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Electrochemical Detector for High-Performance Liquid Chromatography. VI.[1] Application of a Twin Electrochemical Detector

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The fundamental characteristics of a twin electrochemical detector were evaluated to investigate its suitability as a detector for high-performance liquid chromatography. This novel detector was proved to have the following advantages compared to the conventional three-electrode detector. 1) Simultaneous determination of reduced and oxidized species is possible, 2) the stability of the electrode is improved by applying a larger potential to the other electrode, 3) the specificity of detection is improved by the re-electrolysis at the second electrode of the reaction product produced at the first electrode, and 4) specific detection of the post-discharging materials and sensitive detection are possible by using appropriate operating circuits.

Keywords——HPLC; electrochemical detector; two working electrode; coenzyme-Q; monoamine

In recent years, there has been great interest in the use of an electrochemical detector (ECD) for high performance liquid chromatography (HPLC), since it permits sensitive and specific detection of electroactive materials. However, usual ECD has the defects that the stability of the working electrode is rather poor in the reduction mode and that the selectivity decreases when large potentials are applied. Although conventional ECD detects oxidized or reduced species, it cannot detect both simultaneously.

To overcome these shortcomings inherent in the conventional ECD, a twin electrochemical detector (T-ECD) having two working electrodes has been reported.[2] However, the fundamental characteristics of the electrodes and the applications to biological materials have not been much investigated. Here we describe the construction and some features of our T-ECD and its use in the HPLC determination of biological materials.

Experimental

Apparatus—— The HPLC system used was a Yanagimoto LC-2000 system with Yanagimoto VMD-501 (T-ECD) and VMD-101 (three electrode) detectors. For ultraviolet (UV) detection, a JASCO UVIDEC-III was used. For chromatographic separation, a Nucleosil-C18 column (15 cm × 4.6 mm i.d., 5 μm) was used. HPLC measurements were performed at ambient temperature.

Construction of the T-ECD——Two working electrodes were made from glassy carbon disks 3 mm in diameter and positioned in series. The distance between the two electrodes was set at 5 mm.

The upstream electrode was named Wl, and the downstream electrode W2. An Ag/AgCl reference electrode (Ref.) was used, and the potentials of the working electrodes were set *vs.* Ag/AgCl. A stainless tube (0.75 mm i.d.) was used as the auxiliary electrode (Aux.), and it served as the outlet for the effluent (Fig. 1).

Materials——Hydroquinone (HQ) and benzoquinone (BQ) were purchased from Tokyo Kasei Co. Coenzyme Q-9 (CoQ-9) and coenzyme Q-10 (CoQ-10) were obtained from Nisshin Chemical Co. 3,4-Dihydroxybenzylamine (DHBA) was synthesized according to the method of Refschauge *et al.*[3] The reduced CoQ-9 (CoQ-9-H_2) and CoQ-10

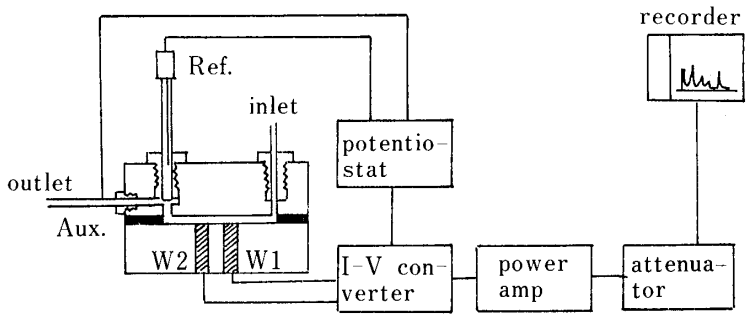

Fig. 1. Construction and Flow Diagram of T-ECD

(CoQ-10-H_2) were obtained by the reduction of CoQ-9 and CoQ-10 with sodium borohydride. 2,3,6-Trimethyl-5-decaprenyl-1,4-benzoquinone (TQ-10) was synthesized according to the method of Rügg *et al.*[4] All other chemicals used were of reagent grade.

Assay Procedure——CoQ-9 in Rat Plasma and Liver: Adult male Sprague Dawley rats weighing 220—300 g were sacrificed by decapitation. The livers were removed, weighed, rinsed with 0.15 M NaCl solution, and homogenized at 4°C with 100 volumes (v/w) of water in a Polytron homogenizer (Hijiri Seiko, Japan) for 20 s. A 0.5 ml aliquot of the homogenate or plasma was placed in a brown glass centrifuge tube and TQ-10 (4 μg) was added as an internal standard. The solution was deproteinized with 2.5 ml of ethanol and extracted with 5 ml of *n*-hexane followed by oxidation of the reduced CoQ-9 with 0.5 ml of 2% (w/v) ferric chloride ethanol solution. Four ml of the *n*-hexane layer was evaporated to dryness under a nitrogen stream at 40 °C. The residue was dissolved in 100 μl of isopropanol and 50 μl of the resultant solution was injected into the HPLC column.

Results and Discussion

Simultaneous Determination of CoQ and CoQ-H_2, and Anodic Determination of CoQ by Re-electrolysis in Biological Materials

Although the simultaneous detection of oxidized and reduced species of 6-hydroxydopamine has already been reported,[2a] applications to biological materials have not been presented. Goto *et al.*[2f,g] also determined biogenic amines in physiological materials by re-electrolysis to improve the selectivity. Here we applied T-ECD to the simultaneous determination of CoQ and CoQ-H_2, and also to the determination of CoQ by anodic oxidation in biological materials.

i) Electrochemical Study of the Electrodes—— A standard mixture containing HQ and BQ as model compounds was separated and the peak currents were measured at various potentials while the potential of W2 ($E2$) was held at +0.7 V. The observed currents for HQ at W1 and W2, and that for BQ at W1 were plotted against the potential of W1 ($E1$) (Fig. 2). Since almost constant currents were obtained at +0.3——0.5 V for HQ (W2) and at −0.1——0.5 V for BQ (W1), the simultaneous determination of the two should be possible. The decreased current for HQ is presumably due to the increase of the diffusion layer caused by the oxidation of HQ at W1. Figure 3 shows the peak current of BQ *vs.* the potential of one electrode, obtained by keeping the potential of the other electrode at +0.7 V. The anodic current detected at W1 was thought to be the flow of a part of the reduction current at W2, caused by the difference of the applied potentials between W1 and W2. The anodic current at W2 is the re-oxidation current of the reductant (HQ) from BQ at W1. It is clear that some undesirable interactions between the two electrodes are present. Nevertheless, linear calibration curves were obtained in the simultaneous determination of CoQ and CoQ-H_2, and in the anodic determination of quinoid compounds.

ii) Simultaneous Determination of Reduced and Oxidized CoQ——CoQ was extracted with a mixture of EtOH–*n*-hexane from the homogenates of various organs in accordance with the reported procedure.[5] Reduced and oxidized CoQ in guinea pig kidney were detected simultaneously by setting $E1$ and $E2$ at +0.7 and −0.3 V, respectively (Fig. 4). Table

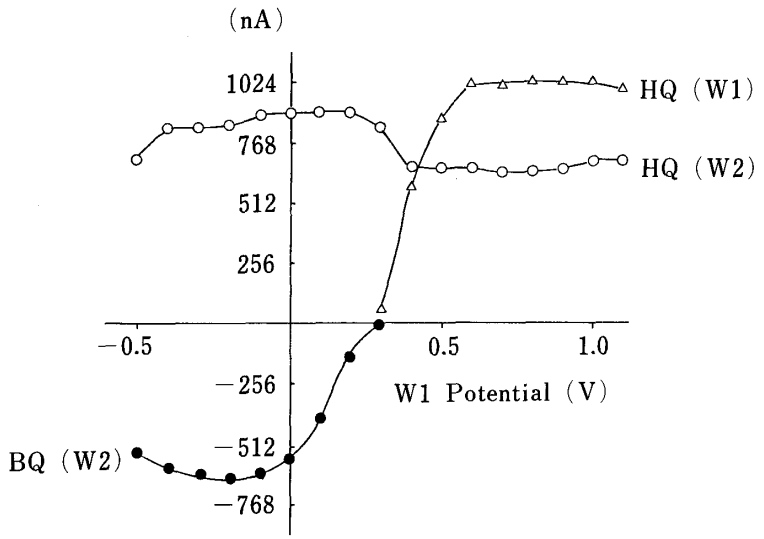

Fig. 2. Hydrodynamic Voltammograms of HQ and BQ ($E2$: +0.7 V *vs.* Ag/AgCl)

HPLC condition: column, Nucleosil-C18 (15 cm × 4.6 mm i.d., 5 μm); eluent, MeOH–H_2O (150 : 850) containing 0.1 M phosphate buffer (pH 3.2); flow rate, 1.0 ml/min. Injected amount: 500 ng.

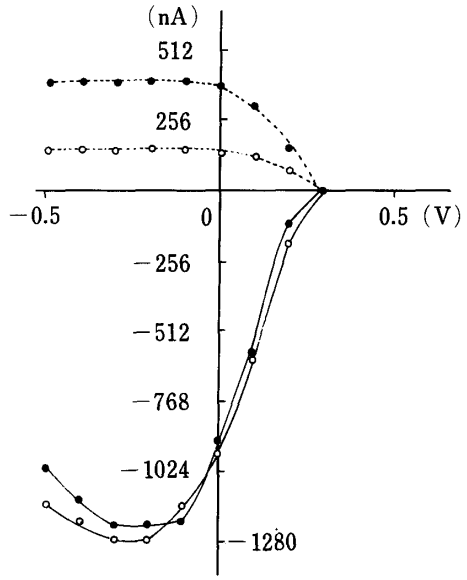

Fig. 3. Hydrodynamic Voltammograms of BQ

The potential of the one working electrode was varied while that of the other was maintained at +0.7 V.
W1 —○—: variable, W1 ---○---: +0.7 V, W2 ---●---: +0.7 V, W2 —●—: variable.

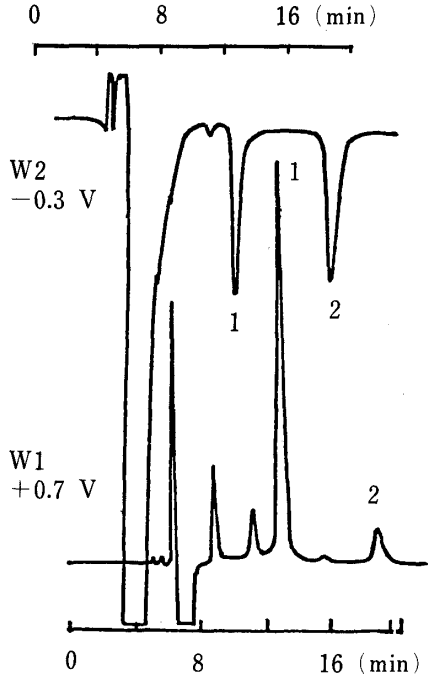

Fig. 4. Simultaneous Detection of CoQ-10 and CoQ-10-H_2 in Guinea Pig Kidney

Mobile phase: MeOH–EtOH–$HClO_4$ (500 : 500 : 1) containing 0.7% $NaClO_4 \cdot H_2O$.
Peak identity: 1, CoQ-10-H_2; 2, CoQ-10.

I compares the present method with T-ECD and with the previous method[5] in the analysis of various tissues for CoQ. The results are in good agreement with each other, and it is clear that the simultaneous determination of reduced and oxidized CoQ in biological materials is possible with T-ECD.

iii) Determination of CoQ by Re-electrolysis——CoQ has been sucessfully determined by cathodic reduction, using a mobile phase flushed with nitrogen gas to eliminate the

Table I. Contents of Reduced and Oxidized Coenzyme-Q in Animal Tissues Determined by the Present and Previous Methods

Animal	Tissue	CoQ	T-ECD (μg/g)	ECD-UV (μg/g)[5]
Rat	Liver	CoQ-9-H_2	100.8	108.3
		CoQ-9	18.5	16.6
Guinea Pig	Heart	CoQ-10-H_2	124.4	122.2
		CoQ-10	102.2	92.8
	Liver	CoQ-10-H_2	48.5	45.7
		CoQ-10	11.6	12.5
	Kidney	CoQ-10-H_2	106.9	106.6
		CoQ-10	36.4	35.9

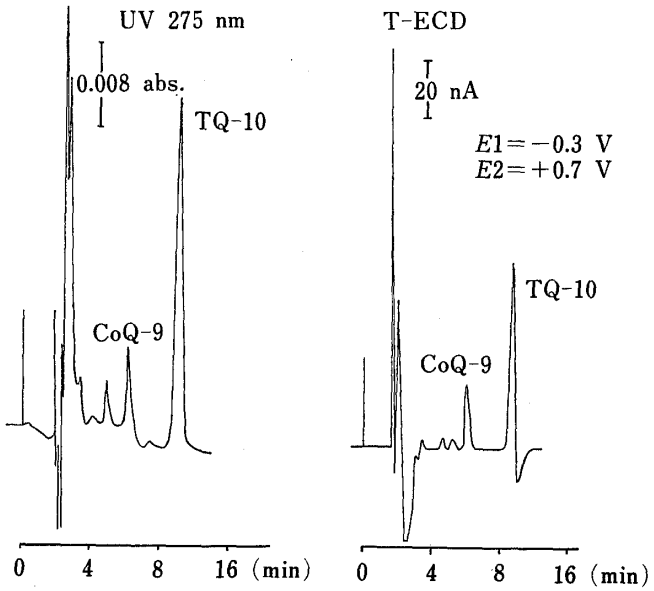

Fig. 5. Chromatogram of CoQ-9 in Rat Liver

Table II. Contents of CoQ-9 in Rat Liver and Plasma

Sample	No.	UV (275 nm)[7]	T-ECD	T-ECD/UV	
Liver	1	207 μg/g	207 μg/g	100%	X=99.5% CV=10.4%
	2	205 μg/g	178 μg/g	86.8%	
	3	208 μg/g	210 μg/g	101%	
	4	189 μg/g	182 μg/g	96.3%	
	5	197 μg/g	232 μg/g	118%	
	6	283 μg/g	268 μg/g	94.7%	
Plasma	1	0.25 μg/ml	0.3 μg/ml	120%	X=107%
	2	0.12 μg/ml	0.10 μg/ml	83.3%	
	3	0.53 μg/ml	0.63 μg/ml	119%	

interference of dissolved oxygen.[6] Electrochemically reduced oxygen at W1 was not detected at W2, *i.e.*, oxygen was not reversibly detected. On the other hand, quinoid compounds showed reversible voltammograms, so the reduced dihydro forms were detected in anodic oxidation without complicated de-aeration of the mobile phase. CoQ was first converted to CoQ-H_2 at W1 ($E1=-0.3$ V) and subsequently the oxidation current at W2 ($E2=+0.7$ V) was detected. CoQ-9 in plasma and liver homogenates was extracted with *n*-hexane–EtOH

followed by the oxidation of reduced CoQ-9 with $FeCl_3$. As can been seen in the chromatogram of rat liver obtained with T-ECD (Fig. 5), interference from biological materials and dissolved oxygen in the sample solution[6] was decreased. The results obtained with T-ECD were in good agreement with those obtained with UV detection[7] (Table II).

Other Applications of T-ECD

i) Suppression of the Increase of Background Current—— The stability of glassy carbon electrodes is rather poor, especially in cathodic operation, so applications to reducible

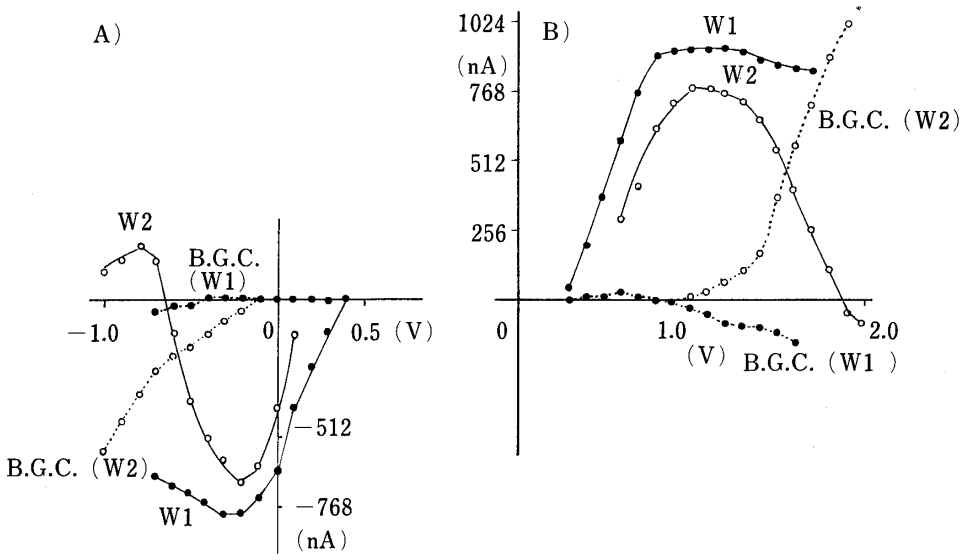

Fig. 6. Hydrodynamic Voltammograms of HQ and BQ

A) Sample, BQ (500 ng). $E2=E1-0.3$ V.
B) Sample, HQ (500 ng). $E2=E1+0.3$ V.
HPLC conditions were as in Fig. 2.

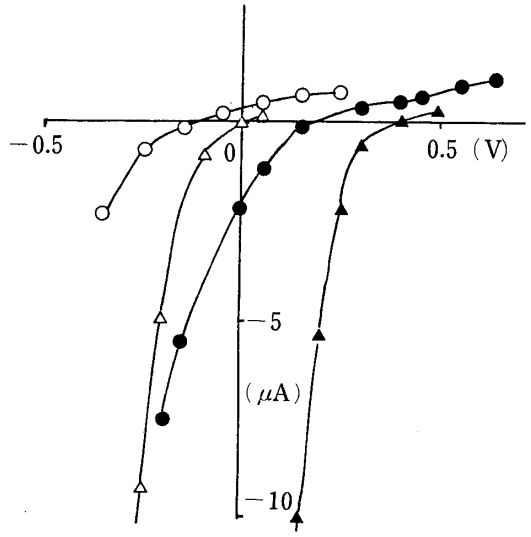

Fig. 7. Effect of Fe^{3+} on the Background Current at W1 and W2 ($E2=E1-0.3$ V)

Mobile phase: 0.1 M phosphate buffer (pH 3.2) in the presence or absence of 0.05% $FeCl_3 \cdot 6H_2O$.
—○—: W1, without Fe^{3+}. —△—: W2, without Fe^{3+}. —●—: W1, with Fe^{3+}. —▲—: W2, with Fe^{3+}.

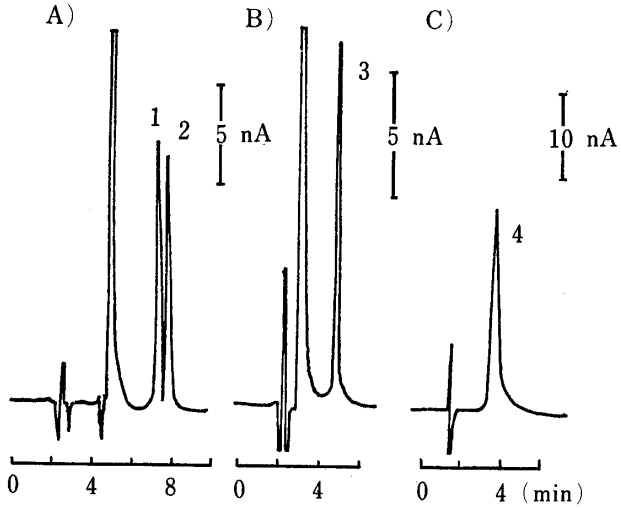

Fig. 8. Chromatograms by Cathodic Detection ($E2$: −1.5 V *vs.* Ag/AgCl)

A) 1, *m*-dinitrobenzene (110 ng); 2, nitrobenzene (110 ng); mobile phase, MeOH–0.043 M $HClO_4$ (600:400) containing 0.01 M $Bu_4N \cdot ClO_4$; $E1$, −1.3 V. B) Benzoyl peroxide (100 ng); mobile phase, MeOH–0.09 M $HClO_4$ (800:200) containing 0.01 M $Bu_4N \cdot ClO_4$; $E1$, −1.0 V. C) Butyl peroxide (1 μg); mobile phase, MeOH–H_2O (400:600) containing 0.01 M $Bu_4N \cdot ClO_4$; $E1$, −0.6 V.

compounds have been limited. In this study, W2 was set at a more negative potential in cathodic reduction, and at a more positive potential in anodic oxidation, than W1 in order to minimize the increase of the background current (B.G.C.) at W1. Figure 6 shows the hydrodynamic voltammograms of BQ, HQ, and B.G.C. obtained at W1 and W2. B.G.C. was greatly suppressed at W1 compared to that at W2, and sensitive detection of HQ and BQ was achieved with W1. Ionic materials are one of the composites causing B.G.C. With the addition of Fe^{3+} to the mobile phase, the B.G.C. at W2 became larger (Fig. 7). That is, ionic materials are adsorbed more at W2, and the B.G.C. at W1 does not increase so much. This method offers an improvement of sensitivity by about one order of magnitude as compared with normal operation in cathodic reduction. In fact, as little as 1 ng of nitrobenzene was detectable (Fig. 8). Figure 8 also shows examples of the detection of peroxides.

ii) Increase of Sensitivity and Selectivity by the Use of Suitable Operating Circuits——By utilizing the outputs at both electrodes, improvement of the sensitivity and selectivity can be expected.

a) Addition: Figure 9 (right) shows a chromatogram of norepinephrine (NE), epinephrine (E), DHBA, and dopamine (DA) obtained by adding the output at W2 to that at W1. The baseline noise was not much affected, and the peak heights were increased about 1.7 times compared to the normal ECD system (left). The detection limit was 2 pg of each amine (injected amount).

b) Subtraction: Normal ECD has the disadvantage that the selectivity decreases at high potentials, and this can be very troublesome in applications to biological materials. However, recording the difference between W1 and W2 outputs resulted in an improvement of selectivity, which permitted the specific detection of post-discharging substances of interest. Figure 10 shows the outputs using a fixed 200 mV "window." At +0.7 V, the limiting currents of DA, DHBA, NE, E, normetanephrine (NM), and 3,4-dihydroxymandelic acid (DOMA)

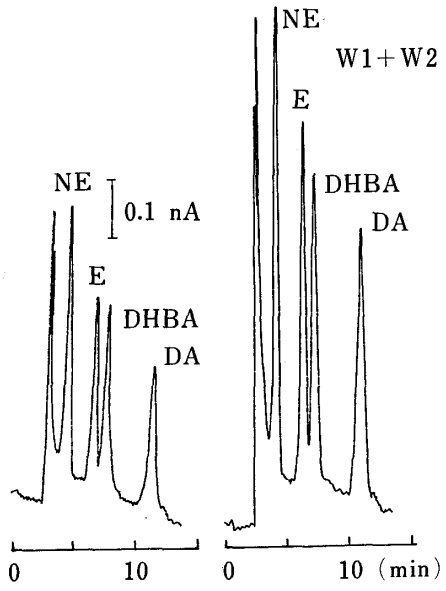

Fig. 9. Chromatograms of Catecholamines (100 pg Each)

Mobile phase: 0.1 M phosphate buffer (pH 3.2). Flow rate: 0.8 ml/min. $E1 = +0.7$ V. $E2 = +0.7$ V.

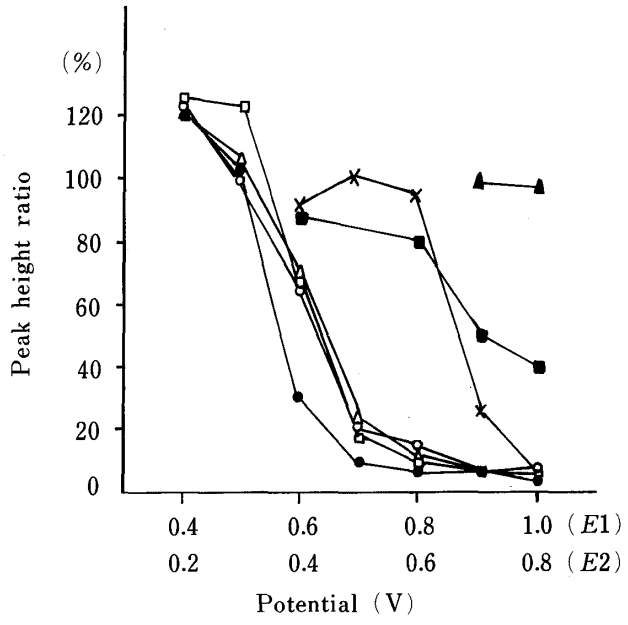

Fig. 10. Relationship between Peak Height Ratio and Applied Potential in the Subtraction Mode ($E1 - E2 = 0.2$ V)

Peak height ratio was calibrated with the limiting current of each material; values exceeding 100% were a result of the re-reduction currents at W2.
—●—, DA; —▲—, Tyr; —□—, DHBA; —○—, NE; —■—, VMA; —×—, NM; —△—, E or DOMA.

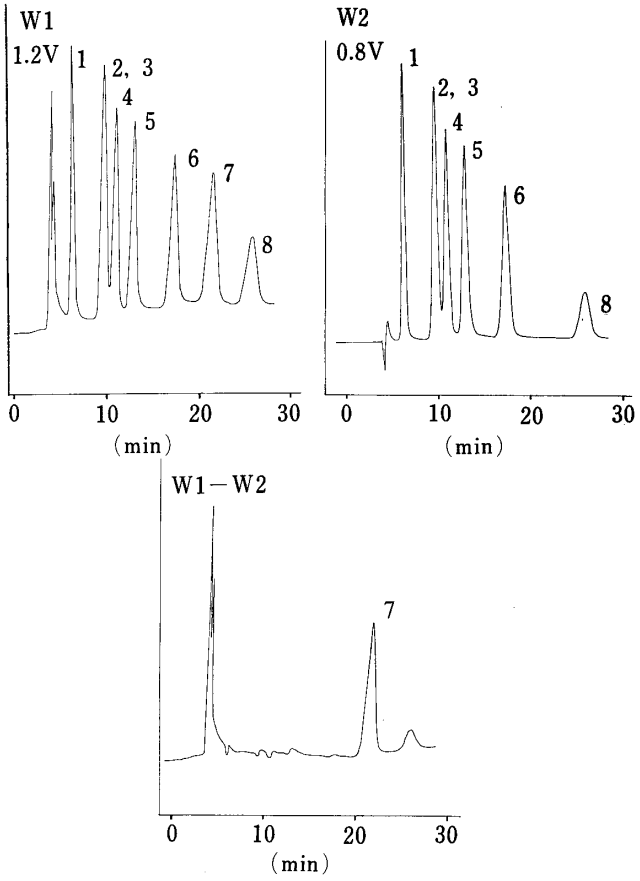

Fig. 11. Specific Detection of Tyr

1, NE; 2, DOMA; 3, E; 4, DHBA; 5, NM; 6, DA; 7, Tyr; 8, VMA. HPLC conditions were as in Fig. 2.

were obtained, so the outputs of these materials decreased. However, tyrosine (Tyr) and vanillylmandelic acid (VMA) did not reach limitting currents, so specific detection of Tyr and VMA was accomplished. Figure 11 shows the standard chromatograms of some monoamines. Specific detection of Tyr was achieved by applying +1.2 and +0.8 V to W1 and W2, respectively. We thus investigated the fundamental features of T-ECD having two working electrodes, and found the following advantages compared to conventional three-electrode detectors. 1) Simultaneous determination of reduced and oxidized species is possible by applying different potentials to the two working electrodes. 2) Quinoid compounds such as CoQ, normally detected in cathodic reduction, can be detected in anodic oxidation with re-electrolysis of the reductant at W1. This means that de-aeration of the mobile phase is not necessary and that interference caused by dissolved oxygen in the sample solution[6] can be eliminated. 3) The stability of the electrode W1 can be improved by applying a large potential to W2, and extension of the applied potential range of glassy carbon can be achieved. 4) The sensitivity is improved by the addition of the outputs from W1 and W2 by the use of suitable operating circuits. 5) Specific detection of post-discharging compounds can be achieved by subtraction excluding interference by the pre-discharging compounds. This overcomes the deficiency of conventional ECD that all the pre-discharging materials are detected under large applied potential. The present method is somewhat related to differential pulse polarography,[8] which is a method of improving the selectivity by applying a pulsed potential to the base potential followed by subtraction of the outputs as in this report.

References and Notes

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