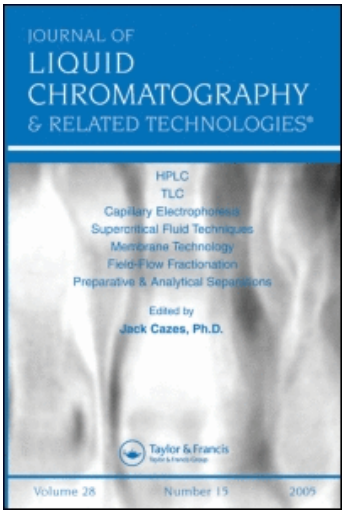

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Dual Electrochemical Detection of Biogenic Amine Metabolites in Micro High-Performance Liquid Chromatography

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DUAL ELECTROCHEMICAL DETECTION OF BIOGENIC AMINE METABOLITES IN MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The dual electrochemical detectors for ordinary and micro high-performance liquid chromatography were briefly reviewed.

The electrochemical behaviors of biogenic amine metabolites were studied by cyclic semi-differential and semi-integral voltammetry with a glassy carbon working electrode. It was found that the electrochemical reactions of many biogenic amine metabolites are quasi-reversible. The dual electrochemical detector based on thin-layer electrolytic cell with two working electrodes (anode and cathode) in series configuration was tested for selective detection of biogenic amine metabolites on their electrochemical quasi-reversibility. The detector was successfully utilized for the simultaneous determination of 3, 4-dihydroxyphenylacetic acid, homovanillic acid and 5-hydroxyindole-3-acetic acid in human urine directly injected by micro high-performance liquid chromatography.

INTRODUCTION

Electrochemical detection in high-performance liquid chromatography (HPLC) has become very popular for the determination of trace amounts of organic substances in biomedical and environmental samples (1-3). An innovative

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approach to electrochemical detection involves the use of two working electrodes oparated simultaneously at different potentials. Several dual electrochemical detectors have recently been developed which provide for enhanced performance (4-21). They can basically be classified into the three types in configuration of the two working electrodes with respect to the flow axis, as shown in Figure 1. In the "parallel-adjacent" configuration, the working electrodes are placed adjacent to each other on one side of the rectangurar thin-layer channel. In the "series" configuration, the working electrodes are positioned along the flow stream on one side of the channel. In the "parallel-opposed" configuration, the working electrodes are placed opposed to one another on both sides of the channel.

Dual electrochemical detection in HPLC

All dual electrochemical detectors can simultaneously provide two chromatograms of both oxidations or both reductions or oxidation and reduction by using the same or different material and size for each working electrode. Glassy carbon is most widely used as the material of working electrodes.

Parallel-ajacent type

The parallel-adjacent dual electrochemical detector (PADEC) is analogous to the dual-wavelength UV absorbance detector, and can provide useful qualitative information from peak current ratios at different potentials. By using PADEC, Roston and Kissinger performed the identification of phenolic constituents in commercial beverages and benzene metabolites by comparison with the standards (8, 16). Shoup and Mayer used PADEC for additional information on peak identity in determination of environmental phenols and biogenic amines and their metabolites (14, 18).

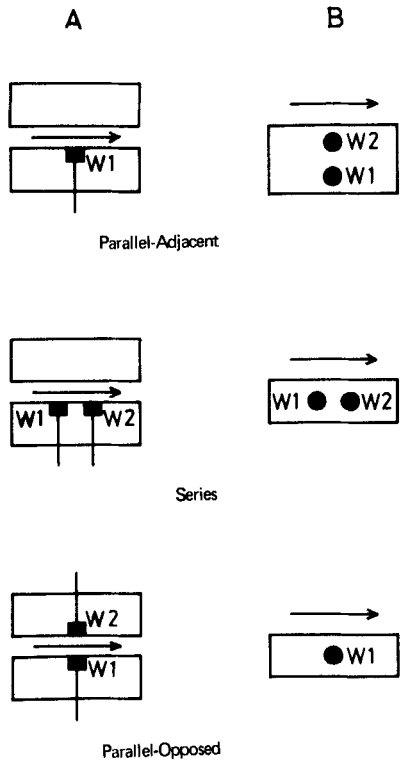

FIGURE 1. Three types in configuration of dual electrochemical detector. (A) Front view, (B) side view. W1 and W2 represent the two working electrodes. The arrows show the direction of flow.

Series type

The series dual electrochemical detector (SDEC) is analogous to the fluorecence detector, and the product of electrode reaction at the upstream working electrode is detected at the downstream working electrode. Blank employed SDEC for instrumental separation of compounds which overlap chromatographically, but have differing electrochemical formal potentials (4). Schieffer reported a series dual coulometric-amperometric detector (6). The upstream coulometric cell was held at lower

potential than the downstream amperometric cell to completely oxidize and make undetectable other species oxidizable at potentials lower than that of the analyte.

MacCrehan and Durst employed SDEC for the downstream oxidative detection of reduction products from the larger upstream mercury amalgam working electrode and demonstrated the detection of analytes with high redox potentials with good sensitivity and selectivity (7). Bratin and Kissinger used SDEC for elimination of oxygen interferences in reductive electrochemical detection (10). Allison and Shoup employed SDEC with mercury amalgam electrodes for simultaneous determination of thiols and disulfides in human blood and citrus leaf homogenate by using their catalytic oxidation of the mercury surface (19).

Roston and Kissinger employed SDEC for the downstream reductive detection of oxidation products from the upstream working electrode and estimated the collection efficiency, the magnitude of fraction of upstream products that are converted at the downstream working electrode, to be less than 0.37 (11). Mayer and Shoup used SDEC for assay of biogenic amines and their metabolites in brain tissue (18).

Parallel-opposed type

The parallel-opposed dual electrochemical detector (PODEC) is analogus to the photomultiplier tube and the product of the electrode reaction at one working electrode can diffuse to the opposite working electrode where starting material may be created. Fenn et al. explored the possibility of PODEC to improve detection limit of catecholamines in blood plasma at flow rates below 0.2 ml/min (5). Kurahashi used PODEC for selective detection of the analyte from peak current difference at different potentials (12). Inoue et al. employed PODEC to lower the background current (17). They applied the same potential to the two working electrodes and monitored the current from only one working electrode. Weber and

Purdy derived the theory concerned with the currents from a coulometric PODEC and approximately confirmed the theory by using a ferricyanide and ferrocyanide redox couple (15). They switched the working electrodes in and out of the current-to-voltage conversion circuit to defeat the large noise current generated by low cell impedance and obtained picogram detection limits for 2,4-toluenediamine in an aqueous methanol solvent.

Dual electrochemical detection in micro HPLC

Goto et al. developed a sub-microliter SDEC suitable for micro HPLC which was used for the downstream reductive detection of oxidation products from the upstream working electrode (9, 13). The detector was successfully utilized for the selective detection of catecholamines in human urine based on their electrochemical reversibility. We obtained the collection efficiencies of 0.68 to 0.78 for catecholamines at a flow rate of 8.3 µl/min. These values are much higher than those of 0.30 to 0.31 obtained in SDEC at a flow rate of 1.6 ml/min in the ordinary HPLC (18).

For slower flow rates, catalytic amplification of detector response for reversible and quasi-reversible analytes may be achieved by recycling the redox couple between the two working electrodes in PODEC. Goto et al. recently developed a coulometric PODEC with small working electrodes for micro HPLC (20, 21). The current amplification in the detector at flow rates of 1.4 to 11.2 µl/min was investigated by using ferricyanide as analyte. The effective current amplification efficiency, the ratio of anodic (or cathodic) current to coulometric current for oxidant (or reductant), of 19.5 was observed for ferricyanide at the flow rate of 1.4 µl/min (21). The collection efficiencies from 0.98 to 0.84 were obtained in the flow rate range from 1.4 to 11.2 µl/min (21). The detector was successfully utilized for the selective and sensitive detection of catecholamines in human serum by micro HPLC (20, 21).

In the present paper, the electrochemical behaviors of biogenic amine metabolites are investigated and their dual electrochemical detection with SDEC is tried for urine analysis by micro HPLC.

MATERIALS AND METHODS

Apparatus

A cyclic voltammetric instrument (Bioanalytical Systems Co., Model CV-1B) and a home-made analogue semi-differ-integrating circuit were used for cyclic semi-differential and semi-integral voltammetric measurements (22-25). Two x-y recorders (Yokogawa Co., Model 3086) were used to simultaneously record the cyclic semi-derivative and semi-integral voltammograms. A glassy carbon disk of 3 mm diameter was used as the working electrode. A silver/silver chloride electrode and a platinum wire were used for the reference and the counter electrode, respectively.

The micro HPLC system used is schematically shown in Figure 2. A micro feeder (Azuma Denki Co., Model MF-2), a micro syringe (Terumo Co., Model MS-CAN 100) and a three way valve were used to feed the mobile phase. A micro sample injector (Jasco, Model ML-422) with 0.3 μl loop was used for sample injection. The twin-electrode thin-layer electrolytic cell in series configuration as shown in Figure 2 of the previous paper (9) was used for dual electrochemical detection. The thin-layer cavity was constructed of two fluoro-carbon resin blocks separated by a PTFE sheet 50 μm thick and 2 mm wide. Two working electrodes were made with glassy carbon disks of 3 mm diameter contained in one of the blocks. The reference electrode, silver/silver chloride electrode, was held in a cylindrical hole in the other block. A platinum tube served both as the counter electrode and the exit line. A dual potentiostat (Nikko Keisoku Co., Model DPGS-2) was employed to control independently the potentials of the two working electrodes and to

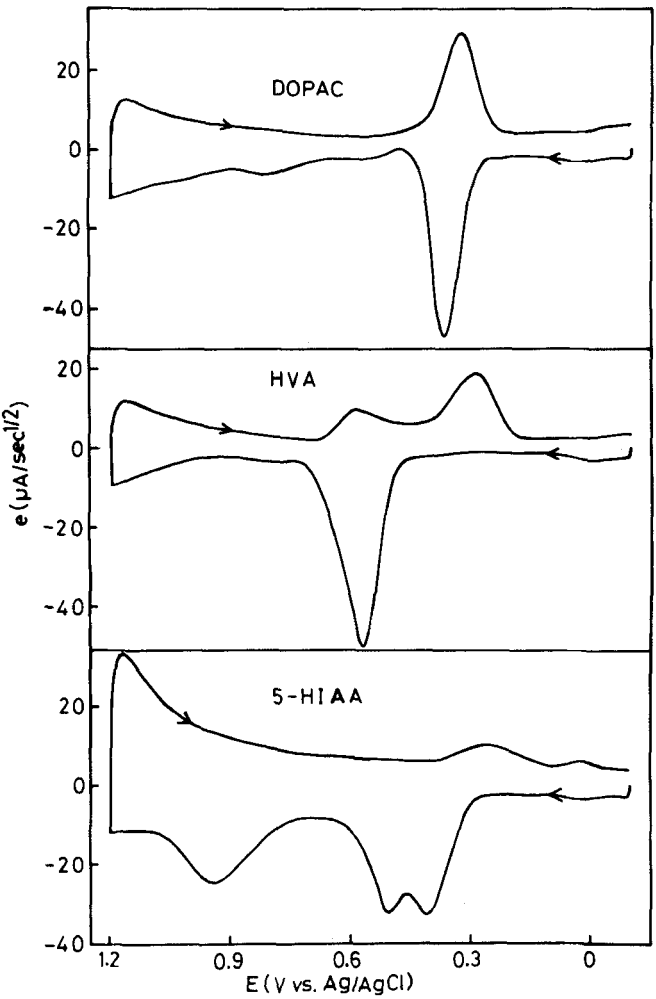

FIGURE 2. Block diagram of the micro HPLC system with series dual electrochemical detector. 1 = Micro feeder, 2 = micro syringe, 3 = three-way valve, 4 = mobile phase, 5 = micro sample injector (0.3 μl), 6 = micro guard column, 7 = micro separation column, 8 = series twin-electrode thin-layer electrolytic cell, 9 = dual potentiostat, 10 = dual pen recorder, 11 = waste.

measure the currents. The anodic and cathodic chromatograms were simultaneously recorded on a dual pen recorder (Yokogawa Co., Model 3056).

The micro guard column and micro separation column were made by packing ODS (Yanapak ODS, 10 μm) in a PTFE tube 2.0 cm x 0.5 mm i. d. and 16.5 cm x 0.5 mm i. d., respectively.

Chemicals

Analytical reagent grade chemicals were used without further purification. All solutions were prepared from distilled and deionized water. For standard samples, 3, 4-dihydroxyphenylacetic acid (DOPAC), vanillylmandelic acid (VMA), homovanillic acid (HVA), serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) were dissolved in Britton-Robinson (B-R) buffer of pH 1.8 to prepare the stock solutions. The mobile phase used for analysis was B-R buffer of pH 3.6 containing 10 % methanol, 50 mM sodium perchlorate and 0.1 mM EDTA (disodium salt).

Procedures of urine analysis

Typically only 0.3 μl of supernatant of raw human urine was injected into the micro HPLC system. The biogenic amine metabolites were separated at the flow rate of 8.3 μl/min. For dual electrochemical detection, the upstream working electrode was held at + 0.80 V (vs. Ag/AgCl) while the downstream working electrode was done at - 0.05 V. The quantitation was performed selectively by using the response of the downstream electrode.

RESULTS AND DISCUSSION

Electrochemical behaviors of biogenic amine metabolites

By means of the cyclic semi-differential and semi-integral voltammetry (22-25), the electrochemical behaviors of biogenic amine metabolites were studied in

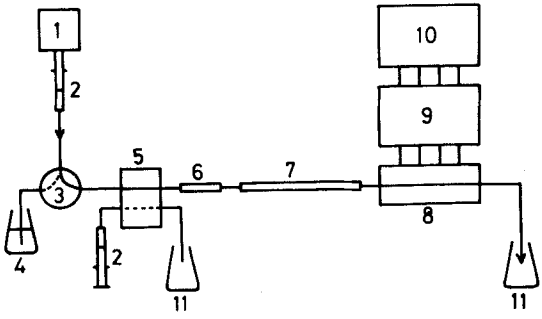

FIGURE 3. Cyclic semi-derivative voltammograms of 1.0 mM DOPAC, HVA and 5-HIAA in the B-R buffer of pH 3.6 containing 10 % methanol, 50 mM sodium perchlorate and 0.1 mM EDTA (disodium salt) at a scan rate of 100 mV/sec.

the mobile phase of micro HPLC, the B-R buffer of PH 3.6 containing 10 % methanol, 50 mM sodium perchlorate and 0.1 mM EDTA (disodium salt). Figure 3 shows the cyclic semi-derivative voltammograms, which are the semi-derivative of current, e, versus electrode potential, E, curves, for DOPAC, HVA and 5-HIAA. VMA and 5-HT showed the roughly similar cyclic semi-derivative voltammograms as HVA and 5-HIAA, respectively. All the species investigated showed oxidation and re-reduction peaks. It is interesting that three successive oxidation steps were observed for 5-HIAA, while only one oxidation step was substantially observed for DOPAC and HVA. On the other hand, two re-reduction steps were observed for HVA and 5-HIAA, while only one re-reduction step was observed for DOPAC. These facts indicate that the electrode reaction of DOPAC is nearly reversible, while those of HVA, VMA, 5-HIAA and 5-HT are quasi-reversible in this medium. For selective detection of biogenic amine metabolites, the potentials (vs. Ag/AgCl) of + 0.80 V and - 0.05 V were chosen as the suitable potentials of the upstream and down stream working electrode, respectively, from Figure 3.

Figure 4 shows the semi-integral voltammograms, the semi-integral of current, m, versus E curves, of 1.0 mM

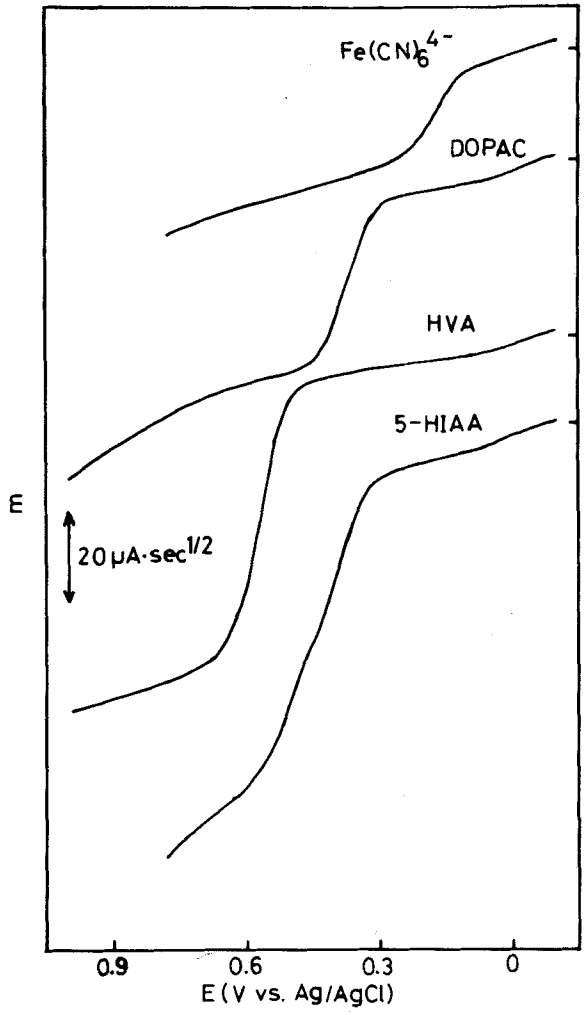

FIGURE 4. Semi-integral voltammograms of 1.0 mM $Fe(CN)_6^{4-}$, DOPAC, HVA and 5-HIAA in the B-R buffer of pH 3.6 containing 10 % methanol, 50 mM sodium perchlorate and 0.1 mM EDTA (disodium salt) at a scan rate of 100 mV/sec.

each of biogenic amine metabolites and ferricyanide for anodic process. It is clear that the electron transfer number for each oxidation reaction at + 0.8 V is two for DOPAC, four for HVA and four for 5-HIAA, on comparison of their wave heights with that of ferricyanide whose electrode reaction is one electron transfer, in the semi-integral voltammograms.

Chromatography and quantitation

The retention of biogenic amine metabolites in reversed-phase chromatography was investigated in order to attain a good separation. Figure 5 shows the effect of pH of the mobile phase on retention time. The retention of three biogenic amine metabolites investigated decreased with increasing pH, while that of 5-HT tended to increase with increasing pH. In this study, pH 3.6 was chosen as the suitable pH value of mobile phase for urine analysis.

Figure 6 shows typical chromatograms of a standard solution of VMA, 5-HT, DOPAC, 5-HIAA and HVA by the micro HPLC system with SDEC. Parts A and B are, respectively , the anodic and cathodic chromatograms. The peak separation is satisfactory, and both the anodic and cathodic peak currents were linear with the amounts of species injected, with correlation coefficients better than than 0.99, as shown in Table 1. The detection limits (S/N = 2) of biogenic amine metabolites by this system were 10 pg for DOPAC, 20 pg for 5-HIAA and 20 pg for HVA, respectively, and the range of linearity was about 1000. The relative standard deviations for repetitive determination of 3 ng level by using the cathodic response in the system were 1.5 % for DOPAC, 1.8 % for 5-HIAA and 1.2 % for HVA, respectively.

The collection efficiencies in the system were found to be 0.61 for DOPAC, 0.20 for 5-HIAA and 0.30 for HVA, respectively, at the flow rate of 8.3 µl/min. It should be noted that these values are much larger than those of 0.31 for DOPAC, 0.05 for 5-HIAA and 0.05 for

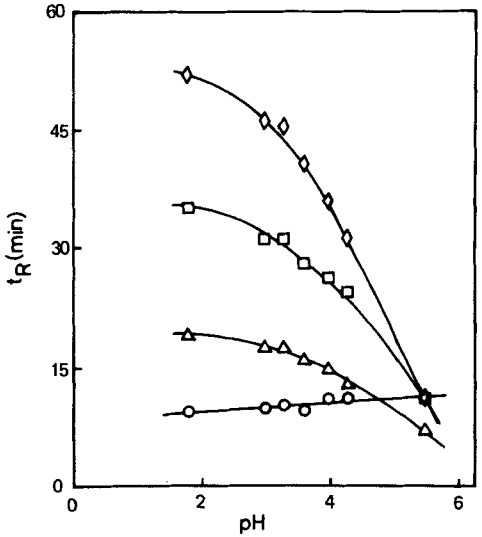

FIGURE 5. Effect of pH of the mobile phase on the retention time of 5-HT(O), DOPAC(Δ), 5-HIAA(□) and HVA(◊). Mobile phase: B-R buffer containing 10 % methanol. Solid sodium hydroxide was used to get the desired pH. Flow rate of mobile phase: 8.3 µl/min. Separation column: silica-ODS (16.5 cm x 0.5 mm i. d.).

TABLE 1

Relationship between Anodic and Cathodic Peak Height and Amount of Species Injected
Potentials (V vs. Ag/AgCl): anode + 0.80, cathode - 0.05, flow rate of mobile phase: 8.3 µl/min.

Species		Relationship*	Correlation coefficient
VMA	Anodic	y = -11.00x - 0.96	0.998
	Cathodic	y = 0.76x + 0.37	0.991
5-HT	Anodic	y = -7.25x	1.000
	Cathodic	y = 1.08x + 0.07	0.999
DOPAC	Anodic	y = -7.49x - 0.05	1.000
	Cathodic	y = 4.53x + 0.23	1.000
5-HIAA	Anodic	y = -6.63x - 0.28	0.999
	Cathodic	y = 1.30x + 0.28	0.998
HVA	Anodic	y = -3.33x - 0.17	0.999
	Cathodic	y = 0.97x + 0.13	0.999

* y = peak height measured in nA, x = amount of species measured in ng.

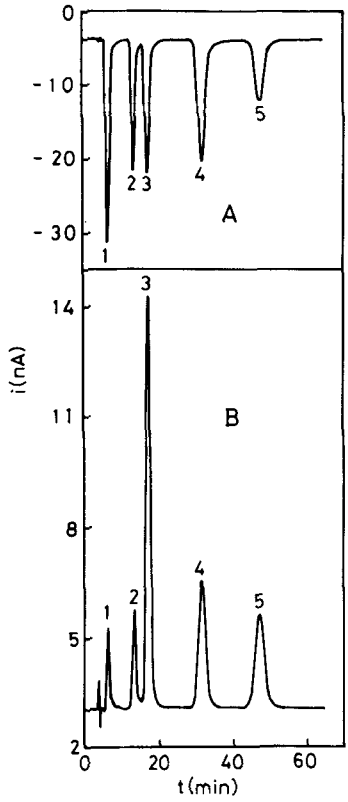

FIGURE 6. Typical chromatograms of a standard solution by the micro HPLC system with SDEC. (A) Anodic response, (B) cathodic response. Peaks: 1 = VMA, 2 = 5-HT, 3 = DOPAC, 4 = 5-HIAA, 5 = HVA. Potentials (V vs. Ag/AgCl): anode + 0.80, cathode - 0.05. Mobile phase: B-R buffer of pH 3.6 containing 10 % methanol, 50 mM sodium perchlorate and 0. 1 mM EDTA (disodium salt). Flow rate of mobile phase: 8.3 µl/min.

HVA, respectively, obtained in SDEC at the flow rate of 1.6 ml/min (18).

Selective detection of DOPAC, 5-HIAA and HVA in human urine

Typical chromatograms for the simultaneous determination of DOPAC, 5-HIAA and HVA in 0.3 µl of human urine directly injected without any pretreatment in the micro HPLC

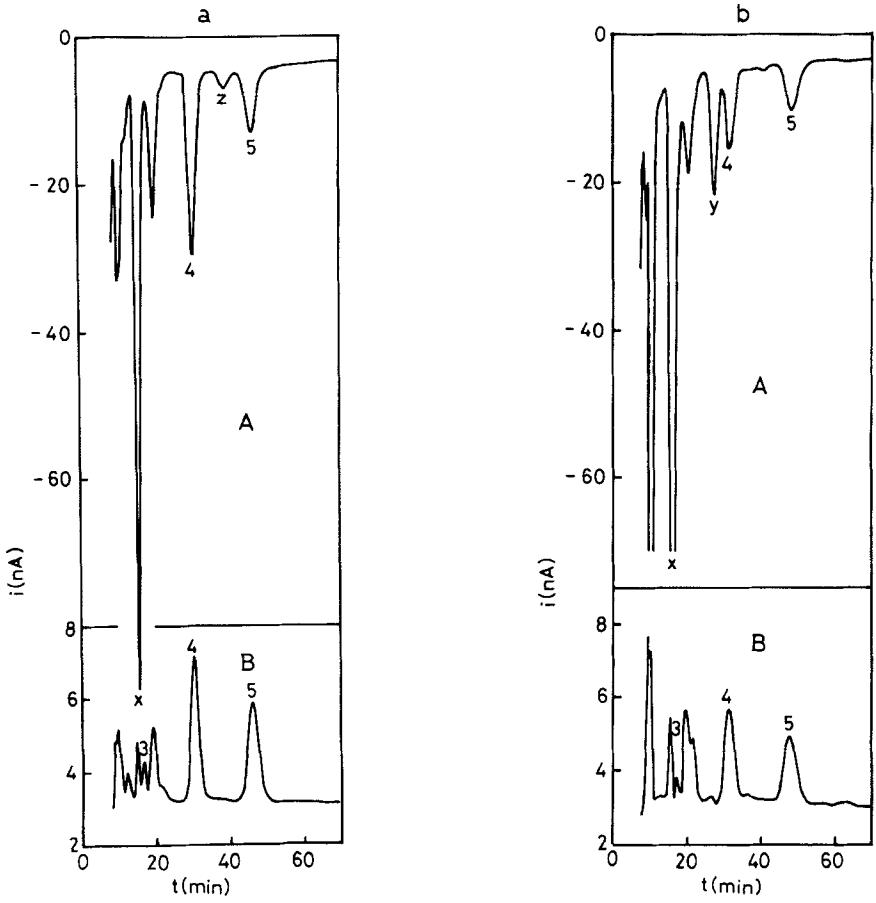

FIGURE 7. Chromatograms of directly injected urines from three healthy individuals to the micro HPLC system. (A) Anodic response, (B) cathodic response. Peaks: 3 = DOPAC, 4 = 5-HIAA, 5 = HVA. Potentials (V vs. Ag/AgCl): anode + 0.80, cathode -0.05. Sample: 0.3 µl of human urine. Other conditions are the same as in FIGURE 6.

system are shown in Figure 7. Parts A and B are, respectively , the anodic and cathodic chromatograms. Figure 7 a, b and c correspond to the urine from three different healthy individuals, respectively. Peaks 3, 4 and 5 are due to DOPAC, 5-HIAA and HVA in urine, respectively. These were identified by the retention time and/or the peak current

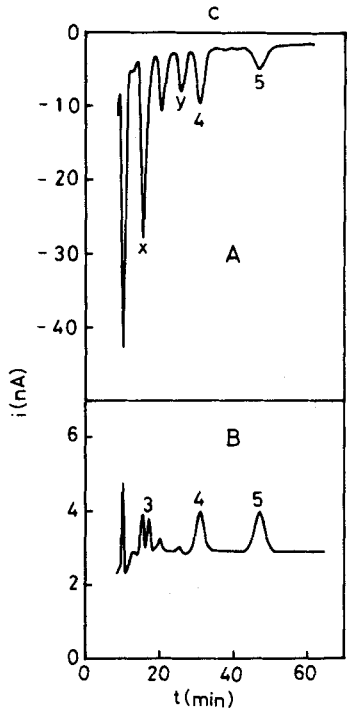

FIGURE 7C

TABLE 2

Analytical Results of DOPAC, 5-HIAA and HVA in Human Urine from Healthy Individuals

Sample number	Concentration (µg/ml)		
	DOPAC	5-HIAA	HVA
1	0.29	9.53	8.77
2	0.24	5.93	6.13
3	0.25	2.40	1.73
4	0.31	8.00	8.27
5	0.28	19.00	5.93
6	0.47	4.07	4.33
7	0.41	2.10	3.30

ratio of cathodic to anodic by comparing with the standards. Of paticular interest is the peaks of x, y and z in parts A of Figure 7. By recording the cathodic response, it was shown that there were essentially no cathodic peaks corresponding to the anodic peaks of y and z, suggesting that the compound or compounds producing the anodic peaks are irreversibly oxidized. Since the main compounds responsible to the anodic peak of x were also irreversibly oxidized, DOPAC could be selectively detected on the cathodic chromatograms, as shown in part B in Figure 7.

Human urine from seven healthy individuals was analyzed from the linear regression equations in Table 1 using the cathodic chromatograms. The results are shown in Table 2. The concentrations for DOPAC, 5-HIAA and HVA in Table 2 are within the range of results reported in the literature for normal human urines (26-29).

The present system appears to be the first method which simultaneously determines DOPAC, 5-HIAA and HVA with resonable precision in human urine directly injected without any pretreatment into the micro liquid chromatograph.

CONCLUSION

The series dual electrochemical detector with anode and cathode is a powerful tool for selective detection of reversible and/or quasi-reversible species for micro HPLC, because the collection efficiency increases with decreasing the flow rate of mobile phase. The parallel-opposed dual electrochemical detector with anode and cathode may provide an enhancement in sensitivity by recycling oxidation and re-reduction between the two working electrodes at slow flow rates of mobile phase. Thus the PODEC is the most advantageous type of detector for reversible and/or quasi-reversible species in micro HPLC (20, 21).

The simultaneous determination of DOPAC, 5-HIAA and HVA in healthy human urine could be performed on direct injection of only 0.3 µl by using the micro HPLC system with SDEC.

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