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Note

Simple high-performance liquid chromatographic method for the simultaneous measurement of vanillylmandelic acid and homovanillic acid

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Concentrations of catecholamines and related metabolites in urine can be determined by several techniques, including classical spectrophotometric methods [1,2], gas chromatography with electron-capture detection (GC–ECD) [3] and, more recently, high-performance liquid chromatography (HPLC) with UV [4] or electrochemical detection [5]. However, some of these techniques require extensive treatment of the urine and others are too complicated for routine analyses.

Catecholamine excretion during stress is a subject of increasing interest for physiologists [2,6]. The increased excretion of catecholamines and related metabolites due to toxic chemical exposure is also of great concern among toxicologists [7,8]. Unlike clinical investigations, the changes in monoamines in urine in physiological and toxicological studies are usually very small; therefore, a sensitive and reliable method of analysis is desirable.

We report here an accurate and rapid procedure for the simultaneous determination of urinary 4-hydroxy-3-methoxymandelic acid (VMA) and 4-hydroxy-3-methoxyphenylacetic acid (HVA). The simplicity of this procedure favours multiple sample analyses.

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EXPERIMENTAL

Reagents

Methanol, glacial acetic acid, orthophosphoric acid, sodium chloride, sodium hydroxide and sodium acetate (anhydrous) were purchased from E. Merck (Darmstadt, F.R.G.). Dowex-1 (chloride form, 100–200 mesh), 4 hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylacetic acid and 3-hydroxy-4-methoxybenzoic acid (isovanillic acid) were purchased from Sigma (St. Louis, MO, U.S.A.).

Chromatographic conditions

The HPLC mobile phase was 1 mol/l acetic acid reagent prepared by mixing 57 ml of glacial acetic acid and 50 ml of methanol and diluting to 1 l with distilled water. The pH was adjusted to 3 with 10 *M* sodium hydroxide solution.

An internal stock standard solution was prepared by dissolving 5 mg of isovanillic acid in 1 ml of methanol plus 9 ml of deionized water. A working standard solution of 20 μg/ml was prepared daily by diluting the stock solution 25-fold with sodium acetate buffer (100 mmol/l, pH 6.1).

A stock solution of VMA and HVA was prepared by dissolving 5 mg of each in 5 ml of 0.1 *M* hydrochloric acid. This stock solution is stable for several months at 4°C. Working standard solutions were prepared daily by diluting the stock solution 500-, 200-, 100-, 50- and 25-fold with a 1.5 *M* solution of sodium chloride in 50% methanol to obtain concentrations of 1, 2, 5, 10 and 20 μg/ml, respectively.

The anion-exchange column was prepared by using approximately 500 mg of Dowex-1 in a 20 mm × 4 mm I.D. column. Preconditioning was achieved by rinsing twice with 2 ml of distilled water.

Apparatus

A Gilson Model 302 liquid chromatograph with a 5SC pump head (Gilson, Villiers-le-Bel, France) and a Rheodyne Model 7125 sampling valve (Rheodyne, Cotati, CA, U.S.A.) was used. The instrument was operated isocratically and a Model LC-4B electrochemical detector with a glassy carbon electrode and an Ag/AgCl reference electrode was used (Bioanalytical, West Lafayette, IN, U.S.A.). A CR3-A integrator (Shimadzu, Kyoto, Japan) was used for peak-area integration. The chromatographic conditions are given in the figure legends.

Extraction procedure

Specimens of 24-h urine were collected and preserved with 20 ml of 6 *M* hydrochloric acid (pH < 2) and stored at −4°C.

A 2-ml urine sample was adjusted to pH 6 with 1 *M* sodium hydroxide solution and 2 ml of sodium acetate buffer containing 20 μg/ml of internal standard were added. The sample was mixed thoroughly and allowed to percolate through the ion-exchange column and the effluent was discarded. The column was washed with 2 ml of phosphoric acid (10 ml/l) followed by 2 ml of deionized water, and both washings were discarded. This process reduced the front peak interferences. The VMA and HVA analytes were eluted twice with 2 ml of 1.5 *M* sodium chloride

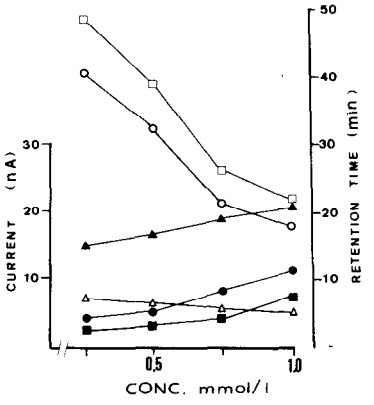

Fig. 1. Effect of acetic acid concentration on retention (open symbols) and detection (current, closed symbols) of VMA (△), HVA (○) and internal standard (□).

solution containing 50% methanol. The addition of methanol to the eluent improved the recovery of HVA. The collected eluents were pooled, and a 5-μl aliquot was injected into the liquid chromatograph.

Clinical studies

Healthy subjects. The reference population consisted of volunteer teaching and laboratory staff of the National University of Singapore, who were deemed healthy on routine physical examination. Exclusion criteria included evidence of pulmonary disease, cardiac disease, neurological disease and hypertension (defined by a systolic blood pressure over 90 mmHg) and those who were under medication.

Hypertensive patients. The population defined as hypertensive consisted of patients undergoing physical examination with a systolic blood pressure greater than 90 mmHg and suspected of having neuroblastoma or pheochromocytoma. The 24-h urine specimens from these patients were submitted to the Clinical Biochemistry Laboratory Department of Pathology, Singapore General Hospital, for analysis. For the present study, specimens were randomly selected from this group of patients.

RESULTS AND DISCUSSION

Analytical variables

Composition of mobile phase. The aqueous mobile phase contained only acetic acid and methanol (95:5, v/v). Higher concentrations of methanol resulted in shorter retention times for HVA and poor resolution. The use of this mixture is an attractive alternative to recently reported methods using phosphate and alkylsulphonate, which cause column instability [9]. The effects of acetate concentration on retention time and detector response were also investigated. In contrast to the increase in detector response, the retention time decreased with increasing acid concentration (Fig. 1). These findings were used to select the optimal concentration of acetic acid for the separation of a mixture of VMA and HVA. No ion-pair reagents are needed in the present method. Complex mobile

TABLE I

RESULTS OF RECOVERY STUDIES

Original levels: VMA, 2.5 μg/ml; all samples were processed as described under Experimental.

Compound	Added (μg/ml)	*n*	Mean recovery (%)
VMA	2	5	94
	5	6	86
	10	9	91
	20	8	97
Mean			92
HVA	2	5	97
	5	9	96
	10	9	97
	20	3	88
Mean			95

phases increase the complexity of HPLC procedures [10]. An additional advantage of using acetic acid is that it does not react with the silica stationary phase. When phosphate buffer was used, the life of the column was reduced because the solubility of silica increases with increasing pH [11,12].

Column temperature. The optimal column temperature was found to be 35°C; at this temperature the band spreading was minimal. Higher temperatures resulted in baseline noise.

Sensitivity and analytical recovery. The detection limit was expressed as peak height relative to the baseline noise level when urine samples were analysed. Using the above conditions, VMA and HVA at concentrations of 0.08 and 0.1 mg/l, respectively, could be detected.

The accuracy of the assay method was assessed by spiking urine samples and also by the internal standard method. Isovanillic acid was used as an internal standard to reduce the variability between ion-exchange columns and to improve the reproducibility. The calibration graphs were linear for absolute amounts of VMA and HVA in the 2 range 0–100 ng.

The results in Table I show the recoveries of VMA and HVA with respect to the internal standard, which were ca. 93% for VMA and 95% for HVA. These values are higher than the 72% and 60% reported recently for VMA and HVA, respectively [13].

The coefficients of variation (C.V.) for within-day and between-day analyses are given in Table II. The data indicate that the precision of the method is good. The precision is better than that for other reported HPLC studies (C.V. 7.8–10.9% at 10–86 μmol/l) [12,14].

Comparison with other methods

The present procedure for the determination of VMA was compared with that of Pisano et al. [1] and Minami and Mori's methods [2]. Close agreement (cor-

TABLE II

PRECISION OF VMA AND HVA MEASUREMENTS

Compound	Concentration (μg/ml)	Coefficient of variation (%)	
		Within-day ($n=5$)	Between-day ($n=5$)
VMA	2	1.3	4.5
	10	3.4	4.7
HVA	2	3.6	6.7
	10	3.7	4.9
IVA*	20	2.4	5.4

*Isovanillic acid.

relation coefficient, $r=0.87$) was observed between the HPLC method and Pisano et al.'s spectrophotometric method (Fig. 2). However, the VMA levels measured by the spectrophotometric method are higher than those measured by HPLC, because spectrophotometric assays are less specific as other closely related compounds such as vanillin and 5-hydroxyindoleacetic acid interfere with the measurements [15]. The C.V. values for Pisano et al.'s method varied between 16 and 18% for VMA for concentrations in the range 2–20 μg/ml. Gross contamination is a common phenomenon when spectrophotometric methods are used for the determination of VMA [12]. A poorer correlation was found between the HPLC method and the spectrophotometric procedure used by Minami and Mori [2]. The correlation coefficient is 0.69 ($y=0.63x+1.43$). We also noted abnormally high absorbances for blank and test solutions in some analyses. Results analysed by spectrophotometric methods could therefore be very misleading [16]. When VMA was assayed by the two different spectrophotometric procedures the correlation was poor ($r=0.58$, $y=0.756x+3.14$).

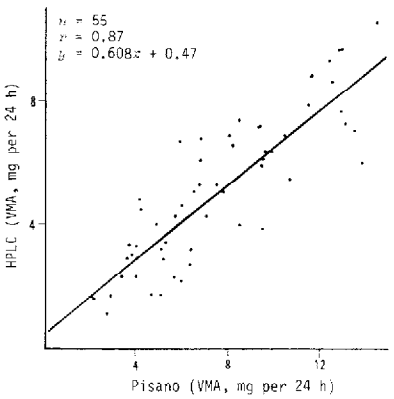

Fig. 2. Comparison of the HPLC method and the spectrophotometric method of Pisano et al. [1]. Regression analysis gave a correlation coefficient (r) of 0.87.

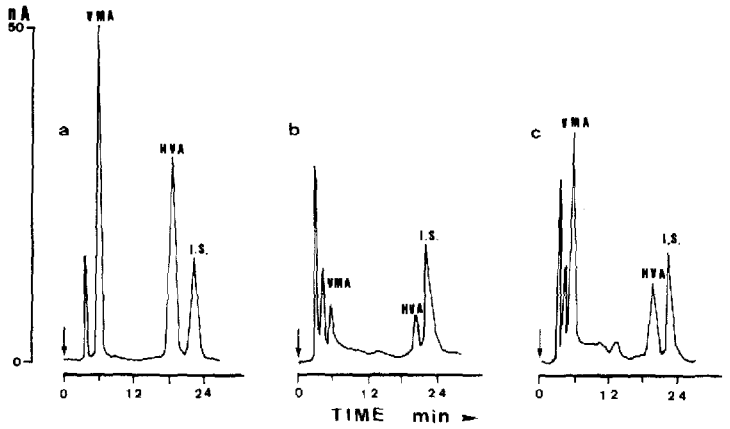

Fig. 3. (a) Chromatogram of a standard mixture containing 20 μg/ml each of VMA, HVA and internal standard (I.S.). (b) Chromatogram of a urine extract from a healthy subject. (c) Chromatogram of a urine extract from a hypertensive patient. Column, Whatman Partisil 5 ODS-3 RAC (10 cm×9.4 mm I.D.) with a Chrompack guard column; mobile phase, 1 *M* acetic acid (pH 3)–methanol (95:5); flow-rate, 1.5 ml/min; temperature, 35 °C; detector potential, 0.8 V vs. Ag/AgCl; sensitivity, full scale 50 nA; attenuation, 10 mV; chart speed, 10 cm/h.

Interference studies

Ion exchange has been shown to be more selective than and superior to solvent extraction (e.g., with ethyl acetate) for the determination of organic acids [17]. Anion-exchange chromatography as used in this study removes most of the interfering substances and fewer disturbances were observed in subsequent analyses.

No drugs were used directly for testing interferences. However, many of the urine samples analysed were collected from patients under medication and the results obtained did not show interferences. Norepinephrine, epinephrine or dopamine did not interfere.

Analysis time and column maintenance

The average sample preparation time was about 60 min per fifteen samples. The analysis time was 20 min (with an internal standard). The high recoveries and precision of the procedure suggest that the assay can be performed without an internal standard. Efficient separation of VMA and HVA requires less than 20 min (Fig. 3). Given the relatively short analysis time, about thirty samples could be analysed per day when an automatic sample injector is used.

At the end of each working day, the column was washed with distilled water for about 15 min. This procedure considerably prolonged the life of the column. When phosphate buffer was used for VMA and HVA analysis, the maximal column life was about 100 analyses [12]. The retention time and batch variations also increased when phosphate buffer and ion-pair reagents were used [18]. Using the HPLC procedure we analysed over 450 urine specimens without significant changes on the chromatograms.

Clinical screening

More than 200 samples were analysed by the HPLC method. The average urinary VMA for healthy subjects was 2.4 ± 0.9 mg/day (range 0.4–4.1 mg per day). This value agrees well with published values of 0.4–5.5 mg/l using another HPLC method [9] and 2.9 mg per day using GC–ECD [3]. There is also general agreement for the HVA value obtained by the present HPLC method (3.3 ± 0.8 mg per day) and those determined by another HPLC method (0.7–6.9 mg per day) [9] or GC–ECD (2.0–5.6 mg per day) [19]. The VMA concentration in specimens collected from a hypertensive patient was 5.16 ± 2.38 mg per day and the HVA concentration was 4.83 ± 2.35 mg per day.

CONCLUSION

This investigation has shown that the combination of ion-exchange chromatography with HPLC using an electrochemical detector provides a rapid and reliable purification and assay procedure. This method is accurate, with a constant precision in the range from low to above-normal concentrations. Sample preparation is minimal and the catecholamine metabolites VMA and HVA are separated in less than 20 min. The method is selective and can be used for routine screening for pheochromocytoma and neuroblastoma and also for neurophysiological and toxicological studies.

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