

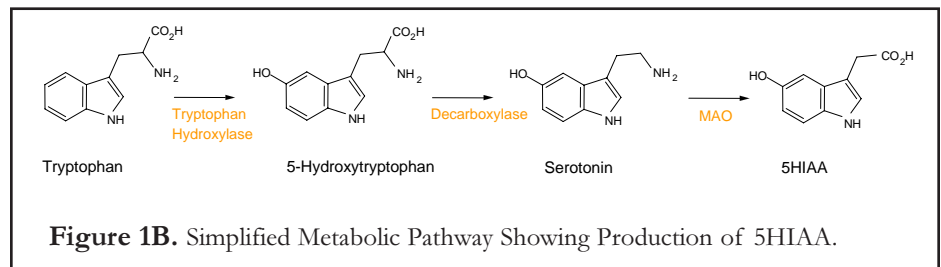
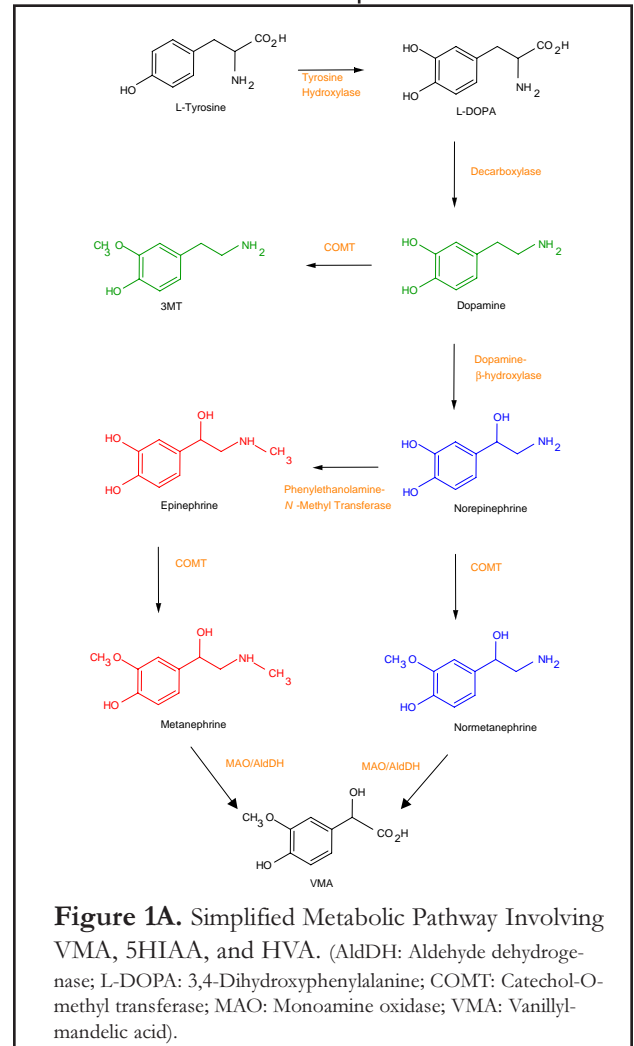
Simultaneous Determination of Urinary VMA, 5HIAA, and HVA Without Extraction

Vanillylmandelic acid (VMA) [3-methoxy-4-hydroxymandelic acid] is a principle urinary metabolite of the catecholamines, norepinephrine and epinephrine, while homovanillic acid [3-methoxy-4-hydroxyphenylacetic acid] is a major metabolite of dopamine. These products are formed primarily by the action of the enzymes catechol-O-methyl transferase (COMT) and monoamine oxidase (MAO) (Figure 1A). 5-Hydroxyindole-3-acetic acid (5HIAA) is the major urinary metabolite of serotonin, formed by the action of MAO (Figure 1B).

Urinary VMA and HVA measurements are useful in the diagnosis and maintenance of tumors of neural crest origin [e.g., pheochromocytoma (in adults), neuroblastoma (in children) and ganglioneuroma (in young adults)] which commonly produce high levels of the parent catecholamines. Urinary 5HIAA measurements are commonly used in the diagnosis of carcinoid tumor and syndrome. Urinary excretion rates of all three analytes is also of great interest in the study of various neuropsychiatric, metabolic, and related disorders and in characterizing the effects of many drugs. Simultaneous measurement of these acid metabolites is often advantageous in terms of increased diagnostic specificity, higher sample throughput, and greater accuracy. Moreover, additional insight can often be obtained regarding metabolic pathway interactions and when atypical tumors are present.

A wide variety of techniques have been used for the measurement of these analytes. High performance liquid chromatography with electrochemical or fluorometric detection are the most common approaches due to their high degree of selectivity. Most techniques are limited to measurement of only one or two of these metabolites per analysis due to the complexity of the sample matrix, and the difference in analyte retention and extraction characteristics.

Time-consuming steps for sample cleanup are often required due to the large number of phenolic, aromatic amine, and heterocyclic aromatic compounds present in urine. An additional limitation of some techniques is that qualitative data relies only on retention time comparison with external standards and some interferences may be concealed.



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Since the early 1990's ESA has offered this procedure for the direct, simultaneous measurement of urinary VMA, 5HIAA, and HVA using a CoulArray® detector-based analytical system. This procedure compliments "Determination of Urinary Metanephrine and Normetanephrine Without Extraction" (ESA Application Note 70-1243) and "Fractionated Urinary Catecholamines" (ESA Application Note 70-2205) which are also extensively used in clinical laboratories. Samples are simply diluted and filtered before separation by binary gradient reversed phase chromatography. The basis of the method is the use of two dimensions of resolution: chromatographic and voltammetric. By using eight serially arranged coulometric sensors, set at incrementally increasing potentials, an additional degree of selectivity is obtained. This provides screening of lower oxidizing interferences and highly specific stepwise oxidation of each analyte. The response behavior across three adjacent sensors can be used to assess resolution and aid in peak identification. This routine procedure provides accurate and precise quantification as well as qualitative peak purity information with minimal sample handling. With the CoulArray system, sample analysis is automated and is complete in 20 minutes.

Samples, Standards and Controls:

Twenty-four hour urine samples were collected into 10 mL of 6 M hydrochloric acid and were stored at 4 °C. A combined stock standard (1 g/L) was prepared by dissolving VMA, 5HIAA, and HVA powders in 50 mM hydrochloric acid containing 100 µM EDTA. The stock standard is stable for up to one month when stored at 4 °C and protected from light. A working external standard (20 mg/L) was prepared by dilution of the stock standard using the hydrochloric acid/EDTA diluent and was stored at 4 °C, protected from light, for up to one week. Lypochek® normal and abnormal controls were obtained from Bio-Rad Laboratories Diagnostics Group. Controls were reconstituted with 50 mM hydrochloric acid and were stored at 4 °C for up to one week.

Reagents:

Use highest purity available. VMA, 5HIAA, and HVA standards were obtained from Sigma Chemical Co. (St. Louis, MO). A MilliQ system (Millipore Corp., Bedford, MA) or equivalent was used as a water source.

Simplified Sample Preparation:

- 1). Add 3.8 mL of water to the top receptacle of a 0.22 µm nylon membrane filter (Centrex®).
- 2). Pipette 0.2 mL of sample, standard, or control, add to filter unit and invert once.
- 3). Centrifuge at 1,500 rpm for 10 minutes.
- 4). Transfer filtrate to autosampler vial and inject 10 µL.

Apparatus:

Gradient Model 5600A CoulArray Clinical System with 2 cell modules each containing 4 electrochemical sensors, two dual piston pumps, gradient mixer, guard column and holder, analytical column and a Model 542 autoinjector.

Column: 150 x 4.6mm I.D., 5 micron; C18.
Mobile Phase: ESA Acid Metabolite Phases A and B.
Temperature: 35° C.
Flow Rate: 1.0 mL/min.
Injection Volume: 10 µL.

Chromatography:

Chromatographic conditions have been optimized for resolution of VMA, 5HIAA and HVA from possible endogenous and exogenous interferences. Binary gradient elution was used with initial isocratic conditions of 6% Mobile Phase B for 2.7 minutes, a linear gradient to 20% B in 1 minute, and a linear gradient to 55% B in 10 minutes before returning to initial conditions. When data acquisition was completed, peak detection, retention time and response ratio comparison, quantitation, and report generation were performed automatically for each sample. The total time for each analysis was 20 minutes.

Detector Potentials:

Cell potentials were maintained at 0, 80, 160, 240, 320, 400, 480 and 560 mV, beginning with the first sensor in the series.

Analysis:

Figure 2 illustrates a multichannel chromatogram obtained from a standard. The retention time (RT) for VMA was 5.0 minutes, for 5HIAA 12.2 minutes, and for HVA 15.3 minutes. The first two electrodes (not shown) were set at potentials below that for the initial oxidation of the metabolites to allow efficient coulometric screening of lower oxidizing interferences (e.g., ascorbate and the catecholamines). The remaining electrodes provide stepwise oxidation of the analytes along their respective current-voltage curves (see Figure 3). VMA responds predominantly at E5 (dominant sensor) while E4 (leading sensor) and E6 (following sensor) can be used for peak

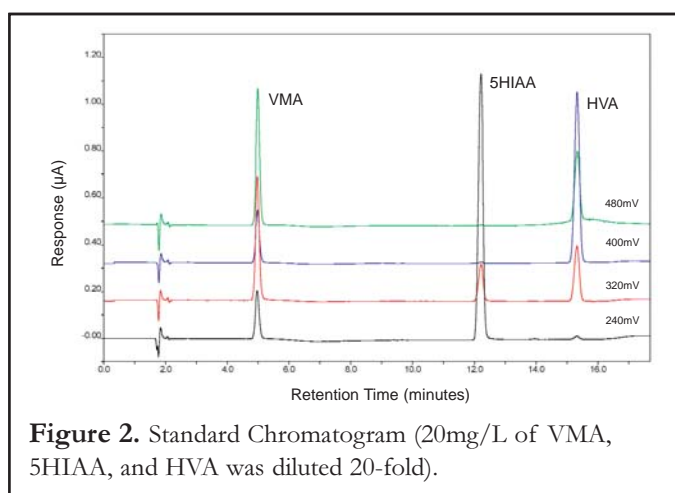


Figure 2. Standard Chromatogram (20mg/L of VMA, 5HIAA, and HVA was diluted 20-fold).

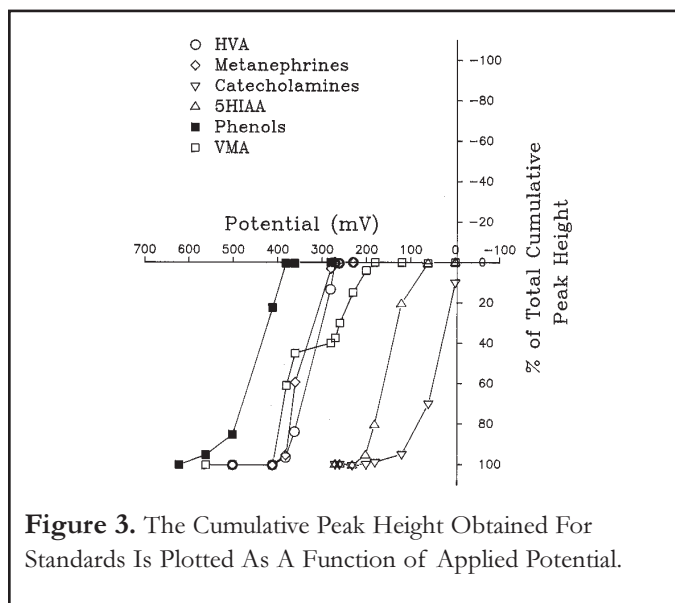


Figure 3. The Cumulative Peak Height Obtained For Standards Is Plotted As A Function of Applied Potential.

ratio comparisons. 5HIAA responds at E3, E4, and E5, and HVA across E5, E6, and E7 (leading, dominant, and following sensors, respectively).

Calculations were performed automatically using the CoulArray for Windows^{®32} software. Peaks were identified by matching the dominant sensor (sensor with the greatest response), retention time and response profile across three adjacent sensors. Quantitation was performed using the sum of peak heights across the three primary sensors.

The response “ratio accuracies” (defined by the CoulArray for Windows software as the sample response ratio/standard response ratio) obtained from the normal patient sample presented in Figure 4 were above 0.90 for all three compounds. These response ratios provided an objective index for examining peak purity in all samples. The closer the ratio accuracy is to 1.00, the greater the similarity in voltammetric behavior between the sample peak and the standard. A low value is a good indicator that an apparently pure chromatographic peak may be comprised of two or more solutes.

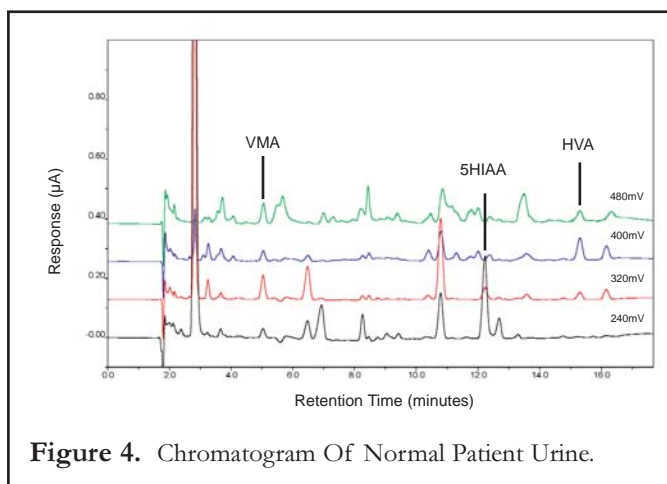


Figure 4. Chromatogram Of Normal Patient Urine.

Method Performance:

Linear response range and lower limit of detection (LOD) were determined by replicate analysis of serially diluted patient samples. The response was found to be linear to 60 mg/L for VMA, 250 mg/L for 5HIAA, and 45 mg/L for HVA, with a lower limit of detection of 0.2 mg/L for all three analytes (signal-to-noise ration 10:1). Table 1 presents linearity data for 6 replicates of five different levels of each analyte.

Clinical method

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Analyte	Slope Mean (SD), n=6	y-Intercept Mean (SD), n=6	Correlation Coefficient Mean, n=6
VMA	0.993	0.68	0.9993
5HIAA	1.000	3.80	0.9988
HVA	0.934	0.68	0.9984

Table 1. Linearity Data For VMA, 5HIAA, And HVA.

Within-run precision was estimated from 10 normal and 10 abnormal controls. Between-run precision was estimated from 63 normal and 63 abnormal controls run on 5 separate days and three separate instruments. All controls were within the target range specified by the manufacturer. The results are summarized in Table 2.

	VMA		5HIAA		HVA	
	Norm	Abnorm	Norm	Abnorm	Norm	Abnorm
Within-Run ^a Mean (mg/L)	3.14	14.9	3.71	31.1	1.79	14.8
CV, % (n=10)	1.60	2.1	1.40	1.8	1.7	2.2
Between-Run ^b CV, % (n=63)	5.50	5.40	11.0	8.7	3.9	4.4

Table 2. Variability In VMA, 5HIAA, And HVA Measured In Urine Controls. (Norm - normal; Abnorm - abnormal; CV, % - percent coefficient of variation; ^aAverage from three runs of ten replicates each; ^bDay-to-day, including within-day, and between three instruments, each run for 5 days.

The presence of interfering drugs, drug metabolites, or endogenous electroactive compounds was assessed by examining the retention time, peak shape, "ratio accuracy", and comparison with other methods. No interferences were evident based on chromatographic and voltammetric data. Results from patient samples were in good agreement with data obtained using Bio-Rad methods as shown in Table 3.

	VMA	5HIAA	HVA
Number of samples	20	81	34
Correlation Coefficient	0.9808	0.9910	0.9965
Slope	0.9475	1.023	1.030
y-Intercept	-0.104	-0.889	-0.954
Standard error of Estimate	0.919	0.201	1.462

Table 3. Comparison Of CoulArray With Three Bio-Rad Methods.

Conclusion:

The use of coulometric array detection with HPLC allowed selective determination of the urinary acid metabolites in routine patients over a wide range of analyte levels. The results from this technique compared well with those from the existing procedures while eliminating most of the sample handling, as well as providing useful qualitative data.

References of Interest:

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Ordering Information:

Description	Part Number
CoulArray, Model 5600A - eight channels	70-4325
CoulArray, Heated Organizer Module	70-4340T
Pump, Model 582	70-4050
Gradient Upgrade	70-4051
Autosampler, Model 542	70-4151
Column, MCM	70-0340
Column, Guard	70-1393
Column, Guard Holder	70-1394
Mobile Phase A	70-4835
Mobile Phase B	70-4836
or Urinary Acid Metabolites Analyzer	70-4247H

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