Urinary excretion of metabolites of catecholamines in normal individuals and hypertensive patients

R. J. GEORGES AND L. G. WHITBY

From the John Bonnett Clinical Laboratories, Addenbrooke’s Hospital, and the Department of Biochemistry, University of Cambridge

SYNOPSIS The 24-hour urinary output of 3-methoxy-4-hydroxy mandelic acid (V.M.A.) has been determined in 20 normal adults, 150 hypertensive patients, and four cases of phaeochromocytoma. In this last group estimations of urinary catecholamines and total urinary methylated amines were also performed and the overall results have been compared with the urinary excretory pattern of catecholamines and their metabolites following the injection of radioactive adrenaline and noradrenaline.

Since the reporting by Engel and Euler in 1950 of an increased urinary output of noradrenaline and adrenaline in two cases of phaeochromocytoma, an extensive literature has accumulated dealing with the development and assessment of biochemical tests for the diagnosis of this condition.

It is now well established that the principal metabolic products of these two catecholamines in man are the 3-O-methylated amines, normetadrenaline and metadrenaline and their conjugates, and 3-methoxy-4-hydroxy mandelic acid (Axelrod, 1959). Methods for the quantitative analysis of all these compounds have been developed in recent years, so that at present it is possible to perform any or all of the following investigations on urine:

1. Group 1, free catecholamines: the urinary excretion of adrenaline and noradrenaline can be individually estimated but the most commonly performed determination is the total excretion of these two compounds; group 2, free plus conjugated catecholamines; group 3, free plus conjugated 3-O-methylated amines, which may be estimated individually or as total excretion as in group 1; group 4, 4-3-methoxy-4-hydroxy mandelic acid (V.M.A.).

The problem confronting the general hospital laboratory is the choice of a reliable method suitable for the examination of large numbers of urine specimens from hypertensive patients suspected of having a possible phaeochromocytoma, since this condition, if recognized, can sometimes be cured by operation.

Of the four types of investigation given above those of the catecholamines (groups 1 and 2) are technically least suitable for routine use and have largely been superseded as a first biochemical investigation by analysis of the metabolites in groups 3 and 4.

For the estimation of total methylated amines a relatively simple method has been described by Pisano (1960) and subsequently evaluated in a series consisting of 91 hypertensive and 30 non-hypertensive patients (Crout, Pisano, and Sjoerdsma, 1961).

A considerable number of methods for the estimation of V.M.A. in urine have been described. Several modifications of the original paper chromatographic method for phenolic acids by Armstrong, Shaw, and Wall (1956) have appeared but these are all at best only semi-quantitative. Methods involving the use either of high-voltage electrophoresis (von Studnitz and Hanson, 1959) or of isotope dilution (Weise, McDonald, and LaBrosse, 1961) are unsuitable for the hospital routine laboratory because they are expensive and time consuming.

Estimation of V.M.A. by conversion to vanillin was first described by Sandler and Ruthven (1959 a and b; 1961) but this technique is tedious since it includes a separation by ion-exchange column chromatography, solvent extraction into ethyl acetate and the evaporation of these extracts to dryness, as well as the use of an autoclave for the oxidation of V.M.A. to vanillin. A somewhat simpler method given by Sunderman, Cleveland, Law, and Sunderman (1960) involves a two-hour period of oxidation by potassium ferricyanide at a strongly acid pH and uses a colorimetric method for vanillin determination which is sensitive to temperature and moisture changes and requires accurately timed readings.

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Recently a much simplified method, involving conversion to vanillin by periodate oxidation, has been published (Pisano, Crout, and Abraham, 1962). This method appeared promising for routine use, but the only figures reported for the 24-hour urinary output of V.M.A. were obtained from 20 cases of essential hypertension. The main objects of the present paper have been to reassess the method of Pisano et al. (1962) and to establish more complete values for normal ranges of V.M.A. excretion.

MATERIALS AND METHODS

The V.M.A. used for the preparation of standards was the A grade obtained from the California Corporation for Biochemical Research. The purity of this material was checked by the following determinations:

**Vanillin content** The optical density at 360 mμ of a solution containing 1 mg./ml. V.M.A. in M potassium carbonate was 0-062, equivalent to 0-5 μg. vanillin or a maximum content of 0-05%.

**Yield of vanillin on oxidation** Oxidation of 5 μg. of the material by periodate as described by Pisano et al. (1962) gave a mean yield of 98-2% of the theoretical quantity of vanillin.

**Chromatographic purity** Paper chromatography of 5 μg. of the material in isopropanol/ammonia/water, 8:1:1 (first direction) followed by benzene/propionic acid/water 2:2:1 (second direction) (Armstrong, Shaw, and Wall, 1956) gave a single compact spot on development with diazotised para-nitroaniline.

**Elementary analysis** Found:— C: 54-58%, H: 5-13% (calculated for C₉H₁₀O₅:— C: 54-55. H: 5-09%).

**3-Methoxy-4-hydroxy mandelic acid assay** The method of Pisano et al. (1962) was followed without modification, but a 20 μg. V.M.A. standard was carried through the entire procedure with each batch of analyses. Results for the tests were calculated from the final optical density at 360 mμ of this standard and were thus corrected for the losses occurring in the various extractions. The percentage recovery of this V.M.A. standard was determined each time by comparing its final optical density at 360 mμ with that of a vanillin standard containing 5 μg./ml. in M potassium carbonate. The overall recovery of 70 standards averaged 85% (S.D. 5-6%, range 69-95%) after correction for aliquots taken.

These aqueous standards, taken through the procedure, provide figures for recoveries which are percentages of theoretical recovery after allowing for the taking of aliquots. In five experiments known amounts of V.M.A. were added to urine samples and the recovery of added V.M.A. was determined by the difference between the V.M.A. contents in each pair of specimens; the mean results for these recoveries did not differ significantly from those obtained with aqueous standards and aqueous standards have been used routinely to provide recovery corrections with each batch of analyses.

**Total methylated amines** These were measured by the method of Pisano (1960). Results for the tests were calculated from the final optical density at 360 mμ of a 30 μg. normetadrenaline standard taken through the chromatographic and subsequent steps of the analyses. The percentage recovery of this standard was determined each time by comparing its final optical density at 360 mμ with that of a vanillin standard containing 5 μg./ml. in 4 N ammonium hydroxide. The overall recovery of nine standards averaged 92-5% (S.D. 5-8%, range 84 to 102%) after correction for aliquots taken.

**Catecholamines** Urinary conjugates were hydrolysed by adjusting to pH 1-1-5 with 6N hydrochloric acid and heating at 100°C. for 20 minutes. The catecholamines were isolated by the method of Bertler, Carlsson, and Rosengren (1958) using Amberlite CG120 cation exchange resin prepared according to the method of Haggendal (1962). Fluorimetric estimation of noradrenaline and adrenaline was carried out by the method of Euler and Lishajko (1961) using an E.I.L. model 27A filter fluorimeter. Primary filters used were a Chance O.X.1 (max. transmission 365 mμ) and a 436 mμ interference filter. The secondary filter combination consisted of a Chance O.Y. 13 together with a Chance O.Y.6 filter.

**Collection of specimens** Twenty-four-hour urine specimens were collected in Winchester bottles containing 10 ml. concentrated hydrochloric acid and stored at 4°C. before analysis. Vanilla essences and bananas were excluded from the diet of subjects for 48 hours before collection of urines, and during the collection period itself.

Creatinine was determined by the Jaffé reaction (Bonsnes and Taussky, 1945).

RESULTS

**Normal adults** Twenty members of the laboratory staff acted as controls. They were ambulant at the time of urine collection and the results of their V.M.A. excretion are shown in Table I.

Expressed as mg./24 hr. the mean excretion for the males (5-91 mg.) is 21% higher than that of the females (4-89 mg.). Expressed as μg./mg. creatinine, however, the mean excretion for the males (3-29 mg.) is 19% lower than that of the females (4-08 mg.). These findings are in agreement with those of Weise et al. (1961). The corresponding percentages calculated from these workers' data are 26 and 13.

Values for the urine blanks given in Table I are all less than 15%, as found by Pisano et al. (1962), and the suggestion of these workers that urine blanks may be omitted in routine work is considered justified. We have found that when V.M.A. solutions containing 10-50 μg./ml. in M potassium carbonate are

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1 Supplied by V. A. Howe & Company Ltd., 46 Pembidge Road, London, W.11.
incubated at 50°C for 30 minutes in the absence of periodate about 3% conversion to vanillin takes place. It seems likely that part of the optical density of a urine blank is due to vanillin being formed from V.M.A. under these conditions. Evidence in favour of this supposition was obtained from the observation that much lower blanks are obtained when the alkaline extracts are left at room temperature instead of being incubated at 50°C. (specimens C and D, Table III).

**TABLE I**

<table>
<thead>
<tr>
<th>Group</th>
<th>No.</th>
<th>V.M.A. (mg./24 hr.)</th>
<th>V.M.A. (μg./mg. creatinine)</th>
<th>Blank O.D. as % Total</th>
<th>Total Metanephrines</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>S.D.</td>
<td>Observed Range</td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>Males</td>
<td>11</td>
<td>5.91</td>
<td>3.9 - 7.5</td>
<td>3.29</td>
<td>2.2 - 4.4</td>
</tr>
<tr>
<td>Females</td>
<td>9</td>
<td>4.89</td>
<td>4.0 - 6.1</td>
<td>4.08</td>
<td>3.0 - 5.5</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>5.45</td>
<td>3.9 - 7.5</td>
<td>3.63</td>
<td>2.2 - 5.5</td>
</tr>
</tbody>
</table>

A further specimen of urine was collected from this patient and the V.M.A. content was found to be 5.8 mg./24 hours. The creatinine content of the two specimens was 2.4 g. and 1.41 g. respectively, giving V.M.A. outputs of 4.9 and 4.1 μg./mg. creatinine which are both within the normal range for V.M.A. output expressed in terms of creatinine excretion. It was felt that the high V.M.A. output in the first specimen was due to inaccurate collection of urine, the period probably having extended considerably beyond the required 24 hours, as evidenced by the creatinine excretion. The output of total methylated amines in the first urine specimen from this patient was 0.6 mg./24 hr. which is at the upper limit of the normal range. This patient is being followed up but, for the present, he is regarded as a case of essential hypertension.

**TABLE III**

<table>
<thead>
<tr>
<th>Case</th>
<th>Sex</th>
<th>24-Hour Volume (ml.)</th>
<th>Creatine Content (g./24 hr.)</th>
<th>V.M.A. (mg./24 hr.)</th>
<th>Total Corrected for Blank</th>
<th>O.D. as % Total</th>
<th>Total Methylated Amines (mg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>F</td>
<td>1,300</td>
<td>1.00</td>
<td>25.4</td>
<td>23.6</td>
<td>7.0</td>
<td>25.4</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>1,920</td>
<td>1.30</td>
<td>30.4</td>
<td>28.6</td>
<td>5.7</td>
<td>28.6</td>
</tr>
<tr>
<td>3</td>
<td>F</td>
<td>1,960</td>
<td>1.33</td>
<td>35.5</td>
<td>33.2</td>
<td>6.4</td>
<td>33.2</td>
</tr>
<tr>
<td>Post-operative</td>
<td>F</td>
<td>800</td>
<td>1.07</td>
<td>7.4</td>
<td>6.8</td>
<td>7.8</td>
<td>6.8</td>
</tr>
<tr>
<td>B</td>
<td>F</td>
<td>3,210</td>
<td>1.32</td>
<td>24.5</td>
<td>23.0</td>
<td>6.5</td>
<td>23.0</td>
</tr>
<tr>
<td>C</td>
<td>M</td>
<td>1,200</td>
<td>0.94</td>
<td>26.5</td>
<td>26.1</td>
<td>1.6</td>
<td>26.1</td>
</tr>
<tr>
<td>D</td>
<td>M</td>
<td>1,500</td>
<td>0.73</td>
<td>15.7</td>
<td>15.1</td>
<td>1.9</td>
<td>15.5</td>
</tr>
</tbody>
</table>

**PHAEOMOCYCTOMA** Urine specimens were obtained from four patients with proven phaeochromocytoma. The analytical results are given in Table III. For case A, three urine specimens before operation and one started 48 hours post-operatively were obtained. In this one case the tumour was analysed for catecholamines and found to contain 1,580 μg. noradrenaline and 130 μg. adrenaline/g. of tissue.

Composite results on 10 specimens of urine (three normals and seven from patients with phaeochromocytoma) in which analysis was carried out for total catecholamines, total methylated amines, and for V.M.A. are given in Table IV; results are expressed
in mg./24 hrs. Under each analytical result, in brackets, is placed a figure expressing the result as a percentage of the combined excretion of the three groups of compound.

DISCUSSION

The results given in Table III for the total methylated amines and for V.M.A. output in phaeochromocytoma are in agreement with the findings of Crout et al. (1961) who reported that in each of 23 cases studied the increase in total methylated amines relative to the normal exceeded that of the 3-methoxy-4-hydroxy mandelic acid. On this basis the determination of the total methylated amines has been favoured for use as a screening test (Varley, 1962).

However, there are grounds for considering this an unsatisfactory choice. The low level of excretion of the 3-O-methylated amines in normal urine results in a much greater relative interference by substances absorbing in the ultra-violet around 330 to 340 mμ. This source of interference is recognized by Crout et al. (1961) who recommend taking readings at 360, 347, and 333 mμ in all cases where the apparent excretion of total methylated amines is over 1 mg./day. It is noteworthy that the mean value for 24-hour output (0.62 mg.) found by Crout et al. (1961) for hypertensive patients without phaeochromocytoma is nearly three times the figure reported by Smith and Weil-Malherbe (1962).

Furthermore it has recently been noted that raised values for total methylated amines can be found in patients treated with α-methyl dopa (Stott, Robinson and Smith, 1963). By contrast, the method for V.M.A. analysis is much less subject to interference from drugs or dietary substances, and in our experience offers a simple and reliable method for the investigation of hypertensive cases suspected of a possible phaeochromocytoma.

The relative proportions of total catecholamines, total methylated amines, and of V.M.A. excreted in single 24-hr. samples of urine from three normal individuals are compared, in Table IV, with the corresponding findings from four patients with phaeochromocytoma. The observed percentage distribution for these groups of compounds in the normals agrees with the ratios calculated from the combined analytical results of other workers who have separately reported values for the normal urinary excretion of catecholamines (Euler, 1956), V.M.A. (Armstrong, McMillan, and Shaw, 1957), and total methylated amines (Pisano, 1960). Table IV also shows that the release of abnormal amounts of catecholamines into the circulation from the adrenal medulla, which occurs in phaeochromocytoma, has resulted in the appearance of an increased percentage of total catecholamines and of total methylated amines in the urine, whereas the percentage contributed by V.M.A. has fallen. In the normal individuals, for instance, the ratio of V.M.A.: total methylated amines varies from 13 to 31:1, and in the patients with a phaeochromocytoma this ratio varies from 3:3 to 10:3:1.

These findings on the relative importance of total methylated amines and of V.M.A. as urinary excretion products of catecholamine metabolism in patients with phaeochromocytoma differ from the results of experiments in which radioactive catecholamines were injected into adults (Goodall, 1959; LaBrosse, Axelrod, Kopin, and Kety, 1961). After

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**TABLE IV**

<table>
<thead>
<tr>
<th>Case</th>
<th>Total Catecholamines (A + N.A.) (mg./24 hr.)</th>
<th>Total Metanephrines (mg./24 hr.)</th>
<th>V.M.A. (mg./24 hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(A + N.A.) (mg./24 hr.)</td>
<td>Total Metanephrines (mg./24 hr.)</td>
<td>V.M.A. (mg./24 hr.)</td>
</tr>
<tr>
<td>Normal cases</td>
<td>1</td>
<td>159</td>
<td>61</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>205</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>212</td>
<td>71</td>
</tr>
</tbody>
</table>
| Cases of phaeochromocytoma | A1 | 2,650 | — | 2.65 (8.2%) | 4.1 (12.7%) | 79.1% (25.4%)
| | A2 | 2,350 | 380 | 2.73 (6.9%) | 6.1 (15.6%) | 77.5% (30.4%)
| | A3 | 4,360 | 110 | 4.47 (10.0%) | 4.5 (10.1%) | 79.9% (35.5%)
| | A Post | 200 | — | 0.20 (2.5%) | 0.4 (5.0%) | 7.4 (92.5%)
| | B | 1,600 | 350 | 1.95 (6.1%) | 5.3 (16.7%) | 77.2% (24.5%)
| | C | 3,150 | 60 | 3.21 (9.9%) | 2.6 (8.0%) | 26.5 (82.2%)
| | D | 820 | 450 | 1.27 (5.9%) | 4.7 (21.7%) | 15.7 (72.4%)
intravenous administration of \(^{3}H\) or \(^{14}C\) catecholamines, approximately equal amounts of total radioactive 3-O-methylated amines and of radioactive V.M.A. were excreted in the urine. It is hoped to extend the present series of analyses to include further patients with phaeochromocytoma, but the discrepancies between the findings summarized in Table IV and the results of infusing radioactive catecholamines suggest the need for caution in equating the urinary excretion patterns of the metabolites of injected adrenaline and noradrenaline observed in the latter experiments with the pattern of inactivation for endogenous catecholamines released into the circulation, for instance, as a result of suprarenal medullary overactivity.

We wish to thank Dr. R. Robinson, Group Pathological Laboratory, Lakin Road, Warwick, for kindly supplying urine specimens from two patients with phaeochromocytoma.

It is a pleasure to acknowledge the skilled technical assistance of Miss D. Bircham and Mr. B. Sloss.

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