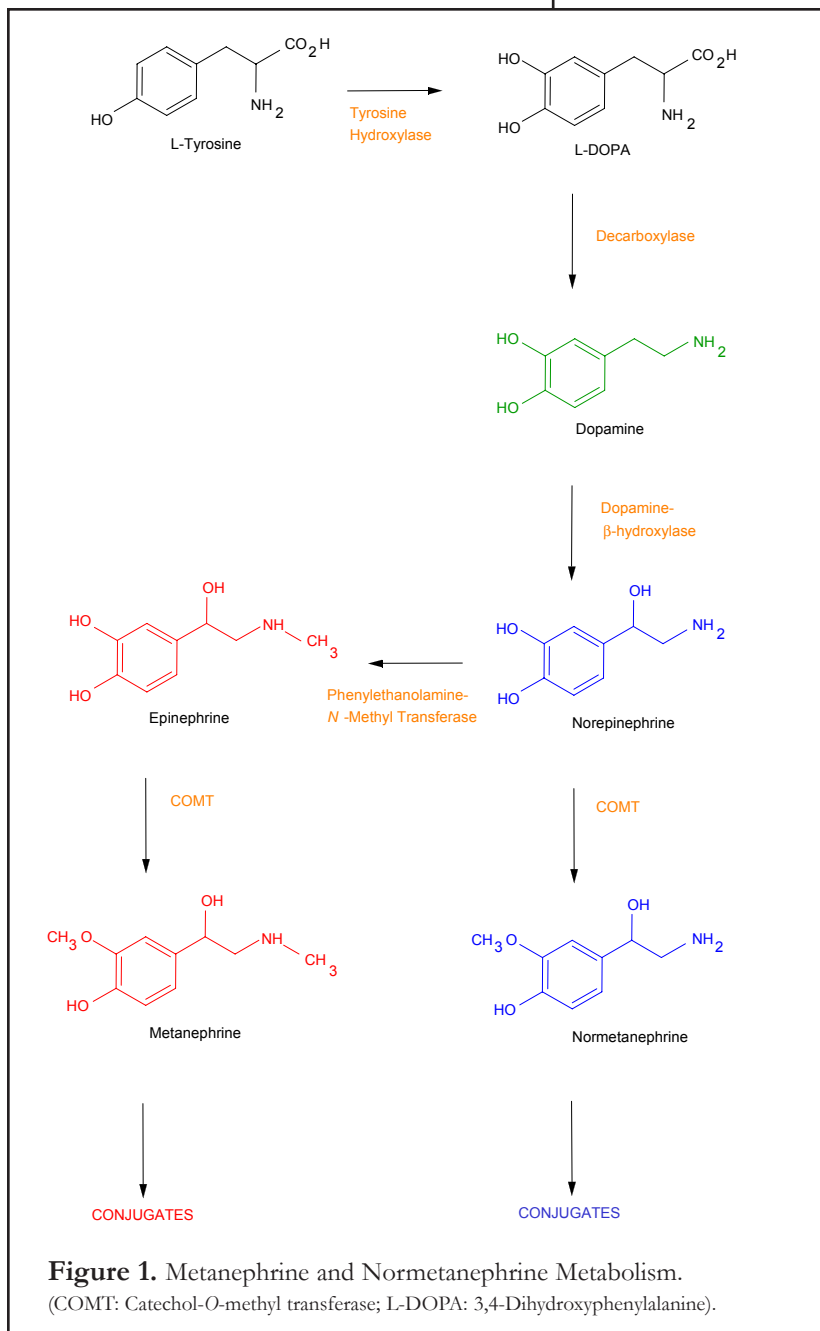


Determination of Urinary Metanephrine and Normetanephrine Without Extraction

Tumors of neural crest origin commonly produce high levels of catecholamines (epinephrine, norepinephrine). Quantitation of metanephrine (MN) and normetanephrine (NMN), the 3-O-methylated metabolites of these compounds, in urine is useful in the diagnosis and management of pheochromocytoma and other neurogenic tumors (Figure 1). These tumors typically produce very serious clinical symptoms and have a high rate of malignancy. In many patients surgical removal of the tumor results in complete reversal of its clinical manifestations while pharmacological treatment by itself is ineffective. For these reasons, although the incidence of pheochromocytoma is relatively low (< 1 %), routine screening of hypertensive patients is a common practice. High performance liquid chromatography with electrochemical detection (HPLC-ECD) has become the technique of choice for the routine clinical measurement of the urinary metanephrines (MN + NMN). Most methods require extensive extraction steps for sample purification before HPLC-ECD analysis and typically utilize detection at a single electrode potential. With these procedures, differences in extraction efficiencies, matrix effects and sample handling errors are potential sources of inaccuracy. Furthermore, qualitative data is limited to chromatographic comparison with external standards and some interferences may be concealed.

Since the early 1990's ESA has offered a procedure for the direct measurement of MN and NMN in hydrolyzed urine using coulometric array detection now available using the CoulArray® detector. After acid-hydrolysis urine samples are simply diluted and filtered prior to automated HPLC analysis. The basis of this method is in the use of two dimensions of resolution - chromatographic and voltammetric. By using eight serial coulometric sensors, set at incrementally increasing potentials a high degree of selectivity is obtained. This detector array allows screening of lower oxidizing interferences and highly specific stepwise oxidation of the metanephrines. The response behavior across 3 adjacent sensors is used to assess resolution and aid in peak identification. This procedure greatly decreases the sample handling requirement, while increasing the qualitative data obtained. With the CoulArray detector, sample analysis is automated and is complete within 20 minutes (Figures 2 and 3).



Determination of Urinary Metanephrine and Normetanephrine Without Extraction

Samples, Standards, and Controls:

Twenty-four hour urine samples were collected from hypertensive patients into 10 mL of 6 mol/L HCl and stored at 4°C for up to one week. Urine-based standards, normal and abnormal controls were reconstituted with 0.05 mol/L HCl and stored at 4°C. Samples and standards were stable for at least one week under these conditions. A composite 10 mg/L standard of each compound in 0.05 mol/L HCl was stored at -20°C for up to 3 months. Standards and controls were included with every 15 samples. The calculations were performed automatically with the CoulArray for Windows^{®32} software.

Samples and Hydrolysis:

Hydrolysis - 2.0 mL of standards, samples, or controls were mixed with 2.0 mL of 0.6 mol/L HCl in glass tubes and placed in a boiling water bath for 30 minutes. After hydrolysis samples were diluted with 5.0 mL of water and passed through 0.22 micron nylon membrane filters by centrifugation (6,000 g, 5 minutes, 4°C). Forty µL of the filtrates were directly analyzed on the CoulArray system.

There is no need for extraction, pH adjustment, or internal standardization.

Apparatus:

Isocratic Model 5600A CoulArray System with 2 cell modules each containing 4 electrochemical detector cells, a dual piston pump and a Model 542 autoinjector.

Column:	250 x 4.6 mm I.D., 5 micron C18.
Mobile Phase:	ESA Ucat/Mets.
Temperature:	37 °C.
Flow Rate:	1.2 mL/minute.
Injection Volume:	40 µL.

Chromatography:

Chromatographic conditions have been optimized for resolution of the metanephrines from possible endogenous and exogenous interferences. When using the ESA Ucat/Mets mobile phase amine compounds are highly protonated while strongly and weakly acidic compounds retain their anionic nature. Under these conditions, a moderately strong hydrophobic eluent combined with an highly concentrated / long carbon chain counterion provides selective retention of amines and very hydrophobic non-ionic compounds on a reversed-phase column.

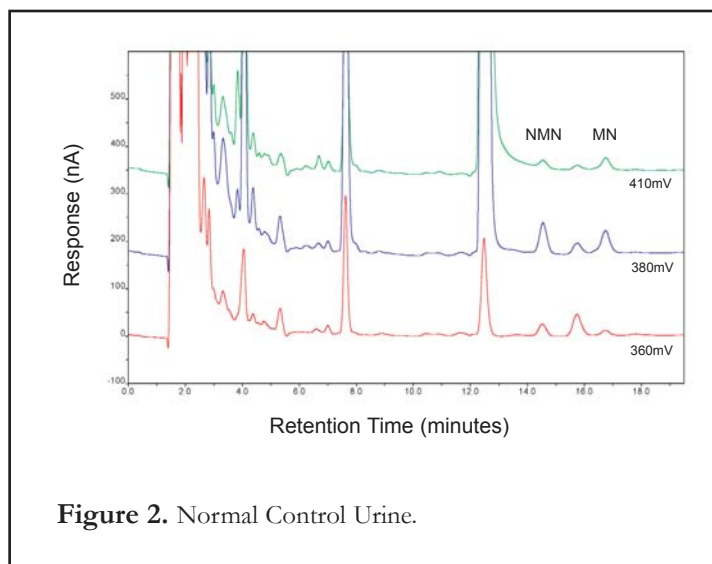


Figure 2. Normal Control Urine.

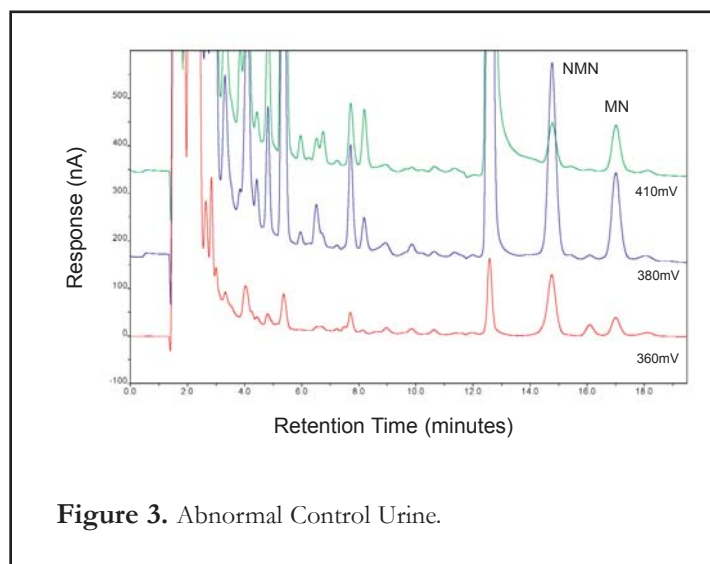


Figure 3. Abnormal Control Urine.

In-line graphite filters were used to protect the analytical column and electrochemical cells from particulates. This configuration allows good long term performance in high volume laboratories. No significant change in column pressure or efficiency was evident after more than 1000 injections of urine filtrates. Retention times typically varied by less than 1% (CV) both within and between runs. Two liter volumes of mobile phase can be recycled on the CoulArray system for up to 1 week.

Detector Potentials:

Cell potentials were typically maintained at 200, 230, 260, 270, 280, 360, 380 and 410 mV, beginning with the first sensor in series. At the completion of each analysis, all cell potentials were increased to 1000 mV for 12 seconds to prevent long term adsorption of material to the electrode surface. The electrodes were allowed to restabilize for 1.5 minutes before the next injection. During this re-equilibration minute period, peak detection, qualitative analysis, quantitation and report generation were performed automatically for each sample. The total time for each analysis was 20 minutes.

Detector Array Resolution:

An array of eight coulometric detectors was used in an oxidative screen mode as described by Matson *et al.*, (1987) and reviewed by Acworth and Bowers (1997). The potentials were optimized for selective measurement of the metanephrines based on their hydrodynamic voltammetric behavior as shown in Figure 4. The first four electrodes were set at potentials below that for initial oxidation of the metanephrines to allow efficient coulometric screening of lower oxidizing interferences (eg. dopamine, hydroxyindoles, and other catechols). The potentials of detectors 5, 6, 7 and 8 (E5 - E8) were chosen such that 15±5, 65±10, 15±5, and 5±3% of the total peak height (current) was obtained at each detector, respectively. Figures 2 and 3 illustrate chromatograms obtained from normal and abnormal controls, respectively. The metanephrine peaks were identified automatically with the CoulArray for Windows³² software through examination of the retention time, the dominant electrode [sensor having the greatest response for each analyte], and the response profile across 3 adjacent sensors. The response profile was compared between standard and sample and "ratio accuracies" were reported. Ratio accuracy (defined as the sample response ratio/standard response ratio x100) provides an objective index for examining peak purity in all samples. Table 1 shows the percent response "ratio accuracies" typically obtained from urine controls and patient samples. The closer the accuracy value was to 100%, the greater the similarity in voltammetric behavior between sample peak and standard. A low value was a good and immediate indicator that an apparently pure chromatographic peak may be comprised of two or more solutes.

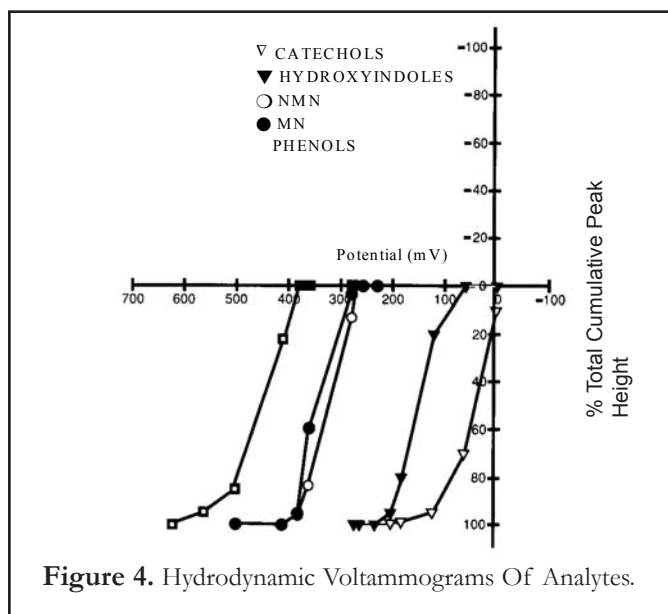


Figure 4. Hydrodynamic Voltammograms Of Analytes.

	Metanephrine		Normetanephrine	
	6/5	6/7	6/5	6/7
Detectors	6/5	6/7	6/5	6/7
Controls:				
Normal	96.5	99.8	95.6	98.7
Abnormal	96.3	95.0	92.9	94.2
Patient Samples:				
(Mean; n=12)	88.0	96.0	88.0	94.0
%CV	5.3	1.8	0.9	3.2

Table 1. "Ratio Accuracy" Data From Patient And Control Urines.

Method Performance:

Precision was examined using commercially available normal and abnormal control urines. Ten replicates were analyzed on each of 3 separate days for within-run studies. Variability between runs was performed by analyzing two or more replicate controls and abnormal controls on 16 separate days.

Good precision was obtained for both analytes at normal and elevated levels as shown below in Table 2. The controls were consistently within the target range specified by the manufacturer.

Determination of Urinary Metanephrine and Normetanephrine Without Extraction

	Normal		Abnormal	
	MN	NMN	MN	NMN
Within-run (n=10)	1.6	2.7	2.4	2.1
Between-run (n=43)	9.2	8.5	7.9	6.9

Table 2. Precision (% CV) Of The Method.

The linear response range was examined by adding equal volumes of aqueous standards (seven levels) or water to patient urine samples. Each sample was analyzed in duplicate in 3 separate runs. The limit of detection was 13 µg/L for MN and 22 µg/L for NMN with linear response to 2.30 mg/L for MN and 3.20 mg/L for NMN. See Table 3.

	Concentration Range (mg/L)	Slope Mean (SD; n=6)	Intercept Mean (SD; n=6)	Standard Error Of Estimate (Mean; n=6)
MN	0.02 - 2.30	0.975 (0.009)	-0.004 (0.0021)	0.018
NMN	0.06 - 3.20	1.011 (0.008)	-0.022 (0.0116)	0.34

Table 3. Linearity Of The Method.

This method has been compared to a routine clinical method which employs a dual column off-line extraction prior to HPLC-ECD analysis. Results of linear regression analysis (CoulArray data as the dependent variable) are: for MN the least squares regression line had a slope (B) of 0.9528, an intercept (A) of -0.0057, and a correlation coefficient (r) of 0.963 for 82 samples. For NMN the relationship was: B=0.9089, A=0.0165, r=0.9768, N=83. Three outliers (residual > 3.5 times the standard error of estimate) were excluded from the metanephrine data and two from the normetanephrine data.

No interferences were apparent with either analyte in samples from patients receiving the following drugs: acetylsalicylate, acetaminophen, alprazolam, buspar, chloral hydrate, cimetidine, clonidine, dexamethasone, dipyridamole, disopyramide, estrogen, flurazepam, hydralazine, hydrocodone, labetalol, metoclo-

pramide, metoprolol, nifedipine, nalbuphine, pentoxifylline, triamterene, and ranitidine. No interferences were found in samples from patients having either elevated serum albumin, serum glucose, urine pH, or 3-methoxytyramine levels. Thirty-eight known endogenous metabolites, including possible "amine-like" compounds, from the tyrosine, tryptophan, and purine metabolic pathways were also tested. Dopamine and synephrine were the only oxidizable compounds that eluted within a ±2 minute retention time range of the metanephrines under these conditions. Dopamine eluted 1.5 minute after MN while synephrine eluted 0.5 minute before NMN. The voltammetric selectivity of the array allowed resolution of these compounds at >1000-fold higher concentrations. Tyramine and octopamine did not interfere with either NMN or MN.

This technique provides on-line resolution of analytes through the combination of chromatographic and voltammetric selectivity. This allows direct measurement of the urinary metanephrines without the extensive sample cleanup necessary with other methods. The CoulArray detector provides objective qualitative data useful in the examination of potential interferences.

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Ordering Information

Description	Part Number
CoulArray, Model 5600A - eight channels	70-4325
CoulArray, Thermostatic chamber	70-1760
Pump, Model 582	70-4050
Autosampler, Model 542	70-4151
Column, Meta-250	70-1956
Mobile phase, Ucat/Mets	70-3067
or Urinary Metanephrine Analyzer	70-4247N



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