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Design and Characterization of Electrochemical Detector for High-Performance Liquid Chromatography and Application to the Determination of Biogenic Amines

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A flow cell electrochemical detector (ECD) using a glassy carbon electrode for high-performance liquid chromatography (HPLC) was designed and characterized. The ECD examined by us showed the same property as the tubular type electrode. The linear dynamic range of norepinephrine (NE), dopamine (DA) and serotonin (5HT) peak current were about 10^4(50 pg—1 μg) and the minimum detectable quantities for NE and DA were 5 and 10 pg.

High-performance reversed-phase chromatography in combination with ECD permitted the simultaneous determination of NE, DA and 5HT in a whole mouse brain and mouse cerebellum. Assay of NE, DA and 5HT gave the mean values of 430, 872 and 749 ng/g wet tissue for whole mouse brain, and 259, 16 and 240 ng/g wet tissue for mouse cerebellum.

Keywords——high performance liquid chromatography; electrochemical detector; reversed-phase column; norepinephrine; dopamine; serotonin

The high-performance liquid chromatography (HPLC) is powerful new technique for analysis of trace compounds. At present, spectrophotometric, fluorometric and chemical reaction detectors are employed for the detection of trace compound in mobile phase. Spectrophotometric detector is easy to use but deficient in specificity. Fluorometric and chemical reaction detectors are used as sensitive tools in trace analysis, but not well suited to routine analysis.

In 1952, Kemula applied a detector cell containing a dropping mercury electrode to liquid chromatography for the first time.[2] However, this system had a limited anodic potential range because of the anodic oxidation of the metal at about 0.2—0.4 V *vs.* S.C.E. Adams and coworkers[3] described the design of the flow cell detector using carbon paste electrode. Also MacDonald and Duke[4] described two designs of low volume solid electrode cell. Solid electrode voltammetry offers considerable promise for the design of detector provided that the problems of nonreproducibility of electrode surface, primarily elimination of adsorption and surface oxide formation can be overcome. The suitability of electrochemical detector to a given problem ultimately depends on the voltammetric characteristics of the objective molecules in a suitable mobile phase and at a suitable electrode surface. Therefore, some efforts are required to simultaneously optimize both the column and detector performance. Recently, Kissinger *et al.* have determined urinary catecholamines by HPLC using carbon paste electrode.[5] Generally, as carbon paste electrode is easy to deform, the

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2) W. Kemula, *Rocz. Chem.*, **26**, 281 (1952).
3) P.T. Kissinger, C. Refschauge, R. Dreiling, and R.N. Adams, *Anal. Letters*, **6**, 465 (1973).
4) A. MacDonald and P.D. Duke, *J. Chromatogr.*, **83**, 331 (1973).
5) *a*) P.T. Kissinger, R.M. Riggin, R.L. Alcon, and L.D. Rau, *Biochem. Med.*, **13**, 229 (1975); *b*) R.M. Riggin and P.T. Kissinger, *Anal. Chem.*, **49**, 2109 (1977).

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reproducibility under the prolonged application and the equal electrochemical properties among individual electrode can not be obtained. Besides, carbon paste electrode can not be used in a nonaqueous solvent. These defects are considered to be overcome by the employment of glassy carbon, but the application of glassy carbon to the flow electrochemical detector (ECD) has scarcely been reported. As electrochemical detector is high sensitive and high selective, it has been usefully applied to the determination of biogenic amines in brain and urine. However, its application to serum has not been described.

The present paper describes the design and the characteristics of ECD cell using glassy carbon electrode and its application for the determination of biogenic amines in whole mouse brain, mouse cerebellum, rat urine and dog serum.

Experimental

Apparatus——A Millton Roy reciprocating minipump equipped with a damping coil system and a Rheodyne loop injector were used. For chromatographic separation, 25 cm×4.6 mm I.D. steinless steel column was packed with LiChrosorb RP-18 (10 μm) or Nucleosil C_{18} (10 μm) using the balanced density packing technique.[6] The design of ECD cell was as shown in "Results and Discussion." In these systems, the cell blocks were made with acrylic resin or fluoride resin and working electrode was made with glassy carbon (Tokai Carbon Co., Ltd.). Reference electrode is Ag/AgCl type and has Vycor porous glass frit junction. A steinless steel tube serves both as exit line and counter electrode. As electrical circuit, a newly designed potentiostat, that had low drift and low noise characteristics, was used. Cell current measuring circuit in the potentiostat was current follower. Cyclic voltammetry was carried out using a Yanaco P8-ES electronic scanner and a Riken Denshi D-51 X-Y recorder. Chronoamperometric measurement was carried out using a Hokuto Denko Model HA 101 potentiostat.

Reagents——All chemicals were of reagent grade except 3,4-dihydroxybenzylamine hydrobromide (mp 185°) which was synthesized according to the procedure of Refaschauge *et al.*[7] Alumina (Woelm neutral) was purified according to Anton and Sayre.[8] The mobile phase was prepared by dissolving 10.4 g of citric acid, 4.75 g of sodium hydroxide, 8.12 g of sodium acetate and 2.08 ml of acetic acid in 990 ml of water and mixing with 10.0 ml of tetrahydrofuran, except the mobile phase used for the analysis of rat urine, which was prepared by dissolving 15.0 g of tartaric acid, 11.5 g of sodium tartrate and 71.0 g of sodium sulfate in 1000 ml of water. These mobile phase were normally vacuum deaerated.

Measurement of the Electrode Area——Based on the chronoamperometry of 4 mM $K_4Fe(CN)_6$ in 2 M KCl aqueous solution at 0.7 V, the observed current time constant, $i \cdot t^{1/2}$, was plotted against time t. $(i \cdot t^{1/2})_0$ was estimated by the extrapolation of the plots to zero time and the electrode area, A was calculated from the following equation[9];

$$A = \frac{(i \cdot t^{1/2})_0}{54.5 \times 10^3 \cdot n \cdot D^{1/2} \cdot C}$$

$(n=1,\ D=0.629 \times 10^{-5}\ \mathrm{cm^2/sec},\ C=4\ \mathrm{mM})$

Assay Procedures

Whole Mouse Brain——Adult male mouse of dd strain (20—30 g) was sacrificed by dislocation. Whole brain (380—450 mg) was removed as rapidly as possible, weighed to the nearest mg, and homogenized in a mixture of 100 μl of 0.1 M EDTA·2Na, 100 μl of 1.1×10^{-5} M 3,4-dihydroxybenzylamine (DHBA) as internal standard and 750 μl of 0.025 N HCl for 3—4 min. 4 g of NaCl and 12.0 ml of butanol were added to the homogenate. The sample was shaken for one hour and centrifuged at 2000 rpm for 5 min. A 10.0 ml aliquot of butanol was transferred to a 40 ml glass-stoppered centrifuge tube containing 200 μl of 0.1 N HCl and 20 ml of heptane. The centrifuged tube was shaken for 10 min, centrifuged at 2000 rpm for 5 min and most of nonaqueous layer was removed with aspiration. A 10—20 μl sample of the aqueous solution was injected into the HPLC. 0.5 ml of working standards (containing 400 ng of norepinephrine (NE), 800 ng of dopamine (DA) and 700 ng of serotonin (5HT) in 1.0 ml of 0.01 N HCl) was added to a mixture of 100 μl of 0.1 M EDTA·2Na, 100 μl of 1.1×10^{-5} M DHBA and 750 μl of citrate buffer (mobile phase) and then mixed. The solution was treated exactly as the brain sample.

6) R.E. Majors, *Anal. Chem.*, **44**, 1722 (1972).
7) C. Refschauge, P.T. Kissinger, R. Dreiling, L. Blank, R. Freeman, and R.N. Adams, *Life Sci.*, **14**, 311 (1974).
8) A.H. Anton and D.F. Sayre, *J. Pharmacol. Exp. Ther.*, **138**, 360 (1962).
9) R.N. Adams, "Electrochemistry at Solid Electrodes," Marcel Dekker, New York, N.Y., 1969.

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Mouse Cerebellum——The cerebellum (about 40 mg) of the mouse mentioned above was weighed and homogenized in a mixture of 100 μl of 0.1 M EDTA·2Na, 25 μl of 1.1×10^{-5} M DHBA and 100 μl of 0.025 N HCl. The homogenate was treated exactly as the whole mouse brain sample and a 20 μl sample of the aqueous layer was injected into the HPLC. Working standards were treated as mentioned above.

Rat Urine——1 ml of rat urine was extracted by the method of Anton and Sayre.[8]

Dog Serum——0.5 ml of dog serum was added to a mixture of 100 μl of 0.1 M EDTA·2Na and 750 μl of 0.025 N HCl and mixed. The solution was treated exactly as the whole mouse brain and 10—20 μl sample of the aqueous layer was injected into the HPLC. All HPLC measurements were performed at 25±0.1°.

Results and Discussion

Design and Characterization of Electrochemical Detector

The construction schemes of three ECD cells for HPLC investigated by us were shown in Fig. 1. Generally, a linear relationships between concentration and current at ECD can be

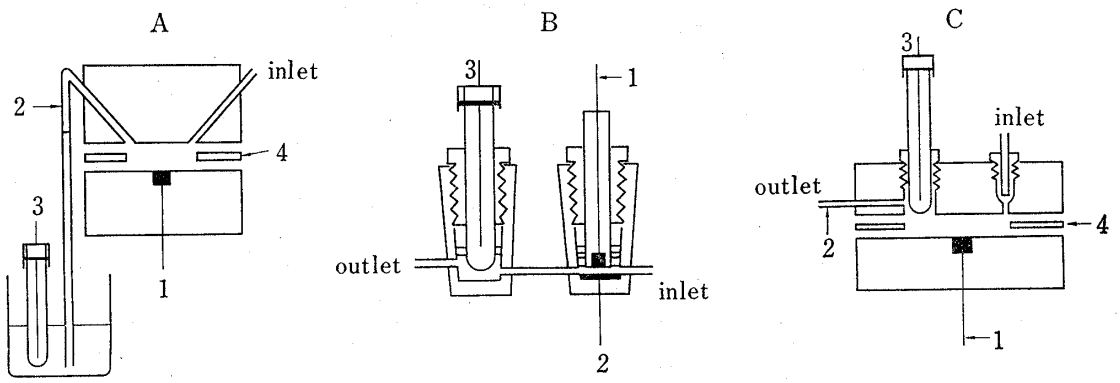

Fig. 1. Constructions of Electrochemical Detector Cells

1: glassy carbon working electrode.
2: auxiliary electrode.
3: reference electrode (Ag/AgCl).
4: Teflon spacer.

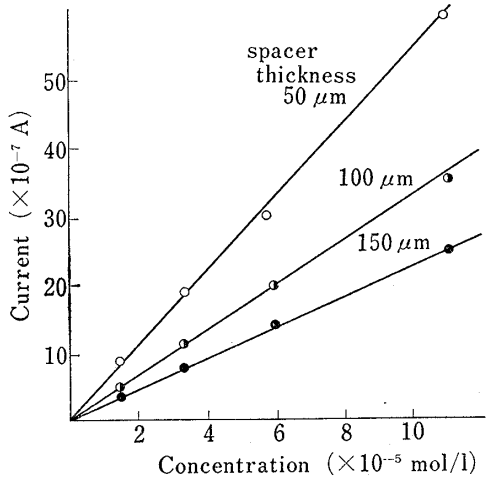

Fig. 2. Dependence of Current upon DA Concentration under Various Spacer Thickness

Flow rate, 0.75 ml/min.

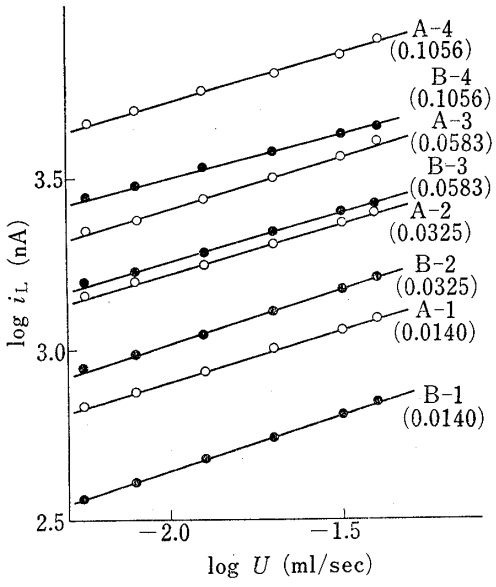

Fig. 3. Dependence of Current upon Flow Rate at Various DA Concentration

A-1—A-4; spacer thickness 50 μm.
B-1—B-4; spacer thickness 100 μm.
Parentheses in figure show the concentration (mM/l).

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expected only for a constant potential of the working electrode, which can be obtained by lowering the cell resistance between the working and reference electrodes, that is, the iR drop as small as possible. Among three cells, the cell C had the highest sensitivity, the smallest peak dispersity, the smallest cell resistance (22 kohm). Consequently, the cell C was chosen and the characteristics of the cell C were examined.

In order to study the relationships between dopamine (DA) concentration and current under various spacer thickness (electrode area, 0.022 cm^2) of the cell C, the detector was connected directly to a reservoir storing the mobile phase, which contained various concentration of DA, and the currents were measured. Generally, $i/(U \cdot C)$[10] represents the static mass flow sensitivity, where i is the current (A), U is the flow rate (1/sec) and C is the concentration (mol/l). In Fig. 2, the sensitivities for 50, 100 and 150 μm spacer thickness were 4.24×10^3, 2.52×10^3, 1.8×10^3 A·sec/mol respectively, from which 50 μm spacer thickness was chosen.

The flow rate dependence of the sensitivity at the 50 and 100 μm spacer thickness (electrode area, 0.022 cm^2) was studied by monitoring the electro-oxidation current for various DA concentration under the same experimental condition as mentioned above. As shown in Fig. 3 which showed log–log plots of current *versus* flow rate, the sensitivity was higher at the 50 μm spacer thickness than at the 100 μm. In addition, peak broadening and background noise were also smaller at the 50 μm than at the 100 μm. For a forced convection electrode in flowing solution, the limiting current (i_L) is defined by the following equation[11]:

$$i_L = k \cdot n \cdot \boldsymbol{F} \cdot C \cdot D^{2/3} \cdot U^x \qquad (1)$$

where k is coefficient dependent on the kinematic viscosity, the geometry and the area of electrode, n is the number of electrons involved in the electrode reaction, $\boldsymbol{F}$ is the Faraday constant, D is the diffusion coefficient, C is the bulk concentration of substance diffusing to the surface, U is the flow rate and x is the value of the exponent of the flow rate. The logarithmic form of equation (1):

$$\log i_L = \log K + x \cdot \log U \qquad (2)$$
$$K = k \cdot n \cdot \boldsymbol{F} \cdot D^{2/3} \cdot C$$

shows that the slope, x, of the graph of log i_L against log U, can be evaluated from several studies at various flow rate. The values of x are known to be 1/3, 1/2 and 3/4 for the tubular electrode,[12] the plate electrode,[13] and the wall jet electrode,[14] respectively. In Fig. 3, the slopes of A-1—A-4 and B-1—B-4 determined by a method of least square were about 0.3. These values were in good agreement with the theoretical value of 1/3, obtained by Blaedel for a tubular electrode.

As a result of a comparison of the electrode area 0.084 cm^2 with 0.022 cm^2, the former

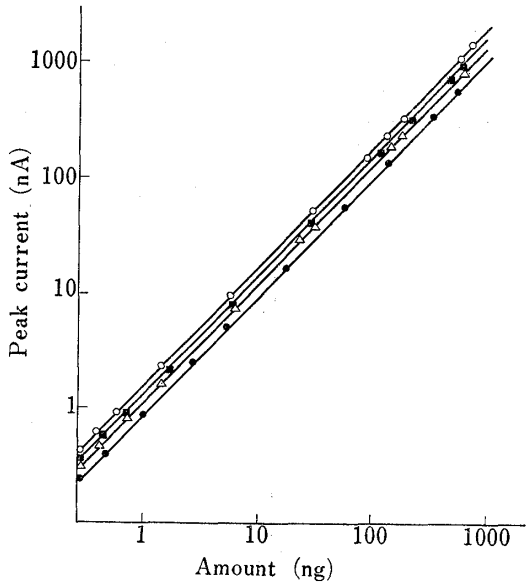

Fig. 4. Peak Currents *versus* Injected Amounts of NE, DHBA, DA and 5HT

Column; LiChrosorb RP-18 (10 μm), 25 cm × 4.6 mm I.D.
Mobile phase; 1%tetrahydrofuran-pH 5.1 buffer.
Applied potential; 0.65 V *vs.* Ag/AgCl.
Flow rate; 0.77 ml/min.
—○—: NE, —■—: DHBA, —△—: DA, —●—: 5HT.

10) H. Lankelma and H. Poppe, *J. Chromatog. Sci.*, **14**, 310 (1976).
11) Z. Feher, G. Naqy, K. Toth, and E. Pungor, *Analyst* (London), **99**, 699 (1974).
12) W.J. Blaedel, C.L. Olson, and L.R. Sharma, *Anal. Chem.*, **35**, 2100 (1974).
13) V.G. Levich, "Physicochemical Hydrodynamics," Prentice-Hall, Englewood, N.J., 1962.
14) J. Yamada and H. Matsuda, *J. Electroanal. Chem.*, **44**, 189 (1973).

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has been 10% higher sensitivity. But the background noise current of the former was about 30% higher.

From the results mentioned above, it was decided to use the cell having the 50 μm spacer thickness, the glassy carbon electrode of 0.022 cm^2 area and 3 μl cell volume.

To examine the detectability and linearity for our ECD, the cell was connected with outlet of reversed-phase chromatographic column and the peak currents were measured with our designed potentiostat after injection of various amount of norepinephrine (NE), DA, serotonin (5HT) and 3,4-dihydroxybenzylamine (DHBA). As shown in Fig. 4, the linear dynamic range of current was about 10^4 (50 pg—1 μg) and the minimum detectable quantities were about 5 pg and 10 pg (signal to noise ratio=2) for NE and DA, respectively. Also, the magnitude of residual current was 4—5 nA. When applied potential was set at 0.65 V *vs.* Ag/AgCl, the current responses (pA/pmol) for NE, DHBA, DA and 5HT separated on reversed-phase column (LiChrosorb RP-18) were 480, 421, 368 and 308, respectively. However, the current responses for NE, DHBA and DA separated on a pellicular strong cation

Table I. Detectability of HPLC with ECD for NE and DA

Investigators	Column for HPLC	Material of working electrode	Current response (pA/pmol)		Detection limit (pg)	
			NE	DA	NE	DA
Present paper	Reversed phase	Glassy carbon	480	368	5(2)[a]	10(2)
	Ion exchange	Glassy carbon	166	84	20(2)	30(2)
Moyer and Jiang[15]	Reversed phase (paired ion)	Carbon paste	203	61	20(2)	—
Riggin and Kissinger[5,16]	Reversed phase (paired ion)	Carbon paste	270	198	100	100
Hashimoto and Maruyama[17]	Ion exchange	Carbon (plate)	963	244	100—150 (2)	500(2)

a) Parentheses show the signal to noise ratio.

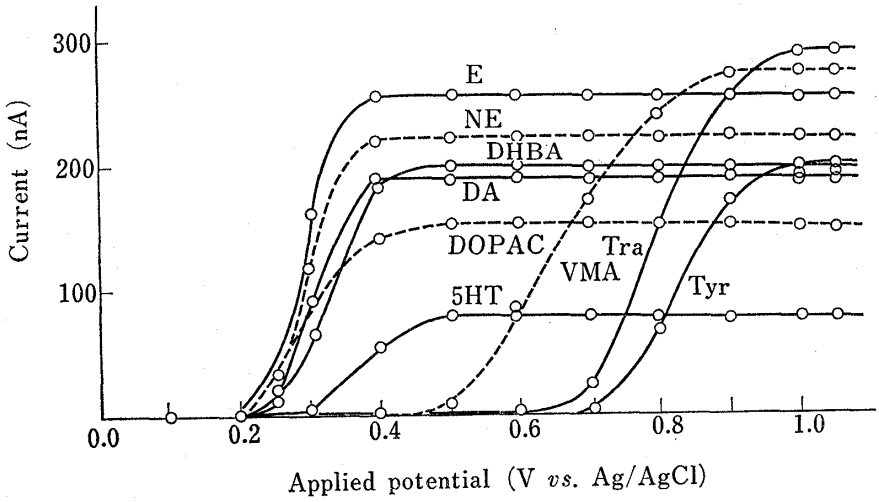

Fig. 5. Current–Potential Curves for Monoamines and Their Metabolites

Injected amount (ng): NE, 65.4; E, 83.8; DHBA, 51.0; Tyr, 78.6; DA, 60.5; Tra, 66.1; DOPAC, 87.5; VMA, 72.0; 5HT, 38.7.

15) T.P. Moyer and N. Jiang, *J. Chromatogr.*, **153**, 365 (1978).
16) P.T. Kissinger, C.S. Bruntlett, G.C. Davis, L.J. Felice, R.M. Riggin, and R.E. Shoup, *Clin. Chem.*, **23**, 1449 (1977).
17) H. Hashimoto and Y. Maruyama, *J. Chromatogr.*, **152**, 387 (1978).

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exchange column (Yanaco pell CX) were 166, 115 and 84, respectively. These discrepancies would be due to the difference of theoretical plate number and resolution for two columns. The results for current response and detection limit of our detector were compared with the reported values of other investigators. As shown in Table I, our detector was superior to the others on both current response and detection limit.

ECD is extremely selective detector because the sensitivity for each compound can be changed by the alteration of the applied potential on the working electrode.[18] Fig. 5 showed the current-potential curves obtained from the chromatographic peak current of the respective biogenic amines at each potential. The limiting currents of NE, DHBA, DA, epinephrine (E) and 3,4-dihydroxyphenylacetic acid (DOPAC) reached plateau at about 0.4 V *vs.* Ag/AgCl. 5HT, 4-hydroxy-3-methoxymanderic acid (VMA), tyramine (Tra) and tyrosine (Tyr) reached plateau at 0.5, 0.9, 1.0 and 1.0 V *vs.* Ag/AgCl, respectively. But in more acidic mobile phase, the oxidation potentials of these compounds were shifted to more positive potential. These biogenic amines can be classified into catechols (NE, DHBA, DA, E, DOPAC), *o*-methoxyphenols (VMA, 4-hydroxyphenylacetic acid; HVA), phenols (Tra, Tyr) and 5-hydroxyindoles (5HT, 5-hydroxyindoleacetic acid; 5HIAA) and each amines are oxidized by two electrons process to produce benzoquinone.[19–22] Catechols are easier to be oxidized than any other compounds described above, and the oxidation potential of *o*-methoxyphenols and phenols are shifted to a more positive potential with decreased aromatic electron density.

Figure 6 illustrated the capability of ECD for selective detection of easily oxidized compound, authentic biogenic amines and their metabolites. At a low potential of 0.65 V *vs.* Ag/AgCl, sensitivity to HVA was decreased and Tra and Tyr peaks could not be detected, but the other compounds could be detected with comparable sensitivity. While at 0.8 V *vs.* Ag/AgCl, all compounds could be detected. As shown in Fig. 6B, the sensitivities of the

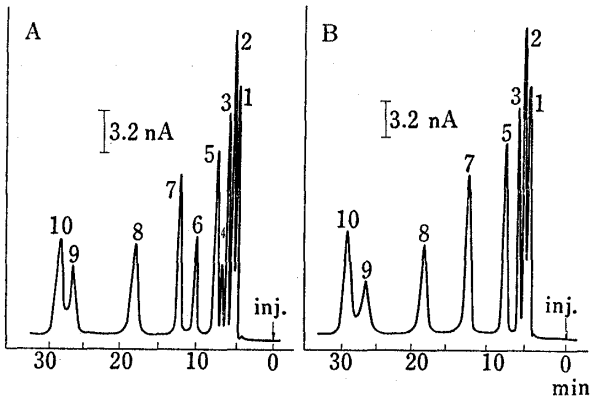

Fig. 6. Chromatograms of Monoamines and Their Metabolites

Column; LiChrosorb RP-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; 1%tetrahydrofuran-pH 5.1 buffer.
Applied potential; A, 0.8 V *vs.* Ag/AgCl,
B, 0.65 V *vs.* Ag/AgCl.
Flow rate; 0.70 ml/min.
1, NE (13.1)[a]; 2, E (16.8); 3, DHBA (10.2); 4, Tyr (15.7); 5, DA (12.1); 6, Tra (13.2); 7, DOPAC (17.5); 8, 5HT (7.7); 9, HVA (15.5); 10, 5HIAA (16.4).
a) Parentheses show the injected amount (ng).

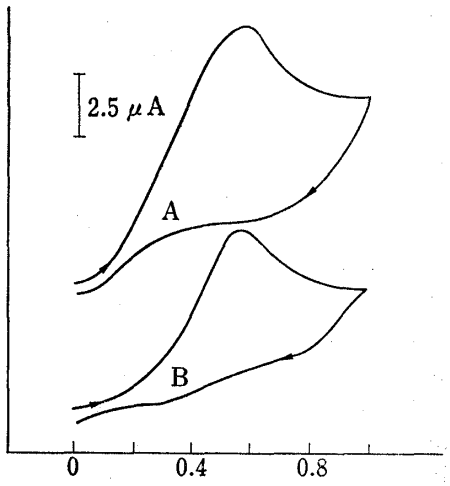

Fig. 7. Cyclic Voltammograms of DA at Sweep Rate of 0.2 V/sec

A; DA separated on column, 0.13 μM.
B; authentic DA, 0.61 mM/l in 1%tetrahydrofuran-pH 5.1 citrate buffer.

18) *a*) C.L. Blank, *J. Chromatogr.*, **117**, 35 (1976); *b*) D.G. Swartzfager, *Anal. Chem.*, **48**, 2189 (1976).
19) *a*) R.N. Adams and M.D. Hawley, *J. Am. Chem. Sci.*, **89**, 447 (1976); *b*) R.N. Adams, *J. Pharm. Sci.*, **58**, 1171 (1969).
20) L. Papouchudo, J. Bacon, and R.N. Adams, *J. Electroanal. Chem.*, **24**, App. 1 (1970).
21) S. Isoe and L.A. Cohen, *Arch. Biochem. Biophys.*, **127**, 522 (1968).
22) S. Garattini and L. Valzelli, "Serotonin," Elsevier Pub. Co., Amsterdam, 1965.

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electroactive biogenic amines (catecholamines, 5-hydroxyindoleamines) under ECD detection were nearly the same, while the sensitivities under fluorometric detection were different among each biogenic amines.[23]

As shown in Fig. 7, the cyclic voltammogram of DA, which was measured in a stopped flow after separation on reversed-phase chromatographic column, gave a single oxidation wave near 0.5 V *vs.* Ag/AgCl. This voltammogram was in good agreement with that of authentic DA in 1% tetrahydrofuran–pH 5.1 citrate buffer. Therefore, the technique of cyclic voltammetry could be used for the peak identification.

The coulometric yield (the percentage of solute actually electrolysed) for DA at flow rate of 1 ml/min was 3—4%. Reproducibility of this electrode was demonstrated by 40 repeated injection of 10 ng DA. The standard deviation of the peak current was approximately 1.8%. This precision is attributed to the flow rate of the mobile phase, the temperature and the stability of the residual current. The adsorption phenomena on the electrode surface were scarcely observed during about 1000 times trials of the biological samples, that was considered to result from continuous cleaning of the electrode surface by the mobile phase.

Application to the Determination of Biogenic Amines

The role of biogenic amines and their metabolites has been extensively studied in relation to the central and peripheral nervous systems. Therefore, it is supposed that the establishment of the simultaneous, simple and accurate method for the determination of biogenic amines in biological samples is desired earnestly by many neuroscientists.

Recently, the catecholamines analyses in biological samples have been performed by HPLC in combination with spectrophotometric,[24] fluorometric detectors.[25] But, these analytical methods admit of improvement on sensitivity and analytical time. Although the gas chromatography–mass spectrometry[26] and radiochemical method[27] have excellent sensitivity and selectivity, these are not suited to routine assays because of very expensive and complicated technique. The most extensive studies for the separation of catecholamines by HPLC have been performed by Kissinger using electrochemical detector.[28] Simultaneous determination of individual catecholamines and 5-hydroxyindoleamines by HPLC has been difficult and the method suited to routine assay has not been established. We applied ECD to simultaneous determination of biogenic amines, NE, DA and 5HT in whole mouse brain, mouse cerebellum, rat urine and dog serum.

For the determination of biological samples, HPLC condition must be settled in consideration of operation time and resolution. First, we examined the separation of NE, DA and 5HT using several packing materials for HPLC, the cation-exchange type (Yanaco pell CX, Partisil PXS, Nucleosil SA) and the chemically bonded reversed-phase type (μ Bondapak C_{18}, LiChrosorb RP-18, Nucleosil C_{18}). Of these packing materials, LiChrosorb RP-18 was definitely superior to the others in operation time and resolution. Also, the reversed-phase packing materials were superior to the cation-exchange one for resolution, sensitivity and biogenic amine metabolites assay.

More recently, Horvath and Molnar[29] demonstrated that reversed-phase columns with neat aqueous mobile phase, which did not contain organic solvent and ion-pairing reagents,

23) K. Mori, *Ind. Health*, **12**, 171 (1974).
24) *a*) J.H. Knox and J. Jurand, *J. Chromatogr.*, **125**, 89 (1976); *b*) I. Molnar and C. Horvath, *Clin. Chem.*, **22**, 1497 (1976).
25) *a*) L.D. Mell and A.B. Gustafson, *Clin. Chem.*, **23**, 473 (1977); *b*) K. Imai, M. Tsukamoto, and Z. Tamura, *J. Chromatogr.*, **137**, 357 (1977).
26) *a*) S.H. Koslow, F. Cattabeni, and E. Costa, *Science*, **176**, 177 (1972); *b*) K. Imai, M.T. Wang, S. Yoshiue, and Z. Tamura, *Clin. Chem. Acta*, **43**, 145 (1973).
27) V. Weise and I.J. Kopin, *Life Sci.*, **19**, 1673 (1976).
28) *a*) R. Keller, A. Oke, I. Mefford, and R.N. Adams, *Life Sci.*, **19**, 995 (1976); *b*) R.E. Shoup and P.T. Kissinger, *Clin. Chem.*, **23**, 1268 (1977).
29) C. Horvath and I. Molnar, *Anal. Chem.*, **49**, 142 (1977).

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could be successfully employed for the separation of acidic, basic and zwitterionic substances, which have been separated using different ion-exchange column, respectively. With aqueous mobile phase, 5HT, HVA and 5HIAA were strongly retained in LiChrosorb RP-18. Accordingly, one part of tetrahydrofuran was added to 100 part of aqueous mobile phase to decrease capacity factor of 5HT, HVA and 5HIAA.

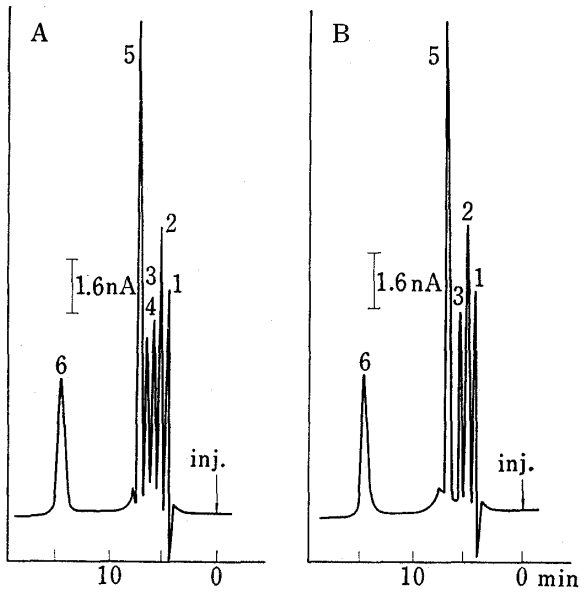

Fig. 8. Chromatograms of Monoamines in Whole Mouse Brain

Column; LiChrosorb RP-18 (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; 1%tetrahydrofuran-pH 5.1 buffer.
Applied potential; A, 0.8 V *vs.* Ag/AgCl,
B, 0.65 V *vs.* Ag/AgCl.
Flow rate; 0.77 ml/min.
1: solvent, 2: NE, 3: DHBA, 4: Tyr, 5: DA, 6: 5HT.

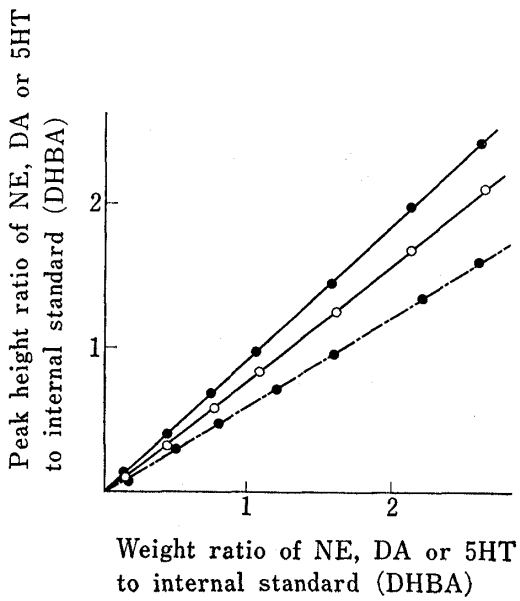

Fig. 9. Calibration Curves of NE, DA and 5HT

—●—: NE, —○—: DA, —●—: 5HT.
Injected amount of NE, DA and 5HT 0.4—10 ng.

Figure 8 illustrated chromatograms of biogenic amines in normal mouse brain at applied potential of 0.65 and 0.8 V *vs.* Ag/AgCl. At 0.8 V *vs.* Ag/AgCl, Tyr peak was detected with comparable sensitivity and found to interfere with the DA determination to a slight extent. But, lowering the potential to 0.65 V *vs.* Ag/AgCl, diminished the Tyr peak. Therefore, the determination of NE, DA and 5HT were carried out at 0.65 V *vs.* Ag/AgCl. These amines in whole mouse brain and mouse cerebellum were extracted according to the modification of the Shore and Olin's method[30] after addition of DHBA as the internal standard and separated on LiChrosorb RP-18 in less than 15 minutes. Percent recoveries for our extraction procedure in the presence of whole mouse brain were 48%, 43% and 40% for NE, DA and 5HT, respectively. These percent recoveries were significantly better as compared with the reported procedures.[30,31] Fig. 9 showed the calibration curves used for the determination of NE, DA and 5HT in whole mouse brain. The results for the determination of NE, DA and 5HT in whole mouse brain and mouse cerebellum were given in Table II, along with the reported value of other investigators. No significant differences were seen between biogenic amines level obtained by us and those reported by other investigators. Accordingly, our methods could be used as the simple routine method for the simultaneous determination of

30) P.A. Shore and J.S. Olin, *J. Pharmacol. Exp. Ther.*, **122**, 295 (1958).
31) S. Sasa and C.L. Blank, *Anal. Chem.*, **49**, 354 (1977).
32) A.S. Welch and B.L. Welch, *Anal. Biochem.*, **30**, 161 (1969).

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Table II. NE, DA and 5HT Concentration in Whole Mouse Brain and Mouse Cerebellum

Tissue	Investigators	Assay procedure	NE (ng/g)	DA (ng/g)	5HT (ng/g)
Whole mouse brain	Present paper	HPLC	430±10[a] (28)	872±22 (28)	749±20 (28)
	Sasa and Blank[31]	HPLC	—	973±16 (40)	805±12 (40)
	Welch and Welch[32]	Fluorometry	350—450 (1000)	700—900 (1000)	600—800 (1000)
Mouse cerebellum	Present paper	HPLC	259±19 (7)	16±3 (7)	240±21 (7)
	Sasa and Blank[31]	HPLC	—	19±2 (6)	240±14 (6)

a) The values are expressed as the mean±standard error of mean for the number of animals in parentheses.

biogenic amines. Although there is no reference to the level of NE in mouse cerebellum, our results were similar to the reported value by fluorometric method for rat cerebellum.[33]

Furthermore, to confirm the application to the biological samples, the biogenic amines in rat urine and dog serum were examined. The catecholamines in unhydrolyzed and hydrolyzed rat urine were extracted according to the method of Anton and Sayre,[7] and separated on Nucleosil C_{18} column with pH 3.0 tartrate buffer containing 0.5 M sodium sulfate as mobile phase. The determination of 5HT in dog serum was performed according to the method used for whole mouse brain. Fig. 10 illustrated chromatograms of catecholamines in normal rat urine at applied potential of 0.7 V *vs.* Ag/AgCl. From Fig. 10, concentration of NE, E, DOPA and DA for unhydrolyzed rat urine were 75, 7, 42 and 170 ng/ml and for hydrolyzed rat urine, 90, 13, 50 and 330 ng/ml, respectively.

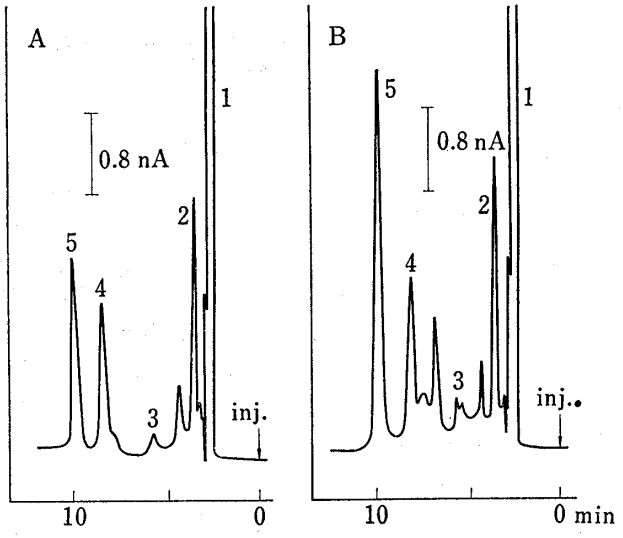

Fig. 10. Chromatograms of Catecholamines in Rat Urine

Column; Nucleosil C_{18} (10 μm), 25 cm×4.6 mm I.D.
Mobile phase; pH 3.0 tartrate buffer (containing 0.5 M Na_2SO_4).
Applied potential; 0.70 V *vs.* Ag/AgCl.
Flow rate; 1.0 ml/min.
A, unhydrolyzed,
B, hydrolyzed.
1; solvent, 2; NE, 3: E, 4: DOPA, 5: DA.

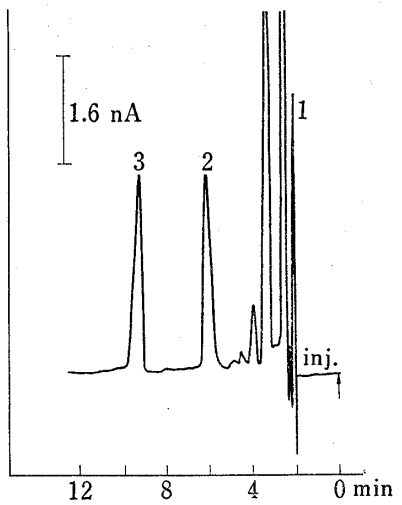

Fig. 11. Chromatogram of 5HT in Dog Serum

Column; LiChrosorb RP-18 (10 μm), 25cm×4.6 mm I.D.
Mobile phase; 1% tetrahydrofuran-pH 5.1 buffer.
Applied potential; 0.65 V *vs.* Ag/AgCl.
Flow rate; 1.5 ml/min.
1: solvent, 2: 5HT, 3: unknown.

33) G.M. Tyce and C.A. Owen, *Life Sci.*, **22**, 781 (1978).

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Figure 11 illustrated chromatogram of 5HT in normal dog serum which could be obtained using only 0.5 ml of serum. Concentration of 5HT in dog serum was 910 ng/ml.

As mentioned above, not only biogenic amines (NE, DA, 5HT) but also their metabolites (DOPAC, HVA, 5HIAA) could be detected by ECD. Therefore, if proper procedure for extraction of their metabolites from biological samples can be used, it is possible to analyse these compounds simultaneously.[34] We believe that in the near future these simple, sensitive and reliable methodology can be extensively used for catecholamines research in pathology as well as in pharmacology.

Also, the ECD described here can be adapted to the determination for other oxidation–reduction compound in nonaqueous mobile phase and those applications will be published elsewhere.

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34) In preparation.

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