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Revised 7 Dec. 2010 rm (Vers. 2.1) **IVD**

NAME AND INTENDED USE

This VMA ELISA kit is designed for in vitro quantitative measurement of vanillylmandelic acid (VMA) concentration in patients' urine. **For *in vitro* use only.**

BACKGROUND

Among spectrophotometric procedures the most widely used procedure was developed by Pisano et al (1) measuring vanillin directly at 360 nm. The colorimetric methods in general, however, suffer from interfering compounds and intensive labor requiring a number of manual steps. The use of paper chromatographic methods (2,3) employing diazotized p-nitroanilline and a thin layer chromatographic (TLC) procedure on silica gel (4) were reported for the analysis of extracted VMA but are considered to be of only historical interest. More recently, high performance liquid chromatography (HPLC) procedures are reported using a variety of detectors including ultraviolet light (5-7), amperometirc detection (8-11), and postcolumn reaction (12, 13). These procedures are considered to be a considerable improvement for overall specificity and sensitivity of analysis.

SUMMARY AND EXPLANATION

Catecholamines include dopamine (found mostly in the central nervous system), norepinephrine (mainly in the sympathetic nervous system) and epinephrine (mainly in the adrenal medulla). They are stored as inactive complex. Released catecholamines, having a short half-life, are taken up by sympathetic nerve endings, or metabolized by the liver and kidney and excreted.
Vanillylmandelic acid (VMA) and 4-hydroxy-3-methoxy-mandelic acid (HMMA) are the end product of both epinephrine and norepinephrine catabolism. Quantitation of the acidic metabolites has long proven to be a reliable diagnostic as well as commonly used follow-up procedure for pheochromocytoma and other catecholamine-, producing tumors (14-16). The prevalence of pheochromocytoma is 0.1% to 0.2% of hypertensive patients (17, 18). Pheochromocytomas have a long record of misdiagnosis due to the metabolic, cardia and gastrointestinal symptoms that can mimic many other diseases. Undetected or mistreated it can be fatal. However, since this is a surgically curable disease, early diagnosis by demonstration of excess VMA excretion is critically important.

PRINCIPLE OF THE ASSAY

The VMA ELISA is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the competition between VMA coated on a microtiter well and that in urine for the monoclonal antibody. Outlined steps are:

1. *Sampling and reaction:* The samples are incubated in the wells with horseradish peroxidase conjugated anti-VMA monoclonal antibody.
2. *Washing:* Unbound VMA and the antibody bound to urinary VMA are removed by washing with 0.9% NaCI solution.
3. *Enzyme Reaction (Color Development):* The amount of bound peroxidase is inversely proportional to the concentration of the VMA present in the urine sample. Upon addition of the substrate (TMB), a blue color is developed, and then it is changed to yellow by adding Stopping Solution. The intensity of this is inversely proportional to the concentration of VMA in the Calibrator or urine sample.
4. *Absorbance Detection:* After addition of Stopping Solution, absorbance is measured at 450 nm. And the readings are converted into the concentrations from the Calibration curve.
   (Refer to the schematic description of Fig. 1)

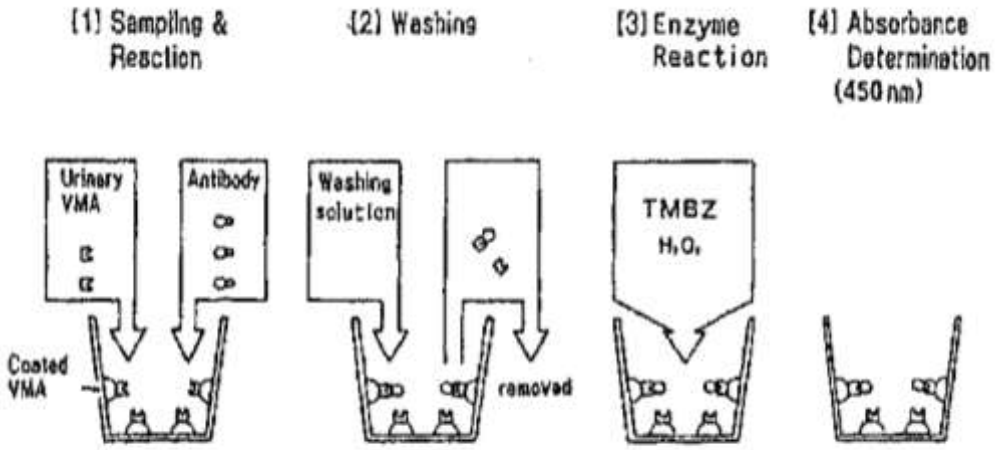

Fig. 1

MATERIALS PROVIDED IN THE KIT

1. VMA Coated **Microwell Plate**:
   1 VMA coated 96-microwell plate.
2. Anti-VMA-**Enzyme Conjugate**:
   Horseradish peroxidase conjugated to an anti-VMA monoclonal antibody, 10 ml.
3. VMA **Color Developing Reagent**:
   Tetramethylbenidine (TMB) solution, 20 ml.
4. VMA/HVA **Stopping Solution**:
   Mixture of Sulfuric acid and Hydrochloric Acids (20 ml)
5. VMA **Calibrator Set of**:
   0, 0.0625, 0.125, 0.25, 1, 2, 4 and 8 µg/ml in phosphate buffered saline, 0.01M, pH 7.4 (1.0 ml each).

MATERIALS REQUIRED BUT NOT PROVIDED

1. Plate reader with 450 nm filter
2. pH meter of pH paper with the range of 5.0-10.0
3. Pipettor with tips for 10, 50, and 100 µl
4. Pipettor with tips for 50 and 100 µl
5. Volumetric cylinders, 10 and 100 ml
6. Volumetric and serological pipettes, 10 ml
7. Disposable test tubes or vials
8. 5N NaOH solution
9. 5N HCI solution
10. Plate washer (optional)
11. Plate shaker (optional)
12. Sodium Chloride or Saline Solution
13. 0.01M phosphate buffered saline, pH 7.4

PREPARATION FOR THE ASSAY

1. Prepare 0.01 M phosphate buffered saline, pH 7.4.
   This solution is used to dilute all unknown urine samples prior to analysis.
2. Before beginning the test, bring all urine samples and reagents to room temperature (15-30°C) and mix well.
3. Set up all reagents and urine samples before running the assay. The entire test procedure must be performed without any interruption in order to get the most reliable and consistent results.

WARNING AND PRECAUTION

1. The VMA kit is designed for *in vitro* only.
2. The components in this kit are intended for use as an integral unit.
3. The components of different lots should not be mixed.
4. Do not use the VMA Calibrators in this kit for other purposes (e.g. HPLC).
5. Use a new pipette tip for each Calibrator or urine sample to avoid cross-contamination.

STORAGE AND STABILITY

1. Store the kits at 2-8°C in refrigerator.
2. Keep microwell plates in dry bag with desiccants. Open the bag only when needed.
3. Expiration dates of the reagents are stated on their labels. Color Developing Reagent should be colorless.
4. Protect the reagents and reaction mixture from exposure to direct sunlight.

SPECIMEN COLLECTION AND HANDLING

A 24-hour urine specimen should be collected with 10 ml of 6 N HCI as a preservative.
Overnight or randomly collected urine should be acidified to a pH between 2 and 3 immediately after collection.
Record the total volume and save 1-5 ml for the analysis of VMA and total creatinine.
All samples should be refrigerated until tested. Centrifuge turbid urine samples containing crystals or sediment.

ASSAY PROCEDURE

A. Preparation of Reagents

Washing Solution:
Dissolve 9 grams of NaCI in 1 liter of deionized or distilled water. Commercially available normal saline can also be used.

B. Preparation of Samples

1. Take 1.0 ml of acidified urine and transfer to a disposable tube in which the pH of urine sample can be readjusted.

2. Bring pH of all samples within the range of 6 and 9 by stepwise addition of small amounts of 5N NaOH (e.g. 5 µl) while checking pH either with a pH meter or using pH paper.
3. Dilute pH re-adjusted samples at a 1:10 ratio with phosphate buffered saline. The pH for diluted samples should be between 7.0 and 8.0.

C. Standard Procedure for the Assay

1. Make work sheet with Calibrators and sample identification.

VMA	Calibrator		Sample		Sample		Sample		Sample		Sample	
	1	2	3	4	5	6	7	8	9	10	11	12
A	0	0	QC1	QC1								
B	0.0625	0.0625	QC2	QC2								
C	0.125	0.125	U1	U1								
D	0.25	0.25	U2	U2								
E	1	1	U3	U3								
F	2	2										
G	4	4										
H	8	8										

2. *Sampling:*
   a. Dispense 50 µl of VMA Calibrators into appropriately designated wells
   b. Dispense 50 µl diluted (1:10) controls or samples to respective wells.

3. *Addition of the Anti-VMA-Enzyme Conjugate:*
   Dispense 50 µl of Anti-VMA-Enzyme Conjugate to each well, using a pipettor.

4. *Antigen-Antibody Reaction:*
   Mix the plate by moving it back and forth slow horizontal movements for a minute. A plate shaker can be used for this purpose also. Allow the plate to stand at 15-30°C, room temperature for 1 hour.

5. *Washing:*
   Wash only once. Removing incubation mixture by decanting the plate into a sink and blotting the plate on absorbent paper.
   Dispense 300 µl of normal saline into each well. Remove saline by decanting the plate and blotting it on absorbent paper. This also can be done by a plate washer.

6. *Enzyme Reaction:*
   Dispense 100 µl of VMA/HVA Color Developing Reagent to the well and allow it to stand at 15-30°C, room temperature for 25 minutes.

7. *Stopping Color Development:*
   Dispense 100 µl of VMA/HVA Stopping Solution to the wells.

8. *Absorbance Measurement:*
   Any microwell reader capable of detecting absorbance at 450nm may be used.

PROCEDURE SUMMARY FLOW DIAGRAM:

MICROWELL PLATE	
⇒ VMA Calibrator, or Control, or Sample, **50 µl**	
⇒Enzyme-Conjugated anti-VMA antibody solution, **50 µl** **(Shake for 1 minute)**	
Antigen-Antibody Reaction	
(At 20-30°C, Room Temp. for 1 hour.)	
WASH	Once with Normal Saline, **300 µl**
⇒ VMA/HVA Color Developing Reagent, **100 µl**	
Enzyme Reaction	
(At 20 – 30°C for 25 minutes)	
⇒ VMA/HVA Stopping Solution, **100 µl**	
READING 450 nm	
The concentration is calculated from the Standard curve.	

CALCULATION OF RESULTS

1. Using semi-log linear paper (Fig. 3) or log-logit paper (fig. 4) the Calibration curve is generated by plotting VMA concentrations on the abscissa and the absorbance on the ordinate. VMA concentration for each unknown sample is obtained from the Calibration curve.

For example:

Description	Absorbance (450 nm)		Average of B/Bo(%)	VMA (µg/ml)
VMA Calibrator (µg/ml)				
0	1.487	1.466	100.0	
0.0625	1.255	1.258	85.1	
0.125	1.037	1.052	70.7	
0.25	0.818	0.839	56.1	
1	0.359	0.378	25.0	
2	0.245	0.261	17.1	
4	0.145	0.145	9.8	
8	0.078	0.084	5.5	
Sample A	1.299	1.218	82.9	0.072 x 10
Sample B	0.769	0.746	51.3	0.285 x 10
Sample C	0.522	0.522	35.4	0.600 x 10

The results obtained above indicate VMA concentration in µg/ml. When the total VMA in 24-hour urine sample is required;

$$\text{VMA (µg/ml)} \times \frac{\text{urine volume (ml)}}{1000} = \text{VMA mg/24 hours}$$

or, when VMA/Creatinine value is required;

$$\text{VMA (µg/ml)} \div \frac{\text{Creatinine (mg/dl)}}{100} = \text{VMA µg/ml Creatinine} \quad \text{or} \quad \text{VMA mg/g Creatinine}$$

2. Calculation can be made with a computer set so as to draw calibration curves based on 4 coefficient log-logit.

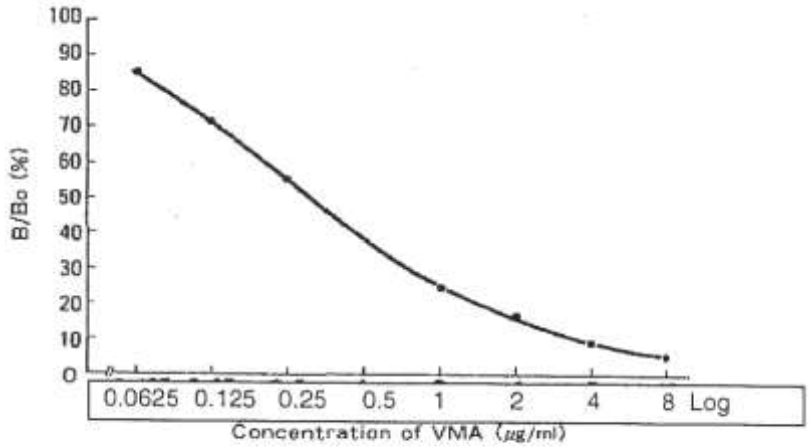

Fig.3 Calibration curve of VMA using semi-log linear paper

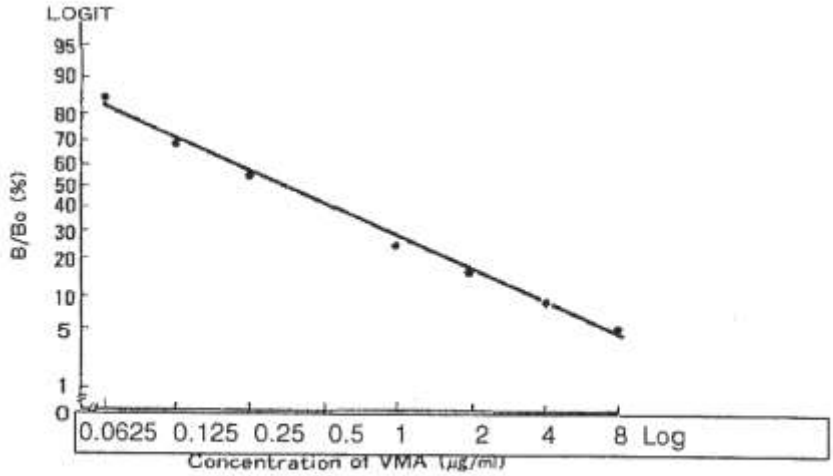

Fig.4 Calibration curve of VMA using log-logit linear paper

NOTE: This is a representative curve and should not be used to calculate results for unknown samples.

PROCEDURAL NOTE

1. It is very important to wash the microwells thoroughly, yet uniformly and remove any residual liquid from the wells to achieve optimal results.
2. Pipette Calibrators or urine samples into the bottom of each well. Vortex-mixing or shaking of wells after each pipetting is not required.
3. Absorbance is a function of the incubation time and temperature. It is, therefore, recommended to ensure the equally elapsed time for each pipetting without interruption.

LIMITATIONS

1. This VMA ELISA kit is designed for the quantitative determination of VMA in urine only.
2. All samples with VMA concentrations greater than 8 µl/ml should be repeated on much larger dilution(s), e.g. 1:20 or more.
3. Interference by Sodium Azide: As Sodium Azide inhibits the enzyme reaction, urine or any buffer used to dilute urine samples containing sodium azide as an antiseptic can not be used.

QUALITY CONTROL

In order to monitor precision of the analytical performance, it is recommended that commercially available urine control samples be included in every run.

EXPECTED VALUES

Each laboratory should determine a normal range to conform to the characteristics of the population being tested. The range given here was determined from 24-hour urine collections on 280 subjects.
Urinary creatinine was measured on Astra to assess the completeness of each collection, and mg VMA per gram creatinine was calculated.

	VMA mg/day	VMA mg/g Creatinine
Number of samples (n)	280	280
Mean Value (x)	7.92	6.87
± 2 S.D. range	0.40 – 15.44	0.00 – 14.37
Reference Range	up to 15.0	up to 14.0
	(Fig. 5)	(Fig. 6)

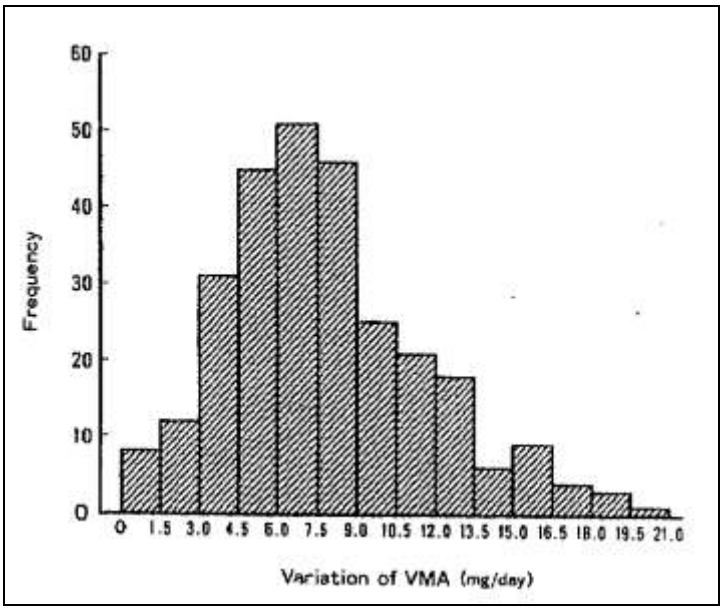

Fig. 5 Distribution of total VMA excretion per day in 280 cases of normal controls.

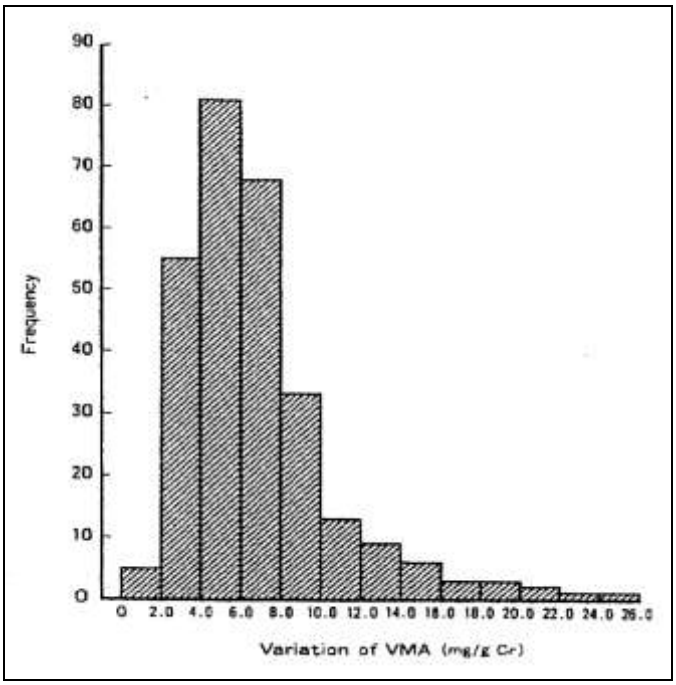

Fig. 6 Distribution of total VMA concentration per day in 280 cases of normal controls.

PERFORMANCE CHARACTERISTICS

ACCURACY

a. Recovery – Recovery studies were performed in urine samples from four healthy adults, to which VMA was added at various concentrations. Recovery was determined by dividing observed value by expected value for each prepared sample.

Sample	Baseline (µg/ml)	Added (µg/ml)	Expected Value (µg/ml)	Observed Value (µg/ml)	Recovery (%)
A	1.76	0.00			
	0.88	9.60	10.48	10.10	96
	1.32	4.80	6.12	5.92	97
	1.54	2.40	3.94	3.90	99
	1.65	1.20	2.85	2.78	98
B	6.16	0.00			
	3.08	9.60	12.68	12.85	101
	4.62	4.80	9.42	9.55	101
	5.39	2.40	7.79	8.00	102
	5.77	1.20	6.97	7.29	104
C	11.71	0.00			
	5.85	9.60	15.45	15.85	102
	8.78	4.80	13.58	18.77	101
	10.24	2.40	12.64	12.61	99
	10.98	1.20	12.18	13.30	109
D	8.43	0.00			
	4.21	9.60	13.81	15.14	109
	6.32	4.80	11.12	11.34	102
	7.37	2.40	9.77	10.34	105
	7.90	1.20	9.10	9.27	101

b. Linearity and Parallelism - Four urine samples and two commercial urine controls Lyphochek 1 and 2 (Bio-Rad) were serially diluted with phosphate buffered saline.
The ratio (B/Bo) of absorbance for each dilution (B) to the absorbance of 0 µg/ml Calibrator (Bo) was calculated and plotted on log-logit paper.

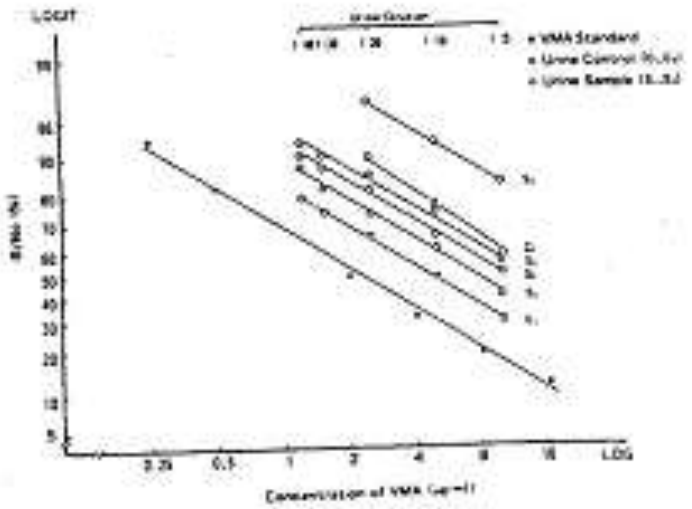

Fig.7 Linearity and parallelism test

PRECISION

a. Intra-assay coefficient of variation was evaluated in three urine samples at different VMA concentrations.

Intra-Assay	Sample A	Sample B	Sample C
N	26	24	24
Mean (µg/ml)	0.96	8.25	11.24
S.D. (µg/ml)	0.06	0.52	0.71
C.V. (%)	6.0	6.3	6.0

b. Inter-assay coefficient of variation was evaluated at three different concentrations, by analyzing the samples in 16 to 19 separate occasions.

Inter-Assay	VMA 1	VMA 2	VMA 3
N	16	19	19
Mean (µg/ml)	1.193	2.821	9.698
S.D (µg/ml)	0.086	0.232	0.686
C.V. (%)	5.7	8.2	7.1

SPECIFICITY

The following substances were tested for cross-reactivity of the assay. Cross-reactivity is expressed in terms of percentage of the concentration of each substance that produced 50% displacement.

Substance	Cross-reactivity (%) VMA
Vanillylmandelic Acid	100
Homovanillic Acid	<0.01
DL-3,4-Dihydroxymandelic Acid	4
3,4-Dihydroxyphenylacetic Acid	<0.01
Metanephrine	<0.01
Vanillylpyruvic Acid	4
Vanillic acid	<0.01
Dopamine	<0.01
5-Hydroxy-3-indolacetic Acid	<0.01
Vanillyllactic Acid	<0.01
3-methoxy-4-hydroxyphenyl Glycol	<0.01

SENSITIVITY

The sensitivity of this test is higher than 0.0625 µg/ml. The minimal detectable concentration of VMA is estimated to be 0.035 µg/ml. The minimal detectable concentration is defined as the concentration of VMA which corresponds to the absorbance that is two standard deviations from the mean absorbance of 20 determinations of zero dose VMA.

SAMPLE STABILITY

Sample stability was studied in two different urine samples at 4°C and -20°C for up to 5 days and 50 days, respectively. The results confirm that VMA is stable at 4°C storage at least for 5 days tested, and for up to 50 days tested when stored at -20°C.

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