Electrophoretic Determination of Vanilmandelic Acid (VMA) in Urine by Direct Application to Cellulose Acetate

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The VMA in urine was quantitated electrophoretically by application of urine to cellulose acetate. Separation of several hydrophenolic acids is distinct. VMA is detected at 0.1-μg. levels. Normal values on 400 specimens was 4.0 ± 2.0 mg./24 hr.

The value of vanilmandelic acid (3-methoxy-4-hydroxymandelic acid, VMA) in urine for the diagnosis of pheochromocytoma is well recognized. The comprehensive review of VMA analysis by Sunderman (1) reiterates that there is a large difference between normal and pathologic 24-hr. urinary excretion of VMA. Such a sharp difference in concentration makes VMA analysis adaptable to a semiquantitative method for detecting abnormal 24-hr. excretions. In normal urine, the VMA concentration averages 3 μg./ml. Patients suffering from hypertension due to pheochromocytoma and children with neuroblastoma have values greater than 12 μg./ml. Such a difference between normal and elevated values means that quantitation need only discover the elevated values with significant confidence.

VMA, which is one of the many hydroxy-phenol acids appearing in the urine, can be separated by electrophoresis. Methods developed by Hermann (2) and Klein et al. (3) require a prior extraction of the urine followed by colorimetric quantitation of the VMA eluted from the electrophoretic support. A direct method using paper electrophoresis (4) appears sensitive to 1.0 μg. of VMA.

In the proposed electrophoretic method using cellulose acetate media, VMA is detected at levels of 0.1 μg. when applying 0.1 ml. of urine to the electrophoretic media; urine specimens containing 1 mg./L. (0.1 μg./0.1 ml.) can be easily recognized. Differences of 0.3 μg. equivalent to 3 mg./L. can be determined semiquantitatively. This precision is more than enough to detect abnormal from normal excretions.

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Apparatus and Reagents

Electrophoresis apparatus  An electrophoresis chamber (Gelman No. 51101) was used with a power supply capable of delivering 250 v D. C. at 25 ma. Cellulose acetate strips were Sephaphore III (Gelman Chemical Co.) measuring 6¼ × 1 in.

Staining trays  Plastic trays measuring 8 × 4 × 1.5 cm. made from 4-mm. thick Plexiglass were used for staining strips.

Hot-air blower  Home style hair dryer is adequate.

Electrolyte buffer pH 4.2  Mix 16 ml. of acetic acid and 3.5 ml. of pyridine; dilute to 1000 ml. with water.

p-Nitroaniline  Dissolve 150 mg. of p-nitroaniline in 1.5 ml. of conc. HCl; dilute to 100 ml. with water. Refrigerate.

Sodium nitrite, 0.1% (w/v)  Refrigerate.

Sodium carbonate, 10% (w/v)  Refrigerate.

Staining solution  Prepare from refrigerated solutions (IMPORTANT). Make fresh solution for each strip of cellulose acetate. To 5.0 ml. p-nitroanaline solution (above), add sufficient 0.1% sodium nitrite to decolorize the solution (approximately 5.0 ml.). Add water to bring volume to 10 ml. Add 10.0 ml. of 10% sodium carbonate; mix and immediately float strip into the staining solution.

Stock VMA standard, 10 mg./100 ml. (w/v)  Weigh 10 mg. of VMA (Nutritional Biochemical) and dilute to 100 ml. with absolute ethanol. Refrigerate.

Working VMA standards  Prepare fresh prior to use. Into two 13 × 100 mm. tubes labeled 4 mg. and 10 mg., add 0.1 ml. and 0.25 ml. of stock VMA standard. Dilute to 1.0 ml. by adding 0.90 ml. and 0.75 ml. of absolute ethanol to the appropriate tubes. Standards are equivalent to 4 mg. and 10 mg. VMA per liter based on the use of 0.1 ml. of urine in the analysis.

Procedure

1. Pick up with a small hemostat, a strip of filter paper (S&S 2043A) cut to 25 × 3 mm. Apply urine in 0.02-ml. aliquots with a Sanz overflow pipet (Beckman Instruments), drying continually under the hot-air blower until 0.1 ml. of urine is soaked into the strip.

2. To filter paper strips identified as 4 mg. and 10 mg. standards, apply 0.04 ml. of working standards, respectively, in the same fashion as the unknown urine.

3. Presoak cellulose acetate strips in the electrolyte buffer. Blot thoroughly and place in electrophoresis cell with strips held taut. It is important that the cellulose acetate strip is free of water drops and that it is held taut in the cell.
4. At cathode end of properly identified cellulose acetate strips, place the filter paper bands containing the dried urine and the standards. With a wet applicator stick, touch strip with a small drop of buffer to dampen the filter paper, making it obviously adherent to the cellulose acetate membrane.

5. Cover cell and let stand for 10 min. Run electrophoresis for 1½–2 hr. at 250 v. across the strips and/or 2 ma per strip.

6. Stain each strip, one at a time, in a separate fresh batch of stain for 30 sec. Place unknowns and standards on white paper.

7. VMA band stains purple and is located 7–9 cm. from the origin. Quantitate unknowns by comparison with the 4-mg. and 10-mg. standards.

8. Calculation: mg. VMA/24 hr. = mg./L. × 24-hr. volume in liters.

**Results and Discussion**

The application of even 0.1 ml. of urine to either a wet or dry electrophoretic media always results in diffusion, overlapping, and poor resolution of the stained hydroxy-phenol acids. The need for adequate resolution necessitates that the sample be applied in either a narrow band or small spot. By drying the urine onto a strip of filter paper the band is held to an acceptable size. The strip containing the urine concentrate is applied to the cellulose acetate support for electrophoresis.

As shown in Fig. 1, there is a distinct difference in staining intensity in the range of 0–1 μg. of VMA, equivalent to 0–10 mg./L. of urine. The semiquantitative difference of the purple-stained VMA band is even more apparent than indicated in the figure. In this range of applied VMA, differences are easily recognized so that the observer can rank
unknowns with a confidence of 0.2 µg. When the quantity of VMA is greater than 1.5 µg., differences are generally difficult to recognize with confidence. Normal urines require at least 100 µl. of sample to obtain sufficient staining intensity for a reliable comparison (Fig. 2).

If an unknown contains more than 1 µg./0.1 ml., it is best to repeat the procedure using a smaller volume of urine. In this way, the unknown is brought into the range where color comparison is most reliably perceived. In Fig. 3, a normal urine has a semiquantitative concentration

![Fig. 2. Rising A.M. urine (s.g. 1.026) applied in 25, 50, and 100 µl. samples; from normal male: deep staining VMA band. VMA, 14 mg./L. Tailing of VMA due to buffer droplets on acetate strip after dampening filter paper strip.](image)

![Fig. 3. Semiquantitative analysis of 24-hr. urine collection (vol., 755 ml.) from normal male (unk.). VMA, 8 mg./L. or 6 mg./24 hr. Distinct vanillic acid band (left edge) on standard strips (variable trace amounts of vanillic acid present in different lots of commercial VMA).](image)

of 8 mg./L. In urine, there are several other phenol acids that stain red, yellow, orange, and purple. The VMA is always obvious and is bracketed by a yellow (cathode side) and a red (anode side) band. Persons taking salicylates show a deep orange-staining salicyluric band located
midway between the application site and the VMA band. Another purple band of vanillic acid of varying intensity—depending upon dietary intake of vanilla flavoring—is located 1–1.5 cm. from the cathode.

During the 18 months that the method has been utilized in this laboratory, normal values on 400 specimens were calculated to be 4.0 ± 2.0 mg./24 hr. The high value was 11.0 mg./24 hr. and the low, 1.0 mg./24 hr. The mean and range of values agree well with a specific vanillin method (5). The separation of VMA by electrophoresis eliminates interference by closely related urinary phenols and permits direct visual semiquantitation by comparison with appropriate standards. By eliminating time-consuming extraction procedures common to most VMA analytic methods, the analyst can screen many more urines in suspect patients. The proposed method has sufficient clinical precision to confidently detect persons having elevated excretions of VMA.

References