TECHNICAL NOTE 73768

Quantification of free metanephrines in human plasma by LC-MS/MS for clinical research

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Application benefits

- Accurate and confident results obtained by implementation of a comprehensive kit for sample preparation
- Robust, sensitive LC and MS platforms enable increased confidence in data

Goal

Implementation of an analytical method for the quantification of free metanephrines in human plasma on a Thermo Scientific™ TSQ Altis™ triple quadrupole mass spectrometer.

Introduction

Plasma free metanephrines (PFM) are the most specific indicators for the diagnosis of extra-adrenal chromaffin tumors. Because catecholamines are metabolized within chromaffin cells to metanephrine or normetanephrine, these metabolites can be used to diagnose pheochromocytoma. The measurement of PFMs (metanephrine (M), normetanephrine (NM), and 3-methoxytyramine (MT)) is challenging because of their polar nature, their low molecular weight, and their low physiological concentration in human plasma.

In this report, an analytical method for clinical research for the quantification of metanephrines in human plasma is reported. Samples were pre-processed by protein precipitation followed by online solid-phase extraction (SPE) and chromatographic separation on a Thermo Scientific™ Vanquish™ Flex Binary UHPLC system. Detection was performed on a TSQ Altis triple quadrupole mass spectrometer with heated electrospray ionization (HESI) operated in positive ion mode by selected reaction monitoring (SRM). Method performance was evaluated using the ClinMass™ LC-MS/MS Complete Kit for Free Metanephrines in Plasma – online analysis from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for all analytes.

Experimental

Target analytes

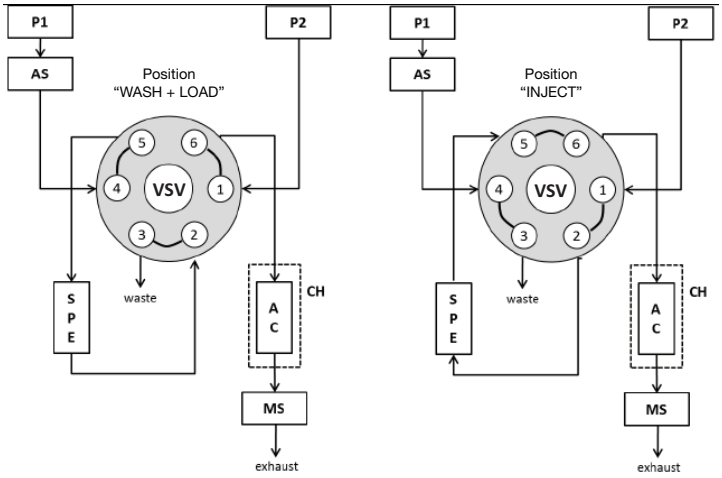
The concentration ranges covered by the calibrators (MS1103 batch #1178) used are reported in Table 1.

Sample preparation

Reagents included five calibrators (including blank) and three controls from RECIPE, as well as three deuterated internal standards for quantification and precipitation solution. Samples of 100 µL of plasma were protein precipitated using 150 µL of precipitating solution containing the internal standards. Precipitated samples were vortex-mixed, kept at room temperature for 5 minutes, vortex-mixed again, and centrifuged. The supernatant was transferred to a clean vial.

Liquid chromatography

Online SPE and LC separation were performed on a Vanquish Flex Binary UHPLC system using mobile phases, an SPE cartridge, and an analytical column provided by RECIPE. A schematic representation of the LC configuration is reported in Figure 1. Details of the analytical method are reported in Table 2. Total runtime was 6.0 minutes.



ABBREVIATIONS

P1 – SPE pump
P2 – HPLC pump
AS – Autosampler
VSV – Vanquish biocompatible switching valve
SPE – SPE column
AC – Analytical column
CH – Column heater
MS – Tandem mass spectrometer

Figure 1. Schematic representation of the Vanquish Flex Binary UHPLC system configuration used for online SPE

Table 1. Analytes, their internal standards, and concentration ranges covered by RECIPE calibrators

Analyte	Internal standard	Concentration range (ng/L)
Normetanephrine	d_3-Normetanephrine	39.90–1974
Metanephrine	d_3-Metanephrine	27.80–1384
3-Methoxytyramine	d_4-3-Methoxytyramine	18.20–1477

Table 2. LC method description

	Time (min)	ASV position	Pump P1 flow rate (mL/min)	L (%)	W (%)	Event SPE column	Pump P2 flow rate (mL/min)	A (%)	B (%)	Event analytical column
Gradient profile	0.00	W+L	0.1	100	0	Loading	0.6	5	95	Equilibration
	0.01		1.0	100	0					
	1.00		1.0	100	0					
	1.01		1.0	0	100	Washing				
	2.00		1.0	0	100					
	2.01	Inject	0.1	0	100	Transfer	0.6	5	95	Loading
	3.50		0.1	0	100					
	3.51	W+L	1.0	0	100	Washing				
	4.50		1.0	0	100		0.6	30	70	Separation
	4.51		1.0	100	0	Equilibration				
	5.60		1.0	100	0		0.6	30	70	
	5.61		0.1	100	0		0.6	5	95	Equilibration
	6.00		0.1	100	0		0.6	5	95	
Injection volume		50 µL	Column temperature							25 °C

Mass spectrometry

Analytes and internal standard were detected by SRM on a TSQ Altis triple quadrupole mass spectrometer with heated electrospray ionization operated in positive ion mode. A summary of the MS conditions is reported in Table 3. Two SRM transitions for each analyte were included in the acquisition method for quantification and confirmation, respectively. SRM transitions with optimized collision energy and RF values are summarized in Table 4.

Method evaluation

The parameters used to evaluate robustness and efficiency of the method included linearity of response within the calibration ranges, accuracy, and intra- and inter-assay precision for all the analytes. Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using quality control samples at three different levels provided by RECIPE (MS11083 batch #1127), prepared and analyzed in replicates of five on three different days. Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at three different levels in replicates of five (n=5). Inter-assay precision was evaluated as the %CV on the full set of samples (control samples at three levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Table 3. MS settings

Source type	Heated electrospray ionization (HESI)
Vaporizer temperature	350 °C
Capillary temperature	350 °C
Spray voltage (positive mode)	2700 V
Sheath gas	40 AU
Sweep gas	0 AU
Auxiliary gas	10 AU
Data acquisition mode	Selected reaction monitoring (SRM)
Source fragmentation	25 V
Collision gas pressure	1.5 mTorr
Cycle time	0.300 s
Q1 mass resolution (FWMH)	0.7
Q3 mass resolution (FWMH)	0.7

Results and discussion

A linear interpolation with 1/X weighting was used for all the analytes. The percentage bias between nominal and back-calculated concentration was always found to be within an acceptable range of ±15% for all the calibrators in all the runs. Representative chromatograms for the lowest calibrator for all the analytes and their internal standards are reported in Figure 2. Representative calibration curves are reported in Figure 3.

The data demonstrate that this method is accurate, with the percentage bias between nominal and average back-calculated concentration for the control samples ranging between -8.6% and 4.2% (Table 5). The %CV for intra-assay precision was always below 10.2% for all analytes. The maximum %CV for inter-assay precision including all analytes was 9.1%. Results for intra- and inter-assay precision are reported in Table 6.

Table 4. SRM transitions, collision energies, and RF lens values

Analyte/internal standard	Quantification			Confirmation			RF lens (V)
	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	Precursor ion (*m/z*)	Product ion (*m/z*)	Collision energy (V)	
Normetaneprhine	166.1	134.1	17	166.1	106.1	19	44
d_3-Normetanephrine	169.1	137.1	18	169.1	109.1	20	44
Metanephrine	180.1	148.1	19	180.1	165.1	18	51
d_3-Metanephrine	183.1	151.1	19	183.1	168.1	18	52
3-Methoxytyramine	151.0	119.1	15	151.0	91.1	20	45
d_4-3-Methoxytyramine	155.1	123.1	16	155.1	95.1	21	47

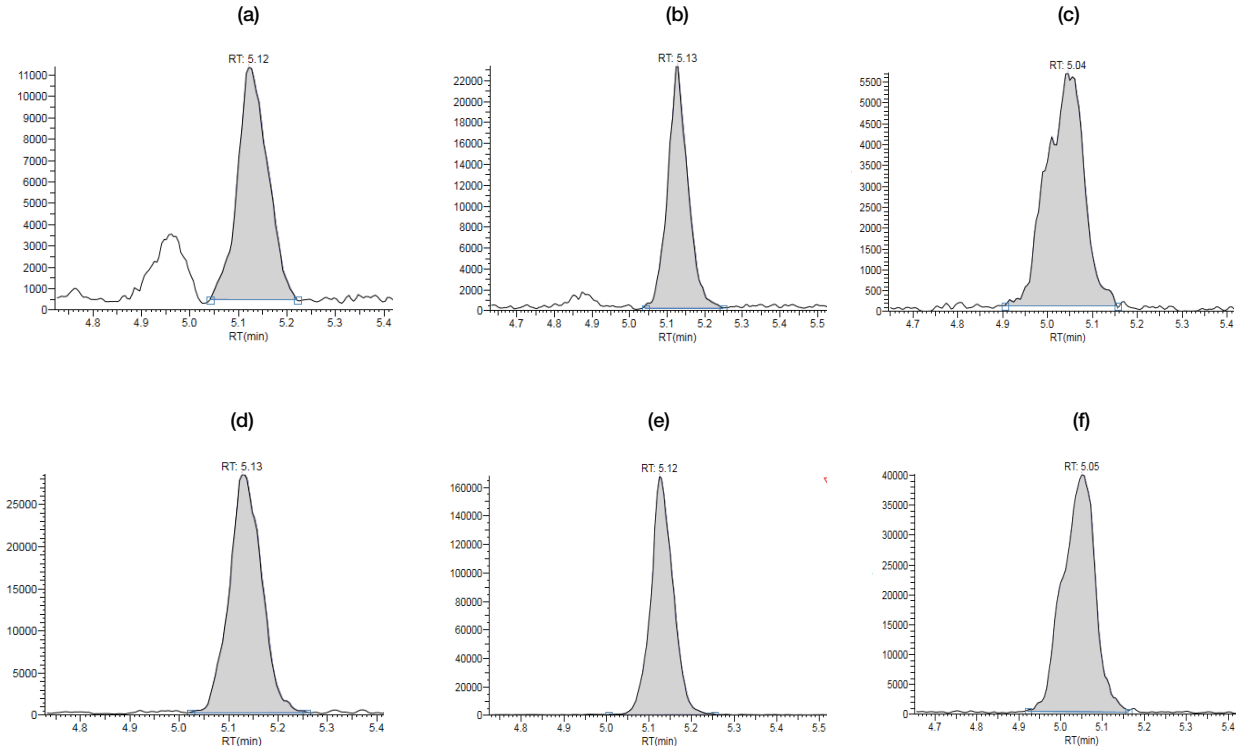


Figure 2. Representative chromatograms of the lowest calibrator for (a) normetanephrine, (b) metanephrine, (c) 3-methoxytyramine, (d) d_3-normetaneprhine, (e) d_3-metanephrine, and (f) d_4-3-methoxytyramine

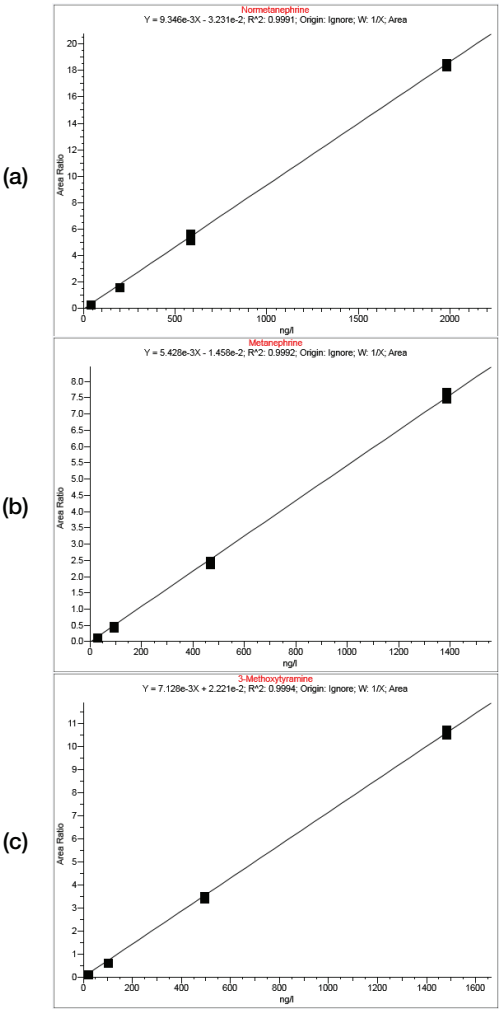


Figure 3. Representative calibration curves for (a) normetanephrine, (b) metanephrine, and (c) 3-methoxytyramine

Table 5. Analytical accuracy results for control MS11083 batch #1127

Analyte	Control	Nominal concentration (ng/L)	Average calculated concentration (ng/L)	Bias (%)
Normetaneprhine	Level I (LOT #1127)	113	118	4.2
	Level II (LOT #1127)	378	368	-2.7
	Level III (LOT #1127)	944	945	0.1
Metaneprhine	Level I (LOT #1127)	54.8	56.0	2.1
	Level II (LOT #1127)	231	214	-7.2
	Level III (LOT #1127)	734	728	-0.8
3-Methoxytyramine	Level I (LOT #1127)	30.6	29.9	-2.2
	Level II (LOT #1127)	196	179	-8.6
	Level III (LOT #1127)	761	760	-0.1

Table 6. Analytical intra- and inter-assay precision results for control MS11083 batch #1127

Analyte	Control	Intra-assay						Inter-assay	
		Day 1		Day 2		Day 3			
		Average calculated concentration (ng/L)	CV (%)	Average calculated concentration (ng/L)	CV (%)	Average calculated concentration (ng/L)	CV (%)	Average calculated concentration (ng/L)	CV (%)
Normetaneprhine	Level I (LOT #1127)	114	5.7	118	7.0	121	6.1	118	6.3
	Level II (LOT #1127)	364	5.1	371	4.3	369	2.9	368	4.0
	Level III (LOT #1127)	909	1.5	988	2.9	937	2.8	945	4.3
Metaneprhine	Level I (LOT #1127)	58.0	9.6	54.7	9.0	55.3	6.4	56.0	8.3
	Level II (LOT #1127)	215	3.0	215	2.2	213	2.0	214	2.3
	Level III (LOT #1127)	723	1.7	737	1.8	724	1.5	728	1.8
3-Methoxytyramine	Level I (LOT #1127)	31.0	9.6	29.3	8.2	29.5	10.2	29.9	9.1
	Level II (LOT #1127)	175	2.9	183	1.2	179	2.1	179	2.7
	Level III (LOT #1127)	747	2.8	776	3.9	758	2.4	760	3.3

Conclusion

A robust, reproducible, and sensitive liquid chromatography-tandem mass spectrometry method for clinical research for quantification of metanephrines in human plasma was implemented. The ClinMass LC-MS/MS Complete Kit for Free Metanephrines in Plasma – online analysis from RECIPE enabled achievement of higher sensitivity and robustness of the method. The method was analytically validated on a Vanquish Flex Binary UHPLC system connected to a TSQ Altis triple quadrupole mass spectrometer. The method described here offers a quick and simple offline protein precipitation with concomitant internal standard addition. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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