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A Sensitive and Interference-Free Liquid Chromatography Tandem Mass Spectrometry Method for Measuring Metanephrines in Plasma

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Abstract

Background: Plasma metanephrines are the primary biomarkers used to aid in diagnosing pheochromocytoma. However, the low physiological levels of metanephrines, physicochemical properties, and potential interferences make it challenging to achieve high sensitivity and specificity. In this report, we developed and validated a sensitive and interference-free liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for measuring plasma metanephrines with simple sample preparation.

Methods: Plasma samples were extracted using weak cation exchange solid-phase extraction cartridges, and analyzed by LC-MS/MS with an analytical cycle time less than six minutes.

Results: Absolute ion suppression and matrix effect were observed, however, were completely compensated for by the internal standards. Epinephrine, an isobaric interferent of normetanephrine, was chromatographically separated, and no interference was observed from other common interferents. The method was linear from 0.08 to 22.2 nmol/L for normetanephrine and 0.03 to 8.2 nmol/L for metanephrine with accuracy ranging from 81 to 107%. No carryover was observed up to 56.8 nmol/L for normetanephrine and 8.7 nmol/L for metanephrine. Intra-assay and total CVs were within 6.8% for normetanephrine and 5.2% for metanephrine for three levels tested. Based on Deming regression, comparison with a reference LC-MS/MS method using patient specimens (n=40) showed a slope of 0.973, intercept of 0.11 nmol/L and correlation coefficient of 0.9936 for normetanephrine and a slope of 1.039, intercept of -0.014 nmol/L, and correlation coefficient of 0.9914 for metanephrine. The mean difference was 3.5% and -1.6% for normetanephrine and metanephrine, respectively.

Conclusion: This LC-MS/MS assay is sensitive and free of interference for quantitation of plasma metanephrines.

Keywords: Plasma; LC-MS/MS; Metanephrine; Normetanephrine; Pheochromocytoma

Abbreviations: HPLC: High Performance Liquid Chromatography; LC-MS/MS: Liquid Chromatography-Tandem Mass Spectrometry; IS: Internal Standard.

Introduction

Pheochromocytomas are catecholamine producing tumors found mainly in the adrenal medulla. These tumors can present as unexplained hypertension and, though rare, can be fatal if not diagnosed and treated appropriately [1-3]. In pheochromocytomas, metabolism of catecholamines to metanephrines is enhanced and therefore metanephrines present a larger increase than catecholamines leading to better sensitivity and specificity [1,3]. Measurement of plasma metanephrines is considered one of the best biochemical markers to exclude or aid in the diagnosis of pheochromocytoma [1-4].

Measurement of metanephrines in plasma is challenging due to the low physiological concentrations and their hydrophilic nature. Methods using high-performance liquid chromatography coupled with electrochemical detection (HPLC-ECD) are commonly used [5]. However those methods require time-consuming sample preparation and relatively long chromatography time with occasional interference from other substances including certain drugs [6]. Immunoassays are also available but display higher imprecision and bias [5]. Assays that use HPLC coupled with tandem mass spectrometry (HPLC-MS/MS) may yield high specificity with simple sample preparation and short chromatography time [7-9]. Matrix effects and interfering substances may compromise the performance of LC-MS/MS methods. In the case of normetanephrine it is important to ascertain that the isobaric epinephrine does not interfere. Lagerstedt et al. [9] used Oasis HLB for solid phase extraction (SPE) to enhance sensitivity and specificity [9] while Oasis weak cation exchange SPE was used by others [7,8]. Since Lagerstedt et al. [9] did not chromatographically separate epinephrine, they assessed and demonstrated no significant interference from epinephrine up to 10.0 nmol/L [9]. No such assessment was found in other works where no chromatographic separation between epinephrine and normetanephrine was demonstrated [7-9]. We aimed to separate normetanephrine from epinephrine chromatographically to completely eliminate this potential interference.

For the measurement of plasma metanephrines in the clinical lab, our overall goal was to develop and validate a robust LC-MS/MS assay, free from interferences, which would achieve the sensitivity required to confidently exclude or aid in the diagnosis of pheochromocytoma.

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Materials and Methods

Chemicals, reagents and solutions

Type I water was from a Millipore Synergy System (Billerica, MA, USA). High Purity Solvent methanol and acetonitrile were purchased from Burdick and Jackson via VWR (West Chester, PA, USA). Ammonium hydroxide (certified ACS Plus) was obtained from Fisher Scientific (Fair Lawn, NJ, USA), and ammonium dihydrogen phosphate (ACS, 98%) was from Alfa Aesar (Ward Hill, MA, USA). Formic acid (for mass spectrometry, ~98%), ammonium formate (LC-MS grade) and sodium metabisulfite were purchased from Sigma-Aldrich (St. Louis, MO, USA). Metanephrine, normetanephrine, epinephrine, norepinephrine, and dopamine were from Sigma-Aldrich, and the isotopically labeled internal standards, metanephrine (α-D2, β-D1) and normetanephrine (α-D2, β-D1) were acquired from Medical Isotopes (Pelham, NH, USA). Oasis WCX SPE cartridges (30 mg, 1 mL) were from Waters (Milford, MA, USA). An Ultra PFP Propyl analytical column (3 µm, 2.1 x 100 mm) and UltraShield UHPLC pre-column filters were purchased from Restek (Bellefonte, PA, USA).

Two stock solutions (from different weighings) were prepared for each analyte, metanephrine and normetanephrine, at 1 mg/mL in 0.1% sodium metabisulfite in water and were diluted 5-fold with methanol to make 1.1 and 1.0 mmol/L sub-stock solutions for normetanephrine and metanephrine, respectively. One analyte sub-stock was used to prepare calibration standard solutions, while the other was used for quality control and other validation samples. The calibration standards were prepared by serial dilution in 10 mmol/L ammonium phosphate in water, pH 6.5, at concentrations of 0.05, 0.2, 0.5, 2.0, 4.1 nmol/L for metanephrine, and 0.1, 0.3, 1.1, 2.7, 10.9, 21.8 nmol/L for normetanephrine. An internal standard (IS) stock solution for each analyte was prepared at 1 mg/mL in 0.1% sodium metabisulfite. A combined working IS solution of metanephrine-IS and normetanephrine-IS was made at 50.7 and 54.6 nmol/L, respectively, in 1mmol/L ammonium formate with 0.1% formic acid in water. All solutions were stored at -70°C until use.

Sample preparation

A solid phase extraction (SPE) using Oasis WCX SPE cartridge was performed according to Peaston et al. [7] with modifications. Briefly, Oasis WCX SPE cartridges were conditioned with 1 mL methanol, followed by equilibration with 1 mL 10 mmol/L ammonium phosphates, pH 6.5. Samples, prepared as a homogenous mixture of 500 µL plasma, calibrator, or quality control sample, 25 µL of IS working solution and 500 µL of 10 mM ammonium phosphate in water, pH 6.5 were applied to the cartridges. Each cartridge was washed with 1 mL of water, methanol, and 0.2% formic acid in acetonitrile, sequentially. After washing, the cartridges were eluted with 500 µL of 2% formic acid in acetonitrile. The eluent was evaporated to dryness under vacuum with medium heat applied in Thermo Scientific Savant. Samples were reconstituted with 100µL 1 mmol/L ammonium formate with 0.1% formic acid in water, and 20 µL was injected for LC-MS/MS analysis.

LC-MS/MS method

This method was developed on an AB SCIEX QTRAP® 5500 LC/MS/MS System (California, USA) coupled to a Shimadzu Nexera HPLC system (Kyoto, Japan). The HPLC system was equipped with an SIL-30AC refrigerated autosampler, four LC-30AD ultra high pressure pumps, and a CTO-20AC heated column compartment. The controlling software was Analyst® 1.5.2. Mobile phase A was 1 mmol/L ammonium formate in water with 0.1% formic acid, and mobile phase B was 1 mmol/L ammonium formate in methanol with 0.1% formic acid. Samples were injected onto an Ultra PFP Propyl analytical column at a flow rate of 0.5 mL/min. After injection, the flow rate was ramped over 2.25 min from 0.5 mL/min to 0.7 mL/min. After 2.25 minutes, flow rate was returned to initial conditions of 0.5 mL/min. Initial mobile phase composition 99:1 (A:B) was used to load samples on the column, and held for 0.25 minutes before stepping to 60:40. Over the next 2 minutes the composition was ramped to 40:60, and then the column was returned to initial conditions of 99:1 to re-equilibrate for 2.75 minutes. The total run time between injections, including autosampler pre-run, was 5.75 min. The MS was set to positive electrospray ionization mode with multiple reactions monitoring (MRM). The IonSpray Voltage was set at 1250 V and the temperature at 700°C. Curtain gas, Ion Source Gas 1, Ion Source Gas 2 and Collision Gas were set at 30, 50, 40 and medium, respectively. Declustering potential (DP), entrance potential (EP), collision energy (CE), and collision cell exit potential (CXP) were set individually for each analyte and are summarized, along with the MRM used for each analyte, in Table 1.

Method validation

Absolute ion suppression was assessed by a post-column infusion experiment [10]. Briefly, a constant flow (5 µL/min) of either normetanephrine-IS (0.2 nmol/L) or metanephrine-IS (0.05 nmol/L) solution was infused into the post-column flow path using a T junction while processed patient samples (3 females and 3 males extracted without IS) were injected. A mixing study was conducted to determine relative ion suppression in plasma and a candidate calibration or diluent matrix in order to verify whether the IS accounted for ion suppression/enhancement by behaving similarly to the analyte [11]. The mixing study entailed extracting and injecting a spiked candidate matrix solution (10 mM ammonium phosphate, pH 6.5 at 1.1 nmol/L for normetanephrine, and 0.3 nmol/L for metanephrine), 6 patient samples (3 males and 3 females), and 1:1 mixtures of each patient sample with the spiked candidate matrix solution. If the response ratio (analyte/IS) of the 1:1 mixture for each patient is within 20% of the theoretical response (a calculated average of the measured ratios for the patient sample and candidate matrix sample), it can be concluded that there is no significant relative ion suppression from plasma or the candidate matrix.

	Q1, m/z	Q3, m/z	Dwell time, msec	DP, volt	EP, volt	CE, volt	CXP, volt
Metanephrine	180.1	148.1	25	123	7	24	17
Metanephrine-IS	183.1	168.1	25	123	7	24	17
Normetanephrine	166.1	134.1	50	95	7	24	13
Normetanephrine-IS	169.1	137.1	50	95	7	24	13

Table 1: MRM transitions and mass spectrometer settings for normetanephrine, metanephrine, and the internal standards.

Interference from lipemic, hemolytic, uremic and icteric plasma samples was evaluated in the same way as relative ion suppression. Two analyte concentrations (low and high) were tested by mixing each of the four possible interferent samples 1:1 with 10 mM ammonium phosphate, pH 6.5 spiked at 0.2 and 1.1 nmol/L for normetanephrine and 0.06 and 0.3 nmol/L for metanephrine, respectively. No significant interference was determined if the response ratio for both levels of the 1:1 mixture was within 20% of the theoretical calculated response ratio from the average of interferent sample and spiked solvent sample. Exogenous compounds that have known interferences with current LC-ECD methods were spiked into 10mM ammonium phosphate, pH 6.5 and extracted before injection. No significant interference was detected if no peak was present at the retention time of normetanephrine and metanephrine.

Patient plasma collected in EDTA purple-top tubes was pooled to assess assay linearity. The patient pool was spiked at a high concentration (23.0 nmol/L for normetanephrine and 9.1nmol/L for metanephrine) and serially diluted to nine subsequent levels with 10mM ammonium phosphate, pH 6.5. Samples were extracted in triplicate, along with one set of calibrators. Analytical recovery and imprecision were evaluated for each concentration. We defined the lower limits of quantification based on the criteria that accuracy was within 100 ± 20%, total coefficient of variation (CV) was within 20%, and signal to noise was greater than 10.

Based on a modified protocol from the Clinical Laboratory and Standards Institute (CLSI) EP10-A3 guideline (Wayne, PA, USA), assay precision was evaluated by running 3 levels of spiked pooled EDTA plasma samples (low, mid, and hi) in the sequence mid-hi-low-mid-mid-low-low-hi-hi-mid twice a day for 5 days. Using EP Evaluator Release 9 (Data Innovations, South Burlington, VT, USA), statistics, including both intra-assay and total CVs, were calculated.

Carryover was evaluated by extracting three independent sets of low and high samples and analyzing them in the sequence low_1-high-low_2, where low_2 is a re-injection of low_1. A passing test meant low_1 is within 20% of low_2, and that low_2 is within 3 standard deviations of the low_1 value. The standard deviation was determined using low_1 values. High samples that were above assay linearity were diluted within the linear range and the values were back calculated.

Using leftover patient specimens for this work was approved by Cleveland Clinic Institution Review Board. Comparison of this LC-MS/MS method and an LC-MS/MS method currently used at NMS laboratories (Willow Grove, PA, USA) was performed by running split patient plasma samples (n=40). Deming regression was applied and statistics were calculated using EP Evaluator Release 9. The two methods are considered comparable if the slope was within 1.0±0.2, an R^2>0.90 and the differences were within ± 20%.

Results and Discussion

Sample preparation

During the development stage, several sample preparation procedures were tested for measuring plasma free metanephrines. Our trials showed, for our purposes, neither protein precipitation [12] nor solid-phase extraction with HLB cartridges [9] achieved high enough recovery to reach our desired limits of quantitation. Another method, currently used in our lab for measuring urine metanephrines, utilizes a complexing reagent before solid-phase extraction [13]. Matrix effects associated with this method in plasma did not allow us to reach appropriate limits of quantitation either. A modified procedure based on Peaston et al. [7] and de Jong et al. [8] using WCX extraction cartridges allowed us to overcome the matrix effects, and attain acceptable limits of quantitation. Therefore, this WCX extraction method was used in the following validation work.

Chromatography

Representative chromatograms for an extracted patient sample are shown in Figure 1. Total analytical cycle time, including autosampler pre-run, was 5.75 min. A noteworthy issue is the known interference between epinephrine and normetanephrine due to their shared mass transitions. As shown in Figure 2, the LC method was able to chromatographically resolve these two peaks thus eliminating any

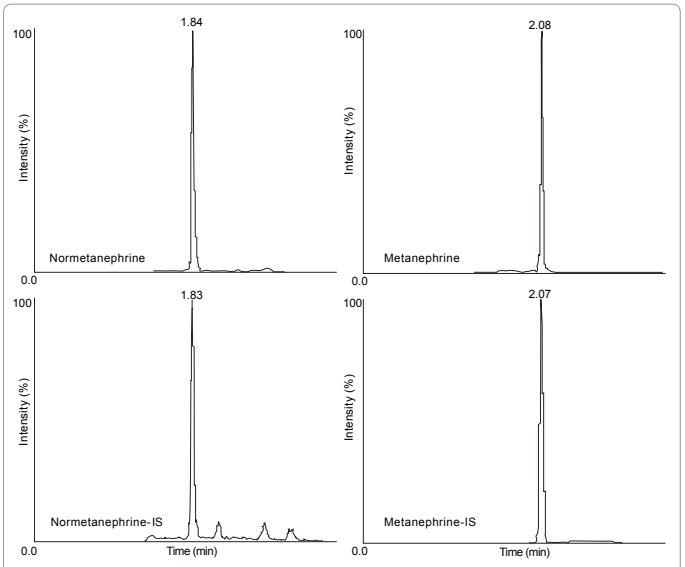

Figure 1: Representative chromatogram of an extracted patient plasma sample. MRM chromatograms of normetanephrine and metanephrine from this patient are shown in the top two panels, and their respective IS chromatograms from the same patient are shown in the bottom two panels.

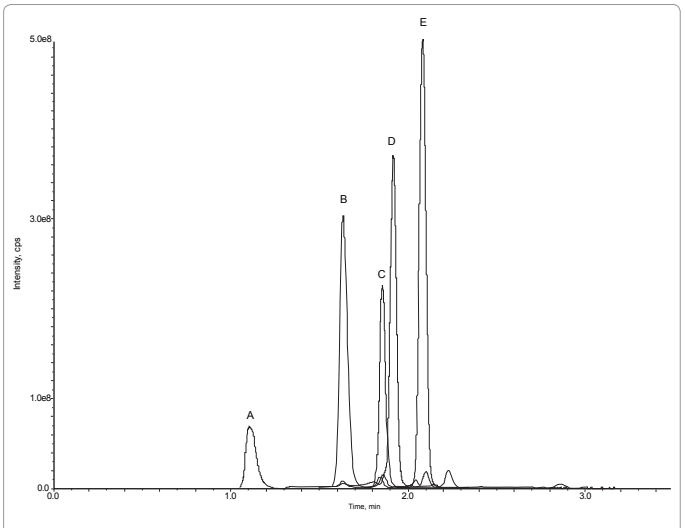

Figure 2: Representative chromatogram of a solvent sample spiked with A) norepinephrine, B) epinephrine, C) normetanephrine, D) dopamine, and E) metanephrine.

possible interference. This was important to us because, although Lagerstedt et al. [9] demonstrated no interference from epinephrine up to 10.0 nmol/L without chromatographic separation in their method, similar evaluation for WCX extraction methods was not available in the literature [7-9].

Assay validation

Significant absolute ion suppression was observed for normetanephrine and, to a lesser extent, metanephrine when 6 extracted plasma samples were injected while infusing normetanephrine-IS or metanephrine-IS by a post-column T-connection. To determine if each analyte's respective IS compensated for this matrix effect, relative ion suppression was investigated. The relative ion suppression test (mixing study) was performed with multiple candidate matrices, including saline, water, 0.1% sodium metabisulfite in water, and stripped blank serum (Seracon II, SeraCare Life Sciences, Milford, MA), however, only 10 mM ammonium phosphate, pH 6.5 was found to have no relative ion suppression for either normetanephrine or metanephrine compared to plasma matrix. Using 10mM ammonium phosphate, pH 6.5, the mean difference between the measured concentration in the 1:1 mixtures and the theoretical calculated concentrations (n = 6) was 1.05%, and -0.64% for normetanephrine and metanephrine, respectively. This indicates that the analytes and their respective internal standards were equally suppressed in patient plasma and 10mM ammonium phosphate, pH 6.5, and therefore this solvent was an acceptable choice as the sample diluent and calibration standard matrix.

Both analytes showed no interference from lipemic (L-index = 43), uremic (U index = 105), icteric (I index = 8) or hemolytic (H index = 199) plasma samples. Exogenous compounds that are known interferences of LC-ECD methods including acetaminophen (20 µg/mL), methyldopa (5 µg/mL), metprolol (200 ng/mL), buspirone (4 ng/mL), labetalol (5 ng/mL), and levodopa (5 ng/mL) were spiked into 10 mmol/L ammonium phosphate, pH 6.5 at the specified concentrations. All samples were extracted, and upon injection, only the sample containing methyldopa showed a peak with the same retention time as normetanephrine, while all other tested drugs did not show any interference to either normetanephrine or metanephrine.

Analyte	Mean, nmol/L	CV, %	Analytical Recovery, %
Normetanephrine	0.1	12.9	81.4
	0.2	4.3	88.5
	0.3	9.6	91.8
	0.7	2.4	91.7
	1.4	6.7	97.3
	2.2	5.4	96.5
	3.1	14.9	105.7
	6.3	6.1	107.0
	11.7	0.7	98.4
	22.2	4.8	93.0
Metanephrine	0.03	7.4	92.0
	0.07	9.6	93.3
	0.1	8.7	95.3
	0.3	3.4	94.7
	0.6	2.3	97.0
	0.9	4.9	99.8
	1.1	2.4	95.4
	2.3	6.7	100.8
	4.6	3.3	100.5
	8.2	6.3	88.9

Table 2: Linearity and recovery data for normetanephrine and metanephrine in EDTA plasma.

	Normetanephrine			Metanephrine		
	Low	Mid	High	Low	Mid	High
N	30	30	30	30	30	30
Mean, nmol/L	1.3	10.1	18.5	0.3	1.3	2.2
Total CV, %	6.8	4.3	5.9	5.2	4.4	4.4
Within Run CV, %	5.6	4.3	5.1	4.2	3.4	2.8

Table 3: Precision data for normetanephrine and metanephrine in EDTA plasma based on the CLSI EP10-A3 protocol.

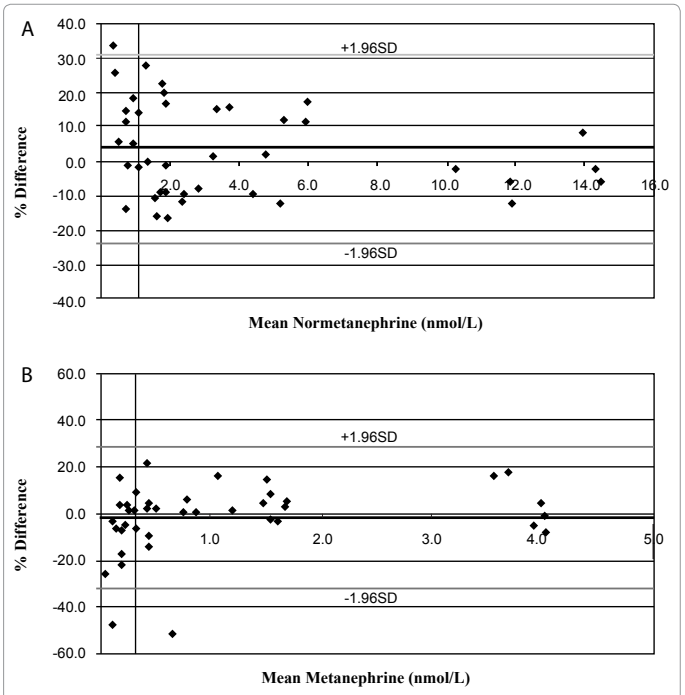

Figure 3: Bland-Altman plot showing method comparison using patient specimens (n=40). The upper reference limit for normetanephrine and metanephrine is 1.1 and 0.3 nmol/L, respectively, and is represented as a vertical line on each graph. The mean difference is shown with a horizontal line and was 3.5% for normetanephrine and -1.6% for metanephrine.

Calibration standards were prepared in 10 mmol/L ammonium phosphate, pH 6.5 and stored at -70˚C prior to use. With quantitation based on peak area ratios of analyte to IS, linearity was established from 0.08 to 22.2 nmol/L for normetanephrine and 0.03 to 8.2 nmol/L for metanephrine with accuracy ranging from 81 to 107% (Table 2). The intra-assay and total CVs were within 6.8% for normetanephrine and 5.2% for metanephrine for all levels tested based on the modified CLSI-EP10A3 protocol (Table 3). No significant carryover was observed up to a tested concentration of 56.8 nmol/L for normetanephrine and 8.7 nmol/L for metanephrine.

EDTA patient plasma samples (n=40) were collected and spiked at varying concentrations, from 0.4 to 14.5 nmol/L for normetanephrine and 0.05 to 4.1 for metanephrine. Deming regression between our new LC-MS/MS method and the NMS LC-MS/MS method showed a slope of 0.973, intercept of 0.11 nmol/L and correlation coefficient of 0.9936 for normetanephrine and a slope of 1.039, intercept of -0.014 nmol/L,

and correlation coefficient of 0.9914 for metanephrine. The mean difference was 3.5% and -1.6% for normetanephrine and metanephrine, respectively (Figure 3).

In conclusion, we have developed a novel LC-MS/MS assay with high sensitivity and specificity for measuring plasma normetanephrine and metanephrine. This method has been fully validated for use in the clinical laboratory.

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