Extraction of Plasma Free Metanephrine (MN) and Normetanephrine (NMN) Using Strata-X-CW and LC/MS/MS on Luna HILIC

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Abstract
In this technical note, we summarize a method for the extraction and concentration of free metanephrines from plasma using a polymeric weak cation exchange resin (Strata™-X-CW) followed by LC/MS/MS analysis using a Luna® 3 µm HILIC HPLC column.

Introduction
Pheochromocytoma is a rare and potentially lethal tumor of hormone affecting chromaffin cells of the adrenal medulla. Pheochromocytoma and other tumors derived from neural crest cells (e.g. paragangliomas and neuroblastomas) secrete catecholamines (epinephrine, norepinephrine, and dopamine). Metanephrine and normetanephrine (collectively referred to as metanephrines) are the stable 3-methoxy metabolites created by action of catechol-O-methyl transferase on epinephrine and norepinephrine, respectively. The metanephrines are secreted along with catecholamines by pheochromocytomas and other neural crest tumors resulting in sustained elevated concentrations of plasma free metanephrine. In 2002 Lenders, et al. reported in the Journal of the American Medical Association that the measurement of plasma free metanephrines is the best tool in chromocytoma research.

Experimental

Chemicals and Reagents

Human plasma disodium EDTA was obtained from Valley Biomedical (Winchester, VA). (±)-normetanephrine-α, α, β-d_3 HCl (NMN-d_3) and (±)-metanephrine α, α, β-d_3 HCl (MN-d_3) with more than 97.5 % isotopic purity were obtained from Medical Isotopes (Pelham, NH). The hydrochloride salts of metanephrine (MN) and normetanephrine (NMN) were obtained from Sigma-Aldrich (St. Louis, MO). All other reagents used were obtained from Sigma-Aldrich and used without further purification. HPLC grade water (Milli-Q, Millipore, Billerica, MA) was used to prepare HPLC mobile phase and for sample preparation. HPLC grade acetonitrile (ACN) was obtained from Honeywell Burdick & Jackson (Muskegon, MI).

Mobile Phase Preparation

100 mM ammonium formate pH 3.2 buffer was prepared by dissolving approximately 6.31 g of ammonium formate in 1 liter water, adding 10.5 mL of concentrated formic acid and mixing well. Mobile Phase A and B were prepared fresh daily.

Sample Preparation

Internal standard (ISTD) was prepared by dissolving 5 mg each of MN-d_3 and NMN-d_3 in 1 mL of methanol/acetonitrile (50/50) solution. After several serial dilutions a final solution of 100 ng/mL was procured. Each sample, standard and QC sample was spiked with 200 µL of the ISTD solution prior to extraction. Stock solutions of MN and NMN were prepared in 50/50 methanol/acetonitrile and used to spike human plasma to create the calibration curve samples and the QC samples. The volume of the spike solutions was always kept at 1 % of the net total plasma sample volume to minimize differences between these samples.

Solid Phase Extraction Procedure

SPE Conditions

Sorbent:	Strata™ X-CW (30 mg/well 96-well plate)
Condition:	400 µL Methanol/Acetonitrile (50/50)
Equilibration:	400 µL Water
Load:	500 µL plasma diluted with 1 mL water
Wash 1:	800 µL Water
Wash 2:	2 mL 50/50 Methanol/Acetonitrile
Dry:	2 min at 10" Hg
Elution:	2 x 200 µL of 5 % Formic acid (freshly prepared) in 50/50 Acetonitrile/Methanol
Evaporate and Reconstitute:	Dry down completely under stream of nitrogen at 45 °C and reconstitute in 100 µL of 95/5 Acetonitrile/100 mM Ammonium formate, pH 3.2

NOTE: Cap the reconstituted extract immediately to prevent evaporation.

Chromatographic Conditions

The chromatographic system consisted of an Agilent 1100 series binary pump equipped with on-line solvent degasser, autosampler, and column temperature module (Palo Alto, California); interfaced with an Applied Biosystems API3000 tandem mass spectrometer with TurboIonSpray® electrospray ionization (ESI) interface. The system was controlled using Analyst 1.41 software.

NOTE: If using an HPLC system with needle wash capability, the needle wash solution should be 95/5 Acetonitrile/Water for best results.

HPLC Conditions

Column:	Luna® 3 µm HILIC
Dimensions:	50 x 2.0 mm
Part No.:	00B-4449-B0
Mobile Phase:	A: Acetonitrile/100 mM Ammonium formate, pH 3.2 (95:5) B: Acetonitrile/Water/100 mM Ammonium formate, pH 3.2 (50:45:5)
Flow Rate:	0.4 mL/min
Inj. Volume:	30 µL of the reconstituted extract

Gradient Time (min)	Flow Rate (mL/min)	A (%)	B (%)
0.0	0.4	100	0
2.5	0.4	100	0
2.51	0.4	0	100
3.0	0.4	0	100
3.01	0.4	100	0
6.0	0.4	100	0

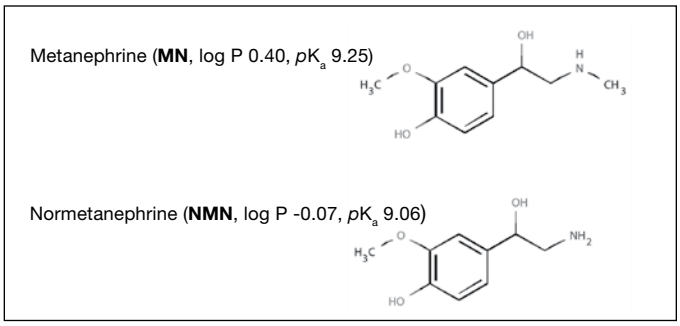
MS/MS Conditions (API3000 w/ TurboIonSpray®)	
Nebulizer Gas Flow (NEB):	9.00
Curtain Gas (CUR):	10.00
Ion Spray Voltage (IS):	3500.00
Temperature (TEM):	550.00
Collision Gas (CAD):	11.00
Declustering Potential (DP):	71 V (MN) 61 V (NMN)
Collision Energy (CE):	23.00 V (MN) 25.00 V (NMN)
Focusing Potential (FP):	370 V
Collision Cell Exit Potential (CXP):	8 V

Analyte	MRM Pair (Q1/Q3)	Dwell Time (sec)
MN	180.4/148.2	150
MN-d_3	183.4/151.2	150
NMN	166.4/134.2	150
NMN-d_3	169.4/137.2	150

Results and Discussion

The structures of metanephrine and normetanephrine are very closely related (**Figure 1**) with both compounds having similar pK_a values. The basic nature of MN and NMN make them ideal candidates for concentration and cleanup from biological sample matrices using solid phase extraction (SPE) with cation exchange functionality. Appropriate choice of the SPE conditions will allow for retention of MN and NMN and removal of potentially interfering plasma matrix components. Strata-X-CW is a polymer-based weak cation exchange sorbent which is ideal for eliminating many matrix components. Both MN and NMN will form relatively strong ionic bonds with the carboxylic acid moieties on the SPE sorbent under neutral pH conditions allowing the use of a strong wash with organic solvent to eliminate hydrophobic matrix components. An initial wash with water removes polar matrix components and salts. Adding 5 % formic acid to the organic elution solvent (50/50 Acetonitrile/Methanol) neutralizes the carboxylic acid groups on the SPE sorbent allowing the plasma metanephrines to elute.

Figure 1.
Structures of MN and NMN with log P and pK_a



The relatively low log P values for MN and NMN indicate that both compounds are relatively polar, especially at low pH, making them ideal candidates for separation using HILIC chromatographic conditions. The HILIC retention mechanism primarily involves partitioning of polar analytes between a water enriched layer of solvent near the sorbent surface and the relatively more hydrophobic bulk eluent. Several other physical processes also play a major role in determining retention and selectivity in HILIC such as ion-exchange, hydrogen-bonding, dipole-dipole, and others. HILIC is generally performed using mobile phases containing high concentrations (>70 v/v %) of acetonitrile, which are ideally suited for use with MS detection.

Under HILIC conditions the more polar compounds elute later in the chromatographic run; this is exactly opposite to what is observed under reversed phase conditions. **Figure 2** shows the chromatogram obtained under HILIC conditions for MN and NMN standards. Note that the more polar NMN (log P -0.07) elutes after MN (log P 0.40). The use of the short 50 mm length Luna HILIC column provides a very fast chromatographic analysis time while providing excellent retention and resolution for MN and NMN.

Calibration curves were generated utilizing an 8-point extracted standard curve with $1/x^2$ weighting over a concentration range of 0.5 to 200 ng/mL for both metanephrine and normetanephrine. The calibration curves for metanephrine (**Figure 3**) and normetanephrine (**Figure 4**), both show good linearity over the entire standard concentration range with R^2 = 0.9993 and 0.9991, respectively.To determine precision and accuracy for this method, QC samples were prepared by spiking the plasma matrix at four different concentrations (0.5, 1.5, 100, and 180 ng/mL) and analyzing in replicate (n=6). The results are summarized in **Table 1** for metanephrine and **Table 2** for normetanephrine and show that accuracy and precision for both MN and NMN are well within the generally acceptable range. The method lower limit of quantitation (LLOQ was determined to be 0.5 ng/mL. Recovery (absolute) was determined by spiking plasma samples at two concentrations, one low (1.5 ng/mL) and one high (180 ng/mL). The results are

(continued on page 4)

Table 1:
Accuracy and Precision for Metanephrine

Sample	Conc. (ng/mL)	Mean (ng/mL)	Std. Dev.	% CV	% Accuracy
QC 1	0.5	0.511	0.0657	12.7	102
QC 2	1.5	1.54	0.0390	2.50	103
QC 3	100	102	2.84	2.75	102
QC 4	180	188	2.12	1.11	104

Table 2:
Accuracy and Precision for Normetanephrine

Sample	Conc. (ng/mL)	Mean (ng/mL)	Std. Dev.	% CV	% Accuracy
QC 1	0.5	0.448	0.0760	17	89.5
QC 2	1.5	1.56	0.120	7.69	104
QC 3	100	102	2.07	2.03	102
QC 4	180	187	2.98	1.59	104

Table 3:
% Absolute Recovery for Metanephrine and Normetanephrine

Spiked conc.	MN	NMN
1.5 ng/mL	87.1 % (N = 7)	72.6 % (N = 8)
180 ng/mL	90.7 % (N = 8)	73.2 % (N = 8)

Figure 2.
LC/MS Chromatogram from SPE Extracts

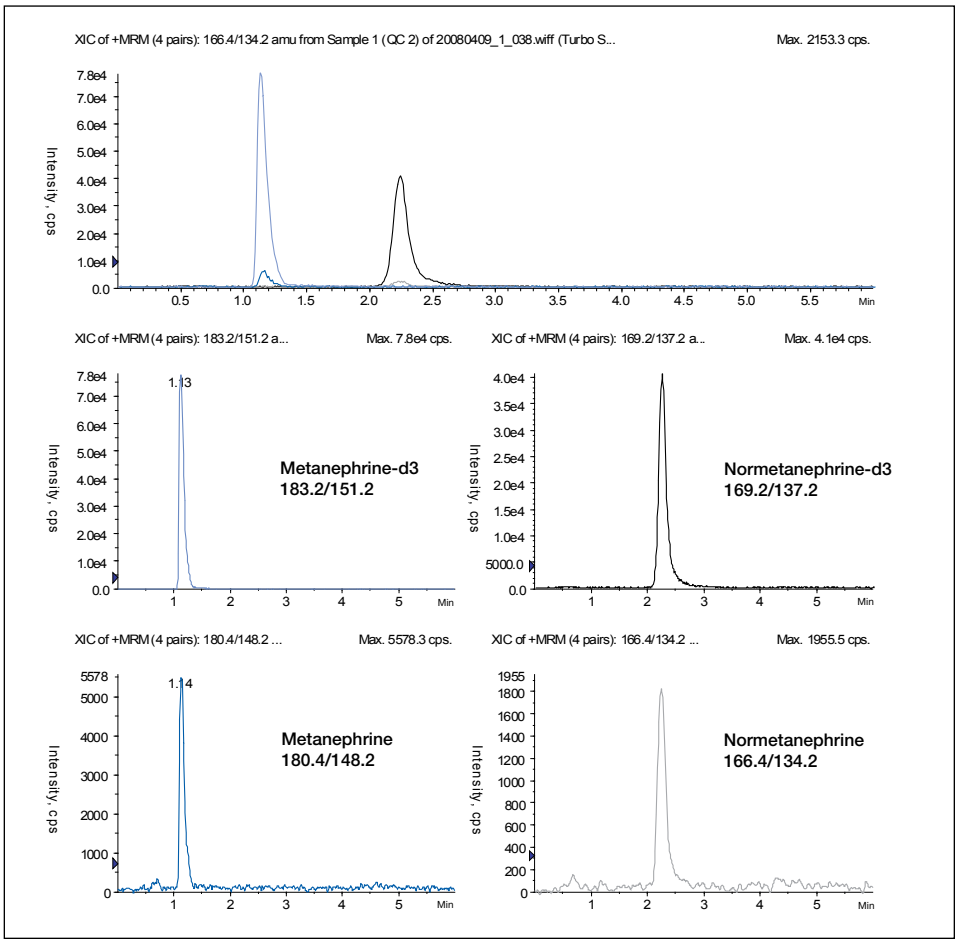


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Figure 3.
Metanephrine Calibration Curve

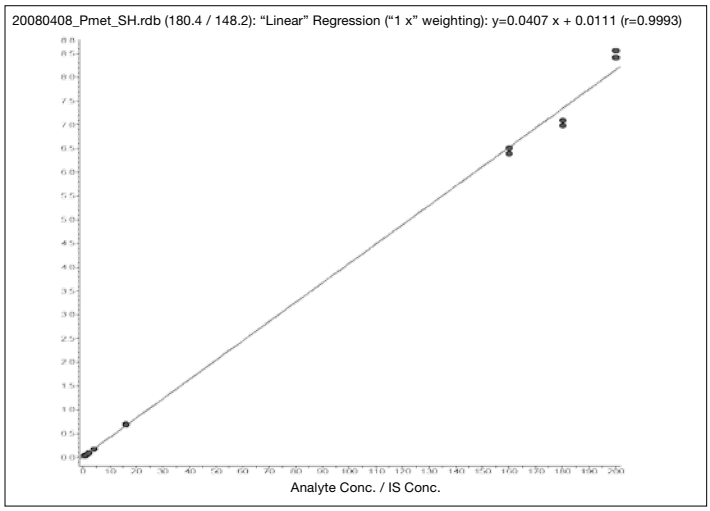
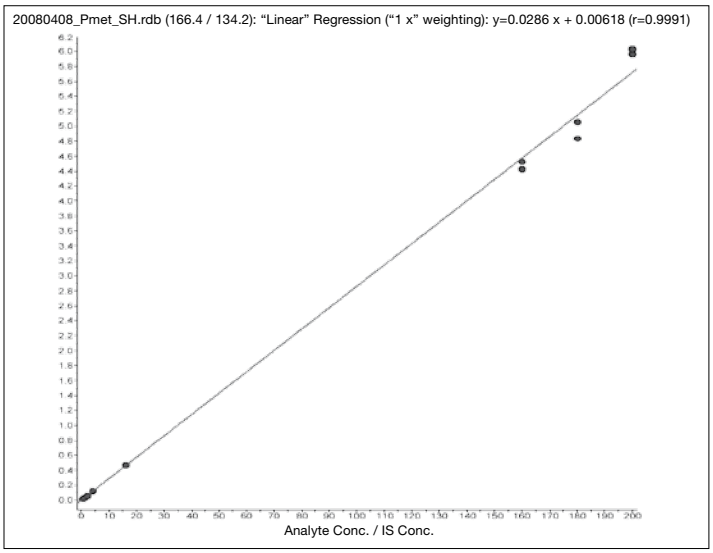


Figure 4.
Normetanephrine Calibration Curve



summarized in **Table 3** and indicate acceptable average sample recoveries at both low and high concentrations.

To explore the effect of the sample matrix on this assay, a reference (REF) was compared with an extracted reference (EREF)MN and NMN spiked at 180 ng/mL in mobile phase (REF) were analyzed and compared with plasma blanks that were extracted per the SPE procedure, blown down to dryness and reconstituted in REF solution (EREF). For NMN and MN the average response for the extracted reference samples were 56 and 66 % of the reference solution, respectively. This result indicates that significant suppression of the analyte signal was observed and illustrates the need to use an internal standard, MN-d_3 and NMN-d_3, for accurate quantitation of plasma metanephrine levels in plasma samples.

The potential for matrix interference attributed to the presence of phospholipids in plasma was also investigated at the LLOQ and found to be minimal.

References

1. J. Lenders, K. Pacak, M. Walther, W. Linehan, M. Mannelli, P. Friberg, H. Keiser, D. Goldstein, G. Eisenhofer; *JAMA* **287**, 1427–34 (2002).

Ordering Information

Part No.	Description	Unit
8E-S035-TGB	Stratax™-X-CW, 30 mg/well 96-WP	2 plates / box
00B-4449-B0	Luna® 3 µm HILIC, 50 X 2.0 MM	ea

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