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AUTOMATED ANALYSIS OF URINARY 3-METHOXY-4-HYDROXYMANDELIC ACID USING ION-PAIR CHROMATOGRAPHY AND FLUORIMETRIC DETECTION

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SUMMARY

A new rapid high-performance liquid chromatographic method, without urine sample pre-treatment and based on isocratic ion-pair elution, fluorimetric detection and column-switching, has been developed for the determination of vanillylmandelic acid. The sensitivity was 0.1 mg/l, and the linearity was excellent in the concentration range tested. For all endogenous substances as well as for all the drugs tested no interferences were observed. Typical concentrations were in the range 0.3–5.5 mg of vanillylmandelic acid per day, depending on the age of subject under investigation.

INTRODUCTION

Vanillylmandelic acid (VMA, 3-methoxy-4-hydroxymandelic acid) is one of the major end-products of catecholamine metabolism. Determination of VMA in urine is of importance for the diagnosis and during the treatment of phaeochromocytoma, neuroblastoma and Parkinson's disease [1,2].

Although a variety of techniques has been applied to the assay of VMA, such as thin-layer chromatography [3], electrophoresis [4], spectrophotometry [5], gas chromatography [6] and gas chromatography–mass spectrometry [7], high-performance liquid chromatography (HPLC) seems to be the method of choice. Various pre-treatment steps, such as anion-exchange, or solid–solvent extraction steps in conjunction with isocratic or gradient elution with or without ion-pair reagents combined with UV, fluorimetric or electrochemical detection, have been described [8–14]. These methods are laborious because the extensive urine sample clean-up required often results in low recoveries and precision. Recently, several HPLC methods using anion-exchange or gradient elution as well as column-switching have been reported for the direct determination of VMA in urine with-

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out sample clean-up procedures [15–17]. Although these methods have simplified the pre-treatment of urine they are either complicated in methodology or not free from interferences.

This paper presents a simple and rapid automatic method for VMA determination in urine based on a simple column-switching technique.

EXPERIMENTAL

Materials

Ethylenediaminetetraacetic acid sodium salt (EDTA) and sodium dihydrogenphosphate were obtained from Merck (Darmstadt, F.R.G.) and tributylammonium hydrogensulphate, VMA, 3-hydroxy-4-methoxymandelic acid (iso-VMA) and hydroxy-3-methoxyphenylethylene glycol (MOPEG) were from Fluka (Buchs, Switzerland). Dopamine, norepinephrine, tyrosine, 5-hydroxytryptophan, DL-3,4-dihydroxyphenylethylene glycol, DL-3,4-dihydroxymandelic acid, DL-normetanephrine, L-β-dihydroxyphenylalanine (L-DOPA), epinephrine, deoxyepinephrine (N-methyldopa), DL-metanephrine and serotonin were purchased from Sigma (Deisenhofen, F.R.G.). Lyphocheck control urines were obtained from Bio-Rad Labs. (Munich, F.R.G.). Metoprolol and 4-hydroxymetoprolol were a generous gift from Astra Chemicals (Wedel/Holstein, F.R.G.). All other drugs used were generous gifts from different pharmaceutical firms.

Instrumental and chromatographic conditions

Two 655A-12 liquid chromatograph pumps, an L5000 LC controller, a 655A-40 autosampler, a D2000 integrator (Merck) and an EL7000 electromagnetic valve (Krannich, Göttingen, F.R.G.) were employed. For urine purification a 25 mm × 4 mm I.D. LiChrospher RP18e pre-column (end-capped, 5 μm) was used,

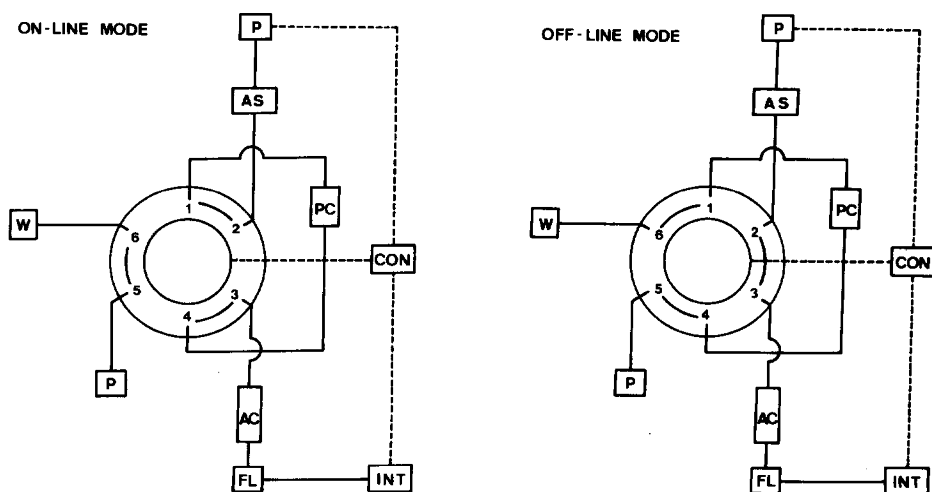

Fig. 1. Diagram of the HPLC column-switching system in on-line and off-line modes for urinary VMA determination. AC = analytical column; AS = autosampler; CON = controller; FL = fluorescence detector; INT = integrator; P = pump; PC = pre-column; W = waste.

and for VMA separation a 125 mm × 4 mm I.D. LiChrospher RP18e analytical column (end-capped, 5 μm, Merck) was used. The effluent was monitored by an F1000 fluorimetric detector (Merck) with an excitation wavelength of 275 nm and an emission wavelength of 330 nm. The flow diagram of the column-switching system in on-line and off-line modes is given in Fig. 1. The aqueous part of the mobile phase was prepared by adding 0.2 g of EDTA, 13.8 g of sodium dihydrogenphosphate and 0.7 g of tributylammonium hydrogensulphate to deionized water, adjusted to pH 5.4 with 13 *M* ammonia, to a final volume of 1 l. The eluent consisted of 96% buffer (v/v) and 4% methanol (v/v). The flow-rate was 1.0 ml/min. Analysis of VMA was performed at room temperature, and diluted samples were kept at 4°C in the autosampler.

Sample preparation

Urine (24 h) samples were collected in plastic bottles containing 10 ml of 8 *M* hydrochloric acid. Samples were controlled for pH and adjusted to pH 3.0–3.5 if necessary, and were frozen at −20°C until analysis. Before analysis the thawed samples were vortex-mixed and centrifuged at 10 000 *g* for 10 min at 8°C. A 50-μl sample of urine supernatant was diluted with 1.5 ml of analytical HPLC buffer, and 30-μl aliquots were injected via the autosampler. Calibration was performed with diluted stock solutions and was checked periodically with Lyphocheck control urines. The VMA stock solution, which was stable for one month at 4°C, was prepared by dissolving 1 mg/ml VMA in 0.01 *M* EDTA pH 3.0. The external standard solutions were always freshly prepared and diluted with the HPLC mobile phase. These solutions were stable for at least 6 h at 4°C.

RESULTS AND DISCUSSION

In the past, VMA analysis required an excessive clean-up procedure with subsequent HPLC elution, or complicated column-switching techniques were necessary to obtain reliable results [16,17]. The aim of this work was to develop a simple, selective and sensitive HPLC system for an automated VMA determination in urine with the following advantages: no sample pre-treatment, isocratic elution profile, high precision, reproducibility and recovery, reduced analysis times and small urine aliquots to avoid impurity problems.

To overcome urine clean-up procedures for VMA analysis, an automated HPLC system with column switching and fluorimetric detection was conceived. Urine samples were pre-separated from lipophilic impurities on a pre-column. Fig. 1 shows both positions of valve switching, in on-line and off-line mode. The samples were injected in on-line mode, chromatographed on the pre-column for a certain period of time, and then VMA was separated on the analytical column. The pre-column was back-flushed in off-line mode ready for the next sample injection.

The first experiments were used to determine the optimal valve-switching times for VMA separation for the best reproducibility and recovery. Therefore VMA standards and an aliquot of diluted urine sample with added VMA were injected via the autosampler. All hydrophilic urine constituents, including VMA, were

eluted from the pre-column for subsequent separation on an analytical column in on-line mode. The highest recoveries of injected VMA were obtained in 1.5 min as the optimal valve-switching time. Several experiments demonstrated that shorter valve-switching times (less than 1.3 min) resulted in significantly lower recovery of VMA. Longer valve-switching times (more than 1.8 min) caused great problems with impurities originating from the complex urine matrix, which interfered with the VMA analysis and resulted in prolonged analysis times.

After valve switching in the off-line mode, lipophilic urine constituents retarded on the pre-column were eluted in the back-flush mode for 8.5 min. This back-flush mode, which was performed with the same eluent, allowed simple and easy removal from strongly retained substances from the pre-column without the need for gradient elution. The retention time of VMA was sufficiently low, at 8.21 min (see Fig. 2), and a baseline–baseline separation of VMA from other co-eluting urine substances was achieved without any clean-up of urine samples. In comparison with the other published VMA determination methods [9, 18, 20] fewer problems with impurities were observed.

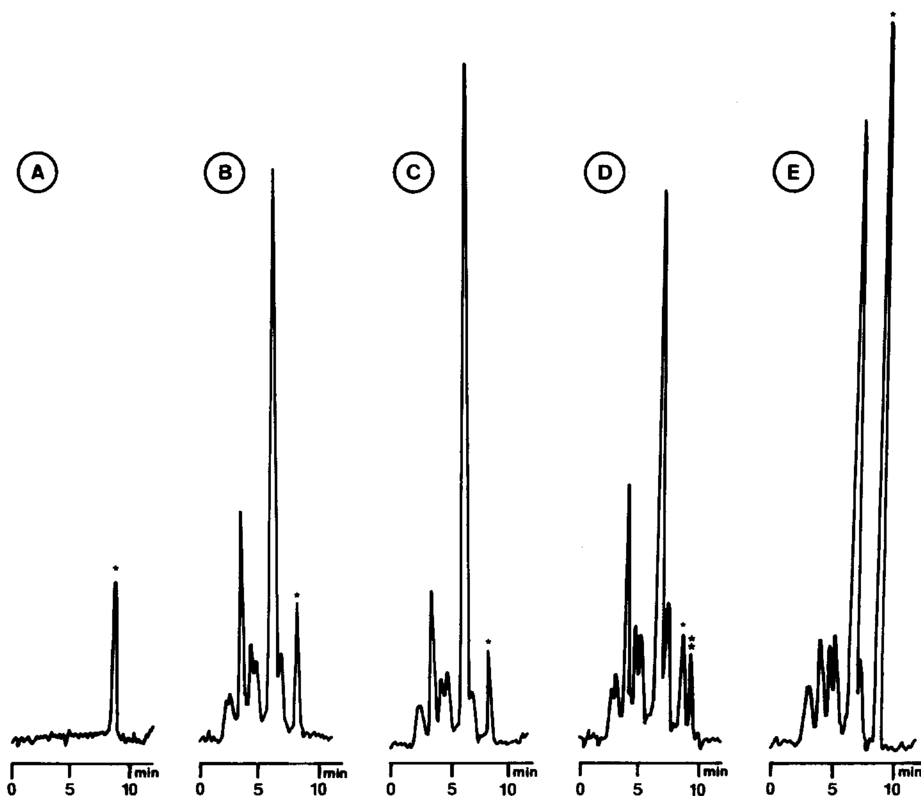

Fig. 2. Chromatograms of different diluted and pretreated VMA samples. (A) External standard sample (4.0 ng/ml VMA); (B) lyphocheck control urine sample (3.6 ng/ml VMA); (C) normal patient urine sample (2.5 ng/ml VMA); (D) urine of a patient treated with metoprolol (2.6 ng/ml VMA); (E) abnormal urine sample from a patient with phaeochromocytoma (20 ng/ml VMA). Peaks: ⋆ = VMA; ⋆⋆ = 4-hydroxymetoprolol.

The cycle time for VMA determination was 11 min, which allowed rapid VMA quantification for diagnostic purposes. Calibration curves for VMA determination were established by the external standard method. Control urines and aqueous VMA solutions in the range 0.5–20.0 mg/l were used. VMA was found to be highly unstable in water, so VMA standards were prepared in a stabilizing buffer that contained EDTA. These diluted VMA standard solutions were stable for more than 6 h at 4 °C. The stability was increased to more than 8 h at 4 °C when VMA was added to blank urine, which indicated that VMA was stabilized by components of the urine. From these results it was concluded that commercially available urine samples were advantageous for VMA control calibration. The calibration curve was linear between 1.0 and 20.0 mg/l VMA. In comparison with literature data [7,10], the detection limit of VMA could be increased to 0.1 ng/ml when 50 μl of urine were used. The sensitivity of VMA determination could be increased further by a lower dilution factor or larger injection volumes.

Reproducibility data for this VMA determination method are given in Table I. The coefficients of variation (C.V.) were in the range 0.7–2.8%. Recovery studies were performed by using different control urines and urines spiked with known amounts of VMA. Recoveries were always more than 95% (Table II). No matrix influence was observed.

Identical precision data were obtained when the internal standard method was

TABLE I

PRECISION AND STABILITY DATA FOR VMA DETERMINATION IN URINE

Sample	VMA concentration (ng/ml)	Coefficient of variation (%)			
		Concentration		Retention time	
		Series ($n=8$)	Day-to-day ($n=10$)	Series ($n=8$)	Day-to-day ($n=10$)
Standard	4.0	2.0	2.8	0.7	1.4
Control urine	3.6	1.4	2.6	0.8	1.5
Patient urine	2.5	1.5	2.7	0.8	1.5

TABLE II

ANALYTICAL RECOVERY DATA FOR VMA IN URINE

Sample	Concentration (mg/l)	n	Mean recovery (%)	Coefficient of variation (%)
Standard	4.0	20	95.5	3.4
Control urine	3.6	10	100.1	2.0
Patient urine	3.0	10	98.8	2.3
Patient urine with added 4.0 mg/l VMA	7.0	10	98.7	2.4

used. Iso-VMA was found to be a suitable internal standard (retention time of 12.32 min), but the analysis time increased (15 min) and interference problems were caused by the longer valve-switching times (2.5 min) that were necessary to elute the internal standard.

Different endogenous substances (Table III) were tested for possible interference. For all the substances tested no interference was observed. Furthermore, none was observed, when drug substances, such as barbiturates, benzodiazepines or antiepileptic, antidepressive, antiarrhythmic drugs or substances of the phenothiazine and β-blocker groups, were tested. An exception was the main metabolite of metoprolol, 4-hydroxymetoprolol. However, this substance could clearly be separated from the VMA peak by optimal choice of eluent and column material (see Table III and Fig. 2D).

TABLE III

RETENTION DATA FOR POSSIBLE ENDOGENOUS INTERFERENCES

	Retention time (min)	Relative retention time
4-Hydroxy-3-methoxymandelic acid (VMA)	8.21	1.00
Norepinephrine	1.48	0.18
Epinephrine	1.48	0.18
3,4-Dihydroxynorephedrine	1.56	0.19
Normetanephrine	1.81	0.22
Metanephrine	1.89	0.23
Deoxyepinephrine (N-methyldopa)	1.97	0.24
Dopamine	2.38	0.29
β-Dihydroxyphenylalanine (L-DOPA)	2.96	0.36
Tyrosine	2.96	0.36
Serotonin	3.37	0.41
3,4-Dihydroxyphenylethylene glycol	4.02	0.49
3,4-Dihydroxymandelic acid	5.25	0.64
5-Hydroxytryptophan	6.24	0.76
4-Hydroxy-3-methoxyphenylethylene glycol (MOPEG)	6.32	0.77
4-Hydroxymetoprolol	8.70	1.06
3-Hydroxy-4-methoxymandelic acid (iso-VMA)	12.32	1.50

TABLE IV

MEAN VMA URINE VALUES OF DIFFERENT AGE GROUPS

Age group	n	VMA concentration (mg/day)	
		Group mean value	Range
Adults (> 15 years)	40	3.8	2.7–5.5
School children (6–15 years)	22	2.4	1.7–3.3
Infants (1–5 years)	12	1.4	0.9–1.9
Babies (0–1 year)	7	0.6	0.3–1.0

This HPLC method allows the determination of VMA without the interruption of medical therapy, and continuous therapy control is possible. In comparison with the other recently published methods, based on sample clean-up procedures such as extraction with ethyl acetate or diethyl ether [12, 18–20], column [9–11, 22] or ion-exchange extractions [21–23], this new method opens up the possibility of direct and automated injection of urine samples. Some methods have already been described that take advantage of direct and automated injection systems [16,17], but two eluents were required for the analysis. In addition, electrochemical detection is in general more difficult in operation, especially in combination with automated systems. This is evident from the reproducibility data, which were less meaningful than those obtained with fluorimetric detection systems.

In general, most of the measured normal VMA samples were in the range 0.3–5.5 mg VMA per day, depending on the age of the patients (Table IV). In the case of urine samples of patients with phaeochromocytoma, values of 20–60 mg VMA per day were found.

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