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Determination of catecholamines in urine and plasma by on-line sample pretreatment using an internal surface boronic acid gel

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ABSTRACT

An automated method of analysis of catecholamines using a new packing material, internal surface boronic acid gel, was developed. The new support is designed with a carboxymethylcellulose-bonded external surface in order to be non-adsorptive to proteins and with a phenylboronic acid-bonded internal surface to retain only catecholamines. This packing support displayed an affinity for basic or neutral catecholic compounds with no protein adsorption and enabled on-line sample pretreatment of catecholamines in urine and deproteinized plasma. The catecholamines were selectively adsorbed on the new material and separated on a reversed-phase or a cation-exchange column. These compounds were then detected electrochemically. The limits of quantitation were 1.5–3.0 ng/ml in urine and 10–15 pg/ml in plasma, at a signal-to-noise ratio of 5.

INTRODUCTION

Catecholamines play an important role in the central nervous system, and their measurement in body fluids such as urine and plasma can help in the diagnosis and treatment of diseases. Determination of catecholamines is widely carried out by HPLC because of its high resolution, sensitivity, precision and availability. However, sample pretreatment is necessary for HPLC analysis of catecholamines in biological fluids. There are two objectives of sample pretreatment: one is to purify the sample and the other is to preconcentrate it to increase sensitivity. Previously, sample pretreatment has been performed mainly by off-line liquid–solid extraction using alumina [1–4] cation-exchange [5–7] or boronic acid gels [8–11]. These off-line sample pretreatments are elaborate, prone to error and time-consuming.

Yamatodani and Wada [12] introduced on-line sample pretreatment for the determination of norepinephrine and epinephrine in plasma using a cation-exchange two-column system. Hansson *et al.* [13] developed a method for the analysis of 3,4-dihydroxyphenylacetic acid (DOPAC) in urine and brain tissue using a column-switching system with a boronic acid silica precolumn and a reversed-phase analytical column. Edlund and Westerlund [14] reported the direct injection of plasma and urine in an automated analysis of catecholamines using three columns, one packed with a boronic acid gel and two packed with reversed-phase material. De Jong *et al.* [15] described a method for the determination of catecholamines in urine and plasma by HPLC after an on-line sample pretreatment using a small alumina or dihydroxyboryl-silica column. Boos *et*

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al. [16] introduced on-line sample processing using phenylboronic acid silica and a reversed-phase column and analysed catecholamines and ribonucleosides in biological fluids.

We determined catecholamines using an on-line sample clean-up technique with a conventional boronic acid gel. Proteins and other matrix compounds in biological samples were adsorbed onto pretreatment columns. In order to prevent this adsorption, we developed a new pretreatment column. In this paper, we describe the synthesis of internal surface boronic acid (ISBA) gel and total automated methods for on-line sample pretreatment of catecholamines in urine and plasma using the ISBA gel as a pretreatment column in combination with an analytical column followed by electrochemical detection.

EXPERIMENTAL

Chemicals and reagents

Norepinephrine (NE) and L-DOPA were obtained from Tokyo Kasei Kogyo (Tokyo, Japan), and 3,4-dihydroxybenzylamine (DHBA) was from Sigma (St. Louis, MO, USA). 3,4-Dihydroxymandelic acid (DOMA), 3-methoxytyramine (3MT) and 3,4-dihydroxyphenylglycol (DHPG) were purchased from Aldrich (Milwaukee, WI, USA). Serotonin (5-HT) and 5-hydroxyindole-3-acetic acid (5-HIAA) were from Techno Chemical (Tokyo, Japan). Glycidoxymethacrylate gel (average particle diameter, 10 μm; exclusion limit of molecular mass, 10 000 as pullulan; epoxy groups, 4 mmol per g of dry gel) was obtained from Hitachi Chemical (Hitachi, Japan). *m*-Aminophenyl boronic acid was from Sigma. Zinc borofluoride solution (45%) was from Hashimoto Chemical (Osaka, Japan). All other chemicals were obtained from Wako. The reagents used were of analytical grade or HPLC grade. Water was purified with a Milli-Q purification system (Millipore, Bedford, MA, USA).

Synthesis of internal surface boronic acid gel

Glycidoxymethacrylate gel (6.0 g) was washed with methanol and water. Carboxymethylcellulose (0.6 g) and 45% zinc borofluoride solution (200 μl) were slurried together with the gel in water (40 ml). The mixture was stirred at 40°C for 2 h. Carboxymethylcellulose that bonded to the surface of the gel was filtered and washed with water.

The gel was then derivatized with *m*-aminophenyl boronic acid (3.0 g) in 60 ml of 1% tetra-*n*-butyl-ammonium solution (pH 11.4) at 40°C for 4 h. The derivatized packing was then filtered and washed thoroughly with water, methanol and 1,4-dioxane. In order to hydrolyse residual epoxy groups, 5.3 g of the gel were slurried in 30 ml of 1 mmol/l sulphuric acid–1,4-dioxane solution at 40°C for 4 h. The resulting ISBA gel was then filtered and washed with methanol and water.

Synthesis of boronic acid gel

Glycidoxymethacrylate gel (4.0 g) and *m*-aminophenyl boronic acid (3.0 g) were slurried in 50 ml of 1% tetra-*n*-butyl-ammonium aqueous solution at 40°C for 4 h. The derivatized packing was then filtered and washed with water, methanol and 1,4-dioxane. Hydrolysis of residual epoxy groups was accomplished using the same technique as described above.

Apparatus

The HPLC system consisted of two modular units from Hewlett-Packard (Waldbronn, Germany): a Model HP1090 Series M liquid chromatograph with autoinjector, DR5 solvent-delivery system, column oven and column-switching valve, and a Model HP79852A pump. The pretreatment column (10 × 4.6 mm I.D.) was packed with ISBA gel. The analytical column for urine samples was packed with Excelpak SIL-C18/5C, 5 μm (250 × 4.6 mm I.D. ; Yokogawa Analytical Systems) and for the plasma samples it was packed with a strong cation-exchange material: Excelpak ICS-C35, 5 μm (150 × 4.6 mm I.D. ; Yokogawa Analytical Systems). A Model HP1049A programmable electrochemical detector (Hewlett-Packard) was used. The detector was set at a potential of 0.75 V *vs.* the Ag/AgCl reference electrode.

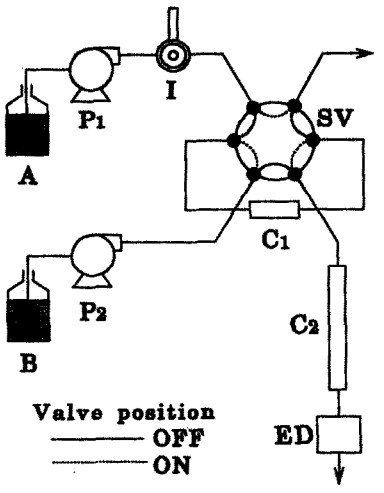

Fig. 1. Schematic diagram of the on-lline sample pretreatment system for analysing catecholamines. A = pretreatment buffer (for adsorption); B = mobile phase (for desorption and separation); P1, P2 = pumps; I = injector; C1 = pretreatment column (ISBA column); C2 = analytical column; SV = switching valve; ED = electrochemical detector.

The column-switching system

The system used for on-line sample pretreatment is shown in Fig. 1. Samples are injected and transported via the switching valve to the ISBA column by a pretreatment buffer. Catecholamines form complexes with boronic acid and are adsorbed on the ISBA column, while the other compounds are eluted in the waste. After washing, the valve is switched to "on" and the eluent is changed from the pretreatment buffer to the mobile phase. The boronate complexes are dissociated and the catecholamines are then eluted to the analytical column.

Urine analysis

A 5-μl aliquot of urine was injected onto the ISBA column. The pretreatment solution consisted of 50 mmol/l phosphate buffer (pH 9.3) at a flow-rate of 1.2 ml/min. The Excelpak SIL-C18/5C column was used as the analytical column. The mobile phase consisted of 50 mmol/l phosphate buffer (pH 3.0), 200 mmol/l potassium nitrate and 0.07 mmol/l EDTA at a flow-rate of 1.2 ml/min. The valve was switched to "on" at the 1.40-min time point and switched to "off" at the 3.80-min time point. The temperature of the column oven was 40°C.

Plasma analysis

A 1.5-ml aliquot of plasma was injected onto the ISBA column. The pretreatment solution consisted of 50 mmol/l phosphate buffer (pH 9.3) at a flow-rate of 1.5 ml/min. The Excelpak ICS-C35 column was used as the analytical column. The mobile phase consisted of 15 mmol/l citric acid, 65 mmol/l sodium nitrate and 0.07 mmol/l EDTA (pH 2.2) at a flow-rate of 1.0 ml/min. The valve was switched to "on" at the 5.00-min time point and "off" at the 6.00-min time point. The temperature of the column oven was 25°C.

Sampling

For the urine sample, urine was collected for 24 h in a receiver filled with 20 ml of 6 mol/l hydrochloric acid. The 24-h urine sample was stored at −20°C after the addition of the internal standard. There are two types of catecholamines in urine: free and conjugated with glucuronic acid or sulphuric acid.

For the analysis of free catecholamines, after thawing the sample was passed through a 0.22-μm pore size filter before injection.

For the analysis of total (free and conjugate) catecholamines, the 24-h urine was acidified to pH 1 with hydrochloric acid and hydrolysed at 100°C for 20 min. After filtering (0.22 μm) the sample was analysed by HPLC.

For the plasma sample, after the addition of the internal standard, 1.5 ml of plasma were deproteinized by 90 ml of 60% perchloric acid and centrifuged at 2000 *g* for 5 min. A 400-μl aliquot of 1 mol/l sodium hydroxide was added to the supernatant and mixed with 50 mmol/l phosphate buffer (pH 8.6) to a total volume of 1.5 ml. The pH adjustment must be done just before the injection, otherwise the catecholamines decompose in a short time (*e.g.* 70% in 30 min, 50% in 60 min).

RESULTS AND DISCUSSION

Under alkaline conditions the carboxy func-

tion that was bonded to the surface of ISBA gel was negatively charged. The anionic proteins were excluded by both ion-exclusion and size-exclusion chromatography. Even though cationic proteins interacted with the surface of the gel by ion exchange, they were also excluded by the ISBA gel. The reason is that the size exclusion of the cationic protein was the dominant factor and the ion exchange with the surface of the gel was diminished. Both the low-molecular-mass anion and cation permeated the pores. However, they were not retained on the ISBA gel because it is impossible to make boronate complexes. On the other hand, the catecholamines that permeated the pores of ISBA gel were retained by forming complexes with boronic acid.

In order to evaluate the ability of the ISBA gel not to adsorb proteins, the recovery of proteins using an ISBA column was compared with a boronic acid (BA) column. On the ISBA column, recovery of human serum albumin (HSA) and γ-globulin was 95% and 88%, respectively, whereas on the BA column recovery of HSA was 89%, and only 46% for γ-globulin.

As reported previously [13,15], the capacity factors (k') for catechol compounds were influenced by the pH value of buffer solutions. The relationship between pH and the capacity factor on an ISBA column for NE was studied (Fig. 2). The influence of pH on the capacity factor for catechol was also similar to NE. The capacity factors increased with pH values higher than 7 and maximum capacity factors were achieved at pH 9–10. Therefore, pH 9.3 was chosen as the pretreatment solution for concentration of catecholamines; pH < 5 was chosen as the pH of the mobile phase for desorption and separation of catecholamines.

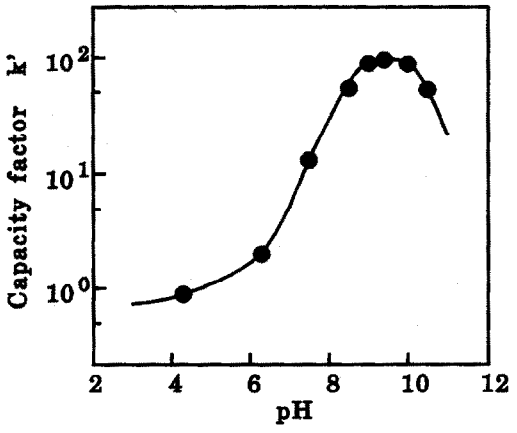

Fig. 2. Relationship between pH and the capacity factor on an ISBA column for NE.

The capacity factors for some catecholamines and related compounds were determined using an ISBA column at a pH value of 9.3 (Table I). Hansson *et al.* [13] reported that compounds containing free 1,2-diols were capable of forming complexes with boronic acid–silica. However, in the case of an ISBA column, only basic or neutral 1,2-diols, such as catechol, NE, E, DA, DHBA and DHPG, were retained, whereas acidic 1,2-diols such as DOMA and DOPAC were not retained. This is the result of the ion exclusion between acidic 1,2-diols and the negatively charged carboxy function, which is bonded to the ISBA gel under alkaline conditions (pH 9.3). Also, DOPA is not retained on the ISBA column under these conditions for the same reason.

TABLE I

CAPACITY FACTORS (k') ON THE ISBA COLUMN FOR THE CATECHOLAMINES AND RELATED COMPOUNDS

Compound	Capacity factor (k')
Phenol	16.5
Catechol	122.3
Resorcinol	14.9
Hydroquinone	11.5
Norepinephrine (NE)	94.3
Epinephrine (E)	130.5
Dopamine (DA)	136.5
DOPA	11.5
3,4-Dihydroxybenzylamine (DHBA)	143.9
3,4-Dihydroxymandelic acid (DOMA)	2.5
3,4-Dihydroxyphenylacetic acid (DOPAC)	2.3
Homovanillic acid (HVA)	0.8
Vanillylmandelic acid (VMA)	0.5
3-Methoxytyramine (3MT)	4.5
Aminophenol	5.4
3,4-Dihydroxyphenylglycol (DHPG)	46.5
Serotonin (5-HT)	23.4
5-Hydroxyindole-3-acetic acid (5-HIAA)	1.5

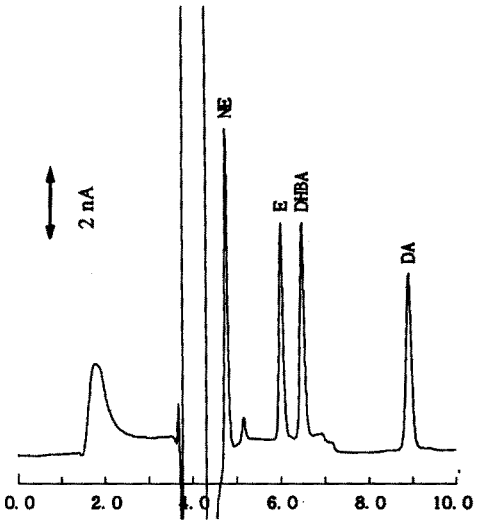

Fig. 3. Chromatogram of a standard mixture of catecholamines using the on-line sample pretreatment system. Standard mixture containing 200 ng/ml each of NE, E, DHBA (internal standard) and DA. For conditions, see Experimental section.

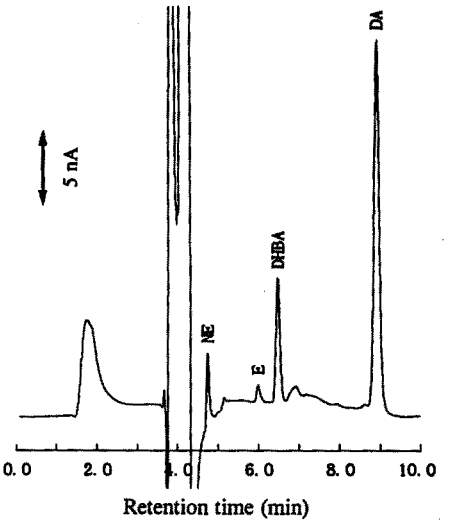

Fig. 4. Chromatogram of total catecholamines in 24-h urine using the on-line sample pretreatment system. Amounts in the urine were NE, 78.3 ng/ml; E, 26.1 ng/ml, and DA, 735 ng/ml. For conditions, see Experimental section.

Fig. 3 illustrates the results of the analysis of a 200 ng/ml catecholamine solution and internal standard (DHBA) using this on-line catecholamine analysis method and electrochemical detection. The relative standard deviation values (R.S.D.) (n = 20) were 2.0, 1.7 and 4.6% for NE, E and DA, respectively. Fig. 4 shows a chromatogram of total catecholamines in 24-h urine. A well-defined chromatogram is obtained without interference from other matrix compounds. Satisfactory reproducibilities were obtained, the R.S.D. (n = 20) being 1.3, 2.6 and 1.5% for NE, E and DA, respectively. The recoveries of catecholamines on addition of 100 ng/ml standard to urine were determined. The results were 95, 100 and 98% for NE, E and DA, respectively (means of six observations). The limits of quantitation of urine were 1.5, 2.3 and 3.0 ng/ml for NE, E and DA, respectively, at a signal-to-noise ratio of 5. To evaluate the accuracy, fifteen samples of hydrolysed urine were analysed. The amounts of catecholamines are in good agreement with the conventional technique, which is alumina extraction in combination with a reversed-phase analytical column and trihydroxyindole (THI) [17,18] fluorescence (FL) detection (Table II).

TABLE II

COMPARISON BETWEEN THE CATECHOLAMINE AMOUNTS IN FIFTEEN URINE SAMPLES DETERMINED WITH THE ON-LINE SAMPLE PRETREATMENT METHOD AND ALUMINA EXTRACTION IN COMBINATION WITH FLUORESCENCE DETECTION

Compound	Concentration (mean ± S.D.) (ng/ml)	
	On-line sample pretreatment	Alumina extraction–THI FL detection
NE	111 + 21	105 + 30
E	26 + 17	21 + 11
DA	597 + 151	648 + 178

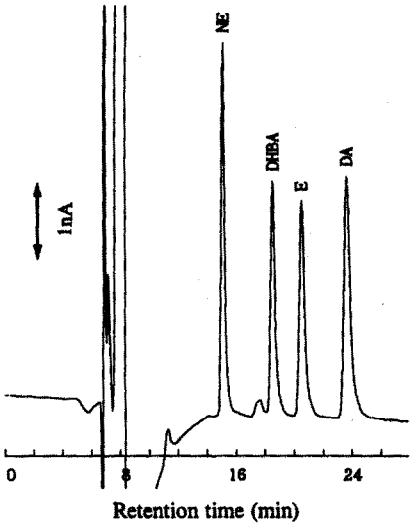

Fig. 5. Chromatogram of a standard mixture of catecholamines using the on-line sample pretreatment system. Standard mixture containing 1 ng/ml each of NE, DHBA, E and DA. For conditions, see Experimental section.

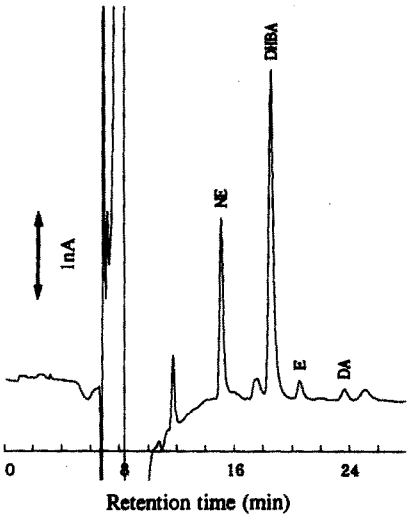

Fig. 6. Chromatogram of catecholamines in plasma using the on-line sample pretreatment system. Amounts in the plasma were NE, 423 pg/ml; E, 47 pg/ml; and DA, 28 pg/ml. For conditions, see Experimental section.

This method was applied to the analysis of catecholamines in plasma. For plasma, with catecholamine concentrations of *ca.* 10–600 pg/ml [19–21], high sensitivity is required. High sensitivity was accomplished by adjusting the sample to pH 8.6 and increasing injection volume to 1.5 ml. A cation exchange was used for the analytical column to separate the NE from the baseline dip. Fig. 5 shows a chromatogram of a 1 ng/ml catecholamine and DHBA. The R.S.D. values (n = 5) for peak areas were 2.9% for NE and 3.5% for E and DA. The calibration graphs for all the catecholamines (100 pg/ml to 10 ng/ml) were linear with correlation coefficients better than 0.99995. Fig.6 shows one resulting chromatogram of plasma in which the amounts of NE, E and DA in plasma were 423, 47 and 28 pg/ml, respectively. The limits of quantitation for NE, E and DA in plasma were 10, 15 and 11 pg/ml, respectively, at a signal-to-noise ratio of 5.

Over several months, more than 300 urine (5-μl aliquots) and 50 deproteinized plasma samples (1.5-ml aliquots) were injected using the same ISBA column without reduction in the chromatographic efficiency.

These results indicate that the proposed method can be used for determination of catecholamines in urine and plasma.

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