A quantitative assay for vanillylmandelic acid (VMA) by gas-liquid chromatography / Sherwin Wilk From the Department of Medicine, the Mount Sinai Hospital, New York, New York and the Department of Chemistry, Fordham University, New York, N.Y. Stanley E. Gitlow and Milton Mendlowitz From the Department of Medicine, The Mount Sinai Hospital, New York, New York Morton J. Franklin and Herman E. Carr From the Department of Psychiatry, Boston University, School of Medicine, Boston, Massachusetts and Donald D. Clarke From the Department of Chemistry, Fordham University, New York, New York
A Quantitative Assay for Vanillylmandelic Acid (VMA) by Gas-Liquid Chromatography

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Until the discovery of the major product of catecholamine metabolism, vanillylmandelic acid (VMA), by Armstrong in 1957 (1), biochemical studies of epinephrine and norepinephrine metabolism were dependent upon determination of the small quantities of these materials excreted unchanged in the urine. The methods designed to measure VMA have proved adequate to detect the gross abnormalities in metabolism usually associated with catecholamine-producing tumors (2-7), but more exact procedures are required for quantitative metabolic studies. Present spectrophotometric and electrophoretic methods are nonspecific in that they yield values for VMA that are higher than those obtained by chromatographic and isotope dilution techniques (5). Although the two-dimensional paper chromatographic system (8, 9) is a reliable quantitative method,

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VMA BY GL CHROMATOGRAPHY

it is cumbersome and yields somewhat variable results. The isotope dilution technique of Weise et al. (10) is probably the most precise method available, but it is laborious, and its application complex for metabolic studies using labeled VMA or its precursors.

The methods of gas-liquid chromatography (GLC) were first applied to this problem by Williams et al. (11–13). The detectors used in their studies, however, were relatively insensitive to the derivatives used.

The electron capture detector was shown to possess exceptional sensitivity for molecules containing electronegative groups (14). Applying this apparatus, nanogram amounts of halogenated derivatives of some biologically important amines were shown by the authors (15) to be detectable. It was, therefore, decided to use the electron capture detector in conjunction with a halogenated VMA derivative in order to formulate an assay procedure that would possess sensitivity adequate for detection of VMA in small volumes of urine.

The cleaving of VMA to vanillin (4, 6, 7) followed by extraction of the vanillin into toluene (7) could solve two basic chromatographic problems. The poorly volatile carboxyl group could be removed, and the resulting toluene extract would be free of interfering background material. To complete the assay, the O-trifluoroacetyl derivative of vanillin, a compound with excellent gas chromatographic properties, could be formed by exposure of the extract to trifluoroacetic anhydride.

METHODS

An arbitrary urine volume containing 3 mg creatinine is placed in a 15-ml ground-glass stoppered centrifuge tube. The urine is saturated with NaCl, acidified with 0.1 vol 3 N HCl and extracted successively with 2, 1, and 1 vol ethyl acetate. The combined ethyl acetate extracts are transferred to a second 15-ml ground-glass stoppered centrifuge tube, reextracted with 1 ml 1 M K₂CO₃, and quantitatively transferred to a 40 ml ground glass stoppered test tube. The VMA is cleaved to vanillin with 0.2 ml 2% NaIO₄ at 50°C for 30 min in a water bath. The tube is then removed and cooled and the K₂CO₃ neutralized with 0.4 ml 5 N acetic acid and 0.6 ml pH 6.2 phosphate buffer (16).

The vanillin is extracted by shaking with 20 ml redistilled toluene for 3 min and the aqueous layer is carefully removed and discarded. The toluene is transferred to a 100-ml round-bottom flask and evaporated to dryness with a flash evaporator. The residual vanillin from the round-bottom flask is transferred to a small screw-cap vial by two successive 1-ml portions of ethyl acetate that has been redistilled over P₂O₅. Finally 0.5 ml trifluoroacetic anhydride (Eastman Organic Chemicals) is added.

²Conditions from this point on must be anhydrous.
The vial is tightly capped and allowed to stand at room temperature for 1 hr. The contents of the vial are then dried under vacuum in a desiccator at room temperature. The derivative is taken up in 1.0 ml redistilled ethyl acetate for chromatography.

A standard can be prepared by placing exactly 1 mg vanillin in a small screw-cap vial, adding 1 ml redistilled ethyl acetate and 0.5 ml trifluoroacetic anhydride, and allowing the mixture to stand for 1 hr at room temperature. After this period of time the contents of the vial are evaporated to dryness under dry N₂ and taken up in 1 ml redistilled ethyl acetate, and 0.1 ml of this solution is diluted to 10 ml with ethyl acetate in a volumetric flask. Injections of dilutions of this standard serve as a check on linearity, and establish the retention time of the derivative under any given set of operating conditions. A standard of trifluoroacetylated p-hydroxybenzaldehyde can be similarly prepared.

Separations are effected on a 6 ft × 4 mm o.d. coiled glass column packed with either 3 or 6% QF-1 coated on Anakrom ABS 60/70 mesh (Analabs). Column temperature is maintained at 155°C, and nitrogen flow is adjusted to 30 ml/min. The meter range is set at 1 × 10⁻⁹ amp, with the high voltage setting at 75 volts on a Packard model 7508 gas chromatograph. Usually a constant volume of 1 μl is injected. A parallel sample containing an added 5 μg VMA per urine aliquot is run through the procedure together with the urine sample to be assayed. A standard curve may be constructed by carrying aliquots of a urine containing increasing amounts of added VMA through the entire procedure. The VMA content of urine samples of 21 normal subjects was determined by this technique.

RESULTS

Recovery studies were performed using 7H₃ VMA. Recovery of vanillin in the toluene layer was 71.8 ± 2.5% (S.D.) and recovery through the entire procedure was 52.0 ± 5.1% (S.D.). A standard prepared by the trifluoroacetylation of vanillin at 4°C was found to yield a single sharp symmetrical peak with a retention time of 2.5 min on the 3% QF-1 column, and a retention time of 4.5 min on the 6% QF-1 column. Preparation at approximately 27°C at times led to the formation of a second peak with a retention time (Rₜ) of 0.87 relative to the first. A standard prepared by the trifluoroacetylation of p-hydroxybenzaldehyde yielded a single sharp symmetrical peak with an Rₜ of 0.47 of the VMA derivative on both the 3 and the 6% QF-1 columns. Repeated injections of 1 μl of sample yielded no greater variability than 1%, and demonstrated that the column does not require priming. Injection of a constant volume of a series of dilutions of a standard resulted in peak heights proportional to

³Avoid conditions of elevated temperatures and high humidity.
the standard concentrations. Such linearity could not be demonstrated with injections of increasing volumes of a single standard.

Blanks in which no trifluoroacetylation was performed yielded no peaks. Blanks formed by carrying water through the entire procedure yielded several peaks, none of which interfered with the VMA peak. When aliquots of a urine containing increasing amounts of added VMA were carried through the entire procedure, the VMA derivative peak height increased in proportion to the amount of VMA added. A standard curve was constructed from these values (Fig. 1). Reproducibility deter-

![Graph](image)

**Fig. 1.** Relation between detector response and peak height of a series of internal standards.

mined by carrying two groups of seven identical samples through the procedure was found to be 10.5%. Using the specific assay conditions described, it was found that 0.2 μg VMA/urine aliquot (or less than 0.1 μg/mg creatinine) could be detected, and that one could discriminate between samples whose VMA content differed by as little as 10%.

A sample of four chromatograms, the first derived from a sample known to contain elevated amounts of VMA, and the last three from normal urine specimens is shown in Fig. 2. The mean VMA excretion of 21 normal subjects was 1.6 μg/mg creatinine (range = 0.3–3.4).

**DISCUSSION**

The trifluoroacetylation of vanillin must be done under anhydrous conditions, special care being taken that the vanillin-containing toluene layer be free of water before evaporation. If a water aspirator is used,
one must use caution to avoid "backing-up" even of minute amounts of water. An added precaution was taken in using redistilled toluene, since evaporation of 20 ml toluene can concentrate impurities that may conceivably react with the anhydride, producing undesired peaks. The most satisfactory solvent for the trifluoroacetylation reaction was found to be redistilled ethyl acetate. Initial work using tetrahydrofuran as solvent led to the formation of a blank peak with retention time identical to that of the VMA derivative. The purity of all reagents should be checked by carrying a water blank through the procedure. The extreme reactivity of the anhydride makes it unnecessary to use a catalyst. No interfering peaks have been found using the reagents suggested. The 6% column afforded total separation of the VMA derivative peak in all urine samples tested. The 3% column gave more rapid results but occasionally incomplete separation. With a GLC instrument containing a dual column oven, operation of both the 3 and the 6% columns was achieved simultaneously.

Under the given set of operating conditions, the O-trifluoroacetyl derivative of vanillin has a mass response of approximately $180 \text{ mm}^2/0.01 \mu g$. This response is about 350 times greater than the methyl-3,4-dimethoxymandelate derivative employed by Williams and Greer (17) and detected by argon ionization. It is also about 300 times greater than unreacted vanillin using electron capture detection. At the operable setting of $3 \times 10^{-10}$ amps, less than one nanogram of derivative is easily seen. The selection of a urine sample containing 3 mg creatinine together with an internal standard of 5 $\mu g$ VMA is purely arbitrary. The final extract can be taken up in a smaller volume than the selected 1 ml of ethyl acetate, and larger amounts than 1 $\mu l$ can be applied to the column. Moreover, one can also operate at the greater sensitivity setting of
$3 \times 10^{-10}$ amp although linearity would have to be demonstrated here. If we take all these factors into consideration, it is perfectly feasible that a modification of the given procedure may be used for the measurement of VMA in biological tissue possessing extremely low concentrations of this substance.

VMA added to urine in varying amounts and carried through the procedure was found to increase the peak height of the VMA derivative in a manner directly proportional to the amount of added VMA. This points to a constant recovery in each sample and a detector response which is linearly proportional to the amount of sample delivered. A curve constructed from these values (Fig. 1) may be used to quantitate unknown urine specimens, but, since detector sensitivity may easily change with use and operating conditions, it is better to assay a duplicate unknown to which VMA is added.

Recovery of VMA as vanillin in the toluene extract is almost identical with results obtained by Connelian and Godfrey (16). The additional losses occur mainly in the final step of the procedure, which requires the removal of unreacted anhydride. The loss of the volatile derivative constitutes the major source of variability in the procedure. Best results were obtained by evaporation under vacuum in a desiccator at room temperature. In this manner a large number of samples may be treated simultaneously, exposed to an identical external environment, and have no possibility of interaction with atmospheric water vapor.

Nuclear magnetic resonance studies were performed on pure vanillin and on a mixture containing about three-fourths derivative and one-fourth vanillin. The NMR spectrum of $O$-trifluoroacetylvanillin was similar to that of vanillin (18). It was evident that the aldehyde group of vanillin was not acetylated in this reaction (Fig. 3), as this derivative showed the presence of an aldehyde proton which was shifted 11 cps to lower field as compared to the aldehyde proton of vanillin.

Trifluoroacetylation at $27^\circ C$ under humid conditions was found at

![Chemical Structures](attachment:image.png)

Fig. 3. Temperature dependence of acetylation of phenolic aldehydes.
times to produce a second peak of retention time of 0.87 relative to the single VMA peak usually obtained. Malkin and Nierenstein (19) have noted that acetylation of phenolic aldehydes at elevated temperatures yields the fully acetylated product. If salicylic aldehyde is warmed for a few minutes with acetic anhydride and anhydrous K₂CO₃, only the 2-acetoxybenzilidine diaacetate is obtained, whereas running the reaction at low temperatures leads to the formation of 2-acetoxybenzaldehyde only (Fig. 4). The fully trifluoroacetylated form of vanillin would be expected to be less stable than the O-trifluoroacetyl form. If a mixture of the two is produced by trifluoroacetylation of vanillin under conditions of elevated temperatures and high humidity and allowed to stand at room temperature, a gradual diminishing of the second peak is observed concomitant with an increase in the height of the O-trifluoroacetylvanillin peak. The fully trifluoroacetylated form disappears completely after 24 hr, whereas O-trifluoroacetylvanillin has been found to be stable for several weeks.

The values for normal VMA excretion obtained in this study agreed favorably with those obtained by Gitlow et al. (5), Weise et al. (10), and Williams and Greer (17). p-Hydroxymandelic acid, which is cleaved by periodate to form p-hydroxybenzaldehyde, and which may constitute a 10% interference in the spectrophotometric assay for vanillin if excreted in amounts equal to VMA (2), is well separated from the derivative of VMA in this assay. As was previously noted, standard trifluoroacetylated p-hydroxybenzaldehyde has a retention time of 0.47 relative to the VMA derivative (Fig. 2). All urines tested show a peak at the retention time corresponding to O-trifluoroacetylbenzaldehyde. This procedure may, therefore, also lend itself to a quantitative assay of p-hydroxymandelic acid.

Vanden Heuvel and Horning (21) have pointed out that quantitative analytical uses of GLC are of great interest at the present time. It is

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**Fig. 4. Formation of O-trifluoroacetylvanillin from VMA.**
hoped that this paper will serve to illustrate one type of approach to the problem. Here derivative formation served a threefold purpose:

1. To increase the volatility thereby enhancing the chromatographic properties of the compound.
2. To increase the sensitivity.
3. In the process, to yield a final extract free of interfering background material.

The major importance of the present study rests in the application of GLC to the problems of a chemical assay in biological material.

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