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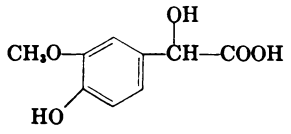
E. THE MEASUREMENT OF 4-HYDROXY-3-METHOXYMANDELIC ACID AND HOMOVANILLIC ACID

M. SANDLER AND C. R. J. RUTHVEN

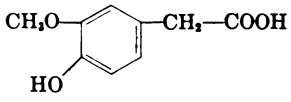
The Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London

The acidic metabolites of the biologically active monoamines are present in considerably higher concentration in urine than in other body sites. It is not surprising, therefore, that despite difficulties in working with such a complex fluid, most attempts at quantification have involved urinary assay.

Since its original identification in human urine (5) 4-hydroxy-3-methoxymandelic acid (VMA) has been measured more commonly than other catecholamine metabolites, having to some extent supplanted catecholamine assay in the diagnosis of catecholamine-secreting tumours (43, 51, 52, 64, 69). Assay of urinary homovanillic acid is now being performed with increasing frequency, because of its value in the detection of dopamine-secreting tumours (23, 25, 31, 32, 47, 54, 64, 67, 81). Both VMA and homovanillic acid are normally excreted in higher concentration than any other catecholamine breakdown product. They can therefore be estimated by procedures which are less dependent upon the skills of the operator and less subject to interference than those for related compounds. Unlike catechols, they are relatively stable (20, 47, 64). They are largely excreted in the free state, so that the need for hydrolysis prior to estimation is avoided.



4-hydroxy-3-methoxymandelic acid (VMA)



homovanillic acid

Two main types of assay procedure have been employed, involving either physical separation of constituents of the same general group or specific reactions for individual compounds. The latter have been reviewed by Ruthven (45). These categories are by no means mutually exclusive. Methods classified among the former include paper, thin-layer, and gas-liquid chromatography and high- and low-voltage electrophoresis. Techniques in this group are often semi-quantitative and are rarely suitable for following small variations in concentration occurring under physiological conditions. Nevertheless, provided the required special equipment is available, they usually possess the virtue of relative simplicity and as a consequence have been widely used. Preliminary extraction of phenolic acids is considered necessary by most authors; ethyl acetate extraction from salt-saturated acidified urine (6, 43, 60) is commonly used. Apart from the special case of gas-liquid chromatography, phenolic acids such as VMA and homovanillic acid are located with a staining reagent, usually diazotized *p*-nitro-

aniline. An approximate assessment of quantity can be made visually, often the only way when overlap of spots occurs. When a clear-cut separation is achieved, it is sometimes possible to elute the azo-dye complex, for example with alkaline methanol (64), and measure its extinction at an appropriate wavelength.

Gödicke and Brosowski (24) have achieved complete separation of VMA from other diazo-reacting substances by one-dimensional chromotography of urine applied directly to a wedge-shaped paper (38), developing with the organic phase of a butanol system. Käser *et al.* (31) have used a similar method. Such an approach is perhaps more successful when few interfering substances are present, as in cerebrospinal fluid (2). Nevertheless, for optimal separation, two-dimensional paper chromatography appears to be mandatory. Following the lead of Armstrong *et al.* (6), most techniques use an isopropanol-ammonia-water (20:1:2) system as first solvent. Whilst benzene-propionic acid-water (2:2:1) (6) or a variation (62) has been widely employed as a second solvent, a less volatile anisole-acetic acid-water (70:29:1) system (43) offers the advantage of needing minimal equilibration. Jacobs *et al.* (29) have devised a system involving two separate one-dimensional runs, which they say gives better separation, even if it is more time-consuming.

Paper chromatographic analyses need up to 24 hr to develop. Thin-layer chromatography, however, can provide results within 2 to 4 hr and is considerably more sensitive. Schmid and his colleagues have developed procedures for the assay of VMA (56, 57) and homovanillic acid (72) with silica gel as support, an isopropanol-ethyl acetate-ammonia-water (45:30:17:8) system to develop, and Gibbs' reagent for location. Annino *et al.* (3) also employed a silica gel system to measure VMA. The procedure of Sankoff and Sourkes (54) for homovanillic acid uses silica gel as support and benzene-acetic acid-water (2:3:1) as developer; the chromogen formed with Folins' reagent is eluted and measured spectrophotometrically, elution of spots being particularly suited to thin-layer chromatography. Using polymide as support, Segura-Cardona and Soehring (58) have devised a thin-layer method for the simultaneous separation of catecholamines and their metabolites, including VMA and homovanillic acid, which employs isobutanol-acetic acid-cyclohexane (80:7:10) as solvent.

Whilst thin-layer chromatographic procedures are of proved usefulness, gas-liquid chromatographic analysis, at least of aromatic acids, is still beset with many problems. Williams and his colleagues (71, 77, 80, 84), who have pioneered these studies, have measured VMA and homovanillic acid with an argon ionization detector, after preliminary ethyl acetate extraction and conversion to their methyl esters. However, esters of VMA tend to trail and are subject to contamination with methyl 3,4-dimethoxymandelate (82). It is probably preferable to convert VMA to this compound completely in order to obtain satisfactory separation, particularly of VMA from hippuric acid (80), even though any 3,4-dihydroxymandelic acid (doma) present is changed to the same compound and will thus reinforce the VMA peak. The presence of dihydroxy-aromatic acids, in fact, tends generally to confuse the picture, giving rise to a mixture of compounds on methylation with diazomethane. By prior acetylation of phenolic groups,

followed by methyl esterification, Williams and Leonard (83) were able to measure protocatechuic acid, 3,4-dihydroxyphenylacetic acid, 3,4-dihydroxymandelic acid, homovanillic acid, and VMA in the same specimen. For measurement of VMA at least, its conversion to the methyl O-methoxyester, with analysis of a duplicate converted to the methyl O-acetoxy ester if 3,4-dihydroxymandelic acid is thought to contribute significantly to the peak, appears to be the method of choice (82). Although gas chromatographic techniques are claimed to be particularly useful in the diagnosis of catecholamine-secreting tumours, it would seem that considerable experience is necessary to achieve meaningful results, particularly if the reliable measurement of VMA and homovanillic acid at normal excretion levels is required. Gitlow *et al.* (22) have overcome problems of resolution encountered during the assay of VMA by gas-liquid chromatography by converting it to vanillin, the trifluoroacetyl derivative of which is detected by electron capture. Whilst this would appear to be an extremely laborious method of achieving an end obtainable by simpler means, its high sensitivity may give the approach a limited place in the analytical armamentarium for the estimation of VMA in body fluids other than urine.

One great drawback of chromatographic techniques in general is that they are prone to interference by diet and drugs. Thus, aspirin, sulpha drugs, penicillin, tea, coffee (4, 61), vanillin-flavoured foods (4, 61), bananas (61, 73) and citrus fruits (63) must all be withdrawn from the diet prior to assay. Similarly, it is necessary to restrict them before attempting any of the electrophoretic separation techniques. Such dietary precautions appear to be particularly necessary if VMA is to be separated from other phenolic urinary constituents, at the pH of 8.6 employed during electrophoresis by Wolf *et al.* (86). Aguayo (1) has also used a pH of 8.6, but the consensus is that a low pH is obligatory for achieving optimal separation. The original high voltage technique of von Studnitz and Hanson (66) was conducted at pH 6, although later modifications (64, 65) employed pH values of 4.6 and 3.6. Both Zeisel (88) and Randrup (42) were unable to obtain satisfactory resolution of VMA and homovanillic acid from other phenolic acids at pH 6. Randrup thought that VMA was likely to be a relatively strong acid by analogy with mandelic acid (pK 3.4); thus its separation from interfering substances might be improved by using a buffer at pH 3. This modification led to a procedure of greater specificity, for now VMA migrated to the anode whilst most other phenolic acids migrated to the cathode. Buffer solutions of low pH have been used successfully also by other authors (16, 55).

One drawback of a low pH system is that migration tends to be low, even when high-voltages are employed, so that special precautions must be taken against overheating and moisture accumulation on the strip (42). The great advantage of using high-voltage electrophoresis (45 to 50 v/cm) (55, 64–66, 88) appears to lie in a shorter running time (4 to 6 hr) compared with a much extended time (about 17 hr) for low-voltage techniques (5 to 10 v/cm) (33). If a cellulose acetate membrane (34) is substituted for paper, a satisfactory separation of VMA from other phenolic acids can be achieved in less than 4 hr at low voltage (1, 16). A further sophistication has been introduced by Eichhorn and

Rutenberg (11, 12), who have employed acetic acid solutions of different strengths in the two electrode compartments. They claim that a conductivity gradient is established, causing buffer to travel towards the anode, in an opposite direction to electro-osmotic flow, so that a counter-current effect is created that increases the mobility of VMA and related compounds towards the anode. Although this method achieves a relatively good separation of homovanillic acid, it does not appear to be suitable for its detection at physiological concentrations. In general, the electrophoretic techniques appear to be more highly sensitive and specific for VMA than for homovanillic acid. This is due, in part, to the diazo reagent used for location forming a more intensely staining chromogen with VMA.

With the expansion of the whole field of catecholamine studies over the past few years, one pressing need remains unfulfilled. A satisfactory screening test for phaeochromocytoma has yet to be devised. Several semiquantitative or quantitative tests for VMA are available, based on a diazo color reaction, preceded by organic extraction from acidified urine (17, 18–21, 37, 85). Although comparatively simple, these tests are relatively nonspecific and particularly susceptible to interference from drugs and diet, so that claims for their usefulness as screening tests are felt to be exaggerated. A commercial kit ("Hycel Pheoset"), marketed in the U.S.A., illustrates most of the limitations of such diazo procedures. VMA level is calculated from the difference between two extinction readings (530 and 420 mμ) of a diazotized chloroform-butanol extract of urine. This difference may sometimes be negative (37, 68). Even when dietary restrictions are adhered to (and these must include drugs such as the muscle-relaxant methocarbamol, for example, which gives a false positive reaction (9)), the procedure gives an upper level of normal 24 hr output of 10 mg, compared with less than 5 mg by an isotope dilution procedure (76). Sunderman (68), after a comparison between this test and a more specific quantitative procedure (69), concluded that it was unreliable as a measure of VMA and unsatisfactory as a screening test for phaeochromocytoma.

The technique of Mahler and Humoller (37), resembling that of the "Pheoset" in principle, gives a similarly high normal range, as does the diazo method of Woiwod and Knight (85), even when modified by Georges and Small (18). One diazo method for VMA, said by the authors to give rise to few false positives, was published by Gitlow *et al.* (21) and soon modified to dispense with the need for dietary control (19, 20). Variations of this method have been described by other authors (17, 87) without any apparent effect on its specificity, which is low. The method is almost as time-consuming as assay procedures based on the conversion of VMA to vanillin, which are considerably more specific. Comparison with a two-dimensional paper chromatographic procedure showed considerable discrepancies between the two methods (87).

Fellman *et al.* (14) have introduced a colorimetric technique based on the reaction of VMA with 4-chloro-*o*-phenylenediamine. The compound also forms chromogens, however, with *p*-hydroxymandelic acid, which is present in appreciable concentration in normal urine (30), and with doma. Indeed, the authors consider it necessary to confirm all abnormally high values by other tests.

The conversion of VMA to vanillin forms the basis of a number of accurate quantitative methods for its estimation. This reaction, a cleavage of the α-β C-C bond, is characteristic of compounds having either a hydroxyl group and an amino group or two hydroxyl groups on adjacent carbon atoms (28). It was first employed for the assay of VMA by Sandler and Ruthven (49), who achieved the conversion by autoclaving at 123°C in dilute acid with alumina as catalyst. Acidic ferricyanide at 37°C was used to effect the conversion by Sunderman *et al.* (69) and by Dauchy and Schwarz (10), whose method is almost identical. The method of Sunderman *et al.* (69), primarily devised for the measurement of VMA in urine, was later adapted to its assay in serum (70). Vanillin formation from VMA in the method of Pisano *et al.* was brought about with alkaline periodate at 50°C, although Brunjes *et al.* (8) found that heating to 100°C was required in their method to remove urinary inhibition of VMA oxidation.

Although alkaline periodate treatment by the method of Pisano *et al.* (41) has always resulted in satisfactory replication in the hands of the present authors, Weil-Malherbe (75) found it gave erratic results. The reason for this discrepancy may be shown by the recent work of Farrand (13), who noted that ethyl acetate, used for the preliminary extraction, is affected deleteriously by daylight and air, so that low and variable conversion of VMA to vanillin results.

Weil-Malherbe's own procedure for converting VMA to vanillin (74) relies on the catalytic effect of cupric ions at pH 10 and at 100°C. The usefulness of this manoeuvre is limited, however, by one unexpected finding: any homovanillic acid present in the urine extract forms vanillin, to the extent of about 10%, when heated with alkaline copper. The mechanism of this reaction is obscure.

Although urinary VMA is excreted as the D-isomer (5), a nonspecific L-mandelic acid dehydrogenase from *Pseudomonas fluorescens*, A-312, has been said by Rosano (44) to convert VMA to vanillin. As nonenzymatic procedures are relatively efficient, the usefulness of the test is likely to be limited.

Preliminary separation of VMA prior to vanillin formation has been achieved in a variety of ways, although organic extractions are employed at some stage in all methods. Sandler and Ruthven (49, 50) and Brunjes *et al.* (8) used an anion exchange resin to eliminate interfering substances from urine, whilst Sunderman *et al.* (69) achieved a similar end by preliminary treatment of the urine sample with florisil.

Several different methods of measuring vanillin have been used. A colour reaction with indole (15) was used by Sandler and Ruthven (49, 50) and later by Sunderman *et al.* (69), while Georges (17a) employed hydrazone formation. Sandler and Ruthven (52) measured vanillin production by differential spectrophotometry (365 and 380 mμ) at alkaline pH, whilst Pisano *et al.* (41) did similarly, but took a reading at only one wavelength (360 mμ).

Although methods depending on vanillin formation have been criticized on the grounds that vanillin-like compounds may be formed from urinary aromatic acids other than VMA (82), in practice even the main candidate for this role, *p*-hydroxymandelic acid, does not interfere appreciably; its derivative, *p*-hydroxybenzaldehyde, exhibits maximum extinction at 332 mμ, whilst the

maximum for vanillin is 350 mμ. Miyake *et al.* (39) suggested that dietary vanillin might cause falsely high values; but ingested vanillin is either metabolized or conjugated (78), and little is excreted in the free form. In fact, dietary restriction is not necessary for any of the methods of VMA assay based on vanillin formation. The three procedures which seem to be most commonly employed (41, 52, 69) all provide a very similar mean excretion value, of about 4 mg per 24 hr, with a small standard deviation, even when the diet is unrestricted. This value is, in turn, very close to that obtained by the isotope dilution procedure of Weise *et al.* (76) which, although protracted and requiring specialized equipment, is useful as a reference method. The present authors now prefer to use the technique of Pisano *et al.* (41) from the viewpoint of speed and general convenience (53).

VMA is not the only catecholamine metabolite which will undergo conversion to vanillin. Weil-Malherbe has already dealt with the metadrenalines (Section III D). 4-Hydroxy-3-methoxyphenylglycol (HMPG) (7), by virtue of its hydroxyl groups on adjacent carbons, can readily be split to vanillin. This reaction forms the basis of an assay procedure recently devised by Ruthven and Sandler (48), the first to be described for measuring endogenous HMPG in normal subjects. After enzymatic hydrolysis (36), urine is passed through a cation exchange column (40) and HMPG is extracted from the effluent with ethyl acetate, to be converted to vanillin with alkaline periodate. Vanillin is measured by differential spectrophotometry. Normal excretion values of 3.0 ± 0.8 mg. per 24 hr have been detected.

As with HMPG, there is only one accurate specific quantitative procedure for measuring urinary homovanillic acid at normal excretion levels, that of Ruthven and Sandler (46, 47), although paper (60) or thin-layer (54) chromatography has been used for this purpose. The procedure involves the purification of homovanillic acid on a silica-gel column followed by its elution, and demethylation to 3,4-dihydroxyphenylacetic acid by autoclaving with a glacial acetic acid-HBr mixture. The product is isolated on an alumina column, eluted and measured colorimetrically. Normal young adults excrete 5.1 ± 1.1 mg per 24 hr. The demethylation step, at least, may well have wider applications. At present we are tentatively exploring its usefulness as a possible means to the quantification of 6-hydroxymelatonin, the major metabolite of melatonin (35).

Still, the method of Ruthven and Sandler (47) for homovanillic acid is protracted and tedious. Since urinary metabolites resulting from coffee (61), salicylates (79) and high vanillin intake interfere with the assay, their restriction is necessary. There exists the need for a simpler test, for homovanillic acid assay provides much useful information in the diagnosis of the neuroblastoma group of tumours (23, 25, 31, 32, 47, 54, 64, 67, 81). Two sensitive procedures, involving conversion to a fluorescent oxidation product, exist for the assay of homovanillic acid in tissues (2a, 59), but it seems doubtful whether either could be adapted for urine. Homovanillic acid is an unremarkable molecule, chemically similar to several other urinary constituents. Unlike VMA, which possesses a β-hydroxyl group, it does not have a characteristic substituent to enable any obvious chemical tricks to be performed. If there is hope for a simple assay procedure in the

future, it lies in Weil-Malherbe's (74) observation of the partial conversion of homovanillic acid to vanillin by an alkaline copper solution; but preliminary observations by the present authors show that there is much work to be done before a definitive method can be produced.

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F. METHODOLOGY OF FLUORESCENCE MICROSCOPY OF CATECHOLAMINES

CH. OWMAN

Department of Anatomy, University of Lund

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