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DETERMINATION OF URINARY 3-METHOXY,4-  
HYDROXYMANDELIC ACID**

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*J Clin Invest.* 1960;**39**(1):221-226. <https://doi.org/10.1172/JCI104022>.

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# THE DIAGNOSIS OF PHEOCHROMOCYTOMA BY DETERMINATION OF URINARY 3-METHOXY,4-HYDROXYMANDELIC ACID \* †

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(Submitted for publication July 21, 1959; accepted September 24, 1959)

In 1957, Armstrong, McMillan and Shaw demonstrated a metabolite of epinephrine (E) and norepinephrine (NE) in normal human urine (2, 3). This substance, 3-methoxy,4-hydroxymandelic acid (VMA, for vanillylmandelic acid), was excreted in abnormally large amounts in three patients with pheochromocytomas (3). Recent studies of sympathomimetic amine metabolism (Figure 1) (4-7) suggested that the urinary excretion of the degradation products of E and NE, such as their 3-methylated derivatives (M and NM respectively) and VMA, might exceed E and NE excretion by some 10- or 20-fold. The diagnosis of pheochromocytoma might therefore be facilitated by analysis of urine for these phenolic compounds.

Armstrong, Shaw and Wall's chromatographic technique for determination of urinary VMA (8, 9) was modified and used for the quantitative determination of VMA excretion in the urines of

15 normal subjects, 36 patients with primary hypertension, and 30 subjects with pheochromocytomas. Correlative E and/or NE determinations were performed on most of these urine samples.

## METHODS

**VMA determination.** Patients receiving no medications and ingesting no fruit, coffee, tea and substances containing appreciable quantities of vanilla for a period of 24 hours, were requested to collect a urine specimen prior to breakfast. An aliquot of urine equivalent to 0.5 mg. of creatinine (technique of Jaffé) was placed in a 15 ml. graduated centrifuge tube, the volume brought up to 2 ml. with water, the pH brought to 2 with a few drops of 3 N HCl, and the tube placed in a boiling water bath for 10 minutes. Following this hydrolysis, the pH was brought to 0.5 to 1.0 with 3 N HCl, 4 ml. of ethyl acetate was added and the tube shaken and centrifuged. The ethyl acetate was removed with a capillary pipette (being careful to avoid aqueous droplets) and the procedure repeated twice with 2 ml. of ethyl acetate. The organic extract was combined in another 15 ml. centrifuge tube, placed in a water bath at 40 to 50° C., and blown to dryness with a stream of air. The sides of the tube were then rinsed with 0.5 ml. absolute ethanol and the contents blown to dryness again. The residue was dissolved in ethanol or ethyl acetate for spotting about 1.5 inches from the corner of a 1 foot square of Whatman No. 1 filter paper. The residue was taken up in a second or third volume of organic solvent in order to insure quantitative transfer to the filter paper. However, the volumes remained small and the spotting procedure was performed slowly enough to keep the spot less than 7 to 8 mm. in diameter. Similar spotting of appropriate quantities of a VMA<sup>1</sup> standard, about six inches above the unknown spot (Figure 2), permitted quantitative estimation of VMA in the aliquot. The filter paper was then stapled in the form of a cylinder, placed in a 6 × 18 inch glass cylinder (possessing a ground glass top which could be sealed by application of silicone grease and a plate glass cover) to which 80 ml. of isopropanol: water: concentrated NH<sub>4</sub>OH (40:9:1) solvent system had been added. About 15 hours later, when the solvent front

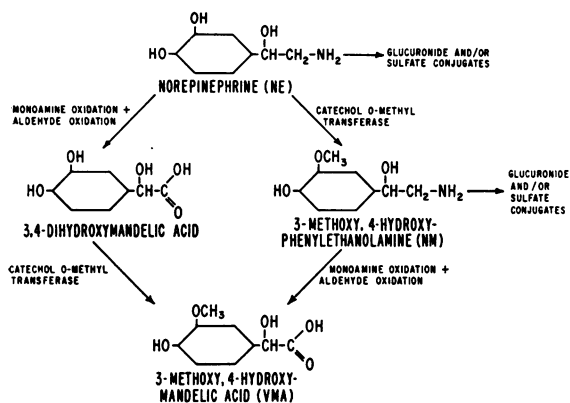


FIG. 1. MAJOR METABOLIC PATHWAYS OF NOREPINEPHRINE

\* This work was aided by grants from the American Heart Association and the National Heart Institute (H 1164), Bethesda, Md.

† A preliminary communication of this work has appeared previously (1).

<sup>1</sup> Kindly supplied by Dr. R. W. Schayer, Merck & Co., Rahway, N. J.

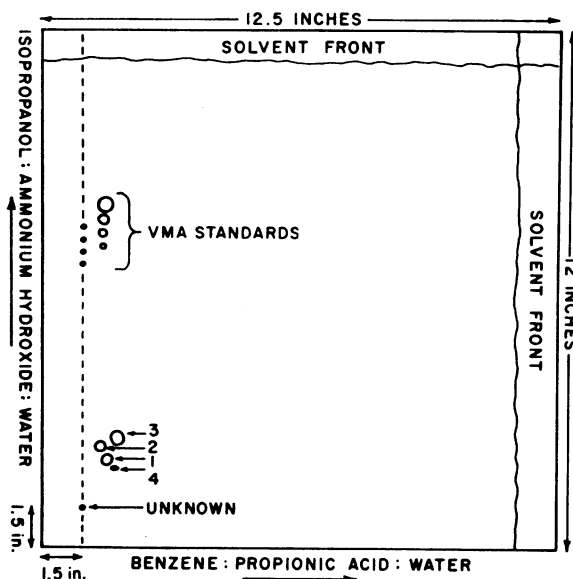


FIG. 2. SAMPLE VANILLYLMANDelic ACID (VMA) CHROMATOGRAM

1, VMA = purple; 2, *m*-hydroxyhippuric acid (8) and *p*-hydroxymandelic acid (9) = salmon; 3, *m*-hydroxyphenylhydracrylic acid (9) = rose; 4, 5-hydroxyindoleacetic acid = brown.

had run 25 to 30 cm., the paper was removed, allowed to dry, and restapled so as to permit chromatography at a 90° angle to the first run in a solvent system of benzene: propionic acid: water (20:14:1). The paper could be removed in about 3.5 hours (25 to 30 cm. movement of the front). It was allowed to dry in the air overnight and then sprayed with diazotized *p*-nitroaniline. The latter was prepared by dissolving 0.5 Gm. of *p*-nitroaniline in a solution of 10 ml. of concentrated HCl in 490 ml. of water and mixing with equal volumes of 0.2 per cent (w/v) NaNO<sub>2</sub> prior to use. Immediately before spraying, this diazotized *p*-nitroaniline was mixed with an equal volume of 10 per cent K<sub>2</sub>CO<sub>3</sub>. VMA appeared as a purple spot with an R<sub>f</sub> of 0.27 (isopropanol: NH<sub>3</sub>) and 0.15 (benzene: propionic acid).<sup>2</sup> Densitometric and subjective comparison of the unknown with VMA standards simultaneously spotted on each chromatogram yielded a reproducible accuracy of ± 10 per cent and a sensitivity of 0.2 to 0.3 μg. Diminishing the urine aliquot to 0.25 mg. creatinine or less improved the accuracy in samples high in VMA content.

E and NE urine analyses were performed within a few days of collection by the techniques of von Euler and Floding (10) and Goldenberg, Serlin, Edwards and Rapport (11), after which the acidified samples from patients with pheochromocytomas were kept at 10° C. for from 3 to 42 months prior to analysis for VMA.

<sup>2</sup> The authors are indebted to Dr. M. D. Armstrong for his instruction in this method (9).

Each patient with pheochromocytoma was surgically proven by removal of the tumor with consequent cure of the hypertension. The patients suffering from primary or essential hypertension fulfilled the usual diagnostic clinical criteria and possessed none of the stigmata of Cushing's syndrome, chronic renal disease, pheochromocytoma, primary aldosteronism, or coarctation of the aorta. These patients had Grade I to III changes in their fundi and none was classified as "malignant" hypertensive. Although some had enlarged hearts and electrocardiographic changes, none had congestive heart failure or azotemia. Two patients with pathologically proven carcinoid syndrome and one with hypertension secondary to chronic renal disease were studied. The effects of phenylephrine, ephedrine, naphazoline and isopropylar-terol on VMA excretion were studied on three normal subjects.

## RESULTS

The urinary VMA excretion of normal subjects varied from 0.8 to 2.0 μg. per mg. of creatinine (Table I). The NE content averaged but 1 per cent of the VMA.

The urinary VMA excretion of the patients suffering from primary hypertension (Table II) varied from 0.7 to 3.0 μg. per mg. creatinine, or approximately 100 times the NE content of these samples. The lowest figure (0.5 μg. of VMA per mg. creatinine) occurred in a specimen from a patient suffering from hypertension secondary to chronic renal disease with azotemia.

The urinary VMA excretion of patients with pheochromocytomata (Table III) varied from 6.0

TABLE I  
Urinary vanillylmandelic acid (VMA), norepinephrine (NE) and epinephrine (E) excretion of normal subjects

VMA*	NE*†	E*†	
1.0			
1.3			
0.8			
1.8			
1.2	0.016	0.003	
2.0			
1.5	0.038	0.005	
1.7	0.012	0.003	
1.2	0.002	0.0005	
1.3	0.004	0	
1.2	0.014	0.001	
1.3	0.002	0.001	
1.2			
2.0			
1.0			
Mean	1.4 (S.D. = 0.38)	0.013	0.002

\* Micrograms per milligram creatinine.

† Technique of von Euler and Floding (10).

TABLE II  
*Urinary vanillylmandelic acid (VMA), norepinephrine (NE)*  
*and epinephrine (E) excretion of patients with*  
*primary hypertension*

VMA*	NE†	E‡
1.3		
1.8		
2.0		
1.0		
2.5		
2.5	0.009	0.003
1.0		
1.2	0.008	0.001
1.0		
1.2		
0.8		
1.3	0.017	0.001
2.5		
1.2		
3.0	0.002	0.003
1.2		
0.7		
1.0	0.009	0.001
1.0	0.018	0.003
1.5	0.022	0
1.0	0.016	0.001
1.3		
1.5		
1.2		
1.3		
1.0		
1.5		
2.5		
1.0		
1.0		
1.0		
0.8		
1.0		
1.7		
1.3		
2.5		
Mean	1.4 (S.D. = 0.6)	0.012
	0.5‡	0.006
		0.002
		0

\* Micrograms per milligram creatinine.

† Technique of von Euler and Floding (10).

‡ Hypertension secondary to chronic renal disease with uremia.

to 40  $\mu$ g. per mg. creatinine (4.4 to 54.7 mg. VMA per 24 hours). The VMA exceeded the NE plus E content (per milligram creatinine) by some 3- to 94-fold (mean = 10). No correlation could be made between the duration of storage of pheochromocytoma urines and their VMA content. Urinary VMA has been found to be stable for many months when kept at pH 3 to 4 at 10° C. Moreover, standard solutions of VMA have remained stable for over a year when kept in this manner.

Two patients with carcinoid syndrome revealed extremely large 5-hydroxyindole acetic acid spots (Figure 2) on the chromatograms, permitting im-

mediate suspicion of the urines as those of patients with carcinoid.

A normal subject given 10 mg. of phenylephrine, 10 mg. of ephedrine, and 0.8 mg. of naphazoline by means of nose drops excreted 1.3  $\mu$ g. of VMA per mg. creatinine following administration of each of these drugs. When 4 mg. of isopropylarterenol was administered by inhalation and when 37.5 mg. was given sublingually, the VMA excretion of two normal subjects remained at control levels.

#### DISCUSSION

Previous studies demonstrated that no more than 1.5 to 4 per cent of parenterally administered NE appeared unchanged in the urine (11, 12). More recently Goodall, Kirshner and Rosen (13) confirmed these findings by recovering only 4 per cent of parenterally administered NE-2-C<sup>14</sup> in the unchanged form in the urine. On the other hand, VMA has been variously ascribed to account for 27 to 36 per cent of the urinary excretion of isotopically-labeled NE (13) and E (5, 7). The results of this study confirm the fact that normal as well as hypertensive subjects excrete many times as much VMA as NE in their urine. Increased excretion of NM, the only other NE metabolite believed to occur in urine in large quantities (6, 13), has also been suggested as a possible means of diagnosing pheochromocytoma (6). Up to this time, however, the procedures for demonstrating NM and M in urine have been inadequate for routine use (14).

The determination of urinary VMA in this study afforded differentiation of normal subjects from those with pheochromocytomas in every instance. Armstrong (9) has drawn attention to the fact that other conditions, such as shock, may be associated with abnormally high VMA excretion. This possibility does not limit the usefulness of this test since its application will be predominantly in the differentiation of primary hypertension from hypertension due to pheochromocytoma. That it serves the latter function is clear since no overlapping occurred between these groups in this study.

Urinary NE excretion of hypertensive patients was previously found to be slightly elevated (11, 12), reduced (15), and normal (16). It is there-

TABLE III  
*Urinary vanillylmandelic acid (VMA), norepinephrine (NE) and epinephrine (E) excretion of patients with pheochromocytoma*

Patient	Date of collection	VMA*	NE*	E*	VMA†	NE†	E†
1	12/55	25	4.82‡	0	>25	>5.3	0
2	11/57	20	3.15	0	24.0	3.78	0
3	8/57	7.5	0.18‡	0.01	4.4	0.11	0.006
4	11/57	7.5	0.61	0.07	12.6	1.02	0.12
5	11/57	12	2.49	0	5.6	1.17	0
6	5/58	15	1.99‡	0	11.0	1.46	0
7	10/57	7.5	0.83	0	5.6	0.63	0
8	1/57	15	0.70	0	16.2	0.76	0
9	5/56	15	0.67	0.27	22.4	1.00	0.41
10	1/57	20	1.19	1.19	23.5	1.40	1.40
11	5/58	7.5	1.32‡	0	6.0	1.05	0
12	10/56	40	7.02	0	13.9	2.44	0
13	1/57	22	6.06	0	8.7	2.40	0
14	3/58	18	2.22‡	0	33.2	4.10	0
15	2/56	20	1.60	0	12.5	1.00	0
16	12/58	16	0.60	0	9.6	0.36	0
17	12/56	7.5	0.20	2.00	18.8	0.50	5.00
18	4/57	6.0	1.73‡	0	8.8	2.51	0
19	11/56	25	0.63	0	27.6	0.70	0
20	7/57	20	2.67	0	19.4	2.58	0
21	10/56	30	2.30	0	50.0	3.83	0
22	9/55	12	0.52	0	6.3	0.28	0
23	5/56	7.5	0.08	0	8.2	0.08	0
24	6/56	20	1.64	0	17.1	1.40	0
25	12/56	10	0.36‡	0.04	9.6	0.35	0.04
26		25					
27	4/56	17.5	0.67	0	20.7	0.79	0
28	10/56	30	0	1.04	54.7	0	1.90
29	10/56	30	0.74	0.37	39.8	0.98	0.49
30	2/59	20	0.14‡	0.08	27.6	0.20	0.11
Mean		17.6 (S.D. = 8.3)	1.63 (S.D. = 1.7)	0.17 (S.D. = 0.45)	18.7 (S.D. = 12.7)	1.45 (S.D. = 1.35)	0.32 (S.D. = 0.97)

\* Micrograms per milligram creatinine.

† Milligrams per 24 hours.

‡ Technique of von Euler and Floding (10); all other catechol determinations done by bioassay (11).

fore interesting that the mean VMA excretions of normal and hypertensive subjects were the same in the present study. However, caution must be exercised in using this as evidence of normal NE metabolism or physiologic function in primary hypertension, since both E and NE give rise to VMA (3, 5, 7), and since the locus as well as the rate of VMA formation are not revealed by these figures. Moreover, it has not been proven that all urinary VMA is of endogenous origin.

Urinary NE excretion has been shown to diminish rapidly within two hours of its intravenous administration (13). On the other hand, Goodall and co-workers (13), found high C<sup>14</sup>-labeled VMA excretion from the first to the twenty-fourth hour after giving a normal subject NE-2-C<sup>14</sup> intravenously. These findings suggest that the VMA excretion of a patient with a pheochromocytoma and paroxysmal hypertension may

be more consistently elevated than the NE excretion. This situation might explain the association of a normal NE excretion and an abnormal VMA excretion in the urine specimen from the patient, No. 23 (Table III). Similarly, Kraupp, Stormann, Bernheimer and Obenaus (17) described a patient with pheochromocytoma who excreted abnormally large quantities of VMA and catecholamines when paroxysmal hypertension occurred, but only large amounts of VMA when normotensive. Moreover, it has been established that increased urinary catecholamine excretion can occur in hypertensive patients in the absence of pheochromocytoma (18) as well as normal catecholamine excretion in the presence of pheochromocytoma (19). It is for these reasons as well as for the relative simplicity of urinary VMA analysis that this test is believed to represent a useful adjunct for the diagnosis of pheochromocytoma.

Such sympathomimetic drugs as ephedrine, phenylephrine, naphazoline and isopropylarterenol failed to affect VMA excretion. Isopropylarterenol does interfere with both the bioassay and fluorimetric techniques for urinary catecholamine determination. Other pharmacologic agents are known to interfere with the fluorimetric analysis of urinary catecholamines (20), but their effect upon VMA excretion was not studied.

The VMA excretions of five patients with pheochromocytomas have been reported by others (3, 5), and show good agreement with the data of this study. Our normal range of VMA excretion is in close agreement with the figures noted by Armstrong and associates (3, 9).

The minor modification of Armstrong's VMA technique (9), by increasing the ethyl acetate extractions from two to three, was required for adequate VMA recovery (over 90 per cent). Another modification, that of subjecting the urine aliquot to mild acid and heat, permitted destruction of the only substance other than VMA which caused a significant purple spot on these chromatograms ( $R_f$  0.35 in  $NH_3$  phase and 0.29 in benzene phase). This procedure did not change the quantitative estimate of either naturally occurring or added VMA in the urine. Not only did this modification permit more simplified reading of the chromatograms, but it also allowed formulation of a new rapid colorimetric procedure which may be used for screening patients with pheochromocytomas from those with primary hypertension (21).

It is noteworthy that the ratio of VMA to NE and E excreted by normal subjects (Table I) and those with primary hypertension (Table II) approximates 100:1, but is only 10:1 in the pheochromocytoma group. The latter ratio agrees more closely with the previously noted data (5-7, 13) derived from the parenteral administration of labeled E and NE. Apparently the body's metabolism of these substances differs at least quantitatively with the route and/or rate of their administration. Relatively rapid introduction of large quantities of E and NE into the systemic veins (as may also occur from a pheochromocytoma) appears to favor urinary excretion of the amine relative to the phenolic acid. Thus, caution must be exercised in drawing conclusions regarding the physiologic degradation of catechola-

mines from studies of their urinary metabolites following parenteral administration.

#### SUMMARY

1. A product of catecholamine metabolism, vanillylmandelic acid, has been chromatographically determined in the urine of 15 normal subjects, 36 patients with primary hypertension, and 30 patients with pheochromocytomas.

2. All patients with pheochromocytomas demonstrated abnormally large amounts of vanillylmandelic acid in their urine.

3. Normal subjects and patients with primary hypertension excreted the same mean quantity of vanillylmandelic acid. No overlapping occurred between these groups and the pheochromocytoma group.

4. This method for urinary vanillylmandelic acid determination is helpful for the diagnosis of pheochromocytoma.

#### ACKNOWLEDGMENT

The authors wish to thank Drs. H. Sobotka, J. D. Chanley and M. Tabachnick of the Department of Chemistry, Mount Sinai Hospital, for the valuable advice and assistance offered during the course of this study.

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