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DEVELOPMENT OF AN ELECTROCHEMICAL DETECTOR FOR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY OF BRAIN CATECHOLAMINES

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SUMMARY

An electrochemical detector that utilizes the reduction–oxidation phenomena of electrochemically active substances has been developed for high-performance liquid chromatography. The construction and application of the detector to brain catecholamines, particularly for norepinephrine and dopamine analysis, are described. The differences between this detector and those reported previously are discussed.

INTRODUCTION

The basic requirements for a modern high-performance liquid chromatographic (HPLC) detector are (1) high sensitivity and high reproducibility, (2) versatility, (3) continuous monitoring of column effluent, (4) independence from column parameters such as mobile phase composition and flow-rate and (5) a wide range of linearity (wide dynamic range). In addition, it must be easy to operate and it must be stable for a long period of operation. However, no such useful and efficient detector has so far been described. Detectors that exhibit high sensitivity lack versatility. For example, ultraviolet (UV) and fluorimetric methods can be sensitive but are of no use when the compounds do not have UV chromophores or do not emit sufficiently strong fluorescence. The refractive index (RI) detector can be versatile but has low sensitivity and hence is not suitable for analyses at the nanomole or picomole level. The advantages and disadvantages of currently available detectors led us to believe that a detector that is highly sensitive and selective will exhibit advantages over those which are more versatile, but less sensitive.

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A polarographic method for the detection of organic molecules has proved fairly sensitive; it is possible to determine 10^{-7}–10^{-8} molar concentrations[1,2]. Several efforts have been made to apply this polarographic detection method to the continuous monitoring of liquid chromatographic effluents. Koen *et al.*[3] developed a polarographic detector and performed the quantitative analysis of some pesticides. They utilized a dropping-mercury electrode as the working electrode. The dropping-mercury electrode is far superior to many other solid electrodes in that a new and clean electrode surface for electrochemical reaction is always available. However, this type of electrode is not easily handled, and furthermore mercury is toxic. Joynes and Maggs[4] also reported the construction and the application of an electrochemical detector (ED) which utilized the dropping-mercury electrode or a carbon-impregnated silicone-rubber membrane as the working electrode. They studied mainly the analysis of inorganic cations and some nitro compounds, and they concluded that the carbon-impregnated silicone-rubber membrane was superior to the dropping-mercury electrode in sensitivity, strength and reliability of response. In 1973, Kissinger *et al.*[5] reported the development of an another type of ED that utilizes carbon paste as the working electrode. With this detection method, they succeeded in determining biogenic samples such as catecholamines[6], ascorbic acid[7] and uric acid[8,9].

We have considered the utility of electrochemical detection in HPLC, as the method has proved highly sensitive. Polarographic detection methods are one of the most sensitive methods for the analysis of organic compounds. Moreover, electrochemical detection is selective rather than versatile, which permits the ready elimination of electrochemically inactive contaminants or impurities contained in samples, reagents or mobile phases. We have therefore developed an ED for HPLC, bearing in mind the need for higher sensitivity, higher reproducibility and simple operation. Our ED is similar in principle, but different in composition and performance, to those described previously[3–5].

CONSTRUCTION OF THE DETECTOR

The fundamental concepts for the construction of the ED were the following: (1) it must be applicable at low picomole levels, because levels of biogenic substances to be measured (in this instance, catecholamines in mouse brain) are lower than the detection limits of the usual detector[10]; (2) it must be stable towards mobile phases containing organic solvents, as reversed-phase chromatographic separation is frequently utilized in order to achieve better separations; (3) it must be easily operable in order to be able to apply it in routine analyses; and (4) it must be simple and easy to maintain. We constructed a suitable ED after several preliminary trials aimed at satisfying the above criteria.

Schematic diagrams of the circuitry of the amplifier and the electrochemical cell are shown in Figs. 1 and 2. The ED consists of a quasi-three-electrode system. An auxiliary electrode and a working electrode are in a flow cell; a reference electrode is installed in the amplifier. A three-electrode system is necessary for instruments that measure nanomole or picomole amounts in order to provide better reproducibility. The electrochemical cell holder was machined from a Teflon block and was stable to reversed-phase chromatographic conditions. The working electrode consists of a surface-polished round carbon plate. An obvious advantage of this working electrode

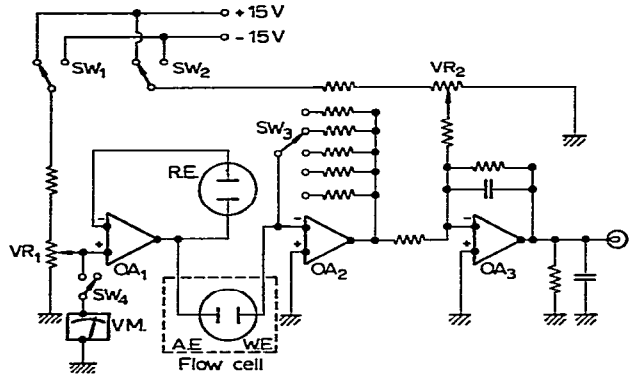

Fig. 1. Circuitry of the electrochemical detector. OA_1, AD-503 JH; OA_2, AD-503 JH; OA_3, Fairchild 741. A.E. = auxiliary electrode; W.E. = working electrode; R.E. = reference electrode.

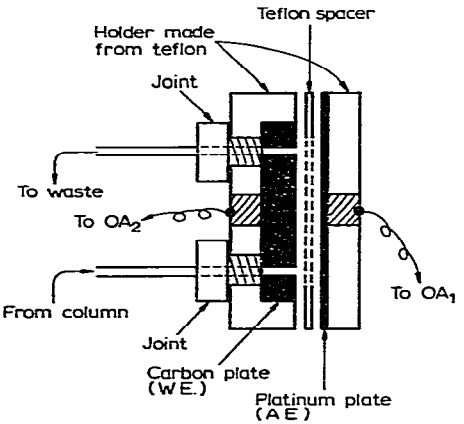

Fig. 2. Construction of the electrochemical cell.

over Kissinger *et al.*'s carbon-paste electrode[5] is that our detector can be operated without concern for the quality of the working electrode even if reversed-phase chromatographic conditions are employed. The auxiliary electrode is a thin platinum plate, which is placed opposite the carbon plate and separated by a 100-μm Teflon spacer. Effluent from the column enters the electrochemical cell through a joint, moves between the two electrodes as a thin film and is passed to waste through another joint. The surface area of the electrode was made as large as possible in order to reduce the electrical resistance between the two electrodes. We had discovered that if the electrical resistance was high, the potential changed when an electrochemically active substance moved through the electrochemical cell, resulting in baseline drift and therefore interference with the analyses.

The amplifier is of a common type with two potentiometers (VR_1 and VR_2), four switches (SW_1, SW_2, SW_3 and SW_4) and one voltmeter (VM). One potentiometer (VR_1) is used to apply the potential to the electrode, and the other (VR_2) is used to eliminate the standing current intrinsically induced by the operation. Switch SW_1

changes the mode of reaction (oxidation and reduction), SW_2 is used to supply a negative or positive voltage to the standing current cancelling circuit, SW_3 is responsible for sensitivity selection and SW_4, which is usually in the off position, is used to monitor the applied potential with the voltmeter. The output level was set at 1 V when the appropriate input current was run (50, 100, 200 and 500 nA and 1, 2, 5 and 10 μA).

METHOD

To demonstrate the applicability of the electrochemical detection method, brain catecholamines, which are important in neurotransmission and have been actively investigated by several methods[11–13], were examined.

The equipment and conditions were as follows. The pump was a Hitachi Model 635. The flow-rate and pressure were 0.5 ml/min and about 25 kg/cm^2, respectively. The column (glass 500 × 2.0 mm I.D.) was dry-packed with Zipax SCX strong cation-exchange resin (DuPont). Samples (2, 5, 10 or 20 μl, depending on the concentration of catecholamines) were injected through a septum injection port. The mobile phase was perchloric acid (0.01 *M*). The potential of the ED was set at +0.80 V *versus* the silver–silver chloride reference electrode and the sensitivity of the detector was set at 50 or 100 nA full-scale, depending on the concentration of catecholamines.

Samples were examined mainly for norepinephrine and dopamine, other biogenic amines such as epinephrine, serotonin (5-hydroxytriptamine) and 5-hydroxyindoleacetic acid being briefly examined. Extraction of catecholamines from mouse brain was performed according to the following general procedure[6]. Mouse brains irradiated with microwaves[14] were homogenized, centrifuged and then the catecholamines were adsorbed on to acid-washed alumina[15]. The catecholamines were eluted from the alumina with 0.1 *M* perchloric acid containing sodium hydrogen sulphite. Full details of the extraction procedure and the results of the analysis will be published elsewhere[16]. 3,4-Dihydroxybenzylamine (DHBA), prepared by demethylation of 3,4-dimethoxybenzylamine (ICN Pharmaceuticals) with 48% hydrobromic acid[5], was used as an internal standard. Reagents and solvents were not purified unless otherwise stated.

RESULTS AND DISCUSSION

The relationship between applied potential and sensitivity was examined first by changing the applied potential from +0.30 to +0.80 V *versus* the reference electrode. Other conditions were held constant. DHBA was used as the sample (100 ng per 2 l). The results are shown in Fig. 3.

The sensitivity of the detector increased proportionally to the applied potential between +0.30 and +0.50 V and was almost constant in the range between +0.70 and +0.80 V. The polarographic determination of DHBA also suggested that an applied potential above +0.70 V is sufficient for the analysis of catecholamines. From these results, the potential was set at +0.80 V *versus* the silver–silver chloride reference electrode.

A typical chromatogram of catecholamines obtained by HPLC using the ED is presented in Fig. 4. Norepinephrine, DHBA and dopamine appeared at 2, 4.5 and

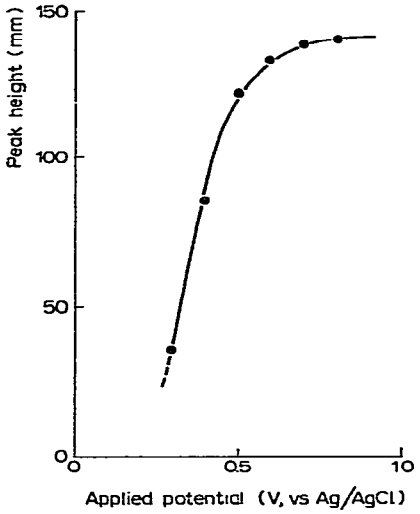

Fig. 3. Relationship between applied potential and sensitivity.

7.5 min, respectively, after injection under the conditions described above. The separation of each peak was almost complete (baseline separation) except for the separation of sulphite ion (added in order to prevent oxidative degradation of catecholamines[5]) and norepinephrine. It should be noted that an unknown peak was observed at about 17 min after injection (not shown in Fig. 4) and was again observed when an empty syringe was passed through the septum. This peak is thought to be due to iron(II) ion, and this assumption was verified by the injection of dilute iron(II) chloride solution into the column. Considerable enhancement of both peak height and peak area was observed.

Calibration graphs for norepinephrine and dopamine are presented in Fig. 5. Both calibration graphs were linear up to 4 ng per injection, with correlation coeffi-

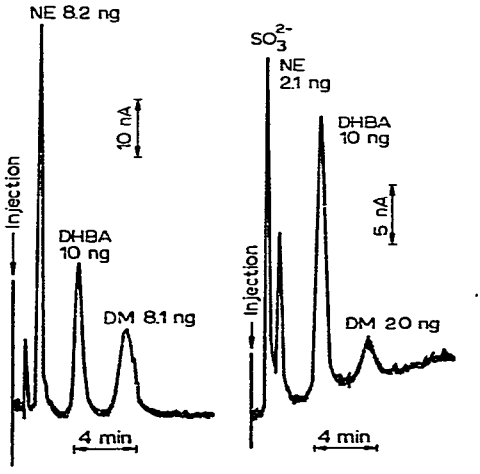

Fig. 4. Typical chromatogram of catecholamines obtained by HPLC with ED.

cients of 0.9997 and 0.9999, respectively. The detection limits were about 100–150 and 500 pg per injection, respectively (signal-to-noise ratio = 2). Therefore, the HPLC method with an ED would permit the determination of the levels of norepinephrine and dopamine in a single mouse brain, as their levels in mouse whole brain have been reported to be 350–450 and 700–900 ng per gram of wet tissue, respectively[10].

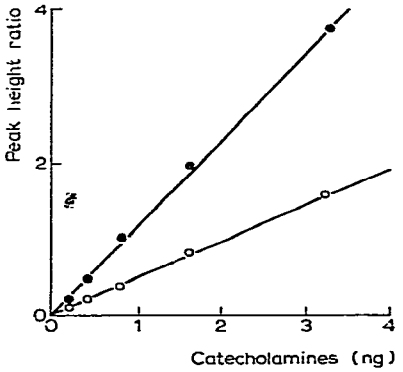

Fig. 5. Calibration graphs for norepinephrine (●) and dopamine (○) by HPLC with ED.

In Table I, the results of analyses of catecholamines in mouse whole brain after two methods of sacrifice (microwave irradiation and decapitation) are presented; no significant differences between these methods were observed[14,16]. However, we have found that the levels of dopamine in rat whole brain found after microwave irradiation were significantly different to those obtained following conventional decapitation[16].

TABLE I

RESULTS OF CATECHOLAMINE ANALYSIS OBTAINED BY HPLC WITH ED

Four mice were decapitated and four were killed by microwave irradiation (4 kW, 0.1 sec). Results are expressed as mean nanograms of catecholamine per gram of tissue ± standard error.

Method of killing	*Catecholamine (ng/g)*	
	Norepinephrine	*Dopamine*
Decapitation	431 ± 18	1088 ± 65
Microwave irradiation	389 ± 32	1268 ± 61

We have found that the electrochemical detection method is superior to other methods with respect to interferences originating from solvents, reagents and impurities contained in tissue samples. Indeed, reagents and solvents (including distilled and deionized water) have no effect on the results of analyses at the levels used (high picogram to low nanogram levels), although they were used without purification. The major interferences encountered were (1) from air bubbles in the detector cell, (2) an extraneous peak due to iron(II) ion and (3) pumping pulsation. The major problem

is air bubbles that result from insufficient deaeration of the mobile phase from an inadequate injection and which cause a sudden drop in the standing current and/or reaction current.

Air bubbles are much more important in electrochemical cells than in cells in UV and RI detectors. Deaeration of the mobile phase must be conducted with the greatest care. The appearance of the extraneous peak due to iron(II) ion may be attributable to the use of a stainless-steel microsyringe. This peak had a relatively long retention time (about 17 min under the conditions described above), compared with less than 10 min for catecholamines and the internal standard. The presence of a peak with a longer retention time is obviously not desirable in routine analyses. It could be made to have a shorter retention time by the addition of a salt such as sodium perchlorate to the mobile phase, but the concentration of the added salt would be critical and the ion(II) ion peak sometimes interfered with the peaks of the catecholamines and the internal standard. The extraneous peak would best be eliminated by using another type of injection system, such as a Teflon loop injector. Pumping pulsations in the electrochemical detection system directly reflected the noise level of the chromatograms and, as a result, it considerably decreased the signal-to-noise ratio. As the noise level of the amplifier itself remained low, even at the highest sensitivity position (when a dummy cell[17] was connected to the amplifier), the major cause for the lower signal-to-noise ratio is pumping pulsation. A 10-m Teflon coil damper (I.D. 0.14 mm) improved the signal-to-noise ratio by a factor of 2.

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REFERENCES

1 K. Hasebe and J. Osteryoung, *Anal. Chem.*, 47 (1975) 2412.
2 S. K. Chang and G. W. Harrington, *Anal. Chem.*, 47 (1975) 1857.
3 J. G. Koen, J. F. K. Huber, H. Poppe and G. den Boef, *J. Chromatogr. Sci.*, 8 (1970) 192.
4 P. L. Joynes and R. J. Maggs, *J. Chromatogr. Sci.*, 8 (1970) 427.
5 P. T. Kissinger, C. Refshauge, R. Dreiling and R. N. Adams, *Anal. Lett.*, 6 (1973) 465.
6 C. Refshauge, P. T. Kissinger, R. Dreiling, L. Blank, R. Freeman and R. N. Adams, *Life Sci.*, 14 (1974) 311.
7 L. A. Pachla and P. T. Kissinger, *Anal. Chem.*, 48 (1976) 237.
8 P. T. Kissinger, L. J. Felice, R. M. Riggin, L. A. Pachla and D. C. Wenke, *Clin. Chem.*, 20 (1974) 992.
9 R. M. Riggin, A. L. Schmidt and P. T. Kissinger, *J. Pharm. Sci.*, 64 (1975) 680.
10 A. S. Welch and B. L. Welch, *Anal. Biochem.*, 30 (1969) 161.
11 P. A. Shore and J. S. Olin, *J. Pharmacol. Exp. Ther.*, 122 (1958) 295.
12 E. L. Arnold and R. Ford, *Anal. Chem.*, 45 (1973) 85.
13 S. H. Koslow, F. Cattabeni and E. Costa, *Science*, 176 (1972) 177.
14 S. T. Weintraub, W. B. Stavinoha, R. L. Pike, W. W. Morgan, A. T. Modak, S. H. Koslow and L. Blank, *Life Sci.*, 17 (1975) 1423.
15 A. H. Anton and D. F. Sayre, *J. Pharmacol. Exp. Ther.*, 138 (1962) 360.
16 Y. Maruyama, N. Iida, M. Kusaka, A. Horikawa, J. Mori and K. Munekiyo, *Progr. Neuro-Psychopharmacol.*, 2 (1978), in press.
17 *LC-2A Electronic Controller, Operations/Maintenance Manual*, Bioanalytical Systems, West Lafayette, Ind., U.S.A., p. 28.