GAS CHROMATOGRAPHY OF URINARY VANILMANDELIC ACID IN PHEOCHROMOCYTOMA

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SUMMARY

The properties and suitability of six different volatile derivatives of 3-methoxy-4-hydroxymandelic acid (VMA) were established on a non-polar and three polar columns, and the optimum conditions for determination of VMA by gas chromatography were established for a patient with pheochromocytoma. The method was then applied to eight other patients with pheochromocytoma. In all patients, VMA was significantly elevated. The advantages and disadvantages of the method are compared briefly with other methods reported in the literature.

INTRODUCTION

Two methods have been described for the gas chromatographic determination of urinary vanilmandelic acid (VMA) in patients with neuroblastoma, a common malignant tumor of childhood[1,2]. These methods should also be applicable to the determination of VMA in pheochromocytoma, a rare, benign, hypertension-producing tumor of adults. However, there is a great difference in the magnitude of excretion of the aromatic acids in the two tumors: in neuroblastoma, VMA may be excreted in amounts up to 1,500 μg/mg creatinine[3]; in contrast VMA is elevated to levels varying from 6–40 μg/mg creatinine (average 18) in 30 cases reported by Gitlow *et al.*[4] compared to a range of 0.8–2.0 (average 1.4) in 18 normal controls. Because a significantly abnormal VMA might be no more than three times the upper limit of normal, it seemed important to establish reliability and specificity of our methods. We have recently had such an opportunity to establish optimum conditions for the determination of urinary VMA by gas chromatography in a patient with pheochromocytoma.

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EXPERIMENTAL PROCEDURES

Gas chromatographic determinations were carried out with a Barber-Colman Model 10 instrument, equipped with a Lovelock argon ionization detection system[5]. Glass U-tubes, 6 feet by 4 mm I.D., were used under conditions described in the tables. The flash-heating zone was maintained at about 50° above column temperature, and the detection cell was maintained at 240°. The detection system was used with a full scale recorded detection of 1×10^{-8} A with an applied voltage of 1,000 V and a 300 Mohm linearizing resistor. Micro-infra-red spectra were performed on NaCl plates with a Beckman IR-5 instrument and a beam condenser (Perkin-Elmer 4×).

RESULTS AND DISCUSSION

Six volatile derivatives of VMA (California Foundation for Biochemical Research, m.p. 128–131°, literature m.p. 129–130° (ref. 6) were considered in the course of this investigation.

I. Methyl 3-methoxy-4-hydroxymandelate is prepared by reaction of VMA with ethereal diazomethane for 15 min at room temperature. This reaction is not optimal for the quantitative determination of VMA because of a small, but variable, yield of methyl 3,4-dimethoxymandelate. This derivative has a retention time nearly identical to methyl hippurate on non-polar columns (SE-30) and an inconveniently long retention time on polar columns (ethylene glycol adipate). Tailing and a relatively low mass response on both polar and non-polar columns indicate significant component loss, probably due to interaction of the hydroxyl group in the 4-position with the active sites in the column support. For these reasons, this derivative has not been considered further.

II. Methyl 3,4-dimethoxymandelate (b.p. 104–105° (756 mm Hg)) is prepared by the reaction of VMA with ethereal diazomethane and methanol (1:1 v/v) overnight at room temperature (Theoretical C 58.5%, H 6.2%; Found C 58.7%, H 6.3%*). The reaction is satisfactory from a quantitative standpoint and has a retention time sufficiently different from methyl hippurate to be usable on both polar and non-polar columns. Comparison of infra-red spectra of this compound with the peak trapped after passing through a non-polar column indicated that no change in the compound had taken place.

This particular reaction has two disadvantages: (1) the lengthy preparation time and (2) the fact that both VMA and 3,4-dihydroxymandelic acid are converted to the same derivative, so that if any of the latter compound is present, the VMA determination is falsely elevated.

III. Methyl α-(3-methoxy-4-acetoxyphenyl)-α′-acetoxyacetate is prepared by reaction of VMA with acetic anhydride (1.0 ml) and trifluoroacetic acid (0.5 ml) at 75° for 30 min followed by evaporation to dryness under a stream of nitrogen and subsequent methylation of the carboxy radical by treatment with ethereal diazomethane for 15 min[2]. This derivative has two advantages over derivative II: (1) it may be prepared in about 1 h, and any 3,4-dihydroxymandelic acid which may be present is converted to a derivative (methyl α-(3,4-diacetoxyphenyl)-α′-acetoxy-

* Performed by Clark Microanalytical Laboratories, Urbana, Ill.

acetate) with a different retention time on both polar and non-polar columns. The reaction is quantitative and the peak is symmetrical with satisfactory mass response. The derivative is a white solid (m.p. 89–92°) and infra-red spectra of this compound and the trapped peak (after passing through a SE-30 column) were identical.

IV. Methyl α-(3,4-dimethoxyphenyl)-α′-acetoxyacetate is produced by the treatment of VMA with diazomethane and methanol overnight followed by treatment with acetic anhydride and trifluoroacetic acid as described above. This derivative suffers from the same disadvantage as derivative II, *i.e.*, the inconveniently long preparation time and the identity of the derivatives, if 3,4-dihydroxymandelic acid is present. Moreover, under these conditions, the reaction did not go to completion with about 10–15% of methyl 3,4-dimethoxymandelate failing to undergo acetylation. For these reasons, this derivative was not considered further.

V. Methyl α-(3-methoxy-4-trimethylsilyloxyphenyl)-α′-trimethylsilyloxyacetate is prepared by the treatment of VMA with ethereal diazomethane for 15 min followed by evaporation to dryness under a stream of nitrogen and treatment with 1.0 ml of tetrahydrofuran (solvent), 1.0 ml of hexamethyldisilazane and 0.5 ml of trimethylchlorosilane (catalyst) overnight at room temperature[7]. Attempts to carry out this reaction rapidly using pyridine as a catalyst[8] were not always reproducible. This reaction produces a derivative with a symmetrical peak and a satisfactory mass response. The derivative is very easily hydrolyzed to the initial compound by traces of water and attempts to dissolve the compound in methanol also resulted in hydrolysis. Moreover, it suffers from the disadvantage of a long preparation time and is unsuitable for quantitative work because of a small, but variable, yield of methyl 3,4-dimethoxymandelate in the first stage of the reaction.

VI. Methyl α-(3,4-dimethoxyphenyl)-α′-trimethylsilyloxyacetate is produced by the reaction of VMA with diazomethane and methanol overnight, evaporation to

TABLE I

RELATIVE RETENTION TIME OF DERIVATIVES OF VANILMANDELIC ACID ON POLAR AND NON-POLAR COLUMNS

	SE-30[1]	*XE-60*[2]	*QF-1*[3]	*EGA*[4]
Methyl hippurate	1.00*	1.00**	1.00***	1.00****
Methyl 3-methoxy-4-hydroxymandelate	1.07	0.95	0.79	1.41
Methyl 3,4-dimethoxymandelate	1.21	0.81	0.80	0.86
Methyl α-(3-methoxy-4-acetoxyphenyl)-α′-acetoxyacetate	2.63	2.15	4.21	1.51
Methyl α-(3,4-dimethoxyphenyl)-α′-acetoxyacetate	1.85	0.99	1.48	0.62
Methyl α-(3-methoxy-4-trimethylsilyloxyphenyl)-α′-trimethylsilyloxyacetate	1.93	0.32	0.58	0.33
Methyl α-(3,4-dimethoxyphenyl)-α′-trimethylsilyloxyacetate	1.55	0.45	0.66	0.23

[1] 5% coated on 100–110 mesh Anakrom AS. Conditions: 185° col. temp., argon inlet pressure 6 psi, outlet pressure atmospheric, flow rate 20 ml/min. * Approximate retention time of methyl hippurate 8 min.

[2] 3% coated on 80–100 mesh GAS-CHROM P, Applied Science Laboratories, Inc. Conditions: 173° col. temp., argon inlet pressure 11 psi, outlet pressure atmospheric, flow rate 32 ml/min. ** Approximate retention time of methyl hippurate 22 min.

[3] 3% coated on 80–100 mesh GAS-CHROM P, Applied Science Laboratories, Inc. Conditions: 170° col. temp., argon inlet pressure 15 psi, outlet pressure atmospheric, flow rate 20 ml/min. *** Approximate retention time of methyl hippurate 10 min.

[4] 8% ethylene glycol adipate coated on 100–110 mesh Anakrom AS. Conditions: 195° col. temp., argon inlet pressure 20 psi, outlet pressure atmospheric, flow rate 70 ml/min. **** Retention time of methyl hippurate 35 min.

dryness under a stream of nitrogen and trimethylsilylation as described above under reaction (V). The reaction is quantitative and the peak produced is symmetrical with a satisfactory mass response. It is similar to derivative V in ease of hydrolysis and injection must be carried out in tetrahydrofuran. This derivative is unsuitable for routine use because of the long preparation time and because the method of preparation cannot distinguish between VMA and 3,4-dihydroxymandelic acid. The relative retention times of these derivatives were established for one non-polar and three polar columns (Table I).

A 24-h urine specimen was obtained from a 28-year old hypertensive female

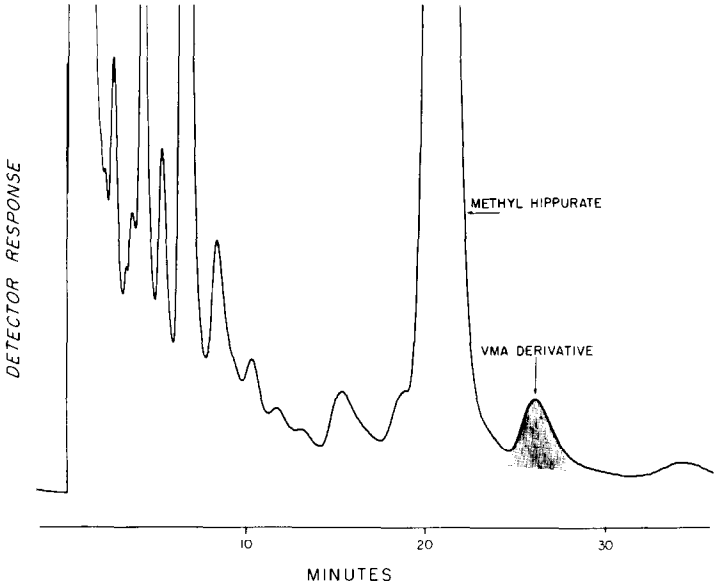

Fig. 1. Aromatic acids extracted from urine (450 μg creatinine equivalent) of a patient with pheochromocytoma and treated with diazomethane and methanol overnight. Conditions: 5% SE-30 coated on 100–110 mesh Anakrom AS (acid-washed and vacuum-siliconized) (Analabs, Inc.), column temperature 161°. Argon inlet pressure 6 psi—outlet pressure atmospheric. Outlet flow 20 ml/min. Shaded peak—methyl 3,4-dimethoxymandelate. Mass response of standard 51 mm^2/μg. This peak corresponds to 24 μg/mg creatinine.

two weeks before laparotomy for removal of a typical benign pheochromocytoma. The urine was acidified to pH 1–2 (conc. HCl) and samples were saturated with NaCl and extracted with ethyl acetate as previously described[8].

If the absence of urinary 3,4-dihydroxymandelic acid could be established in this patient, the derivative of choice would be methyl 3,4-dimethoxymandelate. This was done as follows: 3,4-dihydroxymandelic acid (California Foundation for Biochemical Research, m.p. 139–141°, literature melting point 138° (ref. 9)) was converted to methyl α-3,4-diacetoxyphenyl)-α'-acetoxyacetate (procedure III). Samples of urine from the patient were free of any peak at this retention time at levels at which as much as 0.25 μg/mg creatinine would have been detected. VMA was then determined as methyl 3,4-dimethoxymandelate on a non-polar column (see legend of Figs. 1 and 2 for details). A single large peak having the same retention time and same width at half-height as a standard of the derivative authentic VMA was observed.

This peak was trapped and rechromatographed on two polar columns (QF-1 and XE-60). On each column, the trapped peak was found to consist of a single peak with the same retention time and the same width at half-height as the standard methyl 3,4-dimethoxymandelate. The remainder of the trapped peak was treated with hexamethyldisilazane and trimethylchlorosilane overnight as described above. The new derivative had the same retention time and same width at half-height as a standard of methyl α-(3,4-dimethoxyphenyl)-α'-trimethylsilyloxyacetate on two columns (QF-1 and XE-60) thus confirming the identity of the peak as vanilmandelic acid. A post-operative specimen of the patient's urine showed a normal level of VMA.

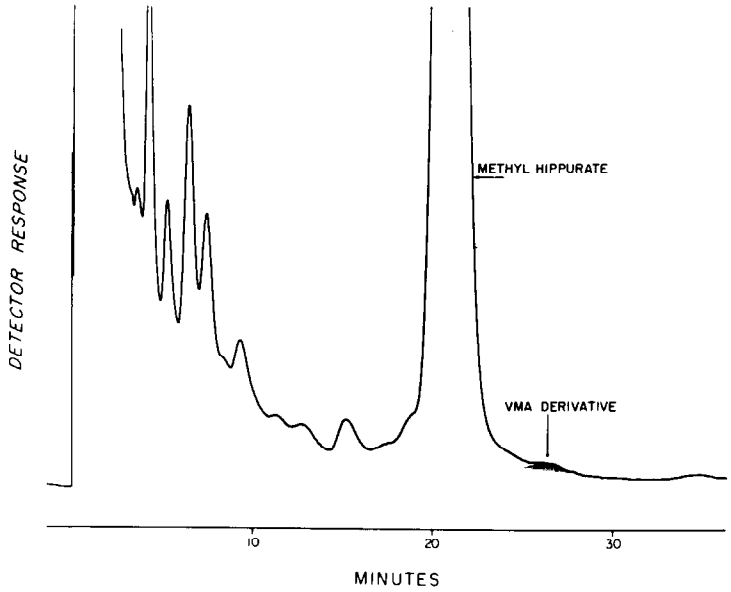

Fig. 2. Aromatic acids extracted from urine (500 μg creatinine equivalent) of a normal control. Treatment and conditions same as Fig. 1. This peak corresponds to 2 μg/mg creatinine.

Urine specimens from 8 patients with pheochromocytoma were received through the courtesy of Dr. Gerald Cohen of the Columbia University College of Physicians and Surgeons. Determination of homovanillic acid (HVA) as methyl homoveratrate and VMA as methyl 3,4-dimethoxymandelate were carried out in duplicate on these eight samples and on eight controls.

Mean HVA excretion for the eight patients with pheochromocytoma was 2.9 μg/mg creatinine (range 1.6–4.8) compared to 3.1 μg/mg creatinine (range 0.5–8.0) for the normal controls. Mean VMA excretion for the patients with pheochromocytoma was 6.9 μg/mg creatinine (range 4.0–9.8) compared to 2.2 μg/mg creatinine (range 1.0–3.5) for the normal controls.

The screening procedures used in most clinical laboratories for the determination of urinary VMA are based on periodate oxidation to vanillin and colorimetry after reaction with one of a variety of complexing agents. These methods are convenient for routine use but are less specific than two-dimensional paper chromatography since many urinary acids contain aromatic nuclei which can be converted to vanillin; an example of such an interferring acid is *p*-hydroxymandelic acid, a normal constituent

of human urine. This problem is important, because there has been reported a case of hypertension in which a VMA screening test was positive for pheochromocytoma; yet, at laparotomy, normal adrenal glands were found and subsequent investigation revealed an elevated urinary level of *p*-sympatol and, tentatively, *p*-hydroxymandelic acid[10]. It was concluded that the latter compound had produced the apparent elevation of VMA. The method described in this paper does not suffer from interference with *p*-hydroxymandelic acid, since methyl *p*-methoxymandelate has a different retention time from methyl 3,4-dimethoxymandelate on both polar and non-polar columns. The gas chromatographic method reported in this paper requires quantitative extraction (90%)[2]. The specificity described in this paper for methyl 3,4-dimethoxymandelate on a non-polar column (SE-30) is complete for the one patient described. The major disadvantage of the method we have described is the fact that 3,4-dihydroxymandelic acid, if present in urine, will give an elevated VMA value, because they are both converted to the same derivative. However, in the normal human less than 2% of infused radioactive epinephrine is excreted as 3,4-dihydroxymandelic acid[11], because o-methylation is the major pathway of metabolism of epinephrine. In six cases of neuroblastoma where much larger amounts of VMA are excreted than in pheochromocytoma, 3,4-dihydroxymandelic acid could not be detected by a procedure where, at least 0.8 μg/mg of creatinine would have been measured[2]. Nevertheless, if a large VMA peak is encountered in a patient with suspected pheochromocytoma, it would be advisable to establish the absence of significant 3,4-dihydroxymandelic acid by the method III described above. A second disadvantage is the $\pm 13\%$ variation in determination of the acid by gas chromatography using the argon ionization detector[12]. This variability can almost certainly be improved by recent advances in inactivation of column supports, as well as by the use of flame ionization detectors.

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