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Determination of free catecholamines in human urine by direct injection of urine into a liquid chromatographic column-switching system with fluorimetric detection

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ABSTRACT

An ion-exchange chromatographic method combined with ion exclusion was developed for the determination of free catecholamines in human urine. Catecholamines were separated by ion exclusion from most acidic and neutral impurities by filtration through an anion-exchange column with a hydrophilic matrix (Asahipak ES-502N) and the excluded catecholamines were separated by ion-exchange chromatography on a column of weakly acidic ion exchanger with a hydrophilic matrix (Asahipak ES-502C), connected in series to the Asahipak ES-502N column with a four-way automatic valve. A sodium succinate–borate buffer of pH 6.7 (0.035 mol of succinic acid, 0.0075 mol of borate and 0.5 mmol of ethylenediaminetetraacetate were dissolved in 1 kg of water and the pH of the solution was adjusted to 6.7 with sodium hydroxide) was used as the mobile phase, and the temperature of both columns was kept at 30°C.

The catecholamines in the eluate were determined fluorimetrically by post-column derivatization with glycylglycine. A diluted urine sample was injected directly onto the first column. The first column was back-flushed with the mobile phase for 52.5 min after the elution of the catecholamines from the first to the second column. Then the columns were washed with the mobile phase for 10 min in the normal direction before the next sample was injected into the first column. Samples could be analysed every 70 min and 5 pmol/ml of epinephrine, 5 pmol/ml of norepinephrine and 25 pmol/ml of dopamine in human urine could be determined.

INTRODUCTION

The determination of catecholamines in human urine by high-performance liquid chromatography consists of three steps, namely prepurification of catechol-

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amines, separation of the catecholamines by ion-exchange or ion-pair chromatography and detection with electrochemical or fluorimetric methods. Various methods for prepurification based on purification on alumina[1,2], phenylboronate[3,4] and cation-exchange columns[5–7] have been automated and the equipment for prepurification has been coupled to high-performance liquid chromatographs[2,4,7]. These automated systems require multiple solvents for the adsorption and elution of the catecholamines and regeneration of the adsorbent in the precolumn, in addition to the mobile phase(s) for the elution of catecholamines from the analytical column, and the systems can be complex and expensive.

On the other hand, we have found that a urine sample filtered through a column of QAE-Sephadex A-25 gave a lower background fluorescence when the sample was analysed on a weakly acidic ion exchanger with a hydrophilic matric (Asahipak ES-502C) using a sodium succinate–borate buffer as described previously[8]. Therefore, we tried to separate a basic fraction containing catecholamines from acidic and neutral components in a urine sample by filtering it through a column of anion exchanger with a hydrophilic matrix (Asahipak ES-502N) with a sodium succinate–borate buffer of pH 6.7. The excluded fraction was transferred to a cation-exchange column (Asahipak ES-502C) using a four-way valve, eluted with the same sodium succinate–borate buffer as that used for filtration and detected fluorimetrically. This method requires a single buffer for exclusion and elution of catecholamines from Asahipak ES-502N and Asahipak ES-502C. Therefore, the whole process can be easily automated and catecholamines in a diluted acidic urine sample could be successfully determined.

EXPERIMENTAL

Materials

Epinephrine bitartrate, norepinephrine bitartrate and dopamine hydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.) and glycylglycine from Nakarai Chemicals (Kyoto,Japan). Other chemicals were of analytical-reagent grade from Yashima Pharmaceutical (Osaka, Japan). Stock solutions of amines (1 m*M*) were prepared in 0.01 *M* hydrochloric acid, and were diluted in 0.035 *M* succinate buffer (pH 3.5) containing 0.5 m*M* ethylenediaminetetraacetate to give standard solutions of various concentrations.

Apparatus

The liquid chromatograph with automatic column switching valves involved two constant-flow pumps (Trirotar III and V; Jasco, Tokyo, Japan), an automatic injector (Model KSST-60J; Kyowa Seimitsu, Tokyo, Japan), two ion-exchange columns (Asahipak ES-502N and Asahipak 502C; Asahi Chemical Industries, Kanagawa, Japan), an automatic six-way valve (Model 821-09; Jasco), an automatic four-way valve (Model MVA-4U7H; Sanuki Kohgyoh, Tokyo, Japan), a dual-head pump (Model SP-024-2; Jasco), a coil of PTFE tubing (50 m × 0.5 mm I.D.), a spectrofluorimeter (Model 821-FP; Jasco) and a recorder (Model RC-125; Jasco). They were assembled as shown in Fig. 1.

Sample injection and the switching events were controlled by a combination of three timer units. The first timer unit (T_1), equipped with a programmable timer (Model KS-1500; Koizumi Computer, Kobe, Japan), repeats on and off modes of

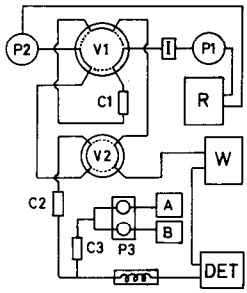

Fig. 1. Diagram of column-switching equipment. P_1 = Trirotar III; P_2 = Trirotar V; P_3 = dual-head pump; I = Automatic injector; V_1 = six-way automatic valve; V_2 = four-way automatic valve; C_1 = Asahipak ES-502N column (10 × 0.76 cm I.D.); C_2 = Asahipak ES-502C column (10 × 0.76 cm I.D.); C_3 = Shodex DS-613 column (15 × 0.6 cm I.D.); DET = spectrofluorimeter and recorder; R = mobile phase reservoir; A = reagent A reservoir; B = reagent B reservoir; W = waste. Full line in V_1 represents position B and dotted line position A, and full line in V_2 represents position L and dotted line position R. The temperature of C_1 and C_2 was kept at 30°C.

electric supply (100 V a.c.), at preset time intervals to the relay circuit of T_1 which controls the automatic injector and four-way valve (V_2), and to the second and third timer units. The second timer unit (T_2) controls V_2 and the third timer unit (T_3) controls the six-way automatic valve (V_1). T_2 is equipped with a motor timer with a maximum graded time of 6 min (Model SYS-6M; Omron Tateisi Electronics, Kyoto, Japan) and T_3 is equipped a motor timer with a maximum graded time of 12 min (Model SYS-12M; Omron Tateisi Electronics).

Mobile phase and reagents

A sodium succinate–borate buffer of pH 6.7 (0.035 and 0.0075 *M*, respectively, containing 0.5 m*M* ethylenediaminetetraacetate) was used as the mobile phase at a flow-rate of 1.5 ml/min. Reagent A was a solution of 0.1 *M* glycylglycine (pH 6.5) containing 0.1 *M* boric acid and 0.05 *M* tartaric acid and reagent B was 0.25 *M* potassium borate buffer of pH 8.3 containing hexacyanoferrate(III) (0.01%, w/v). They were filtered through a membrane filter (pore size 0.45 μm) and degassed under vacuum before use. Water of ultrapure grade, prepared by reverse osmosis (ROpure 40; Barnstead, Boston, MA, U.S.A.), ion exchange and charcoal adsorption of organic matter (NANOpure-II; Barnstead) was used to prepare these solutions.

Sample preparation

Urine was mixed with 1% of its volume of 6 *M* hydrochloric acid and kept in a refrigerator. It was analysed within 2 weeks. The acidified urine was diluted with 0.5 m*M* disodium ethylenediaminetetraacetate solution. The dilution was 5–20-fold, and the diluted urine was centrifuged at 13 000 *g* for 15 min at 5°C. It was filtered through a disposable membrane filter (ACRO LC3A, 0.45 μm; Gelman Sciences, Ann Arbor, MI, U.S.A.) and poured into a plastic vial.

Column switching and detection

Valves V_1 and V_2 were positioned at B and L (full line in Fig. 1) so that the mobile phase pumped by P_1 flowed through the anion-exchange column (C_1) to waste

TABLE I

TIMING AND SEQUENCE OF EVENTS OF AUTOMATED COLUMN SWITCHING FOR THE DETERMINATION CATECHOLAMINES IN HUMAN URINE

Time (min)	*On–off of timers*			*Position of valves*		*Events*
	T_1	T_2	T_3	V_1	V_2	
0	On	On	On	B	L	Sample injection onto C_1
3	On	Off	On	B	R	Connection of C_1 and C_2
7.5	On	Off	Off	A	R	Backflush of C_1 and elution of basic fraction from C_2
60	Off	Reset	Reset	B	L	End of backflush of C_1
70	On	On	On	B	L	Injection of next sample

and the mobile phase pumped by P_2 flowed through the cation-exchange column (C_2) to the post-labelling detector. When switch T_1 was turned on, sample was injected onto C_1, from which basic compounds were excluded. At 3 min after injection, V_2 rotated to position R (dotted line in Fig. 1) and C_1 and C_2 were connected. At 7.5 min after injection, V_1 rotated to position A (dotted line in Fig. 1). The mobile phase pumped by P_1 eluted the basic fraction transferred to C_2 from C_1, and at the same time C_1 was backflushed by the mobile phase pumped by P_2. At 60 min after injection, V_1 turned back to the original position B and V_2 to position L, and 10 min later the next sample was injected onto C_1 (Table I).

Catecholamines in the eluate from C_1 were determined by fluorimetry. Eluate was mixed with an equal mixture of reagents A and B. Each reagent was pumped at a flow-rate of 0.45 ml/min with a dual-head pump and mixed by using a T-shaped connector. The mixture of reagents was filtered through a guard column (15 × 0.6 cm I.D.) packed with 5-μm Shodex DS-613 polystyrene gel (Showa Denko, Tokyo, Japan) to remove fine particles that would cause background noise when passing through the flow cell. The mixture of reagents and the eluate was heated at 70°C in a PTFE tube immersed in a water-bath. The fluorescence was measured with a spectrofluorimeter with excitation at 350 nm and emission at 500 nm. The width of the slits was 18 nm for excitation radiation and 30 nm for emitted radiation.

RESULTS AND DISCUSSION

Elution patterns of a standard sample and a diluted urine sample are shown in Figs. 2 and 3, respectively. When the concentration of boric acid in the mobile phase was increased, the separation of norepinephrine from metanephrine and the separation of dopamine from the impurities which were eluted just after the dopamine peak was improved. However, under these conditions epinephrine was eluted closer to the main impurity peaks eluted before epinephrine. The separation of epinephrine from impurity peaks also became worse when a mobile phase of lower pH was used. Based on these observations, optimum pH and concentrations of the components of the mobile phase were selected. With some batches of Asahipak ES-502C the separation of dopamine from impurity peaks was unsatisfactory. In that event, the use of a mobile

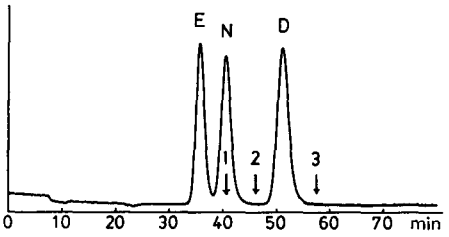

Fig. 2. Elution of standard catecholamines. A 300-μl volume of a solution containing 20 pmol/ml of epinephrine (E), 20 pmol/ml of norepinephrine (N) and 100 pmol/ml of dopamine (D) was injected. Arrows indicate the retention times of (1) metanephrine, (2) normetanephrine and (3) 3-methoxytyramine.

phase of lower succinate concentration (0.030 *M*) and of higher borate concentration (0.008 *M*) gave a better result. When the separation of metanephrine from norepinephrine is needed, the use of a mobile phase with a higher borate concentration (0.011 *M*) and a lower succinate concentration (0.030 *M*) can be used. Metanephrine is eluted after norepinephrine, but the normetanephrine peak overlaps that of dopamine. As the detection limit of metanephrine by the present post-labelling method is 1 nmol/ml, the presence of metanephrine in a urine sample will not affect the determination of norepinephrine by direct injection of a diluted urine sample.

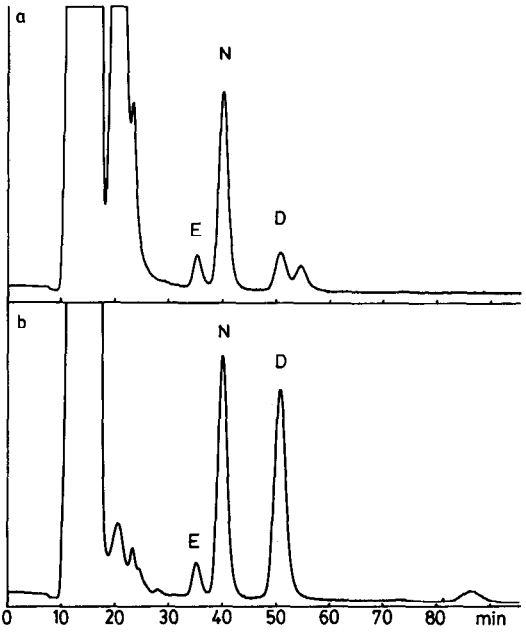

Fig. 3. Elution of diluted urine samples. (a) In the elution patterns of some samples impurity peak(s) were observed just after the dopamine peak, and (b) most of the samples analysed yielded a peak with a retention time of 86 min. When the next sample was injected at 70 min after the first sample, the peak with a retention time of 86 min of the first sample overlapped the large impurity peaks of the second sample.

Urine samples diluted 5–20-fold (pH 2–4) were analysed and 5 pmol/ml of epinephrine, 5 pmol/ml of norepinephrine and 25 pmol/ml of dopamine in urine could be determined. The reproducibility was evaluated by performing six replicate analyse of a normal urine sample diluted 10-fold. The relative standard deviation for epinephrine was 3.2% at a mean concentration of 6.5 pmol/ml, for norepinephrine 1.1% at a mean concentration of 52 pmol/ml and for dopamine 2.7% at a mean concentration of 240 pmol/ml. The correlations between the concentration of catecholamines as determined by the present method (x) and the internal standard method[9] (y) were as follows: for epinephrine, $y = 0.996x - 1.14$ ($r = 0.998$); for norepinephrine, $y = 0.966x + 2.98$ ($r = 0.999$); and for dopamine, $y = 0.932x + 56.1$ ($r = 0.998$) ($n = 17$). These results indicate that the method will be useful for the direct determination of free catecholamines in human urine samples.

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