DETERMINATION OF VANILMANDELIC ACID

by

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ABSTRACT

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Vanilmandelic acid (VMA) or 3-methoxy-4-hydroxymandelic acid is a major metabolite of epinephrine and norepinephrine. Pheochromocytomas, tumors of the chromaffin tissue of the adrenal medulla, are associated with the presence of greatly increased excretion of VMA.

The first method for determination was developed by Armstrong and co-workers in 1957 using paper chromatography. Since then, several other techniques have been described including high and low voltage electrophoresis, gas chromatography, spectrophotometry, enzymology and to a lesser degree, isotope dilution.

Spectrophotometry has become the method of choice in screening purposes because of its simplicity and because it does not require any instrumentation which is not already present in the laboratory.

Commercial kits for VMA determination are presently being marketed. These kits should reduce the time spent on each assay so that results can be reported with minimal delay. This study investigated the methodology of the VMA-Skreen, a kit procedure, and correlated this kit method to a reference method, the Pisano method. The Pisano method was also used in correlation to the method presently used by the Youngstown Hospital Association. Better correlation was observed

between the VMA-Skreen and the Pisano methods, indicating a closer relationship in methodologies.

ACKNOWLEDGEMENTS

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To all of the above, I am deeply grateful.

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LIST OF SYMBOLS

Symbol	Definition
cm	centimeters
g	grams
mg	milligrams
ml	milliliters
mM	millimolar
nm	nanometers
mg/l	milligrams per liter
mg/ml	milligrams per milliliter
rpm	revolutions per minute
ug	micrograms
ug/ml	micrograms per milliliter
ul	microliters
♀	female
♂	male
r	coefficient of correlation

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CHAPTER I

INTRODUCTION AND HISTORICAL REVIEW

Introduction

Vanilmandelic acid (VMA) also referred to as 3-methoxy-4-hydroxymandelic acid (MHMA) was first discovered as present in human urine by Armstrong, Mc Millian and Shaw in 1957, while they were working on a research grant given to them by the National Institute of Health.[1] Armstrong and co-workers, in 1956, had previously identified most of the phenolic acids in human urine by paper chromatography but it was not until 1957 that they were able to identify the compound which they had called compound 10.[2]

They were able to extract compound 10 from urine and found that many qualitative reactions of this compound were similar to those reactions of compounds containing 3-methoxy-4-hydroxyphenyl groups. Based on this and the solubility characteristics of compound 10, as indicated by its chromatographic behavior, Armstrong concluded that VMA was in fact one of the phenolic acids present in human urine and that compound 10 was VMA. The structure of VMA is shown in Figure 1.

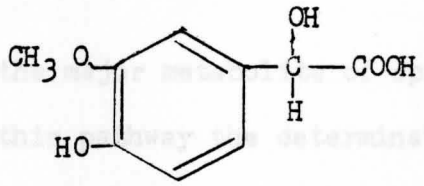


Figure 1: Vanilmandelic acid (VMA)

Concurrent with the discovery of VMA in human urine was the observation that the excretion of this acid was highly elevated in the urine of patients with pheochromocytomas. Pheochromocytomas are tumors of the chromaffin cells that secrete catecholamines and cause hypertension in humans.[3,4,5,6] In about eighty percent of the cases, these tumors are found in the adrenal medulla, but may also be found in other tissues derived from the neural crest cells. The adrenal medulla is primarily an endocrine gland, its cells producing the hormone epinephrine (adrenaline), which they secrete directly into the blood on receipt of efferent impulses via the splanchnic nerves, as a result of stressful stimuli such as fear, hunger or in response to direct stimulation by insulin, histamine or angiotensin.

Epinephrine, norepinephrine and dopamine designated as catecholamines, constitute the major hormonal secretions of the adrenal medulla. These catecholamines are synthesized from the amino acid tyrosine by a series of enzymatic steps shown in Figure 2.

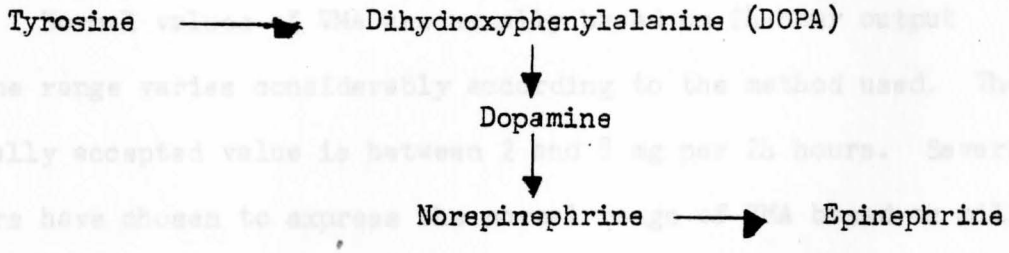


Figure 2: Biosynthesis of Epinephrine.[3]

VMA represents the major metabolite of epinephrine and norepinephrine. Based on this pathway the determination of urinary VMA has been used as a diagnostic test in patients with pheochromocytomas and in some children with neuroblastomas or ganglioneuromas.

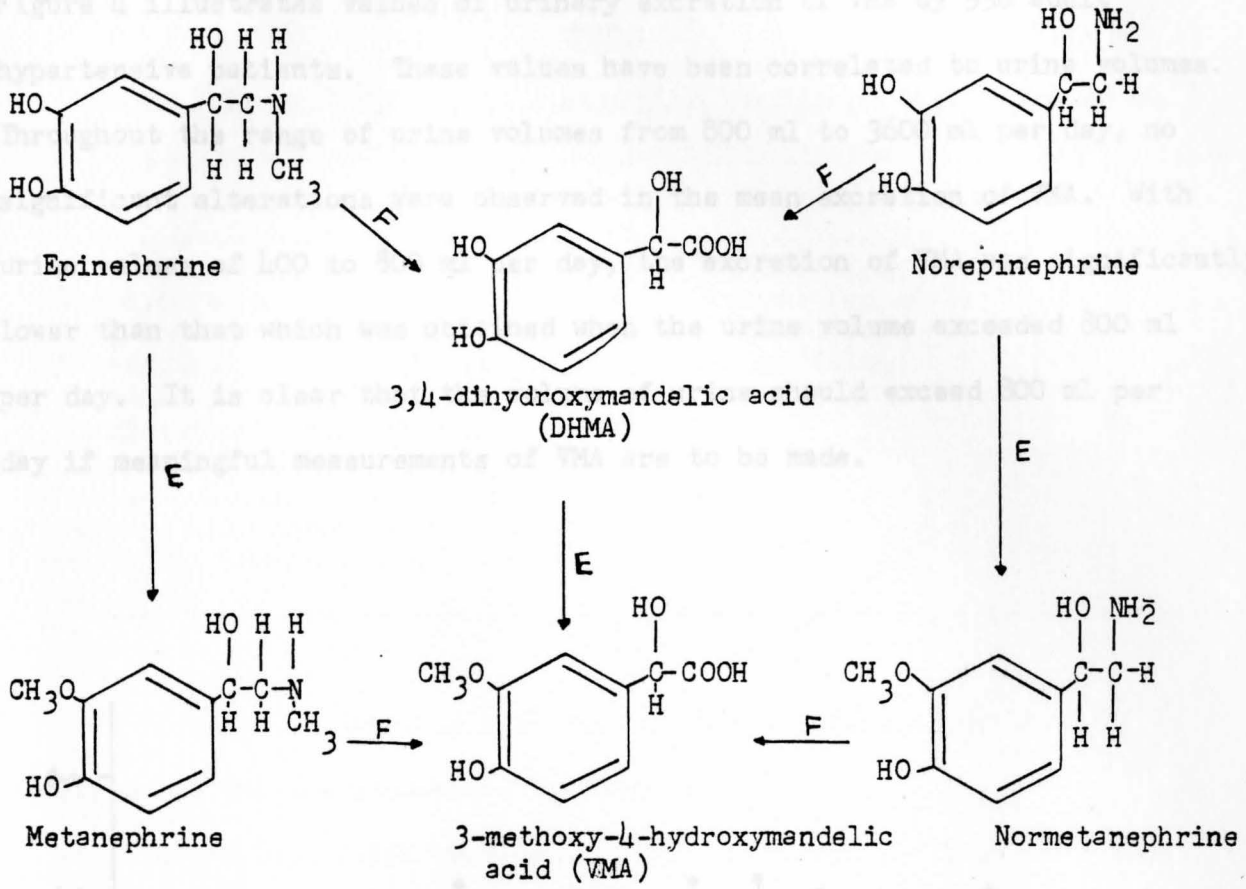
The metabolic pathway of the biosynthesis of VMA was elucidated

by Axelrod in 1959.[7] The pathway is shown in Figure 3. As can be seen from Figure 3, there are two important metabolic events for the ultimate disposition of catecholamines in the body: catechol-O-methylation and oxidative deamination. When oxidative deamination takes place first, epinephrine and norepinephrine are converted to a common metabolite, 3,4-dihydroxymandelic acid (DHMA), which is subsequently O-methylated to VMA. This sequence of reactions is quite controversial but O-methylation is considered to be quantitatively the most significant step and is followed by oxidative deamination.[8]

In pathway one, both epinephrine and norepinephrine are converted to DHMA by the enzyme monoamine oxidase (MAO). The DHMA is subsequently converted to VMA by the enzyme catechol-O-methyltransferase (COMT). In pathway two, the COMT converts epinephrine and norepinephrine to 3-O-methylepinephrine (metanephrine) and 3-O-methylnorepinephrine (normetanephrine) respectively. VMA is the end product of catecholamine metabolism regardless of the pathway taken.[7]

Normal values of VMA are usually based on 24-hour output but the range varies considerably according to the method used. The generally accepted value is between 2 and 8 mg per 24 hours. Several authors have chosen to express the normal range of VMA based on milligrams of creatinine excreted in the urine, since creatinine can be used as a check on the completeness of a 24 hour urine collection.[4,9]

Methods employed in VMA determination are usually one of several types: chromatography, electrophoresis, spectrophotometry and isotope dilution. In general there appears to be agreement in normal values among methodologies of the same type. Weise and co-workers[10] reported that a study done in 1961 revealed that the mean urinary excretion in



F = Monoamine oxidase (MAO)

E = Catechol-O-methyl transferase (COMT)

Figure 3: Metabolism of Epinephrine and Norepinephrine
(reproduced from Tietz p 806)

males exceeded that of females by approximately 0.8 mg per 24 hours. Similar studies have also substantiated the higher excretion of VMA in males over females.

Polyuria has not been accompanied by increased excretion of VMA. Figure 4 illustrates values of urinary excretion of VMA by 558 adult hypertensive patients. These values have been correlated to urine volumes. Throughout the range of urine volumes from 800 ml to 3600 ml per day, no significant alterations were observed in the mean excretion of VMA. With urine values of 400 to 800 ml per day, the excretion of VMA was significantly lower than that which was obtained when the urine volume exceeded 800 ml per day. It is clear that the volume of urine should exceed 800 ml per day if meaningful measurements of VMA are to be made.

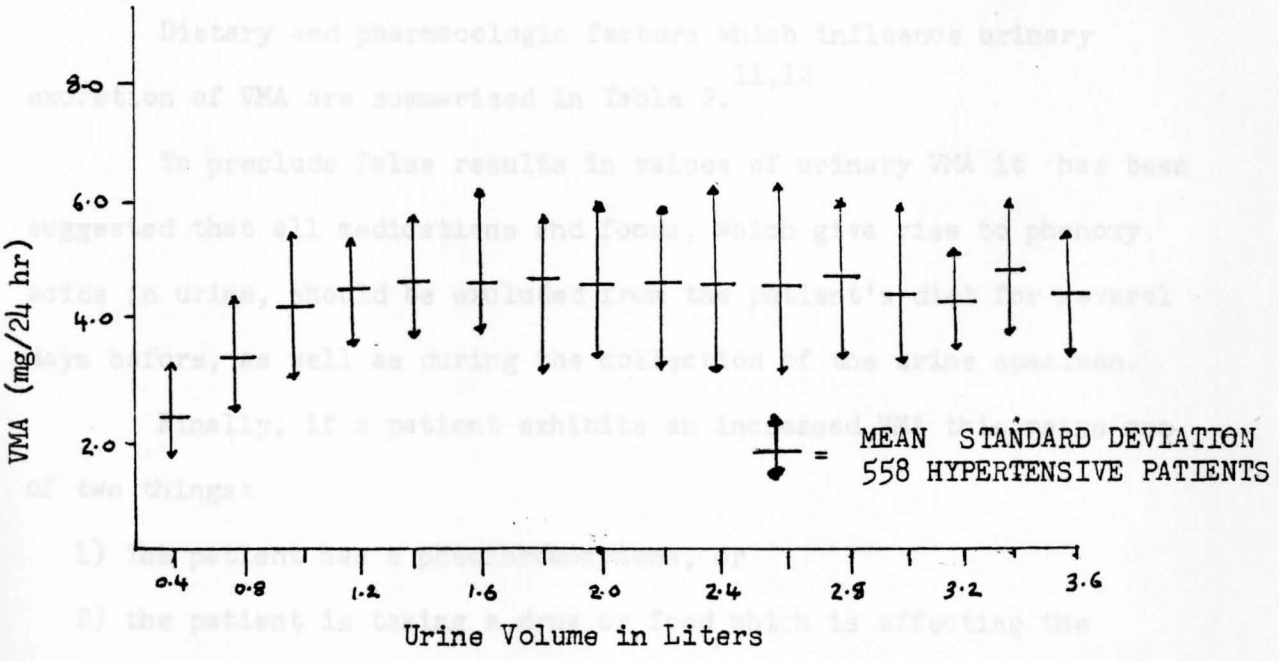


Figure 4: Influence of Urine Volume upon Excretion of VMA. [11]

VMA also exhibits diurnal variation with the peak excretion occuring during the period from 7 AM to 11 PM. Pathologic and physiologic conditions have been reported to be associated with alterations in the urinary excretion of VMA. Table 1 illustrates the conditions.

TABLE 1

PATHOLOGIC AND PHYSIOLOGIC ALTERATIONS IN URINARY EXCRETION OF VMA.[11]

Increased Excretion	Decreased Excretion
Pheochromocytomas	Familial dysautonomia
Neuroblastoma	Transection of cervical spinal cord
Ganglioneuroblastoma	Malnutrition
Parturition, surgery, burns trauma and shock	
Gravitational, thermal and hypobaric stress	
Retinoblastoma	
Carotid body tumor	
Malignant carcinoid	
Acrodynia	

Dietary and pharmacologic factors which influence urinary excretion of VMA are summarized in Table 2.[11,12]

To preclude false results in values of urinary VMA it has been suggested that all medications and foods, which give rise to phenoxy acids in urine, should be excluded from the patient's diet for several days before, as well as during the collection of the urine specimen.

Finally, if a patient exhibits an increased VMA this means one of two things:

1) The patient has a pheochromocytoma, or

2) the patient is taking a drug or food which is affecting the enzyme monoamine oxidase and thereby causing an increased VMA excretion. The assay of VMA can therefore serve as a diagnostic tool in diagnosing a

tumor, in monitoring a patient during therapy or in checking for reoccurance of another pheochromocytoma.

TABLE 2

DIETARY AND PHARMACOLOGIC ALTERATIONS IN URINARY EXCRETION OF VMA

Increased Excretion	Decreased Excretion	No Influence
Bananas	Iproniazid	Glucose
Epinephrine	Chlorpromazine	Coffee
Norepinephrine	Morphine	Citrus fruits
Reserpine	Pentobarbital	Guanethidine
Insulin shock		Meprobamate
		Amphetamine
		-methyl-dopa
		Isopropyl-
		arterenol
		Ephedrine

The normal ranges of VMA are very much dependent on the methodology used. Table 3 indicates the normal ranges based on the different methods already discussed.

TABLE 3

NORMAL RANGES OF VMA BASED UPON DIFFERENT METHODOLOGIES

Authors	Year	Method	Normal range (mg/24 hour)
Armstrong[1]	1957	Chromatography	2.0 - 4.0
Von Studnitz[24]	1960	Electrophoresis	3.2 - 6.4
Sunderman[30]	1960	Spectrophotometry	0.7 - 6.8
Pisano[28]	1961	Spectrophotometry	1.8 - 7.1
Weise[10]	1961	Isotope dilution	2.6 - 4.6
			1.7 - 3.7
Helena Super Z VMA		Electrophoresis	1.0 - 7.0
VMA-Skreen	1978	Spectrophotometry	2.0 - 10.0

The earlier methods, namely chromatography and electrophoresis, have practically been abandoned. It appears as though the trend of recent development has been to produce kits for separation of VMA

followed by quantitation by either simple electrophoresis or spectrophotometry. The kit procedures for VMA determination are relatively new so correlation studies have to be done to compare them with present accepted methods. This new procedure should be monitored closely as to its reliability in regards to its accuracy, sensitivity and specificity. Institutions have therefore been encouraged to report any results by this new method. The method is quite simple and time saving so that results can be obtained with minimum delay.

Historical Review

Eversince Armstrong and co-workers[1] identified VMA, methods for determining its presence in human urine can be placed in several broad categories: chromatography, electrophoresis, spectrophotometry and newer commercial kits. Isotope dilution and enzymatic methods have also been employed but to a lesser degree. This historical report will trace chronologically each method by citing modifications and new methodologies as reported.

Chromatography

The first method in this category was reported in 1957 by Armstrong, Mc Millian and Shaw[1]. They devised a two dimensional paper chromatographic method. The first run was done in isopropyl alcohol - aqueous ammonia - water (8 : 1 : 1) followed by benzene - propionic acid - water (2 : 2 : 1) as the second phase.

Following the lead of Armstrong et al,[1] many researchers made modifications to the original method. Robinson, Ratcliffe and Smith in 1959 used a less volatile second system of anisole - acetic acid - water (70 ; 29 ; 1).[13] This, they suggested, offered the advantage of needing minimal equilibration. Gitlow et al used paper chromatography in much the same manner as Armstrong did.[4] Jacobs and co-workers in 1961 [14] developed a system involving two separate one dimensional runs, which they said gave better separation even though it was more time consuming.

Paper chromatographic analysis needed up to 24 hours to develop. Thin-layer chromatography (TLC), however, could produce results within 2 to 4 hours so that there was a trend to develop procedures for VMA determination based on TLC. O'Neal and others modified the original

method using TLC in 1966.[15] They reported very good reproducibility and found the method to be quite time saving. Other chromatographic techniques were also devised including: gas chromatography,[16,17,18,19,20] microfiber chromatography by Preston[6], chromatography using silica-gel as support by Schmid and his colleagues in 1963.[21]

Most of the methods thus far were qualitative. In 1971 Vahidi et al[22] produced a quantitative method by paper chromatography. More recent work done by Badella et al in 1976 applied Tlc to measure VMA.[23] Brewster and co-workers in 1976 also reported a gas-liquid chromatographic technique measuring both VMA and homovanillic acid (HVA), which is a metabolite of dopamine.[17] These chromatographic techniques were most semi-quantitative and could lead to false positive results and hence unsuitable for routine clinical laboratories. However if the needed equipment was available they very well could be the method of choice.

A great drawback of the above chromatographic techniques was that the results were easily influenced by the patients diet and drug intake.

Electrophoresis

Most of the chromatographic methods previously described were not suited for routine work so that it was considered desirable to devise simpler and quicker methods. Von Studnitz and Hanson in 1959 proposed the first electrophoretic method using high voltage electrophoresis.[24] The first step was to separate the VMA from urine by extracting the VMA with ethyl acetate. The electrophoresis was conducted at 2000 volts with a a current of 100 milliamps for three hours. After the three hours, the electrophoregram was sprayed with para-nitroaniline so as to identify the VMA. Studnitz and Hanson reported normal values which were higher than those

of Armstrong's initial work.[1]

In 1960 Daniel Klein and Joseph M. Chernaik[25] reported a new paper electrophoresis method. They combined Gitlow's[4] conditions for hydrolysis and extraction of urine, with a modification of Studnitz and Hanson[24] electrophoresis and elution method. Essentially, they altered the conditions of the latter procedure by changing the electrophoresis solution to dilute acetic acid. They allowed the separation to be accomplished overnight at low constant current in a Durrum-type paper electrophoresis cell. In so doing , they were able to identify and quantitatively determine VMA.

Randrup in 1962[9] knowing that mandelic acid was an unusually strong aromatic acid with a pK value of 3.4, developed a technique based on a low pH of 3. At about this pH, the VMA would migrate towards the anode, while most of the other aromatic acids in urine would stay near the point of application. This method was in fact a modification of Studnitz and Hanson.[24] The only change was the pH of the electrophoresis buffer.

Eichhorn and Rutenberg proposed a low voltage paper electrophoresis method to determine VMA in 1962.[26] They achieved separation of VMA from all other phenolic acids in approximately 0.05 ml of untreated urine. The basis of this method was to use different concentrations of buffer for the cathode and anode compartments.

A further modification of high-voltage paper electrophoresis was submitted by George A. Herman in 1964.[5] He used cellulose acetate membranes and conventional voltage (200 to 250 volts) and altered the pH and ionic strength of the buffer. Under these conditions, Herman suggested that one may specifically identify and quantitate urinary VMA in four hours or less. Beyond 1964, the work done to develop new electrophoretic methods for the determination of VMA was only slight

modifications of the basic methods mentioned above.

Spectrophotometry

In 1959 Sandler and Ruthven proposed the first spectrophotometric method for VMA determination - as a value in the diagnosis of pheochromocytomas.[3] The principle of this method was to pass urine over a cation exchange resin, then elute the absorbed VMA with acetate buffer followed by extraction with ethyl acetate. After evaporation of the solvent, the VMA was converted to vanillin by heating with dilute sulfuric acid, using activated alumina as a catalyst. The vanillin produced was assayed spectrophotometrically with indole in the presence of ortho-phosphoric acid. Recoveries of VMA added to the urine samples averaged about 90 percent and reproducibility was within plus or minus 10 percent. They modified their initial procedure in 1961.[27] The modification entailed changing the resin from cation form to the anion form. The oxidation to vanillin was now accomplished by autoclaving with dilute acid.

In 1961, J.J. Pisano devised a new method.[28] He proposed to extract the VMA from urine by a single ethyl acetate extraction subsequently converting the VMA to vanillin by sodium periodate oxidation. Toluene was used to extract the vanillin produced during the incubation with sodium periodate. This toluene step Pisano suggested as critical since it left behind the other phenolic acids and compounds which might have been extracted by the ethyl acetate. Vanillin has a maximum absorption between 347-350 nm but due to interference from para-hydroxymandelic acid, Pisano suggested that the vanillin be determined at 360 nm where interference was practically non-existent. Good correlation was obtained when this method was compared to most chromatographic methodologies. There was widespread approval of this method of Pisano and ever since

its inception, it has been used as the method of choice in many institutions.

Most spectrophotometric methods developed after 1962 were slight modifications of the Pisano method. In 1964 Connelian and Godfrey[29] reported a time saving change to the Pisano's method. They proposed to use centrifuge tubes as extraction devices. Sunderman, in 1970, suggested the use of potassium ferricyanide as oxidizing agent and indole-phosphoric acid as the chromogen for vanillin.[30]

Several spot tests were developed to semiquantitate the VMA in urine and then further quantitate the chromogen spectrophotometrically. One such method which found wide adoption was the method suggested by Rogers and co-workers in 1972.[31] They used a drop of 1% 2,4-dinitrophenylhydrazine in 2.5N hydrochloric acid on filter paper. By adding the other reagents necessary to oxidize the VMA, they were able to semiquantitate the VMA based on comparison with standard solutions. This, they suggested be used as a screening test only.

Beyond this, many of the modifications suggested entailed the change of the chromogen used. Knight et al, in 1975, showed that the use of para-nitroaniline as the color producing agent was the most reliable.[32] The reagent, he suggested, was more selective toward possible interfering substances. This was not taken as concrete by many investigators since several authors discredit any method using para-nitroaniline because they claim that there are several other components in urine which form chromogens with para-nitroaniline.

Gumbolt submitted a modification in 1977.[33] He reduced the length of the Pisano method by eliminating several handling steps. This reduction in time was essential for rapid reporting of results.

As can be seen most of the spectrophotometric methods for VMA

determination evolved around the basic Pisano method and also the formation of a chromogen using para-nitroaniline. Many institutions still use the basic Pisano method or some modification of it. It has been taken as a reference method by many standardizing agencies.

Isotope Dilution

This method was not common because of its very involved nature. However, Weise and co-workers did develop this technique in 1961.[10] The procedure is very time consuming and not suited for routine work in a clinical laboratory. Weise reported good correlation to the methods of Armstrong,[1] Sandler and Ruthven,[27] and Studnitz and Hanson.[24]

Enzymatic

The only enzymatic approach was submitted in 1962 by Rosano et al.[34] This method used the enzyme L-mandelic acid dehydrogenase as a catalyst to convert VMA to its keto derivative. The VMA was previously extracted from urine with ethyl acetate. The change from the alpha-hydroxy acid to the keto acid produces an increase in absorbance at 350 nm. The increase in absorbance is proportional to the concentration of VMA present.

Commercial VMA Kits

This methodology is a relatively new field. The first kit to be produced was by the Hycel Company (Houston, Texas 77036). The proprietary kit, called the " Pheoset", is a technique for the rapid estimation of VMA in urine. This procedure was based upon the diazotization of VMA with para-nitroaniline. Many laboratories compared the results obtained from this kit and found poor correlation with the method which they were

using. On the basis of these studies, it appeared that the kit was quite unreliable as a measure of VMA and therefore unsatisfactory as a screening procedure for pheochromocytoma.

Helena Laboratories (Beaumont, Texas 77704) now markets a kit called, "Super Z VMA". Correlation studies with other methods are not presently available. The principle of the procedure is basically electrophoretic using a cellulose acetate plate and running the electrophoresis for 20 minutes at 200 volts.

In 1978, Brinkman Instrument Co. (Westbury, New York 11590) began marketing a kit called "VMA-Skreen". This new kit utilizes a prepared column to separate the VMA which is then determined spectrophotometrically. Brinkman Instrument Co. has reported good correlation to the method of Pisano.

CHAPTER II

STATEMENT OF PROBLEM

The present method which the Youngstown Hospital Association (YHA) uses to quantitate VMA, as an aid in the diagnosis of pheochromocytoma, is very elaborate, time consuming and has been said to exhibit a lack of reproducibility. This research will investigate several simpler, less time consuming and reproducible methods for the determination of VMA. Finally, a recommendation will be made to YHA to replace their method with a method, which based on this research shows better characteristics than their present method.

CHAPTER III

MATERIALS AND APPARATUS

Materials

VMA-Skreen kit obtained from Brinkman Instruments Co. (Westbury, NY 11590)

Super Z VMA kit obtained from Helena Laboratories (Beaumont, Texas 77704)

VMA standard for YHA procedure obtained from Youngstown Hospital Association

VMA standard for Pisano method obtained from Calbiochem (La Jolla, Ca 92037)

Apparatus

Spectrophotometer - Beckman model 26 manufactured by Beckman Instruments (Fullerton, Ca 92634). This instrument was calibrated before use. Linearity was checked and confirmed using known concentrations of potassium chromate. Stray radiation (stray light) was checked and proved to be less than 0.1%. This was done at two wavelengths, first at 370 nm, using potassium chromate (reference 40 mg/l and sample 250 mg/l) and at 220 using sodium iodide (reference 0.011 g/l, and sample 10 g/l). Wavelength calibration was done using a holonium oxide filter. The scan produced by the instrument matched that of holonium oxide.

Densitometer - Helena Laboratories Quic Scan, equipped with a 595 nm filter. Manufactured by Helena Laboratories (Beaumont, Texas 77704)

Mechanical Wrist-action Shaker- Manufactured by Burrell Corp. (Pittsburg, Pa. 15219)

Centrifuge - GLC-1 - (General Laboratory Centrifuge) capable of maintaining 2000 rpm for 5 minutes.

Waterbath - Manufactured by Precision Scientific. The waterbath was calibrated to 50 degrees C.

Reed Pipets - Manufactured by Brinkman Instruments Co. (Westbury, NY 11590) These were used to dispense several of the reagents

Other equipment used:
Vortex- for mixing and extraction
pH meter - used to pH urine samples before refrigeration

CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

VMA-Skreen Kit

VMA-Skreen is a commercial kit manufactured by Brinkman Instruments Co. (Westbury, NY 11590) for the determination of VMA in human urine. In principle it uses a prepacked column for separation of the VMA and followed subsequently with the formation of a chromogen with a diazo reagent. The absorbance of the chromogen is spectrophotometrically read at 570 nm.

The intention is to check the method using the reagents supplied with the kit, then substitute locally made reagents and observe any changes in the performance of the procedure. Substitution of locally prepared reagents will give a substantial reduction in cost both during this research and to any institution which may adopt this procedure.

The chemistry in the procedure is as follows: the VMA is extracted into 12 ml of ethyl acetate from a 2 ml acidified urine sample, pH 1 - 2, saturated with sodium chloride. The extraction takes place by vortexing for one minute.

Five ml of the ethyl acetate layer is added to 0.1 ml of glacial acetic acid. The acidified ethyl acetate aliquot is poured on to separate columns and allowed to drain. The VMA remains on the column while the other aromatic acids pass through the column. After washing the column with a 50:50 solution of ethyl acetate and ethanol, the VMA is eluted with 30 ml of distilled water. 1.0 ml of a 10% solution of sodium carbonate is added and mixed. 1.0 ml of the diazo reagent is then added

and allowed to stand for 5 minutes. At the end of this period the azo-VMA is extracted into 10 ml of ethyl acetate by vortexing each sample for a minute. Five ml of the ethyl acetate layer is then added to 0.2 ml of tetrabutylammonium hydroxide. This produces a purple color which has a optinum absorbance at 570 nm. The sequence of reactions in the diazotization is shown in Figure 5.

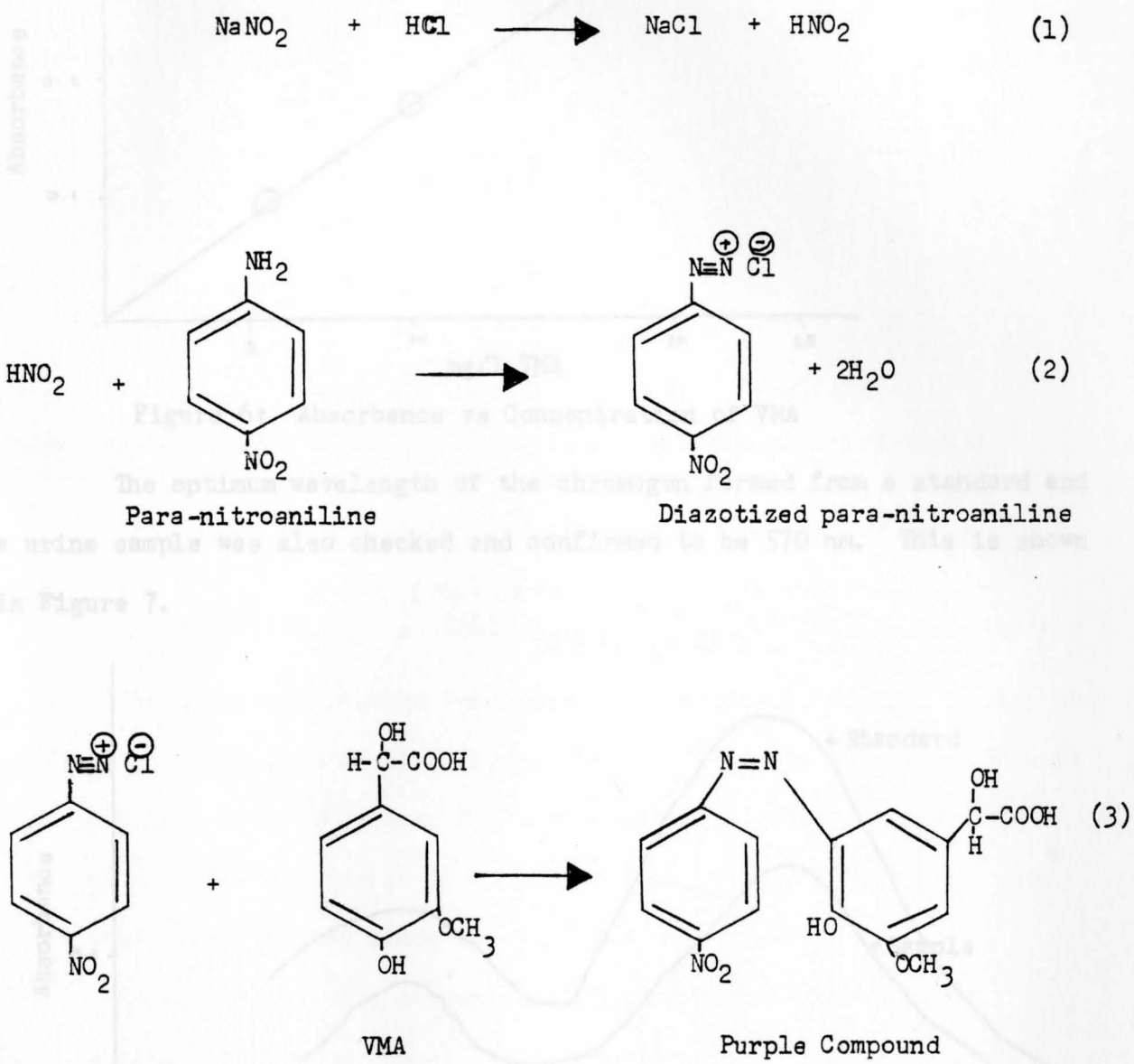


Figure 5: Formation of purple compound from Diazotization of VMA

A check for linearity in the procedure proved to conform to Beer's Law with the range of concentrations tested. The graph of absorbance vs concentration of VMA is illustrated by Figure 6

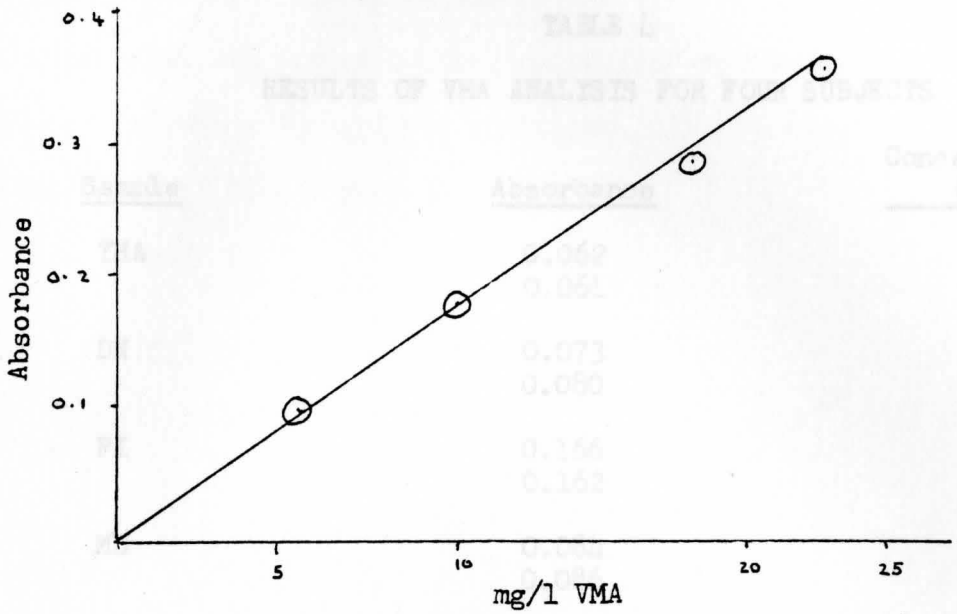


Figure 6: Absorbance vs Concentration of VMA

The optimum wavelength of the chromogen formed from a standard and a urine sample was also checked and confirmed to be 570 nm. This is shown in Figure 7.

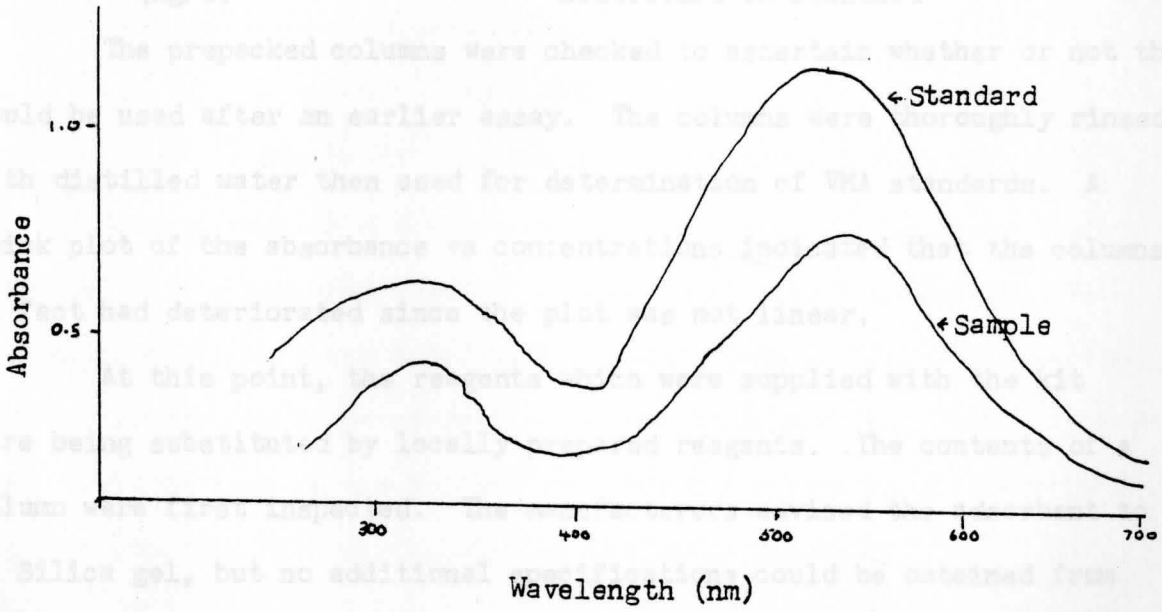


Figure 7: Absorbance vs wavelength

Duplicate assays of a single voided urine sample were analyzed using the reagents supplied with the kit. The results are presented in Table 4.

TABLE 4

RESULTS OF VMA ANALYSIS FOR FOUR SUBJECTS

Sample	Absorbance	Concentration (mg/l)
YHA	0.062	3.5
	0.061	3.5
DN	0.073	4.2
	0.080	4.6
PI	0.166	9.4
	0.162	9.3
MB	0.084	4.8
	0.086	4.9

These concentration values are based on 10 mg/l VMA standard having an absorbance of 0.175.

The values were obtained by using the following equation.

$$\text{Concentration (mg/l)} = \frac{\text{Absorbance Sample} \times \text{Concentration Standard}}{\text{Absorbance of Standard}} \qquad (4)$$

The prepacked columns were checked to ascertain whether or not they could be used after an earlier assay. The columns were thoroughly rinsed with distilled water then used for determination of VMA standards. A quick plot of the absorbance vs concentrations indicated that the columns in fact had deteriorated since the plot was not linear.

At this point, the reagents which were supplied with the kit were being substituted by locally prepared reagents. The contents of a column were first inspected. The manufacturers advised the adsorbent to be Silica gel, but no additional specifications could be obtained from

them. Visual comparison of the kit column material with silica gel G60, distributed by Brinkman Instruments Co., under catalog number 7733, indicated that they were identical. The columns were then packed locally. Cotton plugs were placed at the bottom of each column then silica gel G60. Another plug was placed on the top of the adsorbent so as to keep it in place. A standard curve was again run. It proved to be linear in the range tested. See Figure 8.

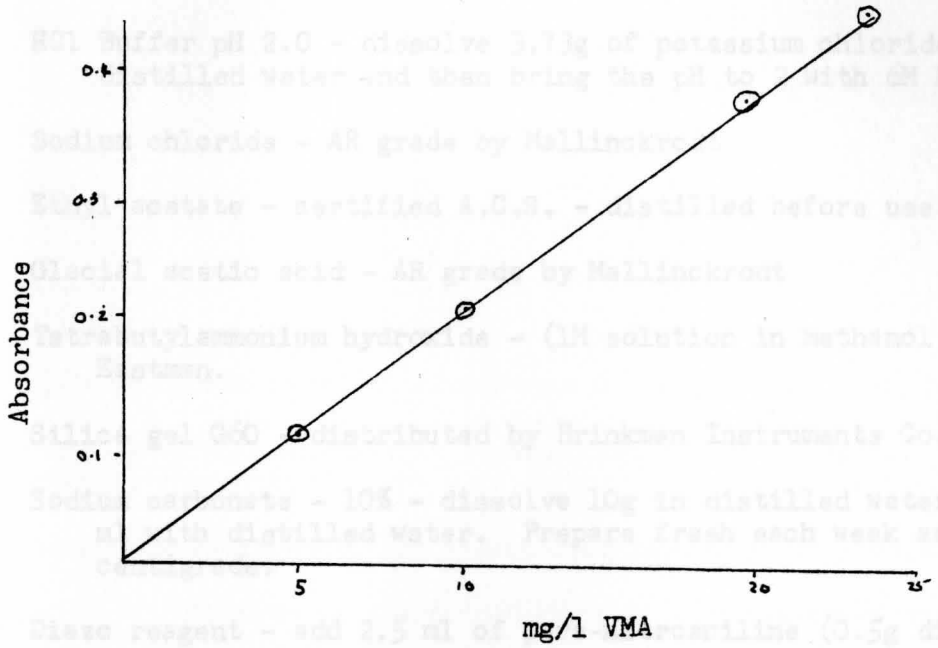


Figure 8: Absorbance vs Concentration of VMA

Several samples which were previously assayed were again assayed with the locally prepared columns. Table 5 presents the results. No remarkable difference was noted with the exception of PI. This, perhaps, could be attributed to deterioration of the urine sample. It now appeared that it was in fact silica gel G60 that was the adsorbent of the columns. The next step was the substitution of the other reagents.

TABLE 5

RESULTS OF SAMPLES USING LOCALLY PREPARED COLUMNS

Sample	Absorbance	Concentration (mg/l)
YHA	0.071	3.5
DN	0.101	5.0
PI	0.157	7.7
MB	0.115	5.7

All of the other reagents were substituted for with the exception of the VMA standard. The preparation of the other reagents is as follows:

HCl Buffer pH 2.0 - dissolve 3.73g of potassium chloride in 250 ml of distilled water and then bring the pH to 2 with 6M HCl

Sodium chloride - AR grade by Mallinckrodt

Ethyl acetate - certified A.C.S. - distilled before use

Glacial acetic acid - AR grade by Mallinckrodt

Tetrabutylammonium hydroxide - (1M solution in methanol 25.9%) produced by Eastman.

Silica gel G60 - distributed by Brinkman Instruments Co., catalog number 7733

Sodium carbonate - 10% - dissolve 10g in distilled water and dilute to 100 ml with distilled water. Prepare fresh each week and store at 4 degrees centigrade.

Diazo reagent - add 2.5 ml of para-nitroaniline (0.5g dissolved in 10 ml of concentrated HCl then dilute to 500 ml with distilled water) to 0.25 ml of 2% sodium nitrite (2G dissolved in distilled water and then dilute to 100 ml with distilled water). Both reagents are then diluted to 10 ml with distilled water.

Several samples were then received from Youngstown Hospital Association Laboratories. These 24-hour urine samples had not been previously assayed for VMA so that no comparison of results could be made. The results are shown in Table 6.

TABLE 6

RESULTS FOR SOME SAMPLES RECEIVED FROM YHA

Sample ID	Absorbance	Concentration (mg/l)	Volume (liters)	Concentration (mg/24 hr)
030952	0.060	3.17	1.35	4.29
14	0.035	1.85	2.80	5.16
030945	0.088	4.66	1.70	7.92
040314	0.073	3.86	1.85	7.15
(above values based on absorbance of 10 mg/l VMA = 0.189)				
045739	0.047	2.54	0.94	2.39
026976	0.115	6.22	0.46	2.86
046556	0.088	4.76	1.03	4.90
047198	0.037	2.00	1.14	2.28
040314	0.040	2.16	1.85	4.00
044149	0.076	4.11	1.41	5.79
(above values based on absorbance of 10 mg/l VMA = 0.185)				
040303	0.019	1.12	2.30	2.57
044610	0.033	1.94	3.70	7.18
030951	0.063	3.71	1.38	5.11
028177	0.075	4.41	1.38	6.09
040399	0.034	2.00	1.94	3.88
040315	0.034	2.00	1.28	2.56
(above values based on absorbance of 10 mg/l VMA = 0.170)				

This data will be used in correlation studies later on in this report.

Concentration (mg/24 hr) = Concentration (mg/l) X Volume (liters) (5)

Performance characteristics of the procedure were next assessed. Twelve specimens of the same 24 hour urine collection from EMM (volume 1760 ml) were assayed to determine the method's reporducibility and standard deviation. This data is shown in Table 7.

TABLE 7

DATA TO ASSESS REPRODUCIBILITY

Specimen	Absorbance	Concentration (mg/l)
1	0.049	5.60
2	0.040	4.58
3	0.044	5.03
4	0.049	5.60
5	0.057	6.51
6	0.060	6.86
7	0.050	5.72
8	0.055	6.28
9	0.061	6.97
10	0.066	7.55
11	0.068	7.77
12	0.065	7.43

(absorbance of 10 mg/l VMA standard which the above values are based on = 0.154 .)

Standard deviation = 1.03 mg/24 hours

Coefficient of variation = 28.1%

Average (mean excretion) = 6.33 mg/24 hours

High and low level recovery studies were also performed using samples from EMM 24 hour urine collection. For the recovery studies the absorbance of 10 mg/l VMA standard was 0.154. Table 8 illustrates the recoveries obtained.

For low level recovery samples 2 through 6 (Table 8) were doped with 5 mg/l VMA. For high level recovery samples 7 through 11 were doped with 25 mg/l VMA.

$$\% \text{ Recovery} = \frac{\text{Concentration (mg/l) of doped sample}}{\text{Concentration of sample 1 + VMA added}} \times 100 \qquad (6)$$

Average % low level recovery = 99.6%

Average % high level recovery = 105.8%

TABLE 8

DATA FOR RECOVERY STUDIES

Sample	Absorbance	Concentration (mg/l)	% Recovery
1	0.060	3.90	-
2	0.134	8.70	98
3	0.131	8.51	96
4	0.138	8.96	101
5	0.141	9.16	103
6	0.137	8.90	100
7	0.461	29.94	104
8	0.464	30.13	104
9	0.475	30.84	107
10	0.463	30.06	104
11	0.489	31.75	110

A time study was done to see whether the intensity of the chromogen changed with time. The absorbance of a sample was monitored continuously over a period of an hour in order to detect any change. The absorbance changed from 0.090 to 0.082 during this time. This does not represent any significant change so that it appears as though the purple chromogen formed is stable for at least an hour.

A urine pool was prepared for quality control studies. Thirty assays of the pool gave an average concentration of 5.92 mg/l with a standard deviation of 0.52 mg/l. The trend of the urine pool is illustrated in Figure 9. Confidence limits were established using the two standard deviation rule. These are illustrated by the dotted line on the graph.

Upper limit - 6.96 mg/l

Lower limit - 4.88 mg/l

The samples from this urine pool will be used later on to establish validity of results by this procedure for future correlation studies.

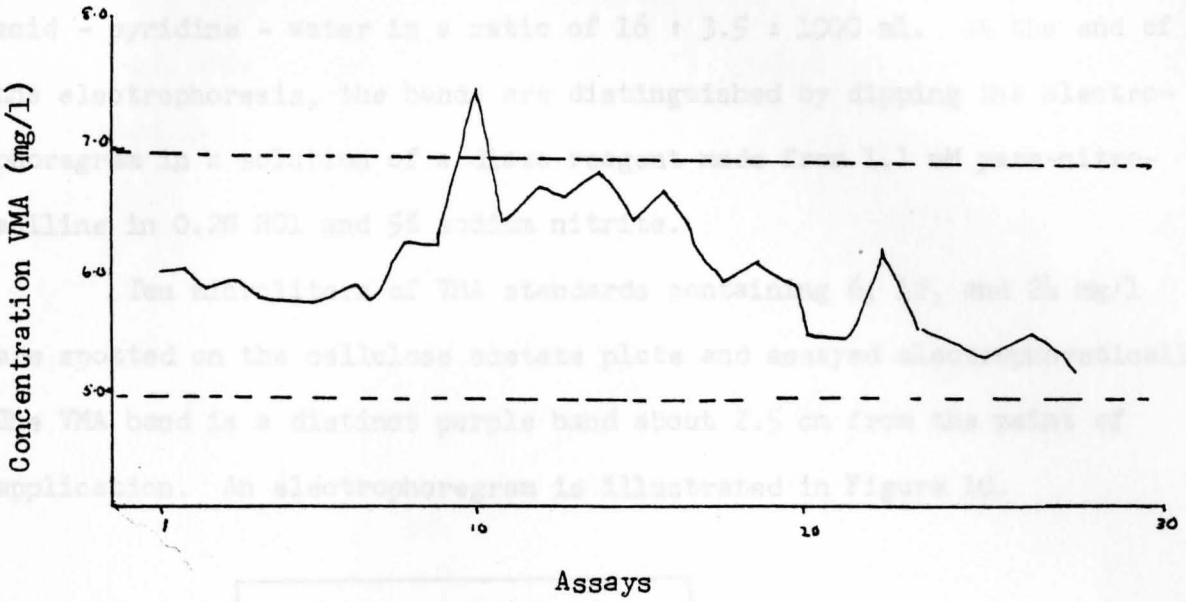


Figure 9: Concentration VMA vs Analysis of Urine Pool over a 25 Day Period

Electrophoresis

Helena Laboratories developed a kit for the determination of VMA, called "Super Z VMA". This electrophoretic method was investigated to determine its reliability. The method entails electrophoresizing on a cellulose acetate plate at 200 volts for 20 minutes using a pyridine buffer at pH 4.2. The pyridine buffer was prepared from glacial acetic acid - pyridine - water in a ratio of 16 : 3.5 : 1000 ml. At the end of the electrophoresis, the bands are distinguished by dipping the electrophoregram in a solution of a diazo reagent made from 1.1 mM para-nitroaniline in 0.2N HCl and 5% sodium nitrite.

Ten microliters of VMA standards containing 6, 12, and 24 mg/l are spotted on the cellulose acetate plate and assayed electrophoretically. The VMA band is a distinct purple band about 2.5 cm from the point of application. An electrophoregram is illustrated in Figure 10.

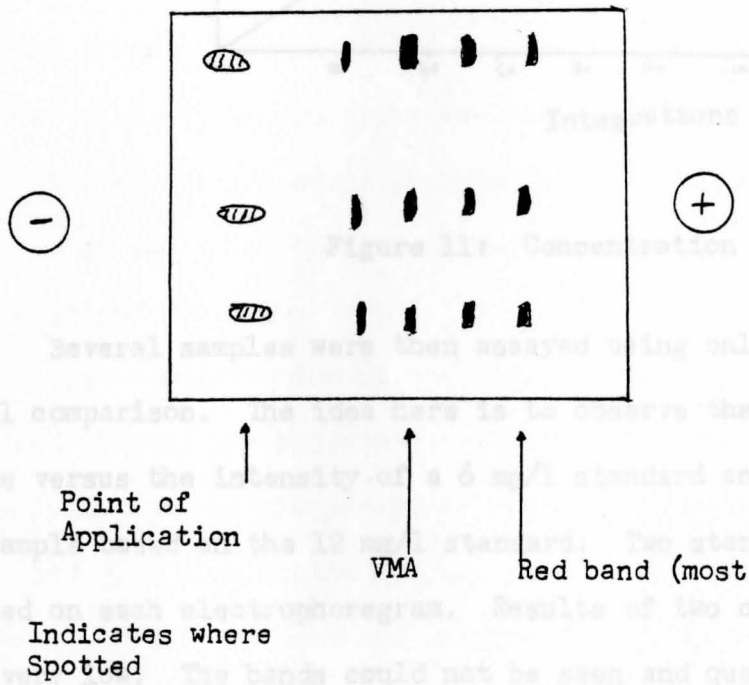


Figure 10: An Example of an Electrophoregram.

Visual comparison of the VMA bands indicates that the 24 mg band was the most intense band followed by the 12 mg band. The 6 mg band could hardly be seen by the naked eye. With the use of a Helena Laboratories Quic Scan densitometer, equipped with a 595 nm filter, a standard curve was drawn. The area under the peaks of each VMA band was calculated by counting the integrations under each peak. These integrations plotted versus concentration produced the graph shown in Figure 11. The VMA peak is the first peak on the densitomer scan. A typical densitometer scan is illustrated in Figure 12.

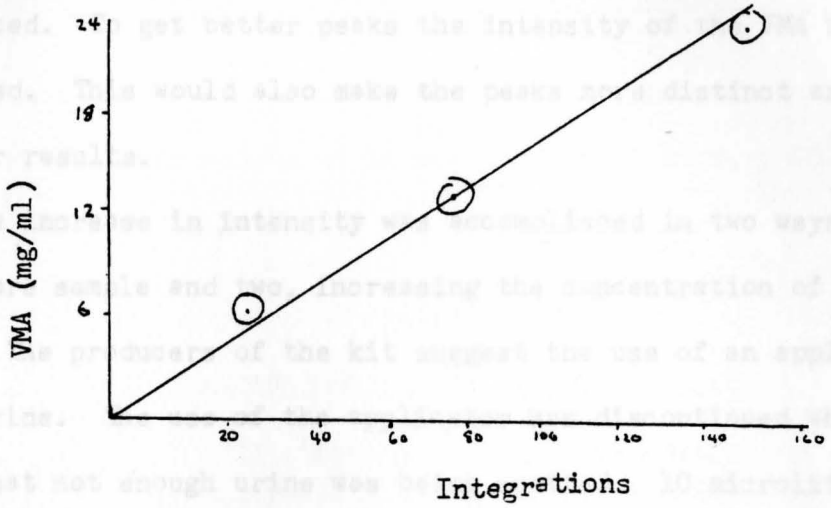


Figure 11: Concentration of VMA vs Integrations

Several samples were then assayed using only one standard for the visual comparison. The idea here is to observe the intensity of the sample versus the intensity of a 6 mg/l standard and then further quantitate the sample based on the 12 mg/l standard. Two standards were therefore spotted on each electrophoregram. Results of two of the samples assayed were very low. The bands could not be seen and quantitation was accomplished with the densitometer. The peak for this low concentration of VMA was not

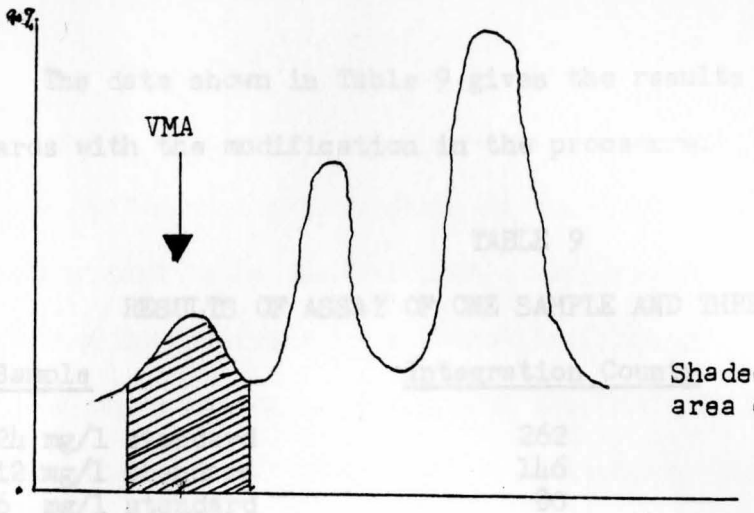


Figure 12: Typical Scan of the Electrophoregram

very prounced. To get better peaks the intensity of the VMA bands had to be increased. This would also make the peaks more distinct and eventually give better results.

The increase in intensity was accomplished in two ways: one, spotting more sample and two, increasing the concentration of the stain solution. The producers of the kit suggest the use of an applicator to spot the urine. The use of the applicator was discontinued when it was realized that not enough urine was being spotted. 10 microliters of the urine was now being spotted in intervals, making sure that the previous application had dried before another portion was added. Drying was done with a hair dryer.

The concentration of the staining solution (diazo reagent) was changed. It was now prepared as described in the VMA-Skreen method. The modification in the procedure resulted in noticeable changes. The bands could be seen easier and the peak heights of the bands could be more easily distinguished and integration of the area was easier.

The data shown in Table 9 gives the results of a sample and standards with the modification in the procedure.

TABLE 9

RESULTS OF ASSAY OF ONE SAMPLE AND THREE STANDARDS

Sample	Integration Counts	Concentration (mg/l)
24 mg/l standard	262	-
12 mg/l standard	146	-
6 mg/l standard	80	-
LA	37	3.0

$$\text{Concentration (mg/l)} = \frac{\text{Integration counts of sample} \times \text{Concentration of standard}}{\text{Integration counts of standard}} \qquad (7)$$

A check for reproducibility and recovery was next done. For recovery a single void sample of LA was doped with 10 mg/l VMA. These results are shown in Table 10.

TABLE 10

DATA FOR REPRODUCIBILITY AND RECOVERY STUDY

Sample	Integration Counts	Concentration (mg/l)	% Recovery
12 mg/l	332	-	-
LA	91	3.29	-
LA (doped)	274	9.90	74.5
(reducing the gain on the densitometer)			
12 mg/l	163	-	-
LA	42	3.09	-
LA (doped)	195	14.35	95.7
12 mg/l	100	-	-
LA	26	3.12	-
LA (doped)	122	14.64	97.6
12 mg/l	115	-	-
LA	43	4.48	-
EMM	32	3.39	-

The integration counts of the standards fluctuated slightly because of the gain on the densitometer. The gain is usually set at a maximum with the most intense band on the plate. This band does not necessarily have to be the VMA band. Good reproducibility and recovery was obtained as indicated by the data in Table 10.

Helena suggests to quantitate the VMA only for those samples with a VMA band more intense than the 6 mg/l standard based on visual comparison. This, therefore, is a quick screening method but leaves a lot to be desired in accurate diagnostic work.

Comparison of results between this electrophoretic method and any other method included in this study can not be made since this method does not rely on a 24 hour urine collection but only on a single void sample, preferably taken just after awakening in the morning.

Youngstown Hospital Association Procedure

The Youngstown Hospital Association procedure is a spectrophotometric one which depends on the reaction of diazotization of para-nitroaniline with VMA. This method is a modification of the VMA test procedure manufactured by Hycel (Houston, Texas 77036).

The principle is as follows: urine is treated with activated magnesium silicate to remove most urinary chromogens. The VMA is then extracted, along with other urinary phenolic acids, into ethyl acetate. It is then extracted from the organic solvent by aqueous sodium carbonate. These steps are done to remove further interfering substances. The VMA is then treated with diazotized para-nitroaniline to form azo-VMA.

The procedure is divided into four basic steps: namely hydrolysis, extraction, color development and sodium hydroxide washings. 10 ml of acidified urine, pH less than 3, from a 24 hour urine collection is pipetted into a 50 ml glass centrifuge tube and 1 ml of concentrated HCl is added. 2 to 3 spoonsful of magnesium silicate is added to the tube which is then shaken by a mechanical shaker for 10 minutes and then centrifuged for 5 minutes at about 2000 rpm. The pigments released during hydrolysis will be absorbed on the magnesium silicate.

The extraction step is accomplished using ethyl acetate. 2 ml of each specimen after hydrolysis (use 2% HCl as blank) is added to 50 ml centrifuge tubes containing 20 ml of ethyl acetate. The standard is a stock solution of VMA containing 10 mg/l. The VMA is extracted into the organic layer after vortexing and centrifugation. The lower layer is removed with a long tip Pasteur pipet and discarded.

2.5 ml of carbonate reagent is added and vortexed for 30 seconds then centrifuged briefly. The upper layer is then aspirated off making

sure that none of the carbonate layer is lost. 2 ml of the carbonate layer is transferred to a 15 ml glass centrifuge tube and to it is added 0.1 ml of the diazo reagent. Mixing is done by inverting the tubes. The tubes are allowed to stand for 5 minutes then 6 ml of ethyl acetate is added to each tube. Vortexing and centrifuging extracts the azo-VMA formed, into the organic layer. The bottom aqueous layer is next removed with a Pasteur pipet. 1 ml of sodium hydroxide reagent is added to each tube. A pink color should appear at this point. This sodium hydroxide wash is repeated three times. On the final wash, the sodium hydroxide reagent is allowed to sit for 5 minutes with the azo-VMA before removal.

After thorough washing, the pink solutions are poured into test tubes containing 2 ml of amino-methanol reagent. The tubes are swirled and allowed to stand for 5 minutes. The absorbance of standard and samples are then read spectrophotometrically at 530 nm against the blank.

Reagents

Magnesium silicate - (florisil) - E. M. Sargent and Co.

Carbonate reagent - dissolve 5g of sodium hydroxide and 25g sodium carbonate in distilled water and dilute to volume of 1 liter with distilled water

Sodium nitrite - AR- Mallinckrodt - dissolve 5g sodium nitrite in distilled water and dilute to 100 ml with distilled water.

Sodium hydroxide reagent - dissolve 17.6g sodium carbonate and 200g sodium hydroxide in distilled water and bring to one liter with distilled water.

Para-nitroaniline solution- 1 ml of 7mM p-nitroaniline plus 47.5 ml distilled water and 2.5 ml concentrated hydrochloric acid.

Diazo reagent- made by adding 1 ml of 5% sodium nitrite plus 8.0 ml p-nitroaniline solution - reagent must be used within one hour.

Amino-methanol reagent - 12.4% (w/w) 2-aminoethanol in methanol

VMA standard - produced by Hycel (Houston, Texas 77036)

The first parameter to be checked by this method was its linearity. A standard curve is illustrated in Figure 13. As shown standards of 5 mg/l through 25 mg/l were assayed. There is a tendency towards linearity up to a concentration of 15 mg/l beyond that the method does not conform to Beer's Law.

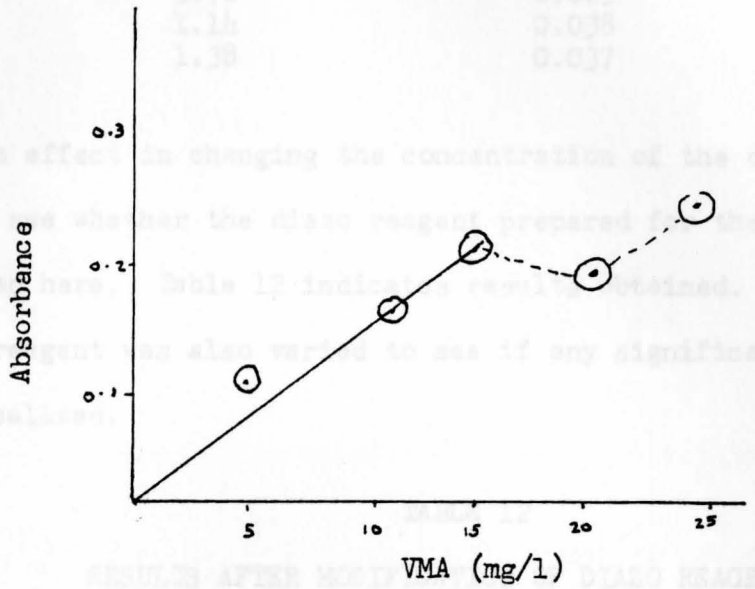


Figure 13: Absorbance vs Concentration of VMA

It has been advised that if the results of a sample exceeds 15 mg/l, a dilution should be done and the diluted sample assayed again and the value obtained be corrected by multiplying by the dilution factor.

Table 11 presents the results of several samples assayed by the VHA procedure. Results are based on a 10 mg/l VMA standard having an absorbance of 0.161.

$$\text{Concentration (mg/24 hr)} = \frac{\text{Abs. sample}}{\text{Abs. standard}} \times \text{Concentration standard} \times 1.1 \times \text{Volume (liters)} \quad (8)$$

TABLE 11

RESULTS OF SAMPLE ANALYSIS BY YHA PROCEDURE

Sample ID.	Volume (liters)	Absorbance	Concentration (mg/24 hr)
030951	1.38	0.028	2.64
046556	1.03	0.033	2.32
044149	1.41	0.044	4.24
044610	3.70	0.023	5.81
047198	1.14	0.038	2.96
028177	1.38	0.037	3.59

The effect in changing the concentration of the diazo reagent was checked to see whether the diazo reagent prepared for the VMA-Skreen could also be used here. Table 12 indicates results obtained. The volume of the diazo reagent was also varied to see if any significant difference would be realized.

TABLE 12

RESULTS AFTER MODIFICATION OF DIAZO REAGENT

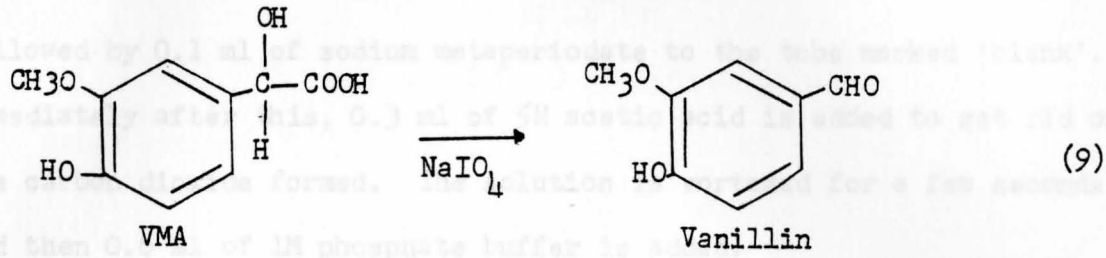
Sample ID.	Diazo Reagent (ml)	Volume (l)	Abs.	Conc. (mg/24 hr)
EMM	0.1	1.58	0.071	5.82
MB	0.1	0.74	0.109	4.19
LA	0.1	1.38	0.107	7.66
EMM	0.2	1.58	0.037	5.06
MB	0.2	0.74	0.059	3.78
LA	0.2	1.38	0.061	7.29

As can be seen from the above data no significant change resulted. The diazo reagent which was prepared for the VMA-Skreen procedure will be used in the YHA procedure from here on in this work.

Values from several other samples were measured by this method, plus values of samples received from YHA, and these will be used later on for correlation studies among the methods discussed in this study.

Pisano Method

The Pisano method is a spectrophotometric method which relies on the oxidation of VMA to vanillin. The reaction of the oxidation is illustrated by Figure 14. The vanillin formed is a measure of the VMA present in the urine.



(9)

Figure 14: Oxidation of VMA to Vanillin

The procedure is as follows: 0.2 percent of the volume of a 24 hour urine collection is placed into three 50 ml glass centrifuge tubes marked, 'test', 'internal standard' and 'blank'. For urine volumes greater than 2500 ml the working aliquot is 5 ml. To the tube marked 'internal standard', 1 ml of 10 ug/ml VMA standard is added. The volumes of all three tubes are diluted to 5.5 ml with distilled water and then acidified with 0.5 ml of 6N HCl. About 3g of sodium chloride is used to saturate the acidified urine samples.

The VMA and other phenolic acids are extracted from the urine samples into 30 ml of ethyl acetate by shaking on a mechanical shaker for 30 minutes then centrifuging for 5 minutes. 25 ml of the organic layer is transferred to a second centrifuge tube and 1.5 ml of 1M potassium carbonate is added. The tubes are then shaken for 3 minutes and centrifuged for 5 minutes. The organic layer is then aspirated off

carefully so as not to lose any of the carbonate layer. 1 ml of the carbonate layer is pipetted into a third centrifuge tube. To the tubes marked 'test' and 'internal standard' is added 0.1 ml of 2% sodium meta-periodate. All three tubes, 'test', 'internal standard' and 'blank', are loosely stoppered and placed in a 50 degree C. water bath for 30 minutes.

After the incubation, the tubes are allowed to cool to room temperature then 0.1 ml of sodium metabisulfite is added to all three tubes followed by 0.1 ml of sodium metaperiodate to the tube marked 'blank'. Immediately after this, 0.3 ml of 5N acetic acid is added to get rid of the carbon dioxide formed. The solution is vortexed for a few seconds and then 0.6 ml of 1M phosphate buffer is added.

The tubes are mixed then 20 ml of toluene is added to each tube. The vanillin formed is extracted into the toluene layer by mechanically shaking for 3 minutes. 15 ml of the organic layer is transferred to another centrifuge tube containing 4 ml of 1M potassium carbonate. The tubes are again shook for 3 minutes then centrifuged for 5 minutes. The organic layer is aspirated off and discarded. The carbonate layer of each tube is transferred to cuvets and read spectrophotometrically at 360 nm against a water blank.

Quite a bit of difficulty was experienced in this procedure initially. In order to assess the validity of the present results, an Ortho I urine control was being assayed. Several analyses were done but none appeared within the range of the urine control (2.6 ± 1.0 mg/l). The first idea was to replace the internal standard. This was done. New VMA standard was obtained from Calbiochem in La Jolla California. This change did not produce a significant improvement. Next, the angle of the arms on the mechanical shaker was changed so that the mixture inside

the centrifuge tubes would have thorough mixing. This was the answer to the problem. When Ortho I assays were done, the values now were falling within the published range of the VMA. This also proved that the shaking technique was creating enough motion necessary for a good extraction. With the new standard and better extraction technique, data for two assays of the Ortho I urine control is shown in Table 13.

TABLE 13

RESULTS OF ORTHO I BY PISANO METHOD

Urine control (1)	A_t = 0.061 A_{st} = 0.140 A_b = 0.025 Concentration = 2.4 mg/l	
Urine control (2)	A_t = 0.080 A_{st} = 0.152 A_b = 0.030 Concentration = 3.5 mg/l	

A_t = Absorbance of test sample

A_{st} = Absorbance of sample with internal standard

A_b = Absorbance of sample blank

$$\text{Concentration (mg/24 hr)} = \frac{A_t - A_b}{A_{st} - A_t} \times \frac{10 \text{ ug}}{1000} \times \frac{100}{0.2} \qquad (10)$$

$$\text{Concentration (mg/24 hr)} = \frac{A_t - A_b}{A_{st} - A_t} \times 5 \qquad (11)$$

Both of the values shown in Table 13 are within the range, so that the modifications made the extraction technique efficient and the procedure ultimately reliable for the quantitation of VMA.

This method will be taken as a reference method in the correlation studies in the next chapter. Several samples were assayed by this procedure and will be used later on.

Reagents - All reagents have to be AR grade

Potassium carbonate - 1M - dissolve 13.8g in distilled water and dilute to 100 ml with distilled water.

Sodium metaperiodate - 2% - dissolve 2g and dilute to 100 ml with distilled water

Sodium metabisulfite - 10% - dissolve 10g and dilute to 100 ml with distilled water

Acetic acid - 5N - 28.6 ml of glacial acetic acid made to volume of 100 ml with distilled water

Phosphate buffer - 1M - pH 7.5
solution 1 : Sodium phosphate dibasic - dissolve 142g in distilled water bring to volume of 1 liter with distilled water
solution 2 : potassium dihydrogen phosphate - dissolve 13.6g in distilled water and bring to volume of 100 ml with distilled water.
To prepare buffer taken 168.2 ml of solution (1) and add 31.8 ml of solution (2) and bring to pH 7.5 by adding solution (2).

VMA - stock solution (1 mg/ml)
weigh out 25 mg of VMA and dissolve and dilute to 25 ml with 0.01N HCl, in a volumetric flask. This reagent is stable for approximately three months when refrigerated.
Working standard (10 ug/ml) - dilute 1 ml of stock solution to 100 ml with 0.01N HCl. Prepare fresh before use.

Ethyl acetate - distill before use

Hydrochloric acid - 0.01N - dilute 0.83 ml of concentrated HCl to 1 liter with distilled water.

CHAPTER V

CORRELATION STUDIES AMONG THE THREE METHODS

One of the statistical tools available to investigate the degree of association between sets of data is " linear regression and correlation". To accomplish this, graphs of results of one method must be plotted vs results of the same sample by a different method. Linear regression results in the "best line" which follows to the equation $Y = aX + b$. One may also obtain the coefficient of correlation which is an indication of the " goodness to fit".

To show the degree of association, twenty-seven samples were assayed for VMA by the three methods discussed in Chapter IV. To ensure the validity of the results by the VMA-Skreen method, a quality control chart was constructed and a control urine sample from the urine pool was run each time samples were being assayed. Figure 15 shows the trend of the value of the urine control sample when assays were made.

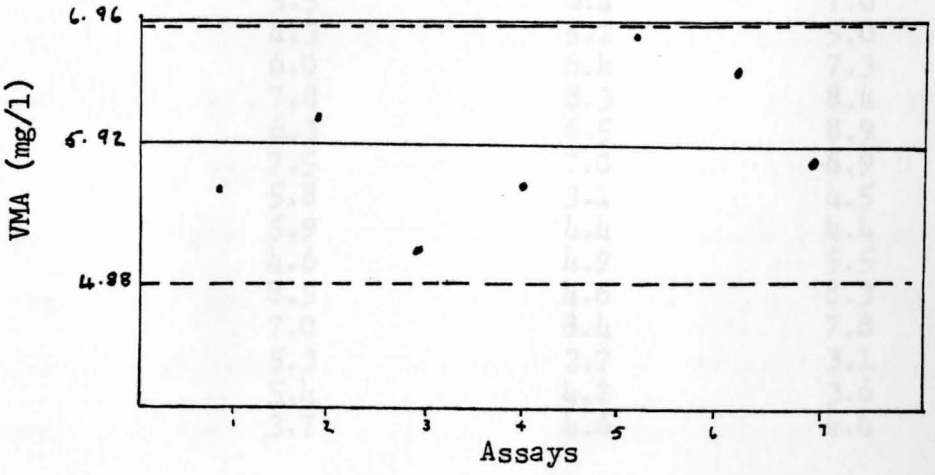


Figure 15: Quality Control Chart for Urine Control

As can be seen the control sample was always within the range of the standard deviation. Based upon this performance, the results obtained by the VMA-Skreen method were taken as valid. Some samples were analyzed locally by the YHA procedure and several others were received after analysis by North Side Hospital. The validity of the results by Pisano method was based on the agreement with the reference value of VMA in Ortho I urine control. Table 14 presents the data which will be used in the correlation study. The values listed are in mg/24 hours.

TABLE 14

DATA FOR CORRELATION STUDY

Sample ID.	YHA	Pisano	VMA-Skreen
030951	2.6	5.4	5.1
046556	2.3	5.5	4.9
044149	4.2	4.8	5.8
044610	5.8	5.1	7.2
047198	3.0	2.7	2.3
028177	3.6	7.3	6.1
1	5.9	7.0	6.7
2	1.9	3.5	3.8
3	4.7	3.1	6.2
4	5.2	5.2	6.0
5	4.8	6.5	6.9
6	6.5	6.1	7.6
7	6.5	6.5	7.2
8	5.5	6.4	7.0
9	4.3	5.2	5.0
10	6.0	6.4	7.3
11	7.8	8.3	8.4
12	6.3	6.5	8.9
13	7.5	7.0	6.9
14	5.8	3.1	4.5
15	5.9	4.4	4.4
16	4.6	4.9	5.5
17	8.5	4.6	8.3
18	7.0	8.4	7.8
19	5.3	2.7	3.1
20	5.4	4.2	3.6
21	3.7	4.6	4.4

Figure 16 illustrates the plot of the values of VMA obtained by the YHA method versus those obtained by the Pisano method. This random scatter of points indicates that there is a lack of relationship between the values.

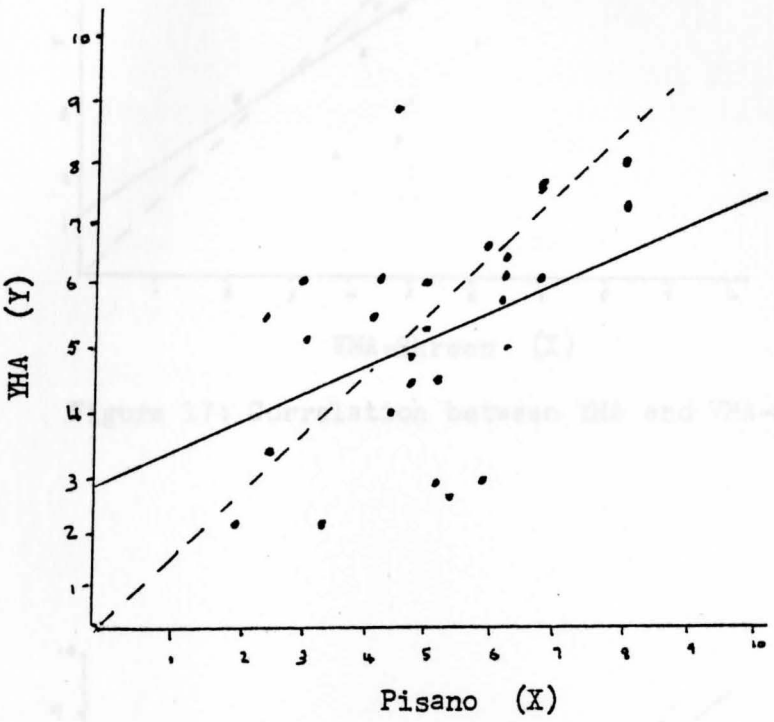


Figure 16: Correlation between YHA and Pisano

Figure 17 illustrates the plot of YHA versus VMA-Skreen and Figure 18 illustrates the plot of VMA-Skreen versus the Pisano method. The dotted line going through the origin in Figures 16 through 18 indicates the region where the points would fall if there had been perfect correlation, that is a coefficient of correlation of 1 and a slope of 1. The solid line indicates the slope of the points plotted. Table 15 summarizes the statistical study among the methods.

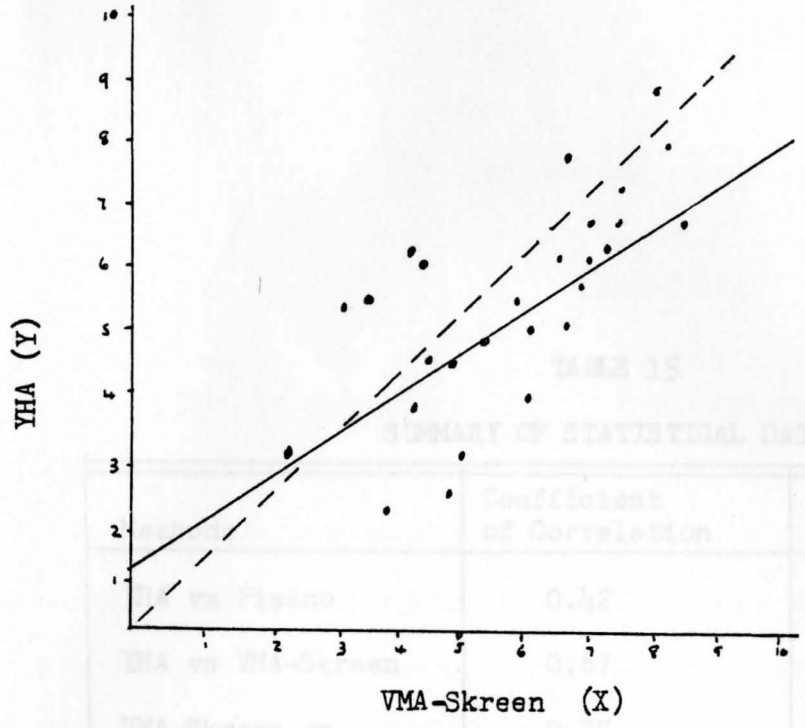


Figure 17: Correlation between YHA and VMA-Skreen

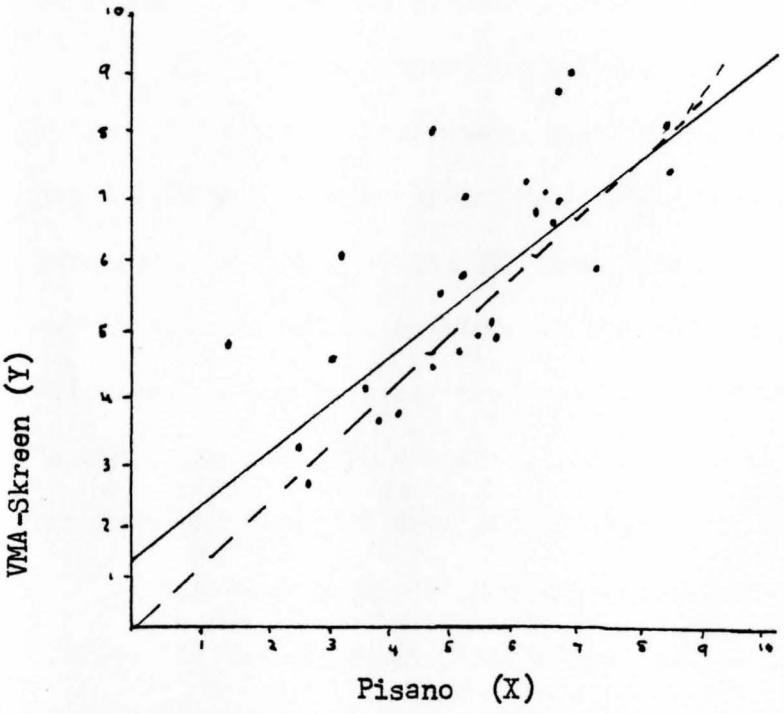


Figure 18: Correlation between VMA-Skreen and Pisano

TABLE 15

SUMMARY OF STATISTICAL DATA

Methods	Coefficient of Correlation	Equation for Regression
YHA vs Pisano	0.42	Y = 0.44X + 2.84
YHA vs VMA-Skreen	0.67	Y = 0.67X + 1.32
VMA-Skreen vs Pisano	0.75	Y = 0.81X + 1.60

CHAPTER VI

DISCUSSION OF RESULTS AND CONCLUSION

Discussion

In this chapter the merit of the VMA-Skreen over the YHA method will be discussed.

The VMA-Skreen method has shown good performance characteristics based on the data given in Chapter IV. Reproducibility of 12 identical samples gave a mean value of 6.33 mg/24 hour with a standard deviation of 1.03 mg/24 hour. This is considered to be good reproducibility but not necessarily excellent. The recovery studies indicated good technique. High level recovery slightly exceeded 100 percent while low level recovery was approximately 100 percent. The overall performance of the VMA-Skreen methodology can be considered good.

In making a comparison between the VMA-Skreen and the YHA procedure , several parameters must be taken into account. Time-wise the VMA-Skreen is more efficient. Eight samples assayed took approximately three hours by the YHA procedure, whereas by the VMA-Skreen method results can be obtained in approximately 1 hour 45 minutes. This time saving is quite critical not from the point of view of a "stat" procedure, since analysis of VMA would not be considered "stat", but from the point of view of efficiency.

The methodology of the VMA-Skreen procedure is relatively simple. On the otherhand, there are several areas which require highly skilled staff in the YHA procedure. One of these areas is in the

aspiration of the organic layer with a Pasteur pipet so as to save all of the carbonate layer. This can be very critical because if some of the carbonate layer is lost, the value of the VMA reported would not be accurate. The value would be decreased. In the VMA-Skreen method the organic layer, which is the top layer, is always used so that the need to aspirate any layer never arises. This minimizes error in this step.

The coefficient of correlation for both methods show a great disparity when compared to the Pisano method, which was taken as the reference method. The VMA-Skreen method is nearest to unity. See Table 15. This indicates that there is closer relationship between the VMA-Skreen and the Pisano method, than there is between the YHA and the Pisano method. The YHA procedure showed very poor correlation to the Pisano method. This can be attributed, in part, to several factors. One of the biggest is probably in the separation of layers when the aqueous layer is to be used.

The YHA procedure proved not to be linear beyond 15 mg/l whereas the VMA-Skreen procedure is linear up to 25 mg/l which was the maximum concentration tested. Beyond 15 mg/l in the YHA procedure, the sample would have to be diluted and assayed again. This would definitely be very time consuming since the sample would have to be run twice before meaningful results could be reported.

The procedure outlined by the VMA-Skreen kit, as shown in this report, is a relatively simple technique, using chemicals, reagents and equipment that are common in an average laboratory. The kit can be purchased on a one time basis and then locally prepared reagents can be substituted. This would not affect the overall performance of the procedure. A strong recommendation would be to check for linearity with

every new bottle of VMA standard used. This VMA-Skreen kit can therefore provide a laboratory with a quick, simple and reliable method for the analysis of VMA as an effective means in differentiating pheochromocytomas from primary hypertension.

The manufacturers suggest that any abnormally high VMA test result received should be investigated for drug interference. A scan of the sample should be compared to a scan of a standard, particularly if the sample has an "off" color. The maximum absorbance of both should be at 570 nm. If the scans are not similar, then drug interference should be suspected.

The normal range by this method is stated as 2 - 10 mg/24 hour, however before adoption of this procedure each laboratory should determine its own normal range by testing a large population of normal in-patient and out-patient hospital subjects.

Helena's " Super Z VMA" has great merit in a screening program. It only requires a single void sample, preferably taken shortly after awakening in the morning, and results can be reported within approximately 30 minutes. With the modification suggested, the VMA bands can be clearly seen so that visual comparison can be made easily. If the need to quantitate arises, a densitometer can be used. It is however strongly recommended that quantitation be accomplished by another methodology and the Helena procedure be used only for rapid screening.

Conclusion

Based on its performance characteristics, its relative simplicity and its better correlation to the Pisano method than the present YHA method, I would recommend to the Youngstown Hospital Association that they investigate the VMA-Skreen methodology in an effort to replace their present procedure. The investigation should include an assessment of all parameters of the method and should be done individually by two qualified technologists. Statistical data between their method and the VMA-Skreen method should be accummulated over an extended period of time so as to get the best possible analysis of correlation.

For a rapid screening method, the Helena " Super Z VMA" is also recommended.

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