of both the output and background current is also very important because it decides the detection limit. The stability is generally much better in coulometric than in amperometric detectors, because the large working electrode surface of the former contacts all of the eluate flow and electrolysis in various part of the flow is averaged. The small surface of the amperometric detector contacts only a

small part of the flow, and is much influenced by the non-uniformity of concentration and the flow-rate. In addition, the boundary line between the working electrode and the supporting material, which is one of the major sources of noise in amperometric detectors, was completely removed in our detector by covering this boundary with the spacer.

Although the amount of residual current was affected by various factors, the effect of the electrode potential was especially large and the residual current steeply increased as the potential rose (see Table I). Therefore, for precise analysis, it was advantageous to use a potential slightly lower than that which results in maximum electrolysis. In the case of HPLC analysis, the amount of a compound is not obtained by the absolute amount of current but by the relative value based on a standard sample. Therefore, the coulometric yield is not required to be 100%, and a yield 80–90% is sufficient without reducing the various advantages of CD.

Table I shows a similar electrolysis yield for each CA (NE, E and DA) at an applied voltage from 0.6 to 1.0 V. When these results were compared with those obtained with a few types of amperometric detector prepared by us (not shown in this paper; also see ref. 67), it was noticed that the change in the electrolysis yield following a change in the applied voltage was much smaller for coulometric than for amperometric detectors. This difference is due to the fact that the former have sufficient capacity for detecting electroactive substances, whereas the latter do not.

A serious disadvantage with CD is the difficulty in making the small detection space. The detector devised by Lankelma and Poppe[64] has a relatively small cell volume: the dimensions of the cell space were 80 × 7 × 0.05 mm (28 μl) or 80 × 7 × 0.1 mm (56 μl). The cell space of our detector is 50 × 10 × 0.03 mm (15 μl). Although this volume is much larger than that of many amperometric detectors, it is comparable with that of many optical detectors, and the broadening of the peaks is probably negligible in most cases of HPLC. An extremely thin spacer (30 μm), used to reduce the volume of the detection passageway, was effective, at the same time, in increasing the electrolysis efficiency.

Most electrochemical detectors for HPLC described in the literature adopt a "three-electrode system" composed of working, auxiliary, and reference electrodes. Such a system has many advantages over the "two-electrode system", composed of working and reference electrodes. However, when the "three-electrode system" was applied to a coulometric detector with a large working electrode made of glassy carbon exhibiting an extremely high level of electronic capacity (*ca.* 100 μF/cm^2), even small noise in the operational amplifier that controls the potential of the working electrode resulted in significant changes in the electrolysis current. In the case of our detector, the capacity of the working electrode is *ca.* 500 μF, and even a noise of 1 μV/sec of the operational amplifier causes a change of 500 pA in the electrolysis current due to charge (or discharge) of the electrode according to the following calculation:

$$i = \frac{dQ}{dt} = C \times \frac{dV}{dt} = 500 \cdot 10^{-6} \cdot 10^{-6} = 500 \text{ pA}$$

where i = charge current and C = capacity of the electrode.

Such large noise was observed not only in the circuitry of our three-electrode

system (Fig. 2B) but also in the circuitry of a few commercial three-electrode systems. For this reason, the old-fashioned "two-electrode system" gave much better results. The "two-electrode system" was very difficult to apply to other coulometric detectors, which have the reference electrode in the flow stream after the very thin flow layer at the large working electrode[64,66], because various extents of potential drop are induced between the reference electrode and the working electrode. In contrast, in our detector, a thin flow layer was formed between the working electrode and CPG plate, and a reference electrode (and also an auxiliary electrode if required) was placed in the electrolyte chamber on the other side of the CPG plate. This CPG plate, which contains numerous pores of 4 nm diameter and has a total pore surface area of 250 m^2/g, contained a large amount of the elution buffer and had high electrical conductivity, and no potential drop was induced between the reference and working electrodes. Because the electrode chamber also contained the same elution buffer, the junction potentials among the chamber, CPG, and eluate flow were all small and stable. The detection limits of the detector for each CA with the HPLC and the flow injection method were 10 and 1 fmole, respectively. The noise and drift in HPLC are mostly produced by non-uniform flow, so the former limit (10 fmole) may approach the latter value (1 fmole) if the HPLC pump is improved.

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