CHROMSYMP. 2439

Determination of catecholamines in urine by liquid chromatography and electrochemical detection after on-line sample purification on immobilized boronic acid

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ABSTRACT

Norepinephrine, epinephrine and dopamine in urine were measured by an automated liquid chromatographic method. After sample purification on a column containing silica–immobilized boronic acid, which showed great affinity for catecholamines at neutral pH, the catecholamines were eluted by backflushing with an acidic mobile phase and transferred to a cation exchanger for separation. Detection was performed electrochemically and the relative standard deviation was 2% for the analysis of endogenous concentrations in human urine.

INTRODUCTION

Liquid chromatography with electrochemical or fluorimetric detection has attained wide acceptance for assay of catecholamines in urine. Most often extensive purification of the complex urine sample is required prior to the chromatographic separation. Extraction methods utilizing organic solvents [1,2], cation-exchange resin [3–5], aluminium oxide [6–9], immobilized boronic acid [10,11] or combinations of these techniques [12–14] have been used. Automated methods have also been applied, using ASPEC (automatic sample preparation with extraction columns, Gilson) or coupled-column systems [15–20]. In this paper we have adapted the same approach for determination of catecholamines as previously demonstrated for vanilmandelic acid (VMA) in urine [21]. α-Hydroxycarboxylic acids, like VMA, bind to boronic acid at a low pH, while catechol compounds containing vicinal *cis*-diols form cyclic boronate esters at neutral pH. By combining affinity to boronate and cation-exchange chromatography a highly selective chromatographic system was obtained, enabling a simple analysis by direct injection of urine samples.

EXPERIMENTAL

Chemicals and reagents

Hydrochloric acid, ethylenediaminetetraacetic acid (EDTA) and all buffer substances were of analytical-reagent grade (Merck, Darmstadt, Germany) and methanol was of high-performance liquid chromatography (HPLC) grade (Rathburn, Walkerburn, UK). Epinephrine (E) bitartrate, norepinephrine (NE) bitartrate, dopamine (DA) hydrochloride, metanephrine (MN) hydrochloride and VMA were obtained from Sigma (St. Louis, MO, USA), α-methyldopamine (MDA) hydrochloride from Merck Sharp and Dohme (Rahway, NJ, USA) and dihydroxyphenylethylene glycol (DOPEG) and 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG) piperazine salt from Regis (Morton Grove, IL, USA).

Chromatographic system

A scheme of the chromatographic system is presented in Fig. 1. It comprised three Model 2150 pumps (LKB, Bromma, Sweden), a Model 465 autosampler with a refrigerated sample tray (Kontron, Zürich, Switzerland) and 0.75-ml injection
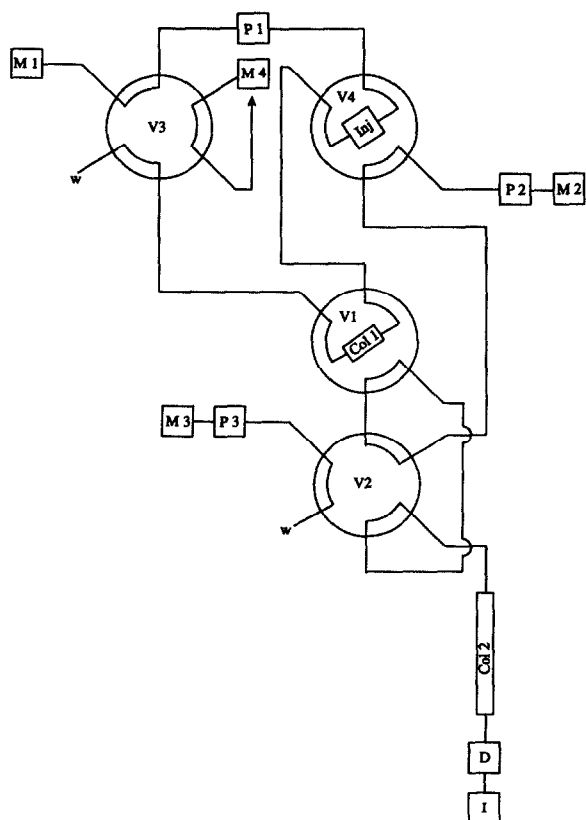
Fig. 1. Coupled-column liquid chromatographic system. P1, P2 and P3 = pumps; M1, M2, M3 and M4 = mobile phases 1 (phosphate buffer pH 7.0, $I=0.01$), 2 (phosphate buffer pH 3.0–3.5, $I=0.1$, containing 0.3 mM EDTA and 5–25% methanol), 3 and 4 (phosphate buffer pH 3.2, $I=0.01$); Inj = autoinjector; Col 1 = SelectiSpher-10 Boronate; Col 2 = Nucleosil 5SA; V1, V2, V3 and V4 = six-port valves; D = detector; I = integrator; w = waste. For further details, see text.

TABLE I

SCHEME OF COLUMN-SWITCHING EVENTS

Time after injection (min)	Switch valve No.	Event
0.0		The sample is injected onto column 1
12.0	1	Columns 1 and 2 are connected. Column 1 is backflushed with an acidic mobile phase and the analytes are transferred to column 2
13.5	2	Columns 1 and 2 are disconnected from each other Column 1 is washed
17.5	1 reset	Column 1 is reconditioned until the next injection
25	2 reset	After the chromatogram is completed the next sample is injected

vials of polypropylene with attached stoppers (Milian Instruments, Geneva, Switzerland), a Model 4270 integrator (Spectra-Physics, San Jose, CA, USA), a Model 7010 six-port valve (Rheodyne, Berkeley, CA, USA) and three Model C6W six-port valves with a high-speed switching unit mounted on an air actuator (Valco, Schenkon, Switzerland). Two of the valves, V1 and V2, were controlled by the integrator and the switching times were 12 and 17.5 min for valve 1 and 13.5 and 25 min for valve 2 (Table I). Valve V3 was controlled by the autosampler and switched after the last set of samples had been injected. The fourth valve, V4, was switched

manually to enable direct injection onto the separation column in recovery studies. The columns were packed with SelectiSpher-10 Boronate (35 × 2.1 mm I.D.) (Skandinaviska GeneTec, Kungsbacka, Sweden) and Nucleosil 5SA (100 × 4.6 mm I.D.) (Machery-Nagel, Düren, Germany).

The neutral mobile phase (pH 7.0; ionic strength, $I=0.01$) contained disodium hydrogenphosphate (2.65 mM) and sodium dihydrogenphosphate (2.10 mM) and the acidic phase (pH 3.2; $I=0.1$) contained sodium dihydrogenphosphate (100 mM), phosphoric acid (15 mM), EDTA (0.3 mM) and methanol (5–25%). For washing of the boronate column a phosphate buffer solution (pH 3.2; $I=0.01$) was used. The water was deionized and filtered through a Milli-Q system (Millipore, Molsheim, France), and prior to use the mobile phases were degassed and filtered through a 0.45-μm MF Millipore filter. The flow-rates were 1.0 ml/min and the eluent from the cation-exchange column was monitored with an ESA Model 5100A Coulochem electrochemical detector (Environmental Sciences, Bedford, MA, USA), with a Model 5011 analytical cell operated at +0.00 V and +0.30 V and a Model 5020 guard cell operated at +0.35 V.

Analytical procedure

Urine samples were acidified with 5 M hydrochloric acid to pH 4 before storage at −20°C. After

thawing, the urine samples were mixed and centrifuged for 2 min at 1000 g. A 150-μl volume of a urine sample or a reference solution of the catecholamines (200 nM) in 1 mM hydrochloric acid was transferred to an injection vial and placed in the autosampler. Addition of a 20-μl volume of an internal standard solution of MDA (10 μM) in 1 mM hydrochloric acid was performed by the autosampler. It was also programmed to adjust the pH of the sample to 7 by adding 80 μl of a solution of 250 mM disodium hydrogenphosphate containing 10 mM EDTA (pH 8) and then mixing the sample by three repetitive pipettings. The injection volume was 20–200 μl.

RESULTS AND DISCUSSION

Retention capacity of boronic acid-substituted silica

In a previous study [21] we used immobilized boronic acid to isolate VMA from urine samples. A complex between the boronate matrix and the α-hydroxycarboxylic acid was formed at low pH. We also studied the retention of related compounds on the boronic acid packing material at pH between 2.1 and 7.7. The results of some representative compounds and of the catecholamines are shown in Figs. 2 and 3. The catecholamines were strongly retained at neutral pH, while the 3-O-methylated amines (MN) and the neutral catechol compounds (DOPEG) were less retarded. The acids (VMA) and the O-methylated neutral compounds (MOPEG) were practically unretained at a neutral pH.

Column switching

For purification and concentration of the urine sample, we utilized the ability of the boronate matrix to strongly retain the catecholamines at pH 7. After injection of the sample, the boronate column was washed for 12 min with a neutral mobile phase. Valve V1 was then switched and the column backflushed with an acidic phase, which rapidly desorbed the catecholamines and transferred them to the cation exchanger, where separation was performed. The time schedule for switching of the valves is reported in Table I. After 0.5–1.5 min of backflushing, the boronate column was disconnected from the separation column by switching of valve V2. While the catecholamines were separated on the cation exchanger and detected, the boronate
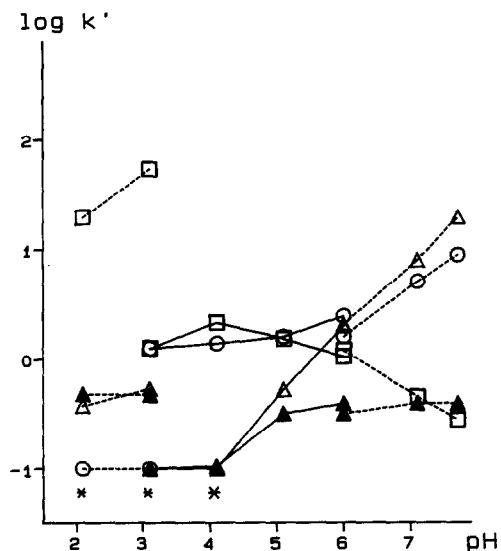
Fig. 2. Influence of pH of the mobile phase on the retention of some catechol derivatives. Stationary phase: SelectiSpher-10 Boronate. Mobile phase: phosphate buffer ($I=0.01$) pH 2.1, 3.1, 6.0, 7.1, 7.7 (----) and citrate buffer ($I=0.01$) pH 3.1, 4.1, 5.1, 6.0 (——); * = log k' values lower than -1. Key: $\bigcirc$ = MN; $\triangle$ = DOPEG; $\square$ = VMA; $\blacktriangle$ = MOPEG.
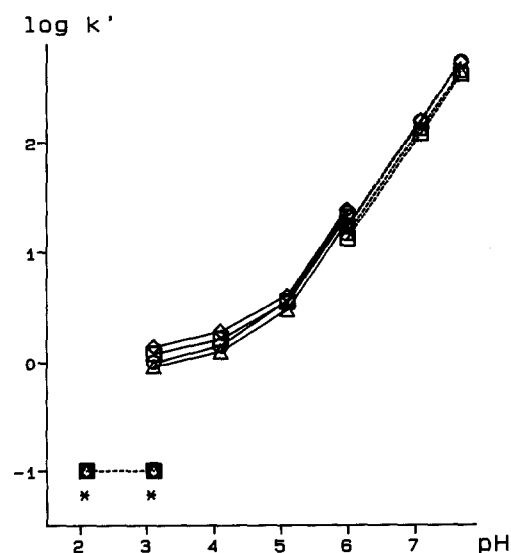
Fig. 3. Influence of pH of the mobile phase on the retention of catecholamines. Conditions as in Fig. 2. Key: $\bigcirc$ = E; $\triangle$ = NE; $\square$ = DA; $\diamond$ = MDA.

column was washed backwards for 4 min with a buffer solution of pH 3 and, after resetting of valve V1, reconditioned with the neutral mobile phase until the next injection. When the chromatogram was completed, valve V2 was reset and the next sample was injected. When all the samples had been chromatographed valve V3 was switched in order to flush the boronate column with a buffer solution of pH 3, until the next set of samples was to be analyzed. This was done to promote a long lifetime of the boronate column. It seems as if the column in the long run tolerated the buffer solution of pH 3 better than the buffer solution of pH 7.

Chromatography

The retention capacity of the boronate phase was tested by coupling the column outlet directly to the detector. Owing to the high affinity of the catecholamines for the boronic acid material at pH 7, the column could be washed for more than 12 min without any loss of analyte. The catecholamines were then desorbed at an acidic pH and eluted onto the cation exchanger. For analysis of catecholamines in biological samples, cation-exchange chromatography has proved to be more selective than reversed-phase chromatography [6,7,14]. Owing to variations in the retention capability between different batches of cation-exchange material, the pH of the mobile phase was varied between 3.0 and 3.5 and the content of methanol used was 5–25%. A more acidic pH of the mobile phase promoted the desorption of the catecholamines from the boronate matrix, but then an interfering peak appeared in the chromatogram. Its capacity factor (k') at pH 1.8 was 10.6 but only 1.9 at pH 2.9. The retention times of the catecholamines were practically unchanged in this pH range. In order to avoid contamination of subsequent chromatograms, owing to incomplete desorption of the boronate column, a 4-min washing step using a buffer solution of pH 3 was introduced after each injection. A chromatogram of a human urine sample obtained after column switching is shown in Fig. 4. The peak eluting between NE and E could easily be moved by adjusting the methanol content of the mobile phase. After a couple of months the cell response of the electrochemical detector had declined, but it was restored by washing with 6 M nitric acid.
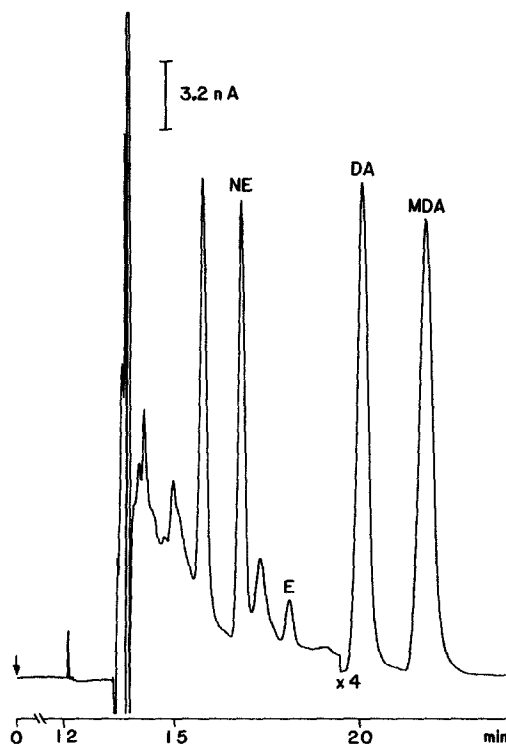

Fig. 4. Chromatogram of a human urine sample containing 220 nM NE, 34.3 nM E and 960 nM DA. Injected sample 120 μl; potential +0.30 V. Mobile phase 2: phosphate buffer pH = 3.2, I = 0.1, containing 0.3 mM EDTA and 21% methanol. Other chromatographic conditions as in Fig. 1.

Stability

It was important that the adjustment of the samples to pH 7 was performed just before injection of the 20- to 200-μl volume, owing to instability of the catecholamines at this pH. By adding 10 mM EDTA to the 250 mM disodium hydrogenphosphate solution (pH 8.0), the catecholamines were kept stable at pH 7 for at least 30 min. The urine samples and the standard solutions in 1 mM hydrochloric acid were stable for more than 24 h in the cold injection vials.

Recovery and precision

Peak areas of standard solutions recorded after injection into the coupled-column system were compared with those recorded after direct injection onto the cation-exchange column. The recoveries ob-

TABLE II

DETERMINATION OF NOREPINEPHRINE, EPINEPHRINE AND DOPAMINE IN URINE SAMPLES BY THE AUTO-MATED COUPLED-COLUMN METHOD (I) AND BY THE EARLIER PRESENTED LIQUID CHROMATOGRAPHIC METHOD (II) [6]

The values are expressed as nmol/mmol creatinine; R.S.D. = relative standard deviation.

Sample	Norepinephrine			Epinephrine			Dopamine		
	I	II	R.S.D. (%)	I	II	R.S.D. (%)	I	II	R.S.D. (%)
1	28.4	27.9	1.2	3.43	3.52	1.8	179	181	0.8
2	23.9	22.4	4.6	4.65	4.55	1.5	137	135	1.0
3	29.8	29.6	0.5	5.29	5.45	2.1	187	187	0.0
4	20.0	20.6	2.1	4.70	3.84	14.2	147	150	1.4
5	28.1	29.4	3.2	3.76	2.95	17.0	106	108	1.3
6	42.6	42.5	0.2	6.15	5.75	4.8	278	276	0.5
7	24.4	24.4	0.0	3.88	3.56	6.1	185	189	1.5
8	14.8	13.9	4.4	2.38	2.29	2.7	104	99	3.5
9	27.7	29.4	4.2	15.4	15.9	2.3	137	146	4.5
10	30.2	30.5	0.7	6.95	6.42	5.6	187	202	5.5
11	21.3	20.9	1.3	4.83	3.96	14.0	172	180	3.2
12	19.0	22.1	10.7	1.37	1.66	13.5	116	132	9.1
13	30.4	30.8	0.9	5.39	5.16	3.1	152	153	0.5
14	19.8	19.8	0.0	3.30	2.90	9.1	163	162	0.4

tained were 98.3 ± 4.1, 97.4 ± 4.7, 100.0 ± 2.7 and $105.0 \pm 2.8\%$ for NE, E, DA and MDA, respectively ($n = 15$). The recovery from urine samples determined by standard addition of 55–220 nM was $100.0 \pm 4.2\%$ for all three catecholamines ($n = 9$). The ratios of the peak height of the analyte to that of the internal standard, MDA, in the reference samples were measured and the median value was used for calculation of the urine concentration. The relative standard deviations for human urine samples containing 220 nM NE, 34.3 nM E and 960 nM DA were 2.0, 2.1 and 1.3%, respectively ($n = 15$), and for aqueous standard samples, at concentrations of 220 nM, the values were 1.9, 1.6 and 0.71% ($n = 15$). The limit of quantitation for epinephrine in human urine was 5 nM with a relative standard deviation of 10% ($n = 10$), and the method was linear up to at least ten times the concentration of the reference solution used in the analytical procedure. The accuracy of the method was tested by analysing some of the samples using our earlier published method [6], which is based on purification by alumina before cation-exchange liquid chromatography. Good agreement between the two methods was found, as shown in Table II.

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