MEASUREMENT OF PLASMA FREE METANEPHRINES USING ON-LINE SOLID PHASE EXTRACTION—HIGH PERFORMANCE LIQUID CHROMTOGRAPHY—TANDEM MASS SPECTROMETRY

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INTRODUCTION

Pheochromocytoma is a rare, catecholamine-producing tumour of the adrenal medulla[1] and its presence must be considered in many patients with hypertension, the latter representing a quarter of the adult population in Western countries[2]. The clinical hallmark is sustained or intermittent hypertension often associated with paraoxysmal symptoms however, pheochromocytoma should also be considered if a patient presents with labile hypertension and hypertension resistant to anti-hypertensive therapies[4].

Many analytes in the catecholamine metabolic pathway have been used to assess the presence of pheochromocytoma in a variety of biological fluids[3], although the diagnosis of pheochromocytoma depends crucially on the demonstration of excess production of catecholamines. This step is problematic with respect to false-negative/positive results due the inadequate specificity and sensitivity of the various biochemical tests[5].

A number of recent studies have demonstrated the higher diagnostic efficacy of plasma free metanephrines (PFM)[2,5-9]. The majority of PFM assays are performed with HPLC using electrochemical detection (HPLC-ECD) that are generally labour-intensive and time-consuming with long run times. Co-eluting interferences from co-prescribed medications are also known to complicate data interpretation.

Enzymatic immunoassays also suffer from interferences and are susceptible to artifacts caused by non-specific binding as well as cross-reactivity. Gas chromatography – mass spectrometry methods address many of these shortcomings however, arduous sample preparation coupled with poor sensitivity mean that there still remains a need for an alternative method of analysis.

A liquid chromatography – tandem mass spectrometry method using off-line solid phase extraction has been published[10]. This method uses relatively large volumes of plasma and a labour-intensive, relatively non-selective sample preparation protocol.

The work presented here describes the use of a completely automated on-line solid phase extraction – liquid chromatography – tandem mass spectrometry (XLC-MS/MS) method for the determination of metanephrine (M) and normetanephrine (NM) in plasma for the diagnosis of pheochromocytoma.

METHODS

Patient Samples

Plasma samples from 6 healthy volunteers were provided by Medeval Laboratories (Manchester, UK) and were used to assess the performance characteristics of the assay and to prepare calibrators. A further 102 plasma samples were used in the preliminary investigation of reference ranges for M and NM. These were collected from patients assumed to be healthy and were provided by UMC Groningen (Groningen, The Netherlands).

Standards, Calibrators & QCs

M and NM were purchased from Sigma Aldrich Ltd (Poole, UK) as D,L-metanephrine.HCl and D,L-normetanephrine.HCl. The deuterated internal standards α,α,β-d3-metanephrine.HCl and α,α,β-d3-normetanephrine.HCl were purchased from Cambridge Isotopes Inc. (Andover, MA, USA) and Medical Isotopes Inc. (Pelham, NH, USA), respectively. Calibrators were prepared by spiking 1mL plasma samples with M and NM (10µL) made up in 0.1M HCl prior to thorough mixing. QC samples were prepared in a similar manner using stock solutions of M and NM that were independent of the those used to prepare the calibrators.

Mass Spectrometry

A Quattro micro tandem mass spectrometer with a ***Z*** *SPRAY* ion source was used for all analyses (Waters Corporation, Manchester, UK). This instrument was operated in positive ionisation mode and was coupled directly to a Symbiosis® Pharma (Spark Holland, Emmen, The Netherlands) on-line solid phase extraction – liquid chromatography system. MS System control and data acquisition was performed using MassLynx v4.0 software with automated data processing by the QuanLynx Application Manager. Control of the Symbiosis system was performed using SparkLink v3.0 software.

In positive ionisation mode, M and NM are protonated to produce ions of the form $[M+H]^+$ at *m/z* 198 and *m/z* 184, respectively. These ions are known to then undergo a facile loss of water[10] and the ion source conditions were optimised for these resulting ions (M = *m/z* 180; NM = *m/z* 166) of the form $[M+H-H_2O]^+$. Upon collision induced dissociation (CID), these precursor ions produced characteristic product ions of *m/z* 148 and *m/z* 134 for M and NM, respectively (Figure 1). Using the information from these experiments, the MS Method shown in table 1 was used to monitor M, NM & their deuterated analogues in MRM mode using a dwell time of 0.07 sec.

On-line Solid Phase Extraction

Sample Volume: 40µL (1:1 dilution of plasma with aqueous IS solution)

Cartridge:	Waters 10mm x 1 mm Oasis® WCX	
Solvation:	1mL Acetonitrile	5mL/min
Equilibration:	1mL Water	5mL/min
Sample Loading:	1mL Water	2mL/min
Wash 1:	1mL Water	5mL/min
Wash 2:	1mL Acetonitrile	5mL/min

Elution Duration: 2 minutes with LC Mobile Phase
Total Extraction Time: 2 min 55 sec including Valve Wash
Total Cycle Time: 7 min 40 sec per sample

LC Conditions

Column: Waters 2.1mm x 50mm HILIC; 3µm

Mobile Phase A: Acetonitrile
Mobile Phase B: 100mM Ammonium Formate @ pH 3

Time(m:ss)	Flow (mL/min)	%A	%B
0:00	0.3	95	5
0:05	0.3	95	5
4:10	0.3	80	20
4:40	0.3	80	20
4:41	0.3	95	5
7:15	0.3	95	5

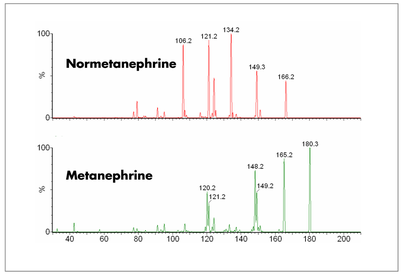

Figure 1. The product ion spectra of normetanephrine (upper) and metanephrine (lower) obtained during the optimization of the mass spectrometer.

Compound	Precursor (m/z)	Product (m/z)	Cone Voltage (V)	Collision Energy (eV)
Metanephrine	180.1	148.1	20	17
d3-metanephrine	183.1	151.1	20	17
Normetanephrine	166.1	134.1	18	16
d3-normetanephrine	169.1	137.1	18	16

Table 1. The Multiple Reaction Monitoring method (MRM) metanephrine, normetanephrine and their deuterated analogues.

	Metanephrine		Normetanephrine	
Patient	**Mean ± Std Dev (nmol/L)**	**%CV**	**Mean ± Std Dev (nmol/L)**	**%CV**
QC Leve1 1	0.29±0.02	5.3	0.76±0.04	5.8
QC Leve1 2	0.82±0.03	3.2	1.57±0.06	3.8
QC Leve1 3	2.93±0.07	2.3	3.56±0.09	2.6

Table 2. The intra-assay performance of the on-line SPE LC-MS/MS assay as shown by QC samples for metanephrine and normetanephrine.

	Metanephrine		Normetanephrine	
Patient	**Mean ± Std Dev (nmol/L)**	**%CV**	**Mean ± Std Dev (nmol/L)**	**%CV**
Patient 1	0.14±0.02	12	0.57±0.06	10
Patient 2	0.16±0.01	8.0	0.41±0.06	15
Patient 3	0.10±0.02	18	0.40±0.03	7.4
Patient 4	0.14±0.01	9.2	0.32±0.05	14
Patient 5	0.14±0.02	14	0.55±0.08	15
Patient 6	0.17±0.02	10	0.41±0.06	15

Table 3. The inter-assay performance of the on-line SPE LC-MS/MS assay as shown by the results for 6 patient samples over seven separate days.

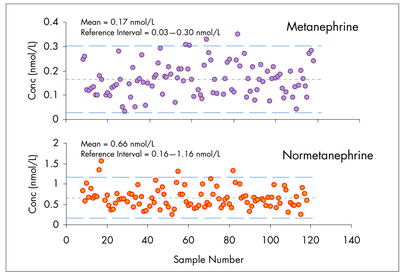

Figure 4. The estimation of reference ranges for metanephrine and normetanephrine was carried out by assaying 102 plasma samples obtained from patients who were assumed to be healthy.

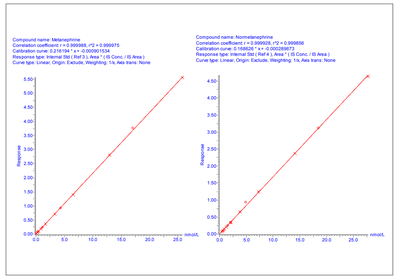

Figure 2. The calibration lines resulting from injection of plasma samples spiked with metanephrine and normetanephrine showing linearity of response over the range 0 to approximately 25nmol/L.

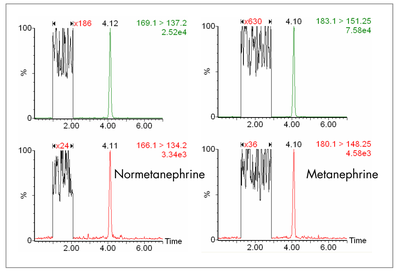

Figure 3. The Multiple Reaction Monitoring Chromatograms for a plasma sample containing 0.16nmol/L metanephrine and 0.38nmol/L normetanephrine with magnified baselines to demonstrate signal-to-noise measurements.

RESULTS

- The calibration lines were linear over the examined range with correlation co-efficients > 0.999 for M and NM (Figure 2).
- The lower limit of quantification (signal-to-noise ratio ≥ 10) for M and NM were 0.04 and 0.16 nmol/L, respectively (Figure 3).
- Extraction recoveries for M and NM were found to be ≥ 90% using the automated Method Development function of the Symbiosis® Pharma system.
- Intra-assay variation was calculated using QC samples at three levels for M and NM. This was found to be < 6% at all levels (Table 2).
- Inter-assay variation was calculated using QC samples at three levels (n=10) and the results from patient samples over several assays (n=7). In both cases, inter-assay precision was found to be ≤ 15% (Table 3).
- Provisional estimation of reference intervals for M and NM was based on the analysis of 102 patient samples who were assumed to be healthy. The reference intervals were calculated using the mean concentrations of M and NM found in the patient samples ± 2 standard deviations (Figure 4)

DISCUSSION

The use of on-line solid phase extraction technology coupled to LC-MS/MS has been shown to provide a PFM assay with improved sensitivity, selectivity and vastly reduced sample handling. Simple dilution of plasma samples with water containing deuterated internal standards followed by centrifugation now replaces tedious off-line extraction methods.

A highly-selective extraction process is achieved using weak cation exchange (WCX) media. Traditionally, strong bases are extracted using strong cation exchange (SCX) media where the base must be eluted via neutralisation. In the case of quarternary amines, this is often not possible and, more commonly, the stabilities of the basic analytes are compromised. Using the Waters Oasis™ WCX media, strong bases bind to the carboxyl ion-exchanger in the cartridge at pH > 5 permitting the cartridge to be washed with water and 100% acetonitrile without elution of the analytes of interest. Elution of the cartridge is then carried using the acidic mobile phase used in the chromatographic method.

The use of HILIC Chemistry for the analysis of polar bases provides LC-MS/MS assays with higher sensitivities than traditional reversed-phase methods when using electrospray ionisation. The analytes of interest elute in high concentrations (circa 75%) of organic solvent where the desolvation process is more efficient.

As a preliminary indication of the validity of the assay, the M and NM levels in the small group of patient samples (n=102) was used to calculate tentative reference intervals (Figure 4). These were found to be in close agreement with those in a previous study[10] that suggests reference intervals of 0.05—0.47 nmol/L and 0.12—1.1nmol/L for M and NM, respectively. It should be noted that specimen collection strategies may have important consequences on the M and NM levels, particularly the position of the patient when blood samples are obtained. Since this information is not known for the samples used in this study, a more controlled study should be undertaken using a larger group of patients to provide reference intervals with greater credibility.

PATIENT CASE STUDIES

During the assay development, plasma samples from two patients, X and Y, displaying signs of hypertension and where the presence of pheochromocytoma was suspected were made available.

Patient X, Male, 69 years.

Hypertensive post Coronary Artery Bypass Graft (CABG). Urinary catecholamine and metanephrine tests were carried a number of times prior to the analysis of plasma samples for free metanephrines, as shown in the table below.

	Total Urinary Catecholamines (µmol/L)	Fractionated Urinary Catecholamines (µmol)		Fractionated Urinary Metanephrines (µmol)	
Analyte	**Epinephrine (E) + Norepinephrine(NE)**	**E**	**NE**	**M**	**NM**
Reference Interval (µmol/L)	**<1.1**	**0.01- 023**	**0.05-0.90**	**<2.0**	**<4.3**
21-Jan-06	>7.0	>1	>1	41.6	0.85
31-Jan-06	1.68	0.55	1.13		
4-Feb-06	1.05	0.33	1	44.6	10.2
6-Feb-06	0.96	0.28	0.7	52.6	11.3

Plasma free metanephrine assays were performed on two plasma samples received from Patient X on 13 and 17-Feb-2006. Levels of M and NM in these samples were found to be 26.4, 13.3nmol/L (M) and 8.31, 6.11nmol/L (NM), on the respective dates. The M/NM ratios in these samples of 3.2 and 2.2 strongly indicate the presence of an adrenal tumour.

M and NM levels determined in plasma from heparinised blood sample and a serum sample showed similar results to those obtained from the EDTA plasma sample taken on the same day (17-Feb-2006).

Patient X is currently awaiting resection of an adrenal mass.

Patient Y, Female, 40 years.

Patient Y was found to have a total urinary catecholamines =1.67µmol/L; urinary E and NE = 0.17, 1.5µmol/L and urinary M and NM = 1.7, 6.0µmol/L. (14-Feb-2006)

Plasma free metariephrine assays were performed on two plasma samples taken on this date. Levels on M and NM were found to be 0.60, 0.59nmol/L (M) and 3.52, 3.52 nmol/L(NM).

Patient Y is currently awaiting an abdominal scan.

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