

Plasma Free Metanephrines

Normetanephrine (NMN), metanephrine (MN) and 3-methoxytyramine (3-MT) are the extra neuronal catechol-o-methyl transferase (COMT) metabolites of the catecholamines norepinephrine, epinephrine and dopamine, respectively (Figure 1). Although elevated catecholamines and their metabolites may be associated with several conditions, laboratory assessment of these compounds is used primarily to rule out the presence of a pheochromocytoma, a catecholamine-secreting tumor of the adrenal medulla. Clinicians rely on clinical laboratories to provide reliable screening tests for these tumors as the underlying cause of a patient's hypertension. Several assays have historically been used to fulfill this need. They have typically included urinary metanephrines, urinary vanillylmandelic acid (VMA) and plasma or urinary catecholamines. Rarely has one of these assays provided the desired specificity and sensitivity needed to reliably detect these tumors and not generate costly false positives. Laboratory measurement of plasma free metanephrines (NMN, MN) has received significant attention lately due to the apparent diagnostic advantages this analysis has over the traditional assays. These advantages stem from the more direct correlation of plasma free metanephrine concentration to the catecholamine and metanephrine production of adrenomedullary and pheochromocytoma cells than from other sources. This results in the ability of plasma free metanephrines to detect all but the smallest of pheochromocytomas, they can be used as an indicator of the disease's progression and yields fewer false positive results that require unnecessary biochemical and imaging studies¹.

High performance liquid chromatography with electrochemical detection (HPLC-ECD) has emerged as the method of choice for the routine clinical measurement of catecholamines and their metabolites due to the technique's inherent selectivity and sensitivity. An ESA detector using patented coulometric electrodes allows for the detection of 100% of the analyte injected which improves both the sensitivity and selectivity over amperometric electrochemical detectors. This method uses the CoulArray detector with three coulometric electrodes arranged in series such that the metanephrines and the internal standard are oxidized at the first electrode, reductively screened at the second and later

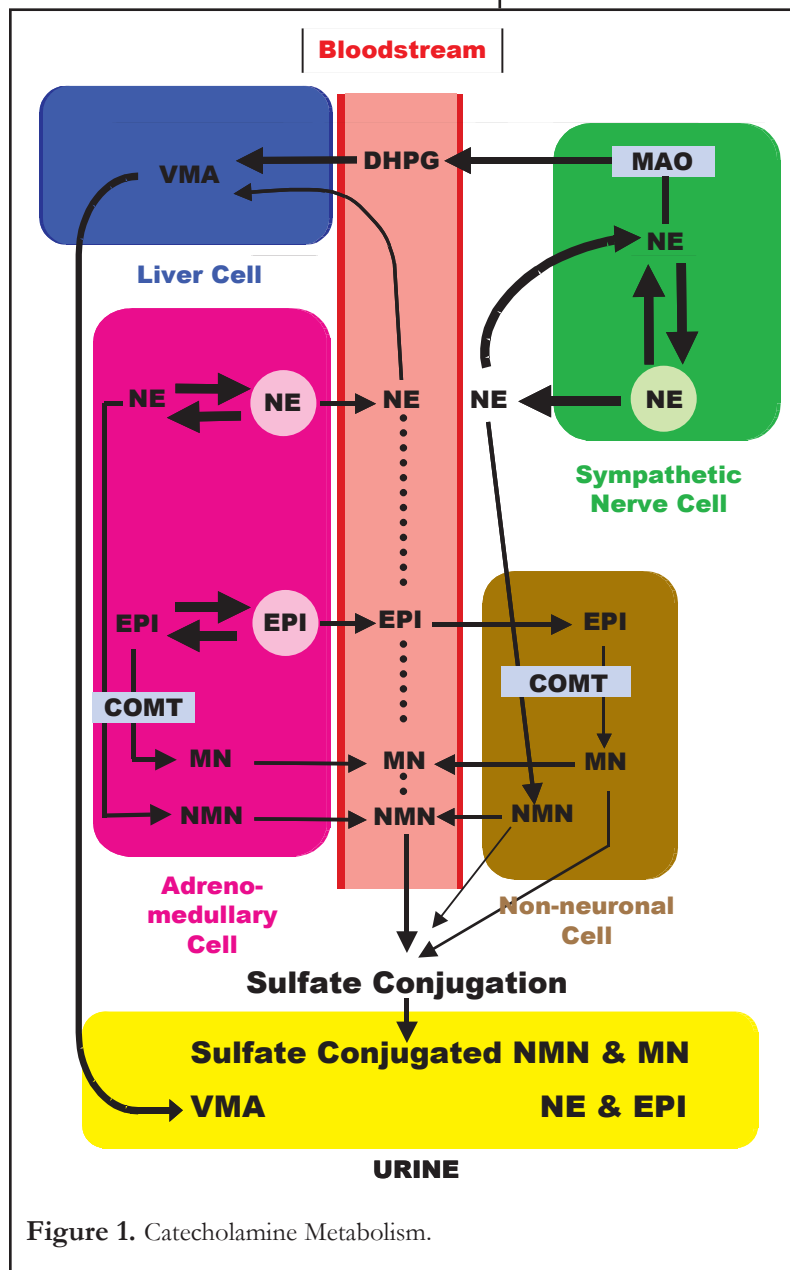


Figure 1. Catecholamine Metabolism.

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reduced at the third electrode. This oxidation / reduction mode of detection eliminates several interfering peaks from the chromatogram that would be present using an oxidation only approach.

Materials and Methods

Samples, Standards and Controls

Blood was collected by routine venipuncture into pre-chilled EDTA anticoagulated vacuum tubes. The whole blood sample was kept on wet ice until centrifuged and the plasma was frozen at -20°C until extraction. All samples were thawed and then centrifuged to remove any fibrin that may clog solid phase extraction (SPE) columns. Instrument calibration was achieved by appropriate dilution and injection of aqueous standards. Control material was prepared by adding metanephrines simulating normal and elevated levels to a plasma pool. The controls were frozen at -80°C until analysis. Standards and controls were included with every 20 samples. Calibration of the assay and full instrument control was achieved with the CoulArray for Windows³² software.

Solid Phase Extraction

As shown in Figure 2, metanephrines were extracted and concentrated from one milliliter of centrifuged plasma by solid phase extraction. This extraction is based on the method described by Lenders². All reagents except HPLC grade water and methanol necessary for the extraction were included in the ESA Plasma Metanephrine Analysis Kit.

After conditioning the SPE columns, sample and internal standard are loaded onto the columns where the analytes of interest were retained. The columns were washed to remove unwanted compounds followed by elution of the metanephrines and the internal standard. The eluates were evaporated to dryness, reconstituted, and injected on the HPLC for analysis.

Apparatus

The isocratic analytical system consisted of a 4-channel CoulArray detector equipped with a 5021 conditioning and 5011 analytical cells, model 582 pump, CoulArray thermal organizer module and a model 542 autosampler.

Column:	250 x 4.6 mm I.D., 5 micron C18
Mobile Phase:	ESA Plasma Metanephrine mobile phase
Temperature:	30°C
Flow Rate:	1.2 mL/minute
Injection Volume:	100 μL

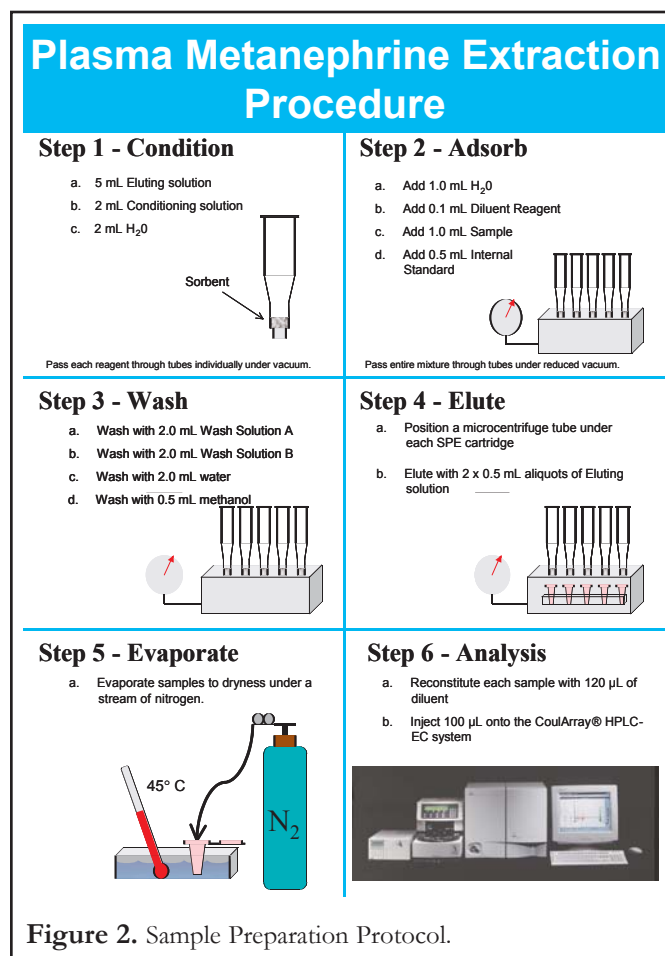


Figure 2. Sample Preparation Protocol.

Cell Potentials:

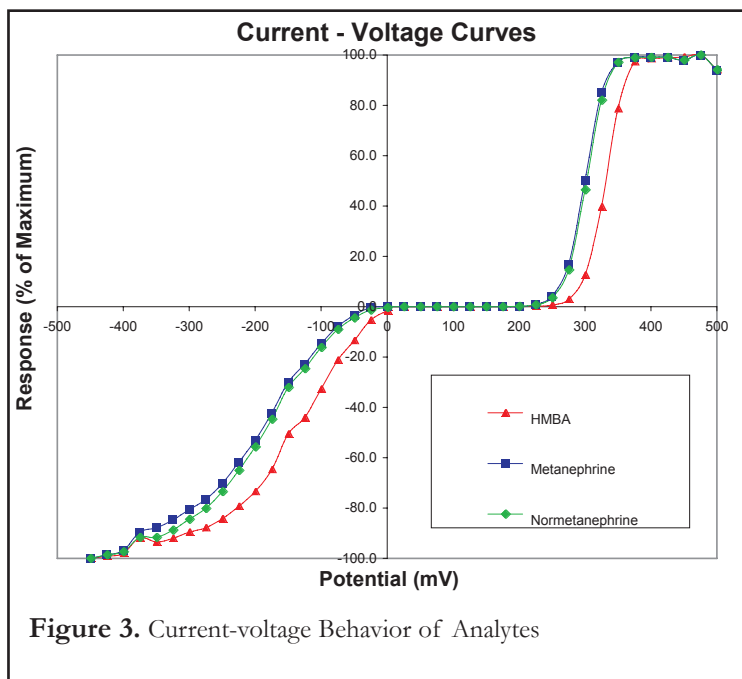
Conditioning Cell 410mV
Analytical Cell
E1 = 0mV, E2 = -325mV

A Turbovap LV[®] evaporation system was used to efficiently evaporate the SPE eluates prior to HPLC analysis.

Chromatography

Chromatographic conditions as well as the solid phase extraction procedure have been optimized to separate the metanephrines from possible endogenous and exogenous interferences and to maximize sensitivity. The 5021 conditioning cell combined with the 5011 high sensitivity analytical cell allow the metanephrines to be detected in the oxidation / reduction mode eliminating many extraneous peaks that would be present using a single electrode via oxidation only. Figure 3 shows the current-voltage curves of the analytes which allow this oxidation / reduction approach to be used. The assay's sensitivity and reliability benefit by having the

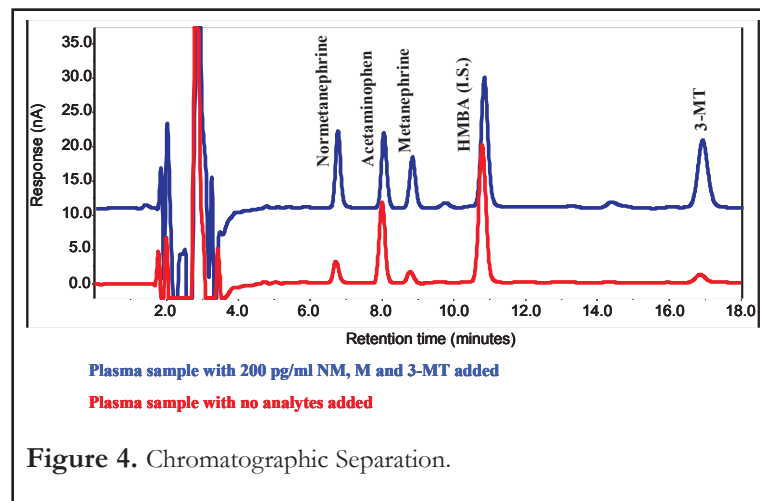
peaks of interest monitored on electrode 2 of the 5011 cell, ESA's most sensitive, maintenance-free coulometric cell. The model 582 dual-piston pump provides one of the most pulse-free chromatographic baselines while contributing very little background current to the electrochemical detector. The chromatographic separation (Figure 4) is achieved via ion-pair reversed phase chromatography on a C18 analytical column with excellent peak shape and longevity.



Results and Discussion

Linearity

Method linearity was evaluated by spiking water or a plasma pool with an aqueous standard resulting in concentrations of 0, 50, 100, 200, 500, 1000 and on occasion 2000 pg/mL. These standards were either injected directly, in the case of the aqueous standards or extracted. In both cases, linearity is the analyte peak height corrected by the internal standard peak height vs. concentration. As can be seen in Figure 5, the linearity curves for plasma and aqueous based standards were virtually identical. For this reason, assay calibration was based upon direct injection of aqueous standards confirmed through the use of controls. Additional linearity data can be found in Table 1.



Aqueous Based Standards			
	Slope	y-Intercept	R ²
Normetanephrine	0.0024	-0.0019	1.000
Metanephrine	0.0019	-0.0016	1.000
Plasma Based Standards			
	Slope	y-Intercept	R ²
Normetanephrine	0.0023	0.0217	0.9975
Metanephrine	0.0018	0.0145	0.9984

Table 1. Linearity Data.

Detection Limits

Limits of detection were determined to be 20 pg/mL and 28 pg/mL for normetanephrine and metanephrine, respectively.

Precision

Within-run precision was evaluated by extracting and analyzing three different plasma samples 11 times each in the same analytical run against the same calibration curve. Within run precision coefficients of variation ranged from 5.0 to 7.6 as shown in Table 2.

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	Within-Run Precision					
	Pool 1 (n=11)		Pool 2 (n=11)		Pool 3 (n=11)	
	NMN	MN	NMN	MN	NMN	MN
Mean	47.8	40.0	53.81	27.91	613.8	327.82
sd	2.72	2.9	2.83	1.95	46.28	20.45
cv %	5.70	7.3	5.25	7.0	7.54	6.24

Table 2. Within-Run Precision.

	Run-to-Run Precision			
	Normal Pool		Abnormal Pool	
	NMN	MN	NMN	MN
Mean (pg/mL)	100.1	78.9	458.7	252.9
sd	7.3	8.2	36.3	21.8
cv %	7.3	10.4	7.9	8.6

Table 3. Run-to-Run Precision.

Run-to-run precision was evaluated by analyzing two plasma pools spiked to contain high normal and abnormal concentrations of the metanephrines. Each of these pools was analyzed 4 times each in 20 separate runs. Run-to-run precision coefficients of variation ranged from 7.3 to 10.4 as shown in Table 3.

Recovery

Recovery studies were performed by spiking a plasma pool with various amounts of NMN and MN. These spike amounts were 50, 100, 200, 500, 1000 pg/mL for both absolute and relative recovery studies. The concentrations of these spiked plasmas were calculated from a calibration curve of directly injected aqueous standards.

The mean absolute recovery of all spike levels run in triplicate were 75.5% and 75.1%, for NMN and MN, respectively. The mean relative recovery of all spike levels run in triplicate were 105.6% and 105.4% for NMN and MN, respectively.

Interferences

The presence of possible interfering drugs and endogenous electroactive compounds was assessed by directly injecting 62 candidates including possible "amine-like" compounds from the

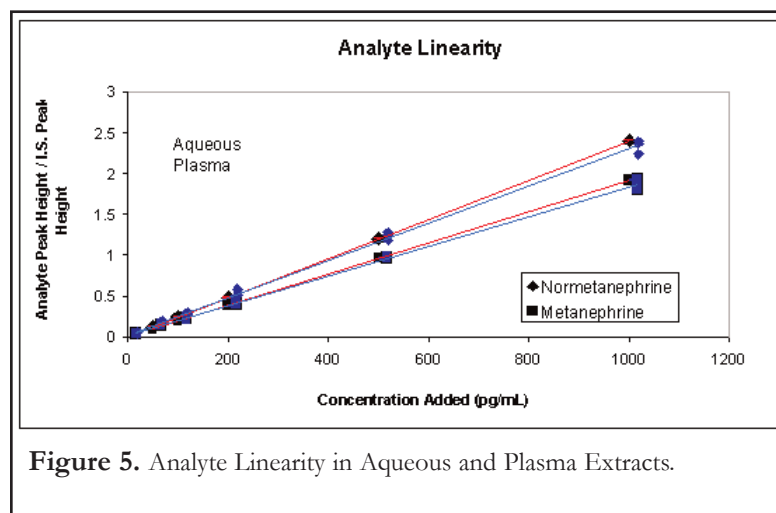
tyrosine, tryptophan and purine metabolic pathways. None of these compounds interfered with the metanephrines or the internal standard even though the vast majority of these do not elute from the solid phase extraction.

Conclusions

The diagnosis of pheochromocytomas has, and will continue to be, an important diagnosis for physicians and clinical laboratories to make. Historically, only after a battery of several biochemical tests have been run have pheochromocytomas been effectively ruled out as an underlying cause of hypertension. Fractionated plasma free metanephrine analysis seems to offer a single assay that can achieve the same diagnostic sensitivity. The ESA CoulArray electrochemical detector and HPLC offer additional analytical selectivity and sensitivity over other HPLC-ECD systems reducing the volume of plasma required.

References

- ¹Eisenhofer, G., *et al.* Pheochromocytoma Improving Diagnosis with Plasma Free Metanephrines. (2001). Clin. Lab. News., February issue.
- ²Lenders, W. M., *et al.*, Determination of Metanephrines in Plasma by Liquid Chromatography with Electrochemical Detection. (1993). Clin. Chem., **39**, 97-103.



Clinical Method

Plasma Free Metanephrines

Ordering Information

Description	Part Number
CoulArray, Model 5600A - 4 channel	70-4320
CoulArray Organizer with Temp. Control	70-4340T
Pump, Model 582 with RS-232 Control	70-4050
Autosampler, Model 542 with Tray Cooling	70-4151
542 Autosampler Start-up Kit	70-4139
Conditioning Cell, Model 5021	55-0450
Analytical Cell, Model 5011	55-0434
Cable, Dual Channel Cell to CoulArray	70-1837
Cable, Single Channel Cell to CoulArray	55-0179
Plasma Metanephrine Extraction Kit	70-5749
Plasma Metanephrine Mobile Phase	70-5627
Plasma Metanephrine Analytical Column	70-5624
Plasma Metanephrine Guard Column	70-5623

The ESA Plasma Metanephrine Analysis Kit has received marketing clearance from the United States Food and Drug Administration through section 510(k) of the Food, Drug and Cosmetics Act 510(k) # K032199.



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