3-Methoxy-4-Hydroxymandelic Acid (VMA) by Microfiber Chromatography

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A method is presented for the rapid, precise, quantitative measurement of 3-methoxy-4-hydroxymandelic acid (VMA) in urine. The method is unique in that it utilizes silica-gel-impregnated glass fiber as a support medium for thin-layer chromatography. An ethyl acetate extract of acidified urine was spotted and chromatographed in an ascending technic using toluene, glacial acetic acid, and ethyl acetate (70:20:5) as a solvent system. The VMA is identified by spraying the chromatogram with diazotized p-nitroanaline. Semiquantitation was made with accuracy by visual inspection and comparison with simultaneously spotted standards. Precise quantitation was made by strip scanning. Diet and a variety of medication did not interfere. The normal range was found to be 0–4 mg./L.

The measurement of abnormal amounts of adrenalin and noradrenalin or their metabolic products in the urine has become an increasingly important tool in the biochemical diagnosis of the catechol-excreting tumors pheochromocytoma and neuroblastoma (1–4). Since these catecholamines are pressor amines, their identification becomes increasingly important in the evaluation of the hypertensive patient, particularly the young hypertensive (5). Unfortunately, less than 10% of endogenous adrenalin and noradrenalin is excreted in the urine in the unaltered form. However, through metabolic pathways utilizing o.-methyltransferase and monoamine oxidase, adrenalin and noradrenalin are converted to metanephrine (M) or normetanephrine (NM) and 3-methoxy-4-hydroxymandelic acid (VMA). Some 40–60% of endogenous adrenalin and noradrenalin is converted to the M or NM, the remainder converted to the VMA (6). During recent years numerous methods have been proposed for the identification of VMA in the urine, including spectrophotometry (7), colorimetry (8), paper electrophoresis (9), gas chromatography (10), high voltage electrophoresis (11),

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thin layer chromatography (12, 13), and paper chromatography (14).
Two commercial kits (Pheoset* and Sigma†) are available for the
colorimetric determination of VMA. The spectrophotometric, gas
chromatographic, and high-voltage electrophoresis methods, while spe-
cific, are not suited for routine clinical use because of the complexity
of equipment and methods required. Paper chromatography, although
precise and rather simple, is time-consuming. The colorimetric pro-
cedures suffer from lack of specificity, and there are numerous inter-
fering chromagens, some of which can be removed through extensive
preparative steps.
Thin-layer chromatography seems to lend itself to the most simple,
precise, and rapid method for identification and quantitation of VMA
in the urine. There is, however, considerable labor and detail required
to prepare adequate thin-layer plates.
This report concerns itself with a method for identification and
quantitation of VMA in the urine using previously prepared silica-gel-
infiltrated glass fiber as a support medium for thin-layer chromato-
graphy.

Materials

Equipment

- Centrifuge tubes, 50-ml., with snap cap and round bottom
- Conical centrifuge tube, 50-ml., with screw cap
- Centrifuge tubes, 15-ml., with glass stopper
- Chromatography chamber (Gelman Instruments)
- Micropipets of 10-μl. capacity and spotting quide (Gelman Instruments)
- Spray bottle
- Glass fiber impregnated with silica gel, 20 × 20 cm. (Gelman Instruments)

Reagents

- Sodium chloride, A.R. (Mallinkrodt)
- Ethyl acetate, A.R. (Mallinkrodt)
- HCl 3N and concentrated
- Solvent system  Tolulene, glacial acetic acid, and ethyl acetate
  (70:20:5)
- Color developer  Diazotized p-nitroaniline
  1. 0.1% (w/v) p-nitroaniline  0.1 gm. p-nitroaniline in 95 ml.
     distilled water and 5 ml. concentrated HCl. Stable in refrigerator for
     up to 1 month.

*Hyce!, Inc., Houston, Tex.
†Sigma Chemical Co., St. Louis, Mo.
2. 0.2% (w/v) sodium nitrite  Add 0.2 gm. sodium nitrite, A.R. (Mallinckrodt), to 100 ml. distilled water. Stable in refrigerator for up to 1 month.

3. 10% (w/v) potassium carbonate  Bring 10.0 gm. potassium carbonate anhydrous, A.R. (Mallinckrodt), to 100 ml. with distilled water. Store in refrigerator; solution is stable indefinitely.

Prepare fresh spray reagent just prior to spraying chromatogram. Add 10 ml. of 0.1% p-nitroanaline to 10 ml. of 0.2% sodium nitrite and mix. Add 20 ml. of 10% potassium carbonate. Spray within 2 min. All reagents should be cold prior to mixing.

Standards  Prepare 2-, 5-, 10-, and 20-mg./100 ml. solutions of 3-methoxy-4-hydroxymandelic acid (Calbiochem) in ethyl acetate. These are equivalent to 0.2, 0.5, 1.0, 2.0 μg./10 μl. spot. They are stable in the refrigerator indefinitely.

Methods

Collection of Specimen

Twenty-four-hour urine specimens were collected with 10 ml. of concentrated HCl used as a preservative. The urine volume was measured and a 50-ml aliquot was stored in the refrigerator until assay. No particular dietary restrictions were enforced, but many of the subjects were receiving drugs, including chlorothiazide, antibiotics, steroids, and salicylates. In addition, 5, 10, and 20 mg. of VMA per liter was added to 24-hr. urine samples of apparently normal individuals.

Extraction of Phenolic Acids from Urine

The pH of the 50-ml. aliquot was adjusted to 1.0–1.5, using a pH meter. Ten milliliters of the adjusted urine was pipetted into a 50-ml. round-bottom centrifuge tube, saturated with sodium chloride (approximately 4 gm.), and mixed for 1 min. on a vortex mixer. After 10 ml. of ethyl acetate was added, the solution was mixed again on the mixer for 1 min. and centrifuged at 2500 rpm for 5 min. The organic phase (upper) was transferred to a 50-ml. conical centrifuge tube. The extraction was repeated with 2 subsequent 10-ml. volumes of ethyl acetate combining all 3 aliquots in the 50-ml. centrifuge tube. The ethyl acetate was evaporated by placing the centrifuge tube in a 60° water bath. A gentle stream of air facilitated evaporation. The residue was resuspended in 1.0 ml. of ethyl acetate. The ethyl acetate extract mixture was divided into 5 aliquots by distributing 0.2 ml. into each of five 15-ml. centrifuge tubes labeled "U," 2, 5, 10, and 20. The tube labeled "U" was put aside for spotting, and the 0.2 ml. of ethyl acetate was evapo-
rated from the remaining 4. The residue in each was resuspended with 0.2 ml. of the 2-, 5-, 10-, and 20-mg./100 ml. standards respectively.

Chromatography

The glass-fiber sheets impregnated with silica gel were used as received from the manufacturer; no previous activation is necessary. Six spots can be conveniently placed in a line 2.5 cm. from the bottom of the sheet, with 3 cm. between spots. Spotting is facilitated by the use of the Gelman-Camag sample spotting guide and micropipet set. Ten microliters of the unknown and each of the 2-, 5-, 10-, and 20-mg. standards plus urine residue were spotted. In addition, 10 μl. of the 10-mg./100 ml. standard in ethyl acetate was spotted as a reference. The spots dried rapidly in air. The bottom reservoir of the chromatography chamber was filled with solvent (approximately 30 ml.), and the spotted sheet was placed in the chamber, with the bottom edge immersed in the solvent trough. The chamber was covered with the glass plate and tilted at a slight angle from the vertical. The solvent system was allowed to migrate for a distance of 12 cm. Migration takes approximately 12 min. at 25°. At the end of the period of migration, the sheet was removed from the chamber and dried in air.

Color Development

The thin-layer sheet was sprayed with freshly prepared diazotized p-nitroaniline. Color development was immediate, reaching a peak in 2–3 min. It then rapidly began to fade. It was suitable for quantitation for approximately 30 min.

Quantitation

Semiquantitation was made by visual inspection, by comparing the VMA spot in the unknown with the VMA spots in the 2-, 5-, 10-, and 20-mg. standards. For densitometry, a horizontal strip 5 mm. wide was cut from the thin-layer sheet to include all 6 VMA spots. The strips were scanned with the Joyce-Loebl Chromoscan (National Instrument) equipped with a digital readout integrator reading in absorbance values. A green (535-mu.) filter and 0.5-mm. slit were used. Scanning was done by reflectance. Concentration of VMA was proportional to absorbance in the 0- to 20-mg. range. A linear calibration curve can be constructed (Fig. 1).

The concentration of VMA in the unknown was calculated as follows:

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\frac{\text{No. pips under U peak}}{\text{No. pips under 5-mg. std peak}} \times 5 = \text{VMA in mg./L.} \tag{1}
\]
Recovery was ascertained by:

\[
\frac{\text{No. pips under internal std peak}}{\text{No. pips under 5-mg. std peak}} \times 5 = \text{Added VMA + U-VMA in mg./L.}
\] (2)

Routinely, comparison of the unknown with the 5-mg. standard was sufficient for quantitation. The internal standard made quantitation more precise in the extremely low range (0.5–2.0 mg./L.), in which case Formula 2 was applied. Internal standards were most useful in the visual semiquantitation. Figure 2 shows a typical scan from a normal subject. The calculated value was 2 mg./L.

**Results**

VMA is clearly separated from the other phenolic acids recovered by ethyl acetate extraction. VMA is identified as a distinct purple spot...
with an Rf of 0.62; o-hydroxyphenylacetic acid migrates ahead of VMA as a rose color. p-Hydroxybenzoic acid can be identified as an orange red spot ahead of the VMA. 5-Hydroxyindoleacetic acid migrates just behind the VMA as a purple brown spot. The color and Rf of the VMA are specific; it can be easily identified by referring to the 10-mg. VMA and ethyl acetate standard.

A total of 25 random normal urines were studied. The average excretion was 2 mg./L. of urine, with a range of 0.5–4.0. There were no false positives noted in the random normals or in patients receiving salicylates, corticosteroid, all diuretics, and a variety of antibiotics. Large intake of coffee, tea, or chocolate prior to urine collection did not interfere. Recovery was 95–100% in urine specimens to which 10 mg. of VMA was added. Recovery was 85–90% in urine specimens to which 20 mg. of VMA was added. There was a high degree of correlation between the visual semiquantitative estimation of VMA and the strip scanning. Normal urine specimens had a barely discernible purple spot at the VMA Rf, while the 2-mg. standard contains a distinct purple spot. Many normal specimens were equal to the 2-mg. standard, but none as dark as the 5 mg. standard spot.

Discussion

Silica-gel-impregnated glass fiber proves to be a highly satisfactory support medium for the thin-layer chromatographic separation of VMA in urine. It lends itself to precise identification and quantitation of VMA. Although quite similar to conventional silica-gel-coated glass technics, the thin-layer solvent systems reported by McGregor (13), viz., isopropanol, 95% ethanol, and 0.1N NH₄OH (5:1:1), and Annino (12), in which n-butanol, acetic acid, and water (9:2:4) were used, showed no separation of VMA.

The normal range of 0–4 mg./L. compares with that reported by McGregor (13) using silica-gel-coated glass. Although precise quantitation is simple with adequate strip-scanning equipment, it does not seem necessary in the ordinary clinical situation. One of the unique values of this procedure is the ability easily to separate normal from abnormal quantities of VMA by visual inspection. The quantitation by strip scanning added little to the evaluation of the stained strips.

Another distinct advantage of this technic is the lack of interference by certain common dietary products, e.g., coffee, chocolate, and many drugs such as salicylates and antibiotics. A commercial kit for VMA assay (Pheoset) has been in continuous use in this laboratory for the past 4 years. In a recent unselected series of 62 examination, 15 gave
falsely elevated values by this method. The interfering chromagens were identified in each case by 2-dimensional paper chromatography, using the technic of Armstrong (14). The most common interfering substances were coffee and aspirin.

The major advantages of silica-gel-impregnated glass fiber over conventional support mediums are the ease in handling, the facility with which strips can be cut and prepared for strip scanning, and the rapidity of the chromatographic portion of the assay. Solvent ascension in this system takes from 10 to 15 min. as compared to a period as long as 2 hr. with conventional thin-layer methods. In addition, activation by heat has not been necessary.

This procedure lends itself well to the routine screening of hypertensive patients for the catechol-excreting tumor. The technic is applicable in almost any routine clinical chemistry laboratory.

References


