TECHNICAL NOTE 66049

Quantification of free metanephrines in human plasma by LC-HRAM mass spectrometry for clinical research

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Application benefits

- Increased accuracy of method by implementation of a comprehensive ClinMass® kit for sample preparation
- Improved selectivity through high-resolution mass spectrometry
- Simple offline sample preparation by solid-phase extraction (SPE) without dilution or evaporation steps

Goal

Implementation of an analytical method for the quantification of plasma free metanephrines on a Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer.

Introduction

The presence of plasma free metanephrines (PFM) in human plasma can potentially indicate the presence of a rare tumor of the adrenal gland, called a pheochromocytoma, or a rare tumor occurring outside the adrenal gland, called a paraganglioma. These tumors produce excess hormones called catecholamines, which are broken down into metanephrines. The analysis of blood or serum for plasma free metanephrines assesses the amount of metanephrine and normetanephrine present. The quantification of PFMs (metanephrine, normetanephrine, and 3-methoxytyramine) is challenging because of their polar nature, low molecular weight, and low physiological concentration in human plasma.

In this method, plasma samples were extracted by offline internal standard addition followed by SPE. Extracted samples were directly injected onto a Thermo Scientific™ Vanquish™ Flex Duo UHPLC system without any need for evaporation and reconstitution. Detection was performed in Full Scan – data-dependent MS^2 acquisition mode using a high-resolution accurate mass (HRAM) Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with a heated electrospray ionization (HESI-II) source. Method performance was evaluated using the ClinMass Complete Kit for Free Metanephrines in Plasma from RECIPE Chemicals + Instruments GmbH (Munich, Germany) in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurements, and intra- and inter-assay precision for each analyte.

Experimental

Target analytes

Target analytes, corresponding internal standards, and concentration ranges covered by the calibrators (MS11013 batch #2420) are reported in Table 1.

Sample preparation

Reagents included five calibrators (including blank) and three controls (MS11083 batch #1229) from RECIPE, as well as three isotopically labelled internal standards for quantification. Samples of 500 µL of plasma were loaded onto conditioned extraction SPE cartridges together with 500 µL of internal standard solution. The loaded sample was washed with three different washing solutions prior to elution using 2 × 100 µL of eluting reagent provided with the kit. The eluate was collected into a clean plate or vial ready for injection.

Liquid chromatography

A Vanquish Flex Duo UHPLC system configured as a dual channel instrument for both LC-only and online SPE applications (Figure 1), was used for chromatographic separation. The LC-only channel was used in this case, utilizing mobile phases and an analytical column provided by RECIPE. Details of the analytical method are reported in Table 2. Total runtime was 4.0 minutes.

Table 1. Target analytes, internal standards, and concentration ranges covered by calibrators

Analyte	Internal standard	Concentration range (ng/L)
3-methoxytyramine	d_4-3-methoxytyramine	13.5–1101
Metanephrine	d_3-metanephrine	27.9–1399
Normetanephrine	d_3-normetanephrine	40.9–1954

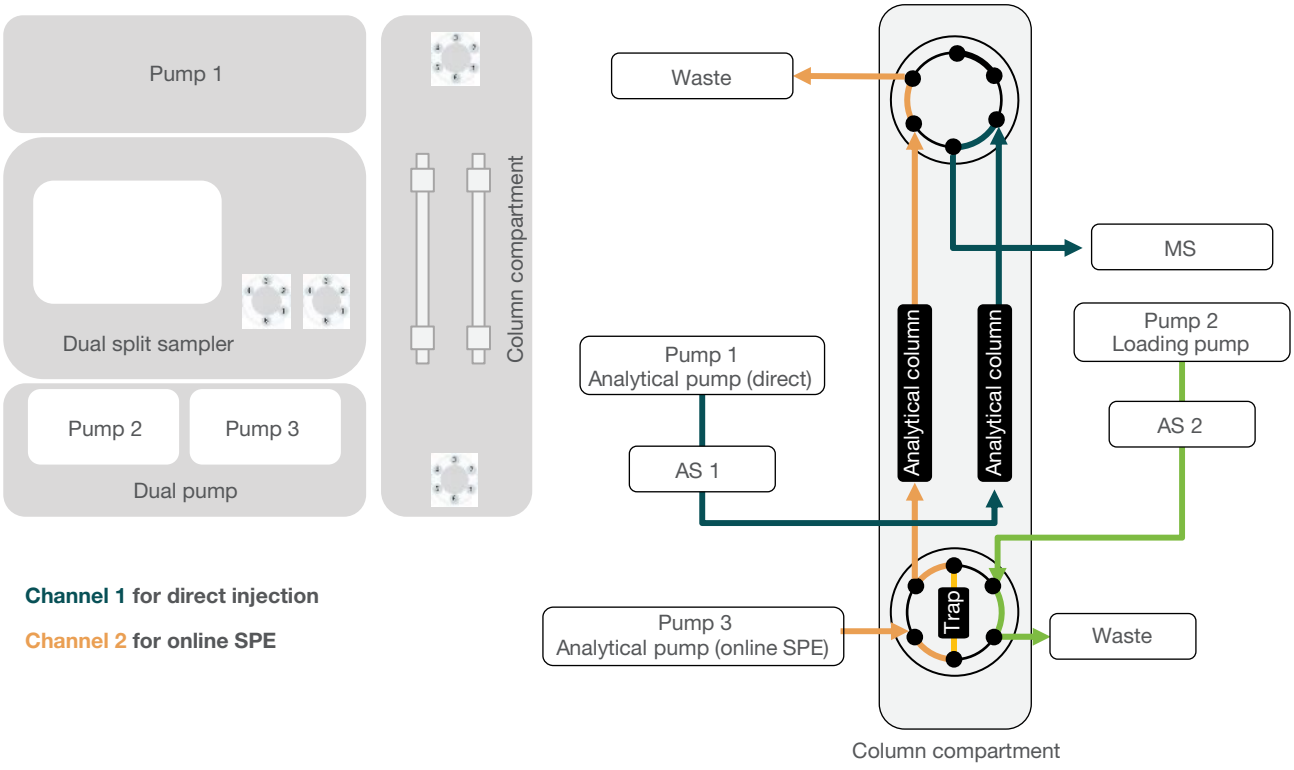

Figure 1. Schematic representation of the Vanquish Duo UHPLC system setup

Table 2. LC method description

Gradient profile			
Time	**Flow (mL/min)**	**A(%)**	**B(%)**
0.00	0.6	5	95
2.00	0.6	30	70
3.00	0.6	30	70
3.01	0.6	5	95
4.00	0.6	5	95
Other parameters			
Injection volume		50 µL	
Column temperature		25 °C	

Mass spectrometry

Analytes and internal standards were detected in Full Scan – data-dependent MS^2 acquisition mode on a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer with heated electrospray ionization operated in positive ion mode. A summary of the MS conditions is reported in Table 3. Two fragments for each analyte were included in the acquisition method for confirmation based on ion ratio.

Table 3. MS settings

Ion source parameters	
Source type	HESI-II
Spray voltage - Positive (V)	3,500
Sheath gas (Arb)	55
Aux gas (Arb)	15
Sweep gas (Arb)	2
Ion transfer tube temp (°C)	275
Vaporizer temp (°C)	450
S-Lens RF level	60
Full Scan settings	
Resolution (at *m/z* 200)	35,000
Scan range (*m/z*)	80–200
AGC target	2e5
Maximum IT (ms)	100
$ddMS^2$ Scan settings	
Resolution (at *m/z* 200)	17,500
Isolation window (*m/z*)	2.0
AGC target	5e4
Maximum IT (ms)	50
Stepped collision energy (CE)	15, 25, 35

Method evaluation

The method performance was evaluated in terms of linearity of response within the calibration ranges, lower limit of quantification (LLOQ), carryover, accuracy, trueness of measurement, and intra- and inter-assay precision for all the analytes.

To determine the LLOQ, the lowest calibrator was diluted 5-fold with blank matrix; a full set of calibrators (four levels), diluted calibrators (two levels), and controls (three levels) were extracted in replicates of five (n=5), injected in a single batch, and all used for the linear interpolation. The LLOQ was set as the lowest level that could be determined with a CV < 20% across the entire batch of samples.

Carryover was calculated in terms of percentage ratio between peak area of the highest calibrator and a blank sample injected immediately after it.

Analytical accuracy was evaluated in terms of percentage bias between nominal and average back-calculated concentrations using the quality control samples at two different levels provided by RECIPE prepared and analyzed in replicates of five on three different days.

Trueness of measurement was also evaluated as percentage bias using certified external quality controls (CP-PM-21 #01 and #02 from RCPAQAP - The Royal College of Pathologists of Australasia – Quality Assurance Programs) prepared and analyzed in replicates of five on a single day.

Intra-assay precision for each day was evaluated in terms of percentage coefficient of variation (%CV) using the controls at two 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 two levels in replicates of five prepared and analyzed on three different days).

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 5.1 software.

Results and discussion

A linear response with 1/x weighting was obtained for all the analytes, not only in the calibration range covered by the calibrators, but also down to a LLOQ reported in Table 4. The percentage bias between nominal and back-calculated concentration was within ±15% for all the calibrators (±20% for the lowest calibrator) in all the runs. Representative chromatograms for both analytes and internal standards are depicted in Figure 2. Representative calibration curves in the concentration range covered by the kit are shown in Figure 3.

No carryover was registered; no peak was detected in the blank sample following the highest calibrator for any analyte.

Experimental data demonstrate the outstanding accuracy of this method with a percentage bias between nominal and average back-calculated concentration for the used control samples ranging between -4.6% and 5.3% (Table 5).

Excellent results were obtained from the evaluation of trueness of measurement, with a percentage bias between -0.1% and 9.4% (Table 6).

The %CV for intra-assay and inter-assay precision was always below 8.7% and 6.9%, respectively (Table 7).

Table 4. Analytes and corresponding LLOQ

Analyte	LLOQ (ng/L)
3-methoxytyramine	13.5
Metanephrine	5.58
Normetanephrine	40.9

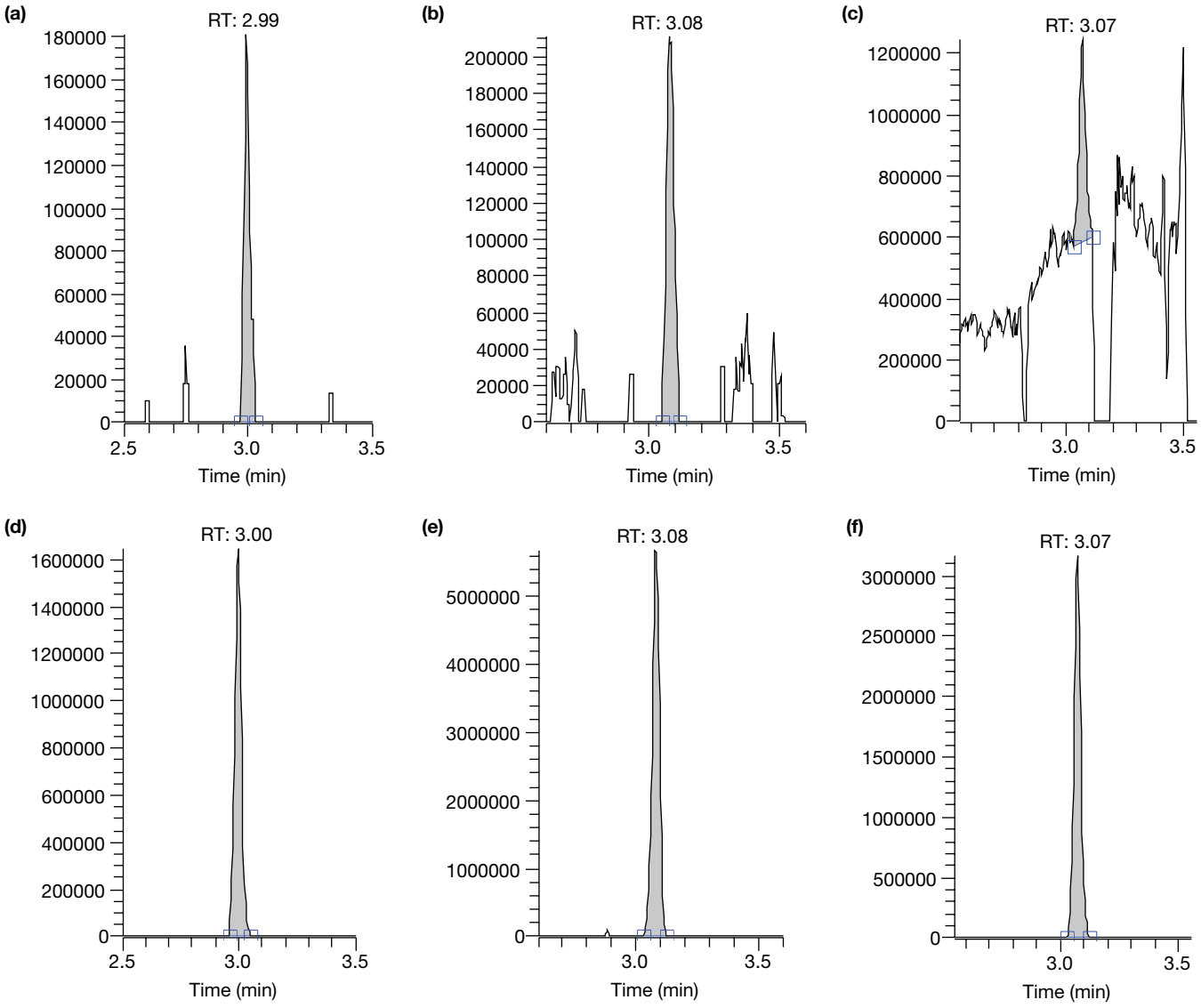

Figure 2. Representative chromatograms at the LLOQ for (a) 3-methoxytyramine, (b) metanephrine, (c) normetanephrine, (d) d_4-3-methoxytyramine, (e) d_3-metanephrine, and (f) d_3-normetanephrine

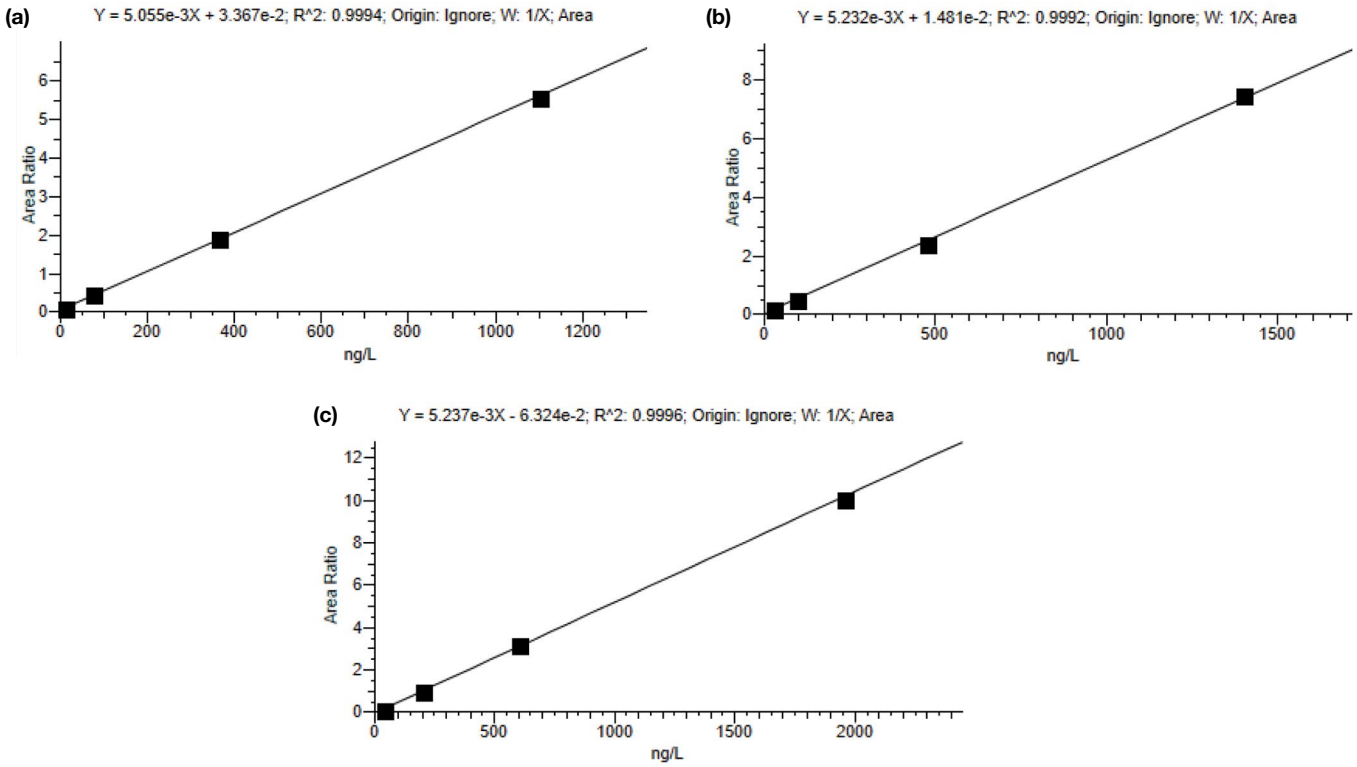

Figure 3. Representative calibration curves for (a) 3-methoxytyramine, (b) metanephrine, and (c) normetanephrine

Table 5. Analytical accuracy results for controls MS11083 batch #1229

Analyte	Level	Nominal concentration (ng/L)	Average calculated concentration (ng/L)	Bias (%)
3-methoxytyramine	Level I	26.2	27.6	5.3
	Level II	177	179	1.2
	Level III	721	709	-1.7
Metanephrine	Level I	57.6	59.0	2.4
	Level II	238	237	-0.5
	Level III	764	729	-4.6
Normetanephrine	Level I	122	125	2.8
	Level II	398	403	1.1
	Level III	991	968	-2.3

Table 6. Analytical accuracy results for external quality controls (RCPAQAP, CP-PM-21 #01 and #02

Analyte	Control	Nominal concentration (ng/L)	Average calculated concentration (ng/L)	Bias (%)
3-methoxytyramine	CP-PM-21-01	196	215	9.4
	CP-PM-21-02	N/A	N/A	N/A
Metanephrine	CP-PM-21-01	249	256	2.7
	CP-PM-21-02	39.2	39.4	0.4
Normetanephrine	CP-PM-21-01	456	455	-0.1
	CP-PM-21-02	53.4	53.4	0.1

Table 7. Analytical intra- and inter-assay precision results for controls MS11083 batch #1229

Analyte	Level	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 (%)
3-methoxytyramine	Level I	25.8	8.7	27.9	1.6	29.1	2.9	27.6	6.9
	Level II	170	3.1	185	3.7	182	5.2	179	5.5
	Level III	700	2.8	724	5.4	702	4.5	709	4.4
Metanephrine	Level I	58.2	2.2	61.6	4.3	57.2	3.6	59.0	4.6
	Level II	238	3.5	239	5.6	233	3.5	237	4.2
	Level III	728	1.6	740	3.1	719	3.6	729	2.9
Normetanephrine	Level I	127	2.8	120	3.8	129	2.1	125	4.1
	Level II	376	2.3	412	2.3	420	2.2	403	5.3
	Level III	943	3.4	984	3.8	978	3.6	968	3.9

Conclusions

An HRAM mass spectrometry-based method using a Vanquish Flex Duo UHPLC system connected to a Q Exactive Plus hybrid quadrupole-Orbitrap MS for the quantification of free metanephrines in human plasma is reported here. The ClinMass LC-MS/MS Complete Kit for Free Metanephrines in Plasma from RECIPE was used. The method incorporates simple offline SPE for sample preparation without the need for evaporation and reconstitution of the eluate. The obtained accuracy and precision demonstrate the power of Orbitrap technology in performing not only accurate qualitative analyses but also routine quantitation with high efficiency. The described method meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and precision.

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