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MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SYSTEM WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR FOR DIRECT INJECTION ANALYSIS OF CATECHOLAMINES IN BODY FLUIDS

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SUMMARY

A new micro high-performance liquid chromatographic system has been developed, which determines catecholamines from directly injected human urine without any sample pretreatment. The system consists of a mixing junction for adjusting the sample to pH 8.5, a micro alumina precolumn for enriching catecholamines in the sample, and a dual electrochemical detector for selectively detecting catecholamines based on their electrochemical reversibility. The system is able to operate with directly injected body fluids and to determine simultaneously all of the four catecholamines with high precision.

INTRODUCTION

Catecholamines play an important role in the central nervous system and in neurological diseases. Consequently, their separation and determination have received considerable attention, and electrochemical methods are proving very useful in such studies [1—5]. Dual electrochemical detectors, having two working electrodes, for high-performance liquid chromatography (HPLC) are especially attractive for selective detection of electroactive species [4, 5]. Micro high-performance liquid chromatography (MHPLC), using packed columns of bore size less than 1 mm, is a very suitable technique for analysis of trace samples of biomedical origin [6]. An amperometric detector based on a thin-layer electrochemical cell with one working electrode suitable for MHPLC was recently described, and successfully utilized for the determination of aminophenol isomers separated by a micro ODS column [7]. In the present work, a thin-layer electrolytic cell with two working electrodes was designed and con-

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structed for use as the detector for MHPLC. A twin electrode detector was used to detect catecholamines selectively, based on their electrochemical reversibility in many electroactive eluates.

In the development of analytical methods using liquid chromatography for biological samples (for example, urine, serum, saliva, plasma), one of the most time-consuming steps, which introduces considerable sources of error, is sample pretreatment and enrichment prior to injection into the chromatograph. Approaches towards on-column sample enrichment for direct injection of body fluids in HPLC have been reported [8—10]. So far, the determination of catecholamines by direct injection of untreated body fluids into a micro liquid chromatograph has not been reported. Thus, we adapted the idea of the use of a precolumn as a protecting device on the micro separation analytical column, following preconcentration of the catecholamines by adsorption on a very small alumina column.

This paper describes a novel analytical system for the analysis of catecholamines by MHPLC with dual electrochemical detection, with micro precolumn sample enrichment, and for direct urine injection without the classical sample pretreatment steps.

EXPERIMENTAL

Sample pretreatment

Typical classical steps in the pretreatment of human urine for subsequent HPLC runs for determining catecholamines with electrochemical detection are the following: urine (5 ml) → stabilize → buffer (pH 6.5) → adsorb (ion-exchange resin) → wash → elute → stabilize → buffer (pH 8.6) → adsorb (alumina) → wash → dry → extract → HPLC [1]. Nearly all steps are susceptible to errors, and waste time and laboratory capacity. The application of the proposed MHPLC system, with micro precolumn and dual electrochemical detector, reduces the number of steps significantly; for example, urine (50–100 μl) → MHPLC.

MHPLC system with micro precolumn and dual electrochemical detector

The direct injection analytical system is shown schematically in Fig. 1. Three micro feeders (Azuma Denki Co., Model MF-2), micro syringes (Terumo Co., Model CAN-1.00) and three-way valves were used to feed the mobile phase, buffer solution and water. A sample injector (Rheodyne Co., Model 7125) with 100-μl sample loop and a six-way valve (Rheodyne Co., Model 50177M) were used for sample injection and alternative connection of the micro precolumn with the sample enrichment system and chromatographic system, respectively. The design of the twin electrode thin-layer electrolytic cell for the dual electrochemical detector 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 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 stainless-steel tube served both as the counter electrode and the exit line. A dual potentiostat

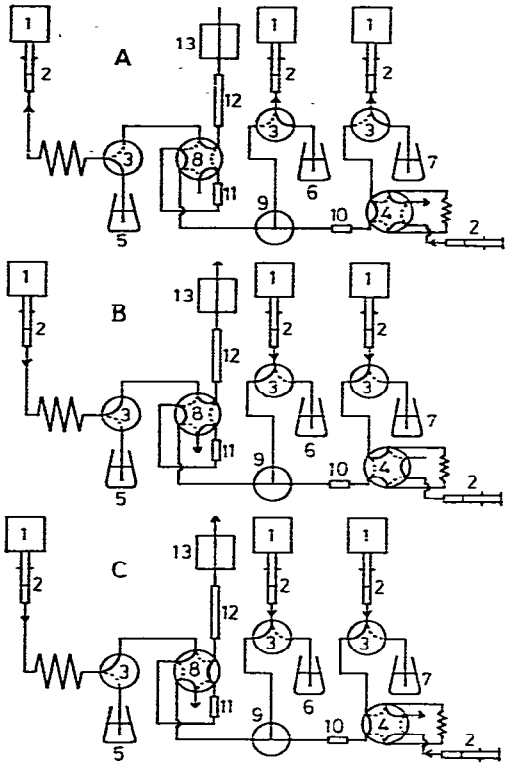

Fig. 1. Block diagram and flow-chart of the MHPLC system with micro precolumn and dual electrochemical detector. 1 = micro feeder, 2 = micro syringe, 3 = three-way valve, 4 = sample injector, 5 = mobile phase, 6 = buffer solution, 7 = water, 8 = six-way valve, 9 = mixing joint, 10 = solution filter,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

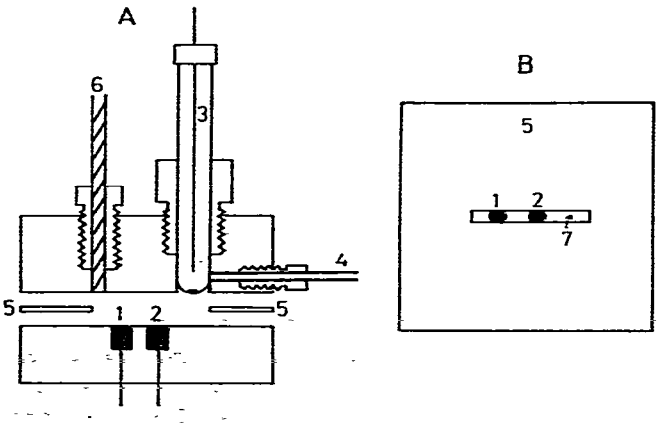

Fig. 2. Construction of twin electrode thin-layer electrolytic cell and connection with micro separation column: (A) side view of cell; (B) top view of spacer. 1, 2 = working electrode (glassy carbon), 3 = reference electrode (Ag/AgCl), 4 = counter electrode (stainless-steel tube), 5 = spacer (PTFE sheet), 6 = micro separation column, 7 = hole.

(Nikko Keisoku Co., Model DPGS-2) was employed to control independently the potentials of the two working electrodes and to measure the currents. The anodic and cathodic chromatograms were simultaneously recorded on a dual-pen recorder (Yokogawa Co., Model 3056).

The micro separation column for analysis was filled by the technique described earlier [11] with ODS (Yanagimoto Co., Yanapak ODS, 5 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, 5 μm) in a PTFE tube 2 cm × 0.5 mm I.D. The solution filter (typical dimensions 1 cm × 0.5 mm I.D.) was made by packing with fine quartz wool.

Reagents

Analytical reagent grade chemicals were used without further purification. All solutions were prepared from distilled and deionized water. For standard samples, noradrenaline, adrenaline, dopamine and *l*-dopa were dissolved in a phosphate buffer of pH 3 to prepare the stock solutions. The mobile phase for analysis was Britton—Robinson buffer (pH 1.8) containing 0.5 m*M* 1-heptanesulfonic acid, sodium salt, as the ion-pair reagent. The buffer solution for pretreatment of the micro precolumn and pH adjustment of the sample was Tris buffer (pH 8.8) containing 0.25% EDTA (disodium salt) and 0.05% $NaHSO_3$ for stabilizing catecholamines.

Procedures

The flow-chart for the direct injection MHPLC system is shown in Fig. 1. At the positions of each valve as shown in part A, the following two procedures are first performed. The mobile phase, buffer solution and water are filled in each respective micro syringe. The first sample of human urine is taken with a 100-μl micro syringe and injected into the sample loop of the sample injector. By switching each valve as in Fig. 1 part B, the procedures for conditioning the micro precolumn followed by sample enrichment and for conditioning the micro separation column are performed at the same time. The micro precolumn is conditioned with the buffer solution of pH 8.8 delivered at a flow-rate of 33 μl/min. The sample is delivered by the water at a flow-rate of 33 μl/min for enrichment by the micro precolumn, solid particles in the sample being removed through the solution filter, and the sample is mixed with a flow of the buffer solution of pH 8.8 in the mixing joint to adjust the sample to pH 8.5. The sample is injected into the micro precolumn for 15 min with a mixed flow of the water and the buffer solution, and then the micro precolumn is washed for a further 15 min with only a flow of water by stopping the flow of the buffer solution. The flow-line from the mixing joint to the micro precolumn was made of a PTFE tube 12 cm × 0.5 mm I.D. to mix the sample with the buffer solution completely. Parallel to the enrichment procedure, the micro separation column is conditioned with the mobile phase delivered at a flow-rate of 8.3 μl/min. Next, by switching the six-way valve, as in Fig. 1 part C, the mobile phase is introduced into the micro separation column through the micro precolumn. In this procedure, the adsorbed compounds are eluted from the micro precolumn and simultaneously separated by the micro separation column. Parallel to the chromatographic procedure, the next sample is taken by

switching the valve of the sample injector again. After the adsorbed compounds are completely eluted from the micro precolumn, the next sample enrichment is performed during the separation process of catecholamines by switching again each valve in Fig. 1 part B. There was no need to change the alumina precolumn in a series of at least 100 analyses with direct injection of the urine samples.

Selective 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 the oxidant is reduced, respectively. The reductant of the reversible or quasi-reversible species is oxidized at the anode placed upstream and the product of this electrode reaction is re-reduced at the cathode placed downstream, while the reductant of the irreversible species is not re-reduced at the cathode. It should, therefore, be noted that only the reversible and/or quasi-reversible species are selectively detected at the cathode.

The separated catecholamines are introduced into the twin electrode thin-layer electrolytic cell, in which the anode and cathode are set at the potentials (V vs. Ag/AgCl) of (+) 0.80 and (+) 0.20, respectively. The catecholamines are selectively detected by monitoring the reduction current at the cathode. The reduction current was measured with one pen of the dual-pen recorder through the electric filter having the time constant of ca. 1 sec to cut out high-frequency noise.

The potentials of the anode and cathode suitable for the selective detection of catecholamines were selected by measuring the cyclic semiderivative of current vs. electrode potential curves by means of semidifferential electroanalysis [12, 13]. The details will be described elsewhere.

RESULTS AND DISCUSSION

Quantitation of catecholamines

The MHPLC system with micro precolumn and dual electrochemical detector was used for the quantitative analysis of catecholamine mixtures. Typical chromatograms of the four catecholamines obtained from a 100-μl injection of a solution of 30 ng/ml of each of noradrenaline, adrenaline and dopamine plus 60 ng/ml of *l*-dopa using the micro ODS column are shown in Fig. 3, in which parts A and B are, respectively, the anodic and cathodic chromatograms. It should be noted that the negative and positive direction peaks correspond to the anodic and cathodic responses, respectively. All the above four catecholamines gave both anodic and cathodic chromatographic peaks. This indicates that their electrode reactions in the Britton—Robinson buffer of pH 1.8 containing 0.5 m*M* 1-heptanesulfonic acid sodium salt used for the mobile phase are reversible or quasi-reversible. The peak separation is good enough and both the anodic and cathodic responses were linear with the amounts of catecholamines injected, as shown in Table I. The procedure of sample enrichment with the alumina precolumn of 2 cm × 0.5 mm I.D. was found to be linear up to the amount injected of ca. 40 ng of each of the four catecholamines. It should be mentioned that the percentage enrichment of *l*-dopa with the micro alumina

TABLE I

RELATIONSHIPS BETWEEN ANODIC AND CATHODIC PEAK HEIGHT AND AMOUNT OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR

Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20; flow-rate of mobile phase, 8.3 μl/min.

Species		Relationship*	Correlation coefficient
Noradrenaline	Anodic	$y = -15.71x + 6.55$	0.999
	Cathodic	$y = 7.33x - 4.39$	0.996
Adrenaline	Anodic	$y = -5.28x + 1.06$	0.997
	Cathodic	$y = 2.41x - 0.77$	0.994
Dopamine	Anodic	$y = -4.82x + 3.88$	0.994
	Cathodic	$y = 2.37x - 1.93$	0.996
l-Dopa	Anodic	$y = -0.21x + 0.37$	0.987
	Cathodic	$y = 0.09x - 0.15$	0.993

*y = peak height measured in nA; x = amount of catecholamine measured in ng.

precolumn in this system is small compared with that of the other catecholamines, as seen in Fig. 3 and Table I. This would be improved by selecting a more suitable buffer solution for adjusting the sample to pH 8.6.

The cathodic responses were found to be about 44, 45, 48 and 42% for noradrenaline, adrenaline, dopamine and *l*-dopa, respectively, of the corresponding anodic responses under the experimental conditions used; i.e. mobile phase flow-rate 8.3 μl/min, anode potential (+) 0.80 V and cathode potential (+) 0.20 V vs. Ag/AgCl. The ratios of cathodic to anodic responses for the four catecholamines were substantially constant in the range of cathode potentials from (+) 0.20 to 0 V under an anode potential of (+) 0.80 V vs. Ag/AgCl. The background current on the cathode tended to increase with decreasing cathode potential. Therefore, the cathode potential of (+) 0.20 V vs. Ag/AgCl was selected for the selective detection of catecholamines in this study.

The cathodic responses reached a high level of precision, as shown in Table II. The relative standard deviations for repetitive determination of catecholamines in the MHPLC system with micro precolumn and dual electrochemical detector were 0.6, 0.9, 1.8 and 5.1% for noradrenaline, adrenaline, dopamine and *l*-dopa, respectively.

Catecholamines in human urine

Typical chromatograms for the determination of catecholamines in 100 μl of human urine directly injected without any pretreatment in the MHPLC system are shown in Fig. 4a and b. Parts A and B are, respectively, the anodic and cathodic chromatograms. Peaks 1, 2, 3 and 4, due to noradrenaline, adrenaline, dopamine and *l*-dopa, are the expected endogenous compounds in the

TABLE II

PRECISION FOR DETERMINATION OF CATECHOLAMINES BY MHPLC WITH MICRO PRECOLUMN AND DUAL ELECTROCHEMICAL DETECTOR

Sample: 100 μl of 40 ng/ml each of noradrenaline, adrenaline and dopamine plus 200 ng/ml of *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20.

Number	Cathodic peak height (inches)			
	Noradrenaline	Adrenaline	Dopamine	*l*-Dopa
1	3.32	1.04	0.97	0.35
2	3.32	1.06	0.99	0.31
3	3.36	1.05	0.96	0.34
4	3.35	1.04	0.95	0.33
Mean	3.34	1.05	0.97	0.33
Relative S.D.	0.6	0.9	1.8	5.1

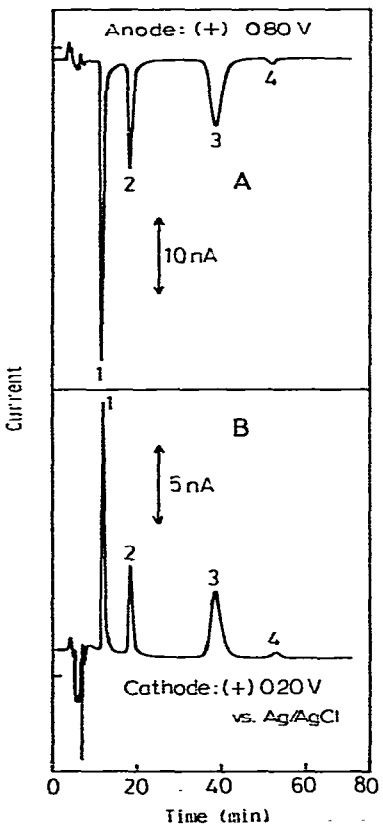

Fig. 3. Typical chromatograms of standard catecholamines by the MHPLC system with micro precolumn and dual electrochemical detector: (A) anodic response, (B) cathodic response. Peaks: 1 = noradrenaline, 2 = adrenaline, 3 = dopamine, 4 = *l*-dopa. Sample: 100 μl of a standard solution of 30 ng/ml each of noradrenaline, adrenaline and dopamine plus 60 ng/ml of *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20.

urine. Their peaks were verified using the standard samples. Of particular interest in part A are the peaks appearing as the shoulder of noradrenaline in Fig. 4b and as the background of *l*-dopa in Fig. 4a. The compound or compounds responsible for these peaks and the peaks appearing after *l*-dopa in part A were not identified. By recording the cathodic current, it was shown that there were essentially no cathodic peaks corresponding to the unknown anodic peaks (see part B), suggesting that the compound or compounds producing anodic peaks are irreversibly oxidized. On the other hand, the compound responsible for the peak appearing after adrenaline in part A of Fig. 4 seems to be one of the metabolites of the catecholamines, because the corresponding cathodic peak appeared in the cathodic chromatograms. It is clear that the proposed dual electrochemical detector can selectively detect catecholamines from many electroactive species co-existent in the urine on the basis of their electrochemical reversibility.

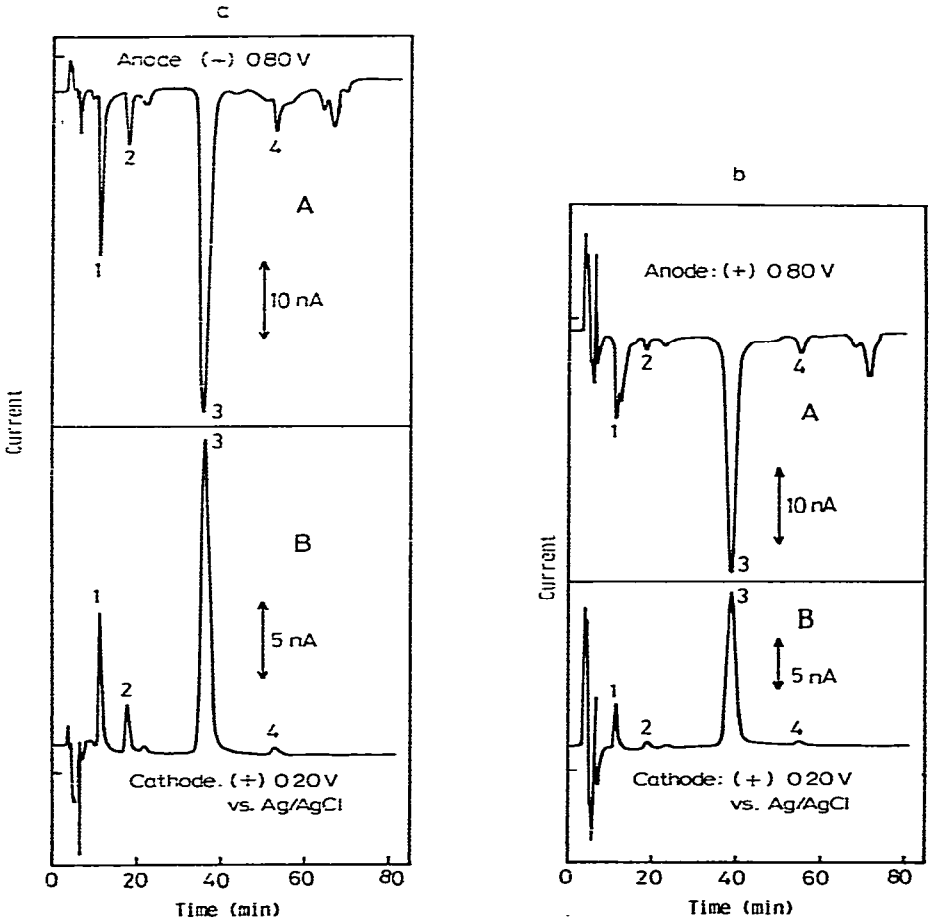

Fig. 4. Typical chromatograms of catecholamines in directly injected human urine: (A) anodic response; (B) cathodic response. Peaks: 1 = noradrenaline, 2 = adrenaline, 3 = dopamine, 4 = *l*-dopa. Potentials (V vs. Ag/AgCl): anode (+) 0.80, cathode (+) 0.20. Sample: 100 µl of human urine.

Human urine from five healthy individuals was analyzed from the linear regression equations in Table I using the cathodic chromatograms. The results are shown in Table III. The concentrations for noradrenaline, adrenaline and dopamine in Table III are within the range of results reported in the literature for normal human urines using HPLC methods with a single electrochemical detector [1] and with a fluorimetric detector [14]. Values for normal urine levels of *l*-dopa do not appear to be available in the literature. This seems to be due to the lack of sensitivity for *l*-dopa in the fluorimetric detection and that the classical pretreatment of urine for electrochemical detection involves ion-exchange procedures before adsorption on alumina, *l*-dopa not being enriched [1]. The values in Table III are somewhat smaller than those obtained using the classical manual method with single electrochemical detection. This indicates that single electrochemical detection tends to overestimate the true values because of lack of selectivity in detection.

The present system appears to be the first method which simultaneously determines all four of these catecholamines in human urine directly injected into the micro liquid chromatograph. Total analysis time for one sample was approximately 1.5 h. One hour is required for the analysis of each additional sample. This time can not be said to be shorter than in the classical manual method, because the manual method may process more samples on a batch-wise basis per working day. However, the sample through-put time in this automated system will be able to be shortened by improving the speed of MHPLC and by arranging for more precolumns to be operated at the same time.

Although this entire article refers to catecholamine analysis in urine, it seems that the system is applicable to other body fluids such as plasma, serum and saliva.

TABLE III

ANALYTICAL RESULTS OF CATECHOLAMINES IN URINE FROM HEALTHY INDIVIDUALS

Sample number	Concentration (ng/ml)			
	Noradrenaline	Adrenaline	Dopamine	*l*-Dopa
1	17	16	101	70
2	17	10	94	65
3	10	7	80	63
4	16	11	86	70
5	16	9	133	60

CONCLUSIONS

The MHPLC system with micro precolumn and dual electrochemical detection is applicable to body fluid samples such as urine without any pretreatment, for the simultaneous determination of four catecholamines with high precision at low concentration. Three processes in this system provide maxi-

mum selectivity for catecholamines. Initially, the compounds are extracted via liquid—solid adsorption on an alumina precolumn. Secondly, separation is effected by means of reversed-phase liquid chromatography. Finally, detection is selectively accomplished by dual electrochemical detection based on their electrochemical reversibility.

Characteristic features of this system are: automated sample pretreatment, direct injection of body fluids (urine, etc.), no internal standard required, high precision, only small amounts of materials for precolumn and separation column and reagents for mobile phase, etc., required. The possibility of further development of this system for clinical purposes seems clear.

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