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Determination of vanilmandelic acid in urine by coupled-column liquid chromatography combining affinity to boronate and separation by anion exchange

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ABSTRACT

An automated liquid chromatographic method for assaying vanilmandelic acid in urine is described. Vanilmandelic acid and potential interfering substances, such as catechol compounds and their metabolites, have been tested for affinity to boronic acid-substituted silica at various pH values. Vanilmandelic acid and the internal standard, isovanilmandelic acid, were bound to the boronate matrix at an acidic pH, whereas for instance catecholamines were unretained and passed through the column. The α-hydroxycarboxylic acids were then desorbed by another mobile phase (pH 6.0) and transferred to an anion exchanger for chromatography and electrochemical detection. A relative standard deviation of 2.8% was obtained for the analysis of human urine samples containing 6.6 μM vanilmandelic acid.

INTRODUCTION

Vanilmandelic acid (VMA), the major metabolite of norepinephrine (NE) and epinephrine (E), is often determined in urine in pathological and clinical studies. Prior to liquid chromatographic (LC) determination a purification step is necessary to remove interfering compounds. This has usually been accomplished by solvent [1–3] or solid-phase [4–7] extraction, but direct injection of untreated urine, using coupled columns, has also been described [8–11].

It is well known that derivatives of boronic acid reversibly form cyclic boronate esters with vicinal *cis*-diols at a pH above 7, and catechol compounds have been selectively extracted from urine and plasma samples [12–15]. It has also been reported that immobilized boronate will bind α-hydroxycarboxylic acids at low pH [16] but to our knowledge no method has been published where this is applied to biological samples. In the present study we used the boronate matrix to selectively extract VMA from urine. By combining affinity to boronate and anion-exchange chromatography a highly selective chromatographic system was obtained.

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EXPERIMENTAL

Chemicals and reagents

VMA, E bitartrate, NE bitartrate, dopamine (DA) hydrochloride, 5-hydroxytryptamine (5-HT) hydrochloride, 5-hydroxyindoleacetic acid (5-HIAA), normetanephrine (NMN) hydrochloride, metanephrine (MN) hydrochloride, 3-methoxytyramine (3-MT) hydrochloride, homovanillic acid (HVA) and 3,4-dihydroxyphenylalanine (DOPA) were obtained from Sigma (St. Louis, MO, U.S.A.), isovanilmandelic acid (iso-VMA) from Aldrich (Milwaukee, WI, U.S.A.), α-methyldopamine (α-MDA) hydrochloride from Merck Sharp and Dohme (Rahway, NJ, U.S.A.), 3,4-dihydroxyphenylacetic acid (DOPAC) from Fluka (Buchs, Switzerland) and 3-methoxy-4-hydroxyphenylethylene glycol (MOPEG) piperazine salt, dihydroxyphenylethylene glycol (DOPEG), dihydroxymandelic acid (DOMA) and dihydroxyphenylethanol (DOPET) from Regis (Morton Grove, IL, U.S.A.).

Hydrochloric acid and all buffer substances were of analytical-reagent grade from E. Merck (Darmstadt, Germany) and methanol of HPLC grade from Rathburn (Walkerburn, U.K.). Water used in the mobile phase was filtered through a Milli-Q system (Millipore, Molsheim, France).

Chromatographic system

A scheme of the chromatographic system is presented in Fig. 1. It was composed of two Model 2150 pumps (LKB, Bromma, Sweden), a Model 460 autosampler (Kontron, Zürich, Switzerland) with a refrigerated sample tray, a Model 7010 six-port valve (Rheodyne, Berkeley, CA, U.S.A.), two Model C6W six-port valves with a high-speed switching unit mounted on an air actuator (Valco, Schenkon, Switzerland), a SelectiSpher-10 Boronate column (35 mm × 2.1 mm I.D.) (HyClone, Lund, Sweden), a separation column (150 mm × 4.6 mm I.D.) with packing material Nucleosil 5SB (Macherey-Nagel, Düren, Germany), a Model 4270 integrator (San Jose, CA, U.S.A.) and an ESA electrochemical detector Model 5100A Coulochem (Environmental Sciences Assoc., Bedford, MA, U.S.A.), with a Model 5011 analytical cell operated at +0.00 and +0.50 V and a Model 5020 guard cell operated at +0.55 V.

The acidic mobile phase (pH 3.0; ionic strength, $I = 0.01$) comprised sodium dihydrogenphosphate (10 m*M*) and phosphoric acid (1.5 m*M*) and the pH 6.0 phase ($I = 0.2$) disodium hydrogenphosphate (18 m*M*), sodium dihydrogenphosphate (146 m*M*) and 5% methanol. Prior to use the phases were degassed and filtered through a 0.45-μm MF Millipore filter.

The flow-rates were 1.0 ml/min and the switching times for valve V2 were 0.5 and 1.5 min and for valve V3 17 and 35 min.

Analytical procedure

Urine samples were acidified with 5 *M* hydrochloric acid to a pH of 4 before

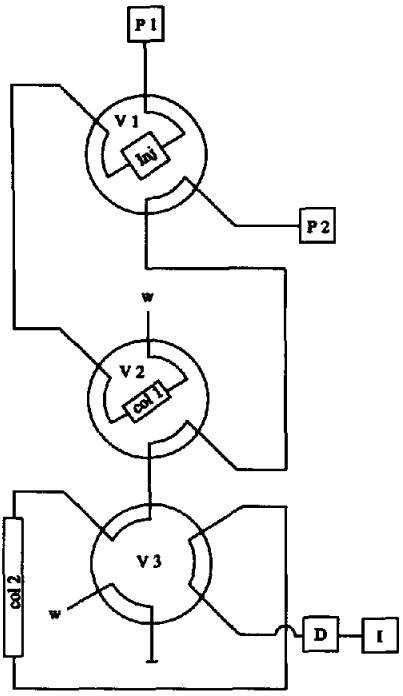


Fig. 1. Coupled-column liquid chromatographic system. P1 and P2 = pumps with mobile phase 1 (phosphate buffer pH 3.0, I = 0.01) and 2 (phosphate buffer pH 6.0, I = 0.2, containing 5% methanol); Inj = autoinjector; col 1 = SelectiSpher-10 Boronate; col 2 = Nucleosil 5SB; V1 = six-port valve, Rheodyne; V2 and V3 = six-port valves, Valco; D = detector; I = integrator; w = waste. For further details, see text.

storage at −20°C. After thawing, the urine samples were mixed and centrifuged for 2 min at 1000 *g*. A 1-ml volume of a solution of iso-VMA (20 μM) dissolved in 0.01 M hydrochloric acid was added as internal standard to 1 ml of a urine sample or a standard solution of VMA (10 μM). After mixing 5–20 μl were injected into the liquid chromatograph.

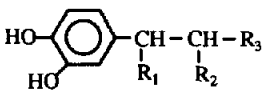
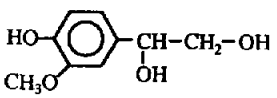
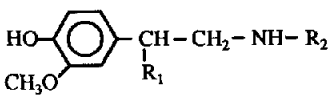
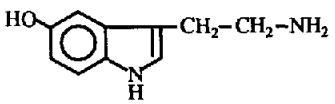
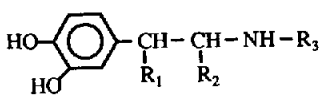
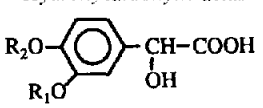
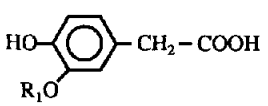
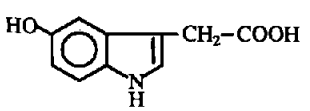
RESULTS AND DISCUSSION

Influence of pH on retention

We studied the retention of VMA and related compounds on the boronic acid packing material at pH between 2.1 and 7.7. Above this pH range both the risk of oxidation of the catechol compounds tested and the dissolution of silica would increase. The compounds tested are presented in Table I and the results of some representative compounds and of the α-hydroxycarboxylic acids are shown in Figs. 2–4. The retention of the substances containing a catechol or an amine function increased with increasing pH, and the catecholamines were strongly

TABLE I

STRUCTURES AND ABBREVIATIONS OF THE COMPOUNDS STUDIED

Structure	Compound	R_1	R_2	R_3
Neutral catechol compounds				
	DOPEG	OH	H	OH
	DOPET	H	H	OH
	DOPA	H	COOH	NH_2
(methylated)				
	MOPEG			
Amines (no catechol)				
	MN	OH	CH_3	
	NMN	OH	H	
	3-MT	H	H	
	5-HT			
Catecholamines				
	E	OH	H	CH_3
	NE	OH	H	H
	DA	H	H	H
	α-MDA	H	CH_3	H
α-Hydroxycarboxylic acids				
	VMA	CH_3	H	
	iso-VMA	H	CH_3	
	DOMA	H	H	
Acids (no α-hydroxy group)				
	DOPAC	H		
	HVA	CH_3		
	5-HIAA			

retained at neutral pH. At pH 3 a difference in retention was seen between the phosphate and citrate buffer. The methylated compound MOPEG, lacking both an amine and a catechol moiety, was practically unretained.

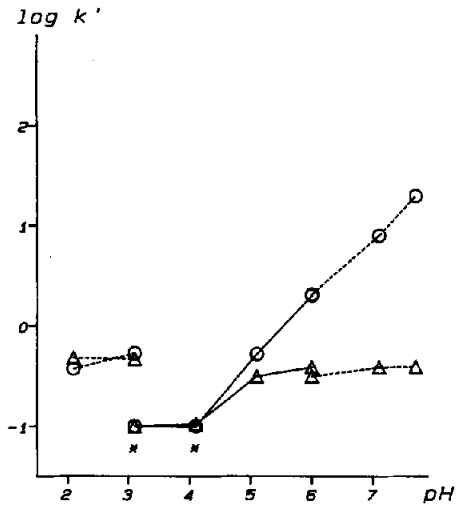


Fig. 2. Influence of the pH of the mobile phase on retention of neutral catechol compounds. Stationary phase: SelectiSpher-10 Boronate. Mobile phase: (---) phosphate buffer (I = 0.01), pH 2.1, 3.1, 6.0, 7.1, 7.7 and (—) citrate buffer (I = 0.01), pH 3.1, 4.1, 5.1, 6.0. * = log k' values lower than −1. Key: ○ = DOPEG; △ = MOPEG.

Contrary to the catecholamines the α-hydroxycarboxylic acids VMA, iso-VMA and DOMA showed high capability to form complexes with boronic acid at low pH using phosphate buffer. However, this was not true for citrate buffer (Fig. 4) and it seems as if citric acid competes with the carboxylic acids for the same binding sites on the stationary phase. The retention of other carboxylic

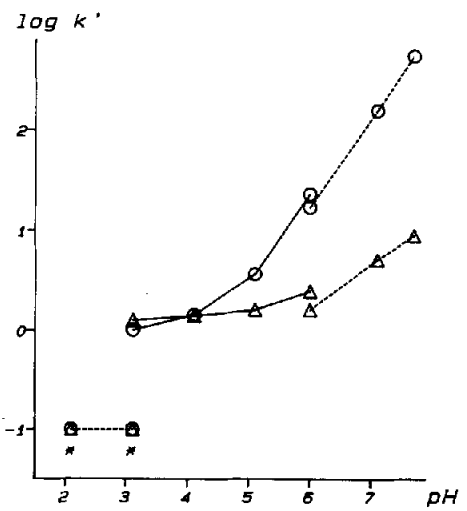


Fig. 3. Influence of the pH of the mobile phase on retention of amines. Conditions as in Fig. 2. Key: ○ = E; △ = MN.

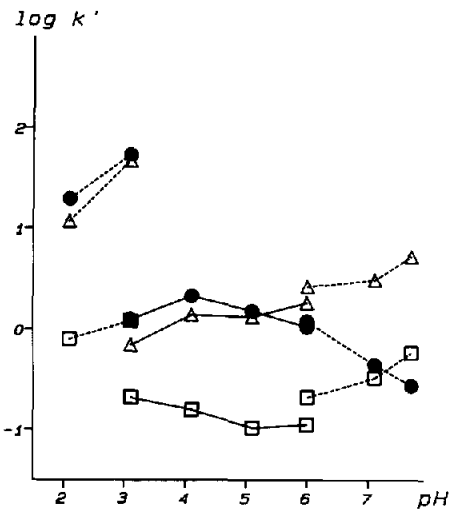


Fig. 4. Influence of the pH of the mobile phase on retention of acids. Conditions as in Fig. 2. Key: ● = VMA and iso-VMA; △ = DOMA; □ = DOPAC.

acids, lacking the α-hydroxy group, was low throughout the pH interval studied, which is a prerequisite for successful clean-up of crude urine samples.

Column switching

The affinity of VMA and iso-VMA to the boronate matrix, using phosphate buffer at pH 3, made it possible to retain them on the pre-column. The time schedule for switching of the valves is reported in Table II. Half a minute after injection valve V2 was switched and the mobile phase, used for the subsequent analytical separation on the anion exchanger, was backflushed through the pre-

TABLE II

SCHEME OF COLUMN-SWITCHING EVENTS

Time after injection (min)	Switch valve No.	Event
0.0		The sample is injected onto column 1.
0.5	V2	Column 1 and 2 are connected in series. Column 1 is backflushed with mobile phase 2 and VMA and iso-VMA are transferred to column 2.
1.5	V2 reset	The columns are disconnected from each other and column 1 is reconditioned with mobile phase 1.
17	V3	After the chromatogram is completed, column 2 is backflushed with mobile phase 2.
35	V3 reset	The flow, through column 2, is restored and the next sample is injected.

column. The sample components were rapidly desorbed and transferred to the anion exchanger where separation was performed. After another minute the valve was switched back in order to recondition the pre-column before injection of the next sample. When the chromatogram was completed, valve V3 was activated to reverse the flow direction of the mobile phase through the separation column. Strongly retarded compounds were forced backwards to waste to avoid disturbances in the following chromatograms. After 35 min the flow was restored and the next sample was injected onto the pre-column. For recovery studies, direct injection onto the separation column was made after switching of valve V1.

Chromatography and detection

An anion exchanger, Nucleosil 5SB, was used for the analytical separation, giving a much better selectivity than a reversed-phase column, Nucleosil 5 C_{18}. The anion-exchange materials, Spherisorb SAX from Phase Sep and Bakerbond from Baker, were also tested but gave too low retention.

A phosphate buffer of pH 6.0 could be used both to desorb VMA from the pre-column and as mobile phase for the separation. The cell response decreased with increasing pH and at pH 7 a 40% lower response was obtained. To get suitable retention times and good resolution between VMA and a compound eluting just ahead of the VMA peak 5% methanol was added to the mobile phase. Chromatograms of a human urine sample recorded after column switching and after direct injection onto the anion exchanger are shown in Figs. 5 and 6. Increased plate heights of 10 to 15% were seen when chromatograms of a standard solution injected into the coupled-column system were compared with those recorded after injection of 5 μl directly onto the anion-exchange column.

Interference by DOMA, which also showed affinity to the boronate matrix, was tested and found negligible. DOMA eluted close to the internal standard but showed a bad chromatographic performance, resulting in a low wide peak. Furthermore, compared with the internal standard, the concentration of DOMA was much lower.

Detection was performed with an ESA electrochemical detector at +0.50 V. After a couple of months the cell response had declined, but could be restored by washing with 6 *M* nitric acid.

Quantification and recovery

The ratios of the peak height of VMA to that of the internal standard, iso-VMA, in the reference samples were measured and the median value was used for calculation of the urine concentrations.

The within-day variability was determined by performing replicate analysis ($n = 10$) of an aqueous reference sample containing 12.2 μM VMA and a urine sample containing 6.6 μM VMA: the relative standard deviations were 1.0 and 2.8%, respectively.

The recovery of the column-switching event was 92.5 ± 3.4% for VMA and

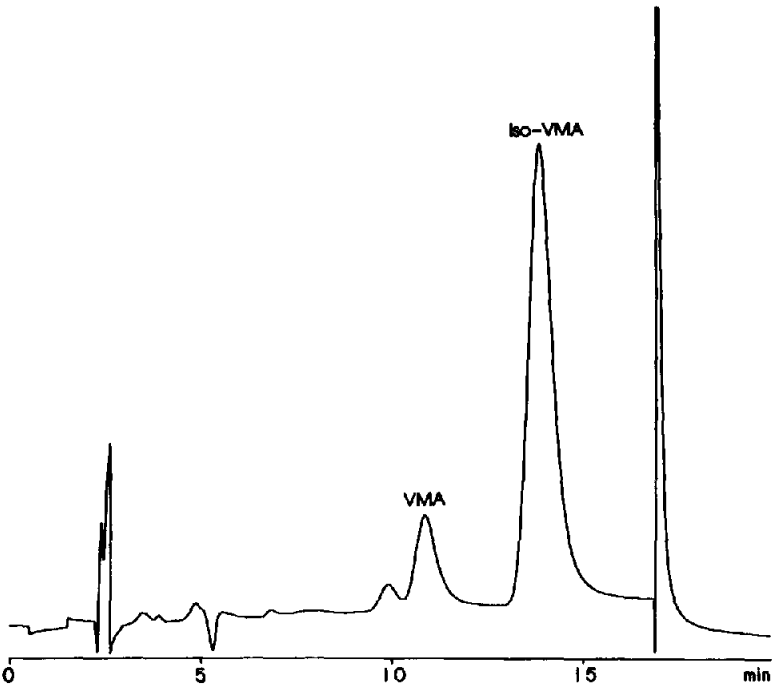


Fig. 5. Chromatogram (after column switching) of a human urine sample containing 6.6 μM VMA. The internal standard solution added contained 49.1 μM iso-VMA. Injected sample volume, 5 μl; potential, +0.50 V; sensitivity, 256 nA full scale. Chromatographic conditions as in Fig. 1.

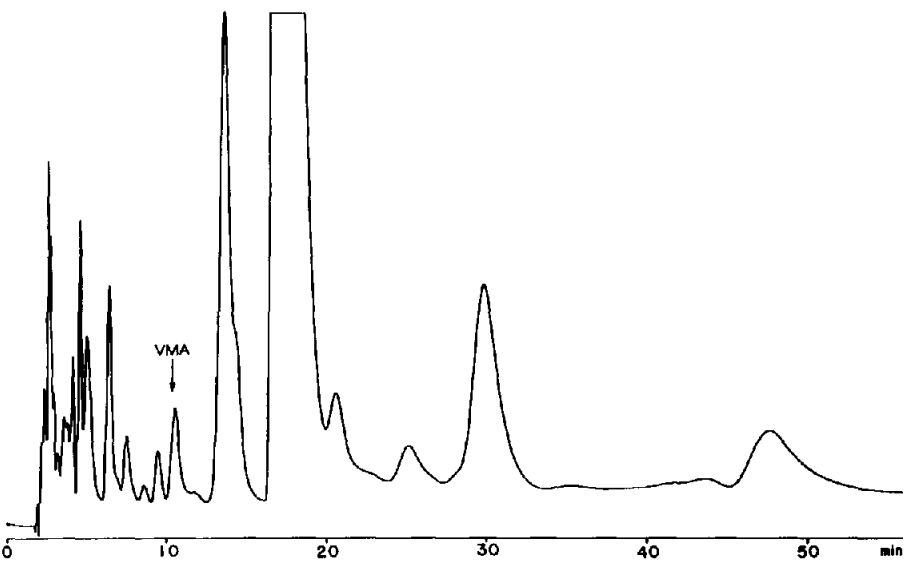


Fig. 6. Chromatogram of the same urine sample as in Fig. 5 after direct injection onto column 2. Other conditions as in Fig. 5.

94.9 ± 2.1% for iso-VMA (n = 10) and was calculated by comparing the peak area of a standard solution after injection into the coupled-column system with the same amount injected directly onto the anion exchanger. The recovery from urine was 102.5 ± 0.6% (n = 4) and was determined by standard addition.

The limit of determination for 5-μl injection was 0.15 μM (signal-to-noise ratio = 10), but could be lowered by larger injection volumes.

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