Journal of Chromatography, 275 (1983) 271—281
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1672

DETERMINATION OF CATECHOLAMINES IN HUMAN SERUM BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTION

MASASHI GOTO*, GONGWEI ZOU* and DAIDO ISHII

Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya-shi 464 (Japan)

(First received November 30th, 1982; revised manuscript received February 9th, 1983)

SUMMARY

A dual electrochemical detector having two working electrodes (anode and cathode) in parallel—opposed configuration suitable for micro high-performance liquid chromatography was developed for the selective and sensitive detection of catecholamines on the basis of their electrochemical reversibility and catalytic amplification by recycling oxidation and re-reduction. The micro high-performance liquid chromatographic system with a micro alumina precolumn for enriching catecholamines and the dual electrochemical detector in parallel—opposed configuration was successfully utilized for the determination of catecholamines in healthy human serum injected directly after ultrafiltration.

INTRODUCTION

An innovative approach to electrochemical detection in high-performance liquid chromatography (HPLC) involves the use of two working electrodes operated simultaneously at different potentials. Several studies have reported the use of such dual electrochemical detection for HPLC [1—6].

In preceding papers [7, 8], a dual electrochemical detector (DECD) having two working electrodes (anode and cathode) in series configuration was designed for use in micro high-performance liquid chromatography (MHPLC), and the MHPLC system with a micro alumina precolumn for enriching and the DECD in series configuration was successfully utilized for the selective determination of catecholamines in healthy human urine directly injected. In the present work, a DECD based on a thin-layer electrolytic cell with two working electrodes (anode and cathode) in parallel—opposed configuration was designed and constructed to gain the catalytic amplification of electrochemical response in MHPLC. The direct injection analysis of catecholamines

*Visiting Scholar from Department of Chemistry, Nanjing University, Nanjing, China.

0378-4347/83/$03.00

in healthy human serum was tried by using the MHPLC system with an alumina precolumn and such a DECD.

EXPERIMENTAL

Apparatus

The direct injection analytical system used is similar to that in the former study for urine analysis [7] and is shown schematically in Fig. 1. The dif-

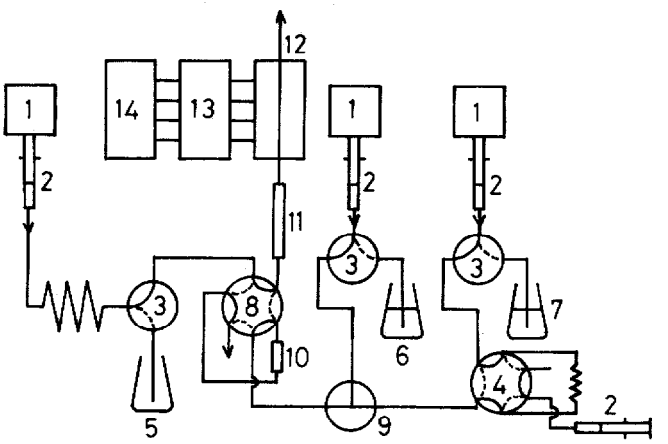

Fig. 1. Block diagram of the MHPLC system with micro precolumn and dual electrochemical detector in parallel—opposed configuration. 1 = Micro feeder, 2 = micro syringe, 3 = three-way valve, 4 = sample injector, 5 = mobile phase, 6 = buffer solution, 7 = water, 8 = six-way valve, 9 = mixing joint, 10 = micro precolumn, 11 = micro separation column, 12 = parallel—opposed twin-electrode thin-layer electrolytic cell, 13 = dual potentiostat, 14 = dual pen recorder.

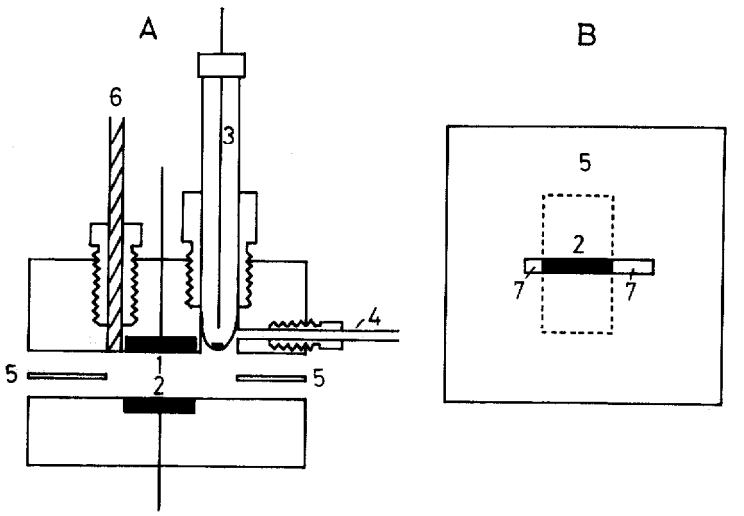

Fig. 2. Construction of parallel—opposed twin-electrode thin-layer electrolytic cell and connection with micro separation column. (A) Side view of cell, (B) top view of spacer. 1, 2 = Working electrode (glassy carbon), 3 = reference electrode (Ag/AgCl), 4 = counter electrode (platinum tube), 5 = spacer (PTFE sheet), 6 = micro separation column, 7 = hole.

ferences are as follows: the solution filter was removed, the pre-concentration way was reversed, the 200-μl sample loop was set and the DECD in parallel—opposed configuration was applied. The design of the twin-electrode thin-layer electrolytic cell in parallel—opposed configuration for DECD and connection with the micro separation column are shown in Fig. 2. The thin-layer cavity was constructed of two fluorocarbon resin blocks separated by a PTFE sheet 50 μm thick and 2 mm wide. Two working electrodes were made with glassy carbon plates 1 cm long and 2 cm wide contained in each block. The reference electrode, silver/silver chloride electrode, was held in a cylindrical hole in one of the blocks. A platinum tube served both as the counter electrode and the exit line.

The micro separation column for analysis was filled with octadecylsilica (Yanapak ODS-T, 10 μm) in a PTFE tube 15 cm × 0.5 mm I.D. The micro precolumn for enrichment was made by packing alumina (E. Merck, LiChrosorb Alox T, 30 μm) in a PTFE tube 2 cm × 0.5 mm I.D. The other instruments used are the same as in the previous paper [7].

Reagents

Analytical-reagent grade chemicals were used without further purification. All solutions were prepared from distilled, deionized water. For standard samples, noradrenaline (NA), adrenaline (AD) and 3,4-dihydroxybenzylamine hydrobromide (DHBA) were dissolved in Britton-Robinson (B-R) buffer pH 1.8. The buffer solution for pretreatment of the micro precolumn and pH adjustment of the sample was 1 *M* Tris buffer pH 8.7 containing 0.25% EDTA (disodium salt) and 0.05% sodium hydrogen sulfite for stabilizing catecholamines. The mobile phase for analysis was the B-R buffer pH 1.8 containing 2 m*M* sodium 1-heptanesulphonate (HSA) for ion-pairing, 0.1 m*M* EDTA (disodium salt) for masking iron ion and 50 m*M* sodium perchlorate for smoothing base currents.

Procedures

The human blood sample was drawn in a serum separation tube by venipuncture. After incubation at room temperature (ca. 20°C) for about 10 min to induce coagulation, the tube was centrifuged, separating the serum from the blood cells. Then, 500 μl of the raw human serum were taken in a test tube, to which had been added 0.5 mg of solid EDTA (disodium salt), 0.5 mg of solid sodium hydrogen sulfite, and 10 μl of 25 pg/μl DHBA standard solution. The former solid was added to complex any possible metal ions present, the latter solid to serve as an antioxidant and the DHBA solution to serve as an internal standard. The test tube was stoppered, shaken for 1 min by hand and a large portion of the mixed sample solution was transferred to an ultrafiltration cell (Millipore, type XX42 013 10). The ultrafiltration was carried out by stirring the solution with a magnetic stirrer under a nitrogen pressure of 2.5 kg/cm^2 in an ice bath. The filtrated serum was taken with a micro syringe and injected into the sample loop (200 μl) of the sample injector. The sample was delivered by water at a flow-rate of 33 μl/min and mixed with Tris buffer (pH 8.7) at the same flow-rate in the mixing joint to adjust the sample to pH 8.6. The sample was injected into the micro precolumn

for 10 min for enrichment with a mixed flow of water and buffer solution and then the micro precolumn was washed for a further 10 min with a flow of water only by stopping the flow of the buffer solution. By switching the six-way valve, the mobile phase was introduced into the micro separation column through the micro precolumn at a flow-rate of 8.3 μl/min. In this procedure, the adsorbed catecholamines are eluted from the micro precolumn and simultaneously separated by the micro separation column.

Selective and sensitive detection of catecholamines

Consider a reversible or quasi-reversible redox couple. The anode and cathode of the twin electrode thin-layer cavity are set at potentials where the reductant is oxidized and its oxidant is reduced, respectively. The reaction product of the anode is detected at the cathode positioned across from the anode. The reversible and/or quasi-reversible species are selectively detected at the cathode from many irreversible species. The selective detection of reversible species from quasi-reversible species may be achieved if the potentials of anode and cathode are properly chosen. The catalytic amplification in electrochemical detection may be obtained by recycling the oxidation and re-reduction between the anode and cathode at low flow-rates. In this manner the number of electrons exchanged in the detection process may be increased, and the sensitivity enhanced.

The separated catecholamines were introduced into the twin electrode thin-layer electrolytic cell in parallel–opposed configuration, in which the lower and upper working electrodes were set at the potentials (V vs. Ag/AgCl) of 0.60 and 0.20, respectively. The catecholamines were selectively detected with high sensitivity by monitoring the re-reduction current at the cathode and determined by comparing with the response of internal standard.

RESULTS AND DISCUSSION

Electrochemical behaviors of catecholamines

The electrochemical behaviors of NA, AD and DHBA on the glassy carbon electrode in the mobile phase used in MHPLC were studied by cyclic semi-integral voltammetry (CSIV) [9, 10] and semi-differential voltammetry (CSDV) [11–14]. CSIV and CSDV measure, respectively, the semi-integral (m) and semi-derivative (e) of current with respect to time vs. applied potential (E) under the same experimental conditions as in conventional cyclic voltammetry. Fig. 3 shows typical cyclic semi-integral and semi-derivative voltammograms of NA as an example. Voltammograms similar to those for NA were observed for AD and DHBA. The anodic and cathodic peak potentials (V vs. Ag/AgCl) in semi-derivative voltammograms were 0.48 and 0.46 for NA, 0.48 and 0.46 for AD, and 0.51 and 0.47 for DHBA, respectively. Therefore, the anode potential of 0.60 V and the cathode potential of 0.20 V were chosen for selective detection of three catecholamines in dual electrochemical detection.

According to the theory [9, 10], the wave height (m_d) in semi-integral voltammogram is represented by $m_d = nAFC\sqrt{D}$, where n is the electron transfer number, A the electrode area, F Faraday's constant, C the concen-

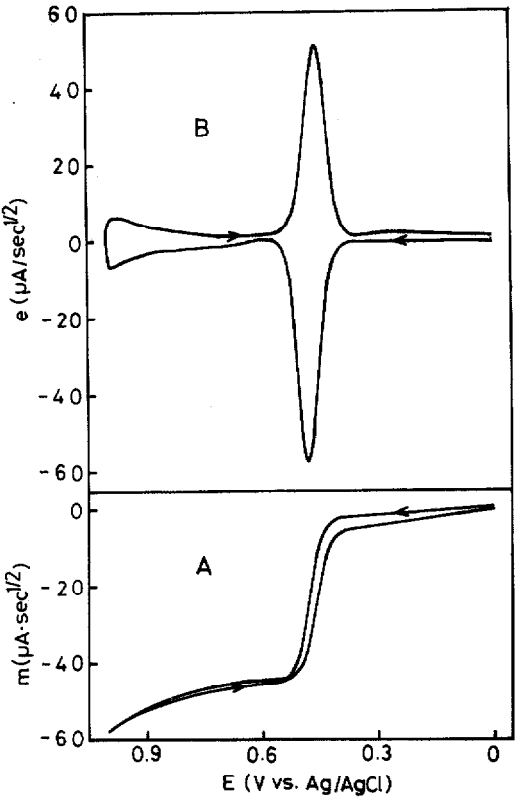

Fig. 3. Cyclic semi-integral and semi-derivative voltammograms of 1.0 m*M* noradrenaline in the B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate at a scan rate of 100 mV/sec. (A) Cyclic semi-integral voltammogram, (B) cyclic semi-derivative voltammogram.

tration and D the diffusion coefficient. The wave heights ($\mu A \cdot sec^{1/2}/mM$) in semi-integral voltammograms for oxidation of NA, AD and DHBA were 41.5, 41.0 and 44.5, respectively, while that for one electron reduction of $Fe(CN)_6^{3-}$ measured in the same medium was 20.9 $\mu A \cdot sec^{1/2}/mM$. It is clear that the three catecholamines investigated show nearly reversible electrode reactions in the medium used and the number of electron transfers involved is two.

Catalytic amplification in parallel—opposed dual electrochemical detection

Micro high-performance liquid chromatograms after precolumn enrichment were measured by using one or two working electrodes of the twin-electrode thin-layer electrolytic cell in parallel—opposed configuration. The flow-rate of mobile phase was 8.3 μl/min, and the applied potentials of the anode and cathode were 0.60 V and 0.20 V, respectively. The anodic peak currents of NA, AD and DHBA in measurements using one working electrode (anode) were amplified to 2.3, 2.8 and 2.7 times, respectively, on measurements using two working electrodes (anode and cathode). It should be noted that these values of amplification are much larger than those (1.1—1.2 times) obtained by using the twin-electrode thin-layer electrolytic cell in series configuration

[8]. The better amplifications are expected at slower flow-rates of mobile phase. The details will be described elsewhere [16].

Quantitation of catecholamines by the internal standard method

The MHPLC system with precolumn and DECD in parallel—opposed configuration was used for the quantitative analysis of the mixtures of NA, AD and DHBA. Typical chromatograms of standard catecholamines obtained from a 200-μl injection of 500 pg/ml of each of NA, AD and DHBA using the micro ODS column are shown in Fig. 4, in which parts A and B are, respectively, the anodic and cathodic chromatograms. All three catecholamines gave both anodic and cathodic chromatographic peaks as expected. The peak current ratios of re-reduction to oxidation of catecholamines were 0.69, 0.67 and 0.74 for NA, AD and DHBA, respectively, under the conditions shown in Fig. 4. These ratios in parallel—opposed configuration coincided with 0.68 and 0.68 for NA and AD, respectively, obtained by using the DECD in series configuration [8]. It is interesting that these ratios, collection efficiencies, in our DECD were much higher than those (< 0.37) observed in the similar series dual electrode detector in conventional HPLC [6]. This seems to

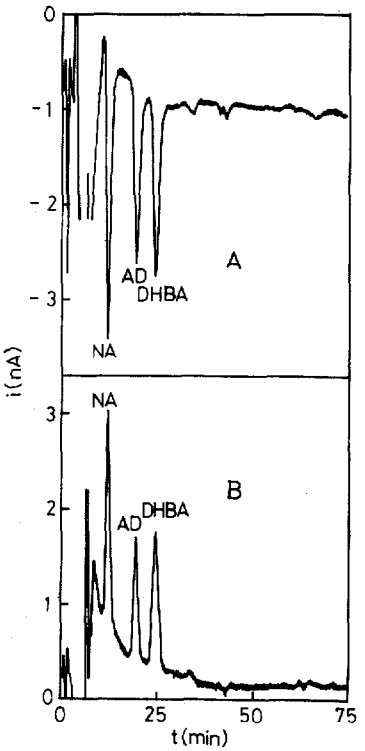

Fig. 4. Typical chromatograms of standard catecholamines by the MHPLC system with micro precolumn and dual electrochemical detector in parallel—opposed configuration. (A) Anodic response, (B) cathodic response. Sample: 200 μl of a standard solution of 500 pg/ml each of NA, AD and DHBA. Mobile phase: B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate. Flow-rate of mobile phase: 8.3 μl/min. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20.

depend on the differences in flow-rates used of 8.3 μl/min and 1 ml/min, and in the thickness of the thin-layer channel of 50 μm and 130 μm, respectively, in the former and latter. The electrolytic efficiency in the DECD in parallel–opposed configuration was about 83% at a mobile phase flow-rate of 8.3 μl/min. The better electrolytic efficiencies are expected at slower flow-rates of mobile phase. The details will be described elsewhere [16].

Both the anodic and cathodic peak height ratios of NA and AD to DHBA were linear with each amount of catecholamines injected, with good correlation, as shown in Table I. The linear dynamic range was about 10^3 and the

TABLE I

RELATIONSHIPS BETWEEN ANODIC AND CATHODIC PEAK HEIGHT RATIOS TO DHBA AND AMOUNT OF CATECHOLAMINES USING THE MHPLC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL–OPPOSED CONFIGURATION

Mobile phase: B-R buffer pH 1.8 containing 2 m*M* HSA, 0.1 m*M* EDTA and 50 m*M* sodium perchlorate. Flow-rate of mobile phase: 8.3 μl/min. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20. Amount of DHBA injected as an internal standard: 200 pg.

Species		Relationship*	Correlation coefficient
NA	Anodic	$Y = -8.10X + 0.01$	0.999
	Cathodic	$Y = 7.81X - 0.07$	0.998
AD	Anodic	$Y = -4.64X - 0.01$	0.999
	Cathodic	$Y = 4.34X$	0.998

* Y = peak height ratio to DHBA; X = amount of catecholamine measured in ng.

TABLE II

PRECISION FOR DETERMINATION OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL–OPPOSED CONFIGURATION

Sample: 200 μl of 500 pg/ml each of NA, AD and DHBA. Potentials (V vs. Ag/AgCl): anode (+) 0.60, cathode (+) 0.20.

Number	Peak height ratio to DHBA			
	NA		AD	
	Anodic	Cathodic	Anodic	Cathodic
1	1.75	1.71	1.03	0.98
2	1.87	1.67	1.05	1.00
3	1.77	1.62	1.05	0.97
4	1.80	1.58	1.04	0.93
5	1.78	1.64	1.00	0.98
6	1.85	1.62	1.06	0.96
7	1.81	1.60	1.03	0.94
8	1.81	1.65	1.02	0.99
Mean	1.81	1.64	1.04	0.97
Relative S.D.	2.2	2.5	1.9	2.5

minimum detectable amount in the proposed system was about 3 pg for NA and AD. This detection limit using the parallel—opposed DECD was about three times better than that [7, 8] using the series DECD. The precision for determination of catecholamines by the proposed system is shown in Table II. The relative standard deviations (%) for repetitive determination at the 100 pg level using the anodic and cathodic responses were 2.2 and 2.5 for NA, and 1.9 and 2.5 for AD, respectively.

Catecholamines in human serum

In order to examine the applicability of the proposed system for completely direct injection analysis of catecholamines in human serum, experiments were carried out to see whether raw human serum can be directly injected into a micro alumina precolumn. We were unable to establish the conditions to preconcentrate catecholamines without stopping up the precolumn. However, satisfactory results were obtained when raw human serum was ultrafiltrated before injection into the precolumn, as described under Procedures. Fig. 5 shows chromatograms of 200-μl injections of ultrafiltrated

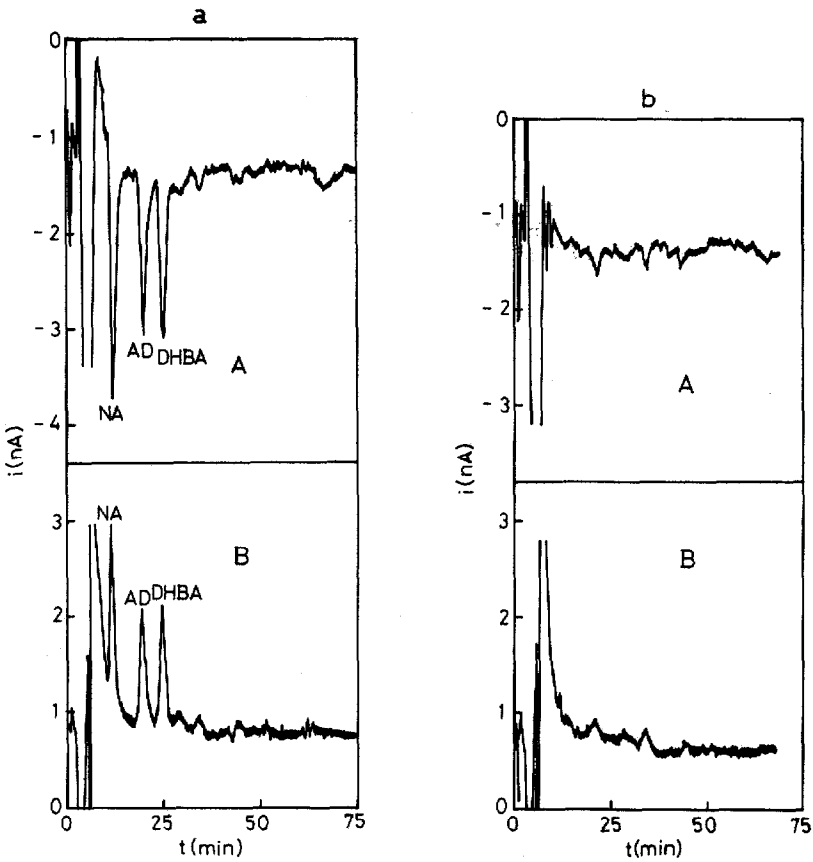

Fig. 5. Chromatograms of ultrafiltrated blank human serum and that spiked with standard catecholamines. (A) Anodic response, (B) cathodic response. Sample: (a) 200 μl of ultrafiltrated blank human serum spiked with 100 pg each of NA, AD and DHBA, (b) 200 μl of ultrafiltrated blank human serum. Other conditions are the same as in Fig. 4.

blank human serum and that spiked with 100 pg each of NA, AD and DHBA. The blank serum was prepared by storing the raw human serum for a long time (about three months) in the freezer at −8°C, which may introduce slow spontaneous oxidation or enzymatic conversion of catecholamines. Parts A and B in Fig. 5 represent, respectively, the anodic and cathodic chromatograms. The chromatograms of blank human serum spiked with catecholamines compare well with those of standard catecholamines (see Fig. 4). This indicates that the component of human serum does not seriously interfere the determination of catecholamines by the proposed system.

Typical chromatograms of catecholamines in human serum from healthy individuals obtained using the proposed procedures of internal standard addition and ultrafiltration, and the MHPLC system with precolumn and parallel—opposed DECD are shown in Fig. 6. Of particular interest in parts A are the peaks appearing as the shoulder of NA in Fig. 6b and the background of AD in Fig. 6a, respectively. By recording the cathodic current, the interferences from the compounds responsible for these peaks could be removed, as shown in parts B, on the basis of their electrochemical irreversibility. The precision for determination of catecholamines in the same healthy human serum by the proposed method is shown in Table III. The relative standard deviations for the determination of catecholamines in healthy human serum

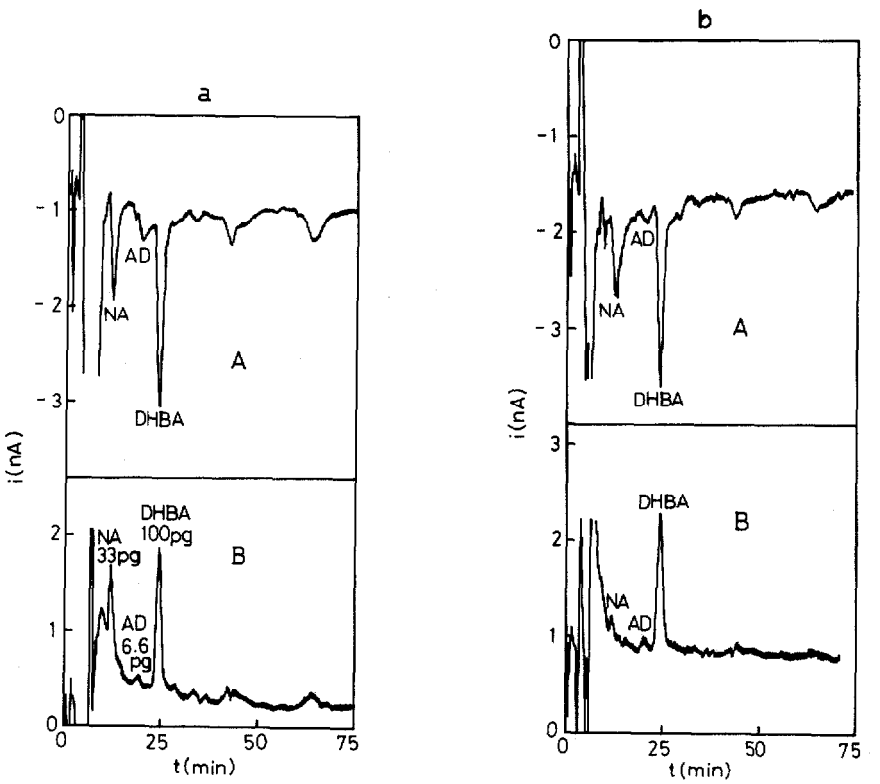

Fig. 6. Typical chromatograms of catecholamines in human serum from healthy individuals by internal standard addition. (A) Anodic response, (B) cathodic response. Sample: 200 μl of human serum spiked with 100 pg of DHBA. Other conditions are the same as in Fig. 4. a and b represent two different individuals.

TABLE III

PRECISION FOR DETERMINATION OF CATECHOLAMINES IN THE SAME HEALTHY HUMAN SERUM BY INTERNAL STANDARD ADDITION USING THE MHPLC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR IN PARALLEL—OPPOSED CONFIGURATION

Number	Concentration (pg/ml)			
	NA		AD	
	Anodic	Cathodic	Anodic	Cathodic
1	162	184	59	42
2	159	178	62	37
3	187	165	67	33
4	160	177	94	38
5	160	169	113	34
6	146	170	89	30
Mean	162	174	81	36
Relative S.D.	8.3	4.0	26.5	11.8

using the cathodic responses were 4.0% for NA and 11.8% for AD. The precision for determination using the cathodic responses was about two times better than that using anodic responses. This depends on the difference in the possibility of interference from other electrochemical species present in human serum. The serum concentrations for NA and AD of the normal sample in Table III were 174 pg/ml and 36 pg/ml, respectively. These values compare well with the normal plasma concentrations (mean ± standard deviation) for NA and AD of 210 ± 100 pg/ml (n = number of samples = 1883) and 38 ± 20 pg/ml (n = 800), respectively, estimated by fluorometric detection [15].

CONCLUSIONS

The DECD in parallel—opposed configuration with anode and cathode is a powerful tool for selective detection of reversible and/or quasi-reversible species from many irreversible species and can provide an enhancement in sensitivity by recycling oxidation and re-reduction between the anode and cathode at slow flow-rates of mobile phase.

The determination of catecholamines in healthy human serum could be performed on direct injection of only 200 μl of an ultrafiltrated sample by using the proposed MHPLC system.

ACKNOWLEDGEMENT

The authors thank Mr. E. Sakurai for his valuable technical assistance.

REFERENCES

1 L. Blank, J. Chromatogr., 117 (1976) 35.

2 R.J. Fenn, S. Siggia and D.J. Curran, Anal. Chem., 50 (1978) 1067.
3 G.W. Schieffer, Anal. Chem., 52 (1980) 1944.
4 D.A. Roston and P.T. Kissinger, Anal. Chem., 53 (1981) 1695.
5 W.A. MacCrehan and R.A. Durst, Anal. Chem., 53 (1981) 1700.
6 D.A. Roston and P.T. Kissinger, Anal. Chem., 54 (1982) 429.
7 M. Goto, T. Nakamura and D. Ishii, J. Chromatogr., 226 (1981) 33.
8 M. Goto, E. Sakurai and D. Ishii, J. Chromatogr., 238 (1982) 357.
9 M. Grenness and K.B. Oldham, Anal. Chem., 44 (1972) 1121.
10 M. Goto and K.B. Oldham, Anal. Chem., 45 (1973) 2043.
11 M. Goto and D. Ishii, J. Electroanal. Chem., 61 (1975) 361.
12 P. Dalrymple-Alford, M. Goto and K.B. Oldham, J. Electroanal. Chem., 85 (1977) 1.
13 P. Dalrymple-Alford, M. Goto and K.B. Oldham, Anal. Chem., 49 (1977) 1390.
14 M. Goto, M. Kato and D. Ishii, Anal. Chim. Acta, 126 (1981) 95.
15 K. Mori, Protein, Nucleic Acid Enzyme, 26 (1981) 1099.
16 M. Goto, G. Zou and D. Ishii, J. Chromatogr., submitted for publication.