

Plasma Free Metanephrine and Normetanephrine Quantitation Using On-line Sample Extraction Coupled with Tandem Mass Spectrometry

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Overview

The goal of this study is to develop a quantitative, sensitive, automated LC-MS/MS method optimizing Thermo Scientific TurboFlow technology for the analysis of metanephrine and normetanephrine in plasma for clinical research laboratories.

Introduction

Metanephrine (MN) and normetanephrine (NMN) (Figure 1) are created by the action of catechol-O-methyl transferase on epinephrine and norepinephrine, respectively. Current clinical research methods usually involve labor-intensive, time-consuming offline sample preparation. In this study, a sensitive, selective LC-MS/MS method was developed using the Thermo Scientific Aria TLX-1 system powered by TurboFlow™ technology for online sample extraction coupled with the Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer. The results demonstrate this method's suitability for clinical researchers to measure MN and NMN.

Methods

Analytes were extracted online from activated charcoal-stripped, acetonitrile-crashed rat plasma. Calibration curves were analyzed using an Aria™ TLX-1 liquid chromatography (LC) system coupled to a TSQ Vantage™ triple stage quadrupole mass spectrometer with a heated electrospray ionization II (HESI II) source. The plasma samples were extracted using a novel Thermo Scientific TurboFlow Cyclone MCX-2 cation exchange column (1 x 50 mm). Chromatography separation was performed using a Thermo Scientific Hypercarb column heated to 65 °C. Mass spectrometry detection was performed under the highly-selective reaction monitoring (H-SRM) mode with positive electrospray ionization. Internal standards used were metanephrine-d₃ and normetanephrine-d₃.

Sample Preparation

A standard stock solution of 1 µg/mL MN and NMN in methanol was prepared. The calibrator preparation method is shown in Figure 2.

Aria TLX-1 System Parameters

Columns

TurboFlow Cyclone MCX-2 cation exchange column (1.0 x 50 mm)

Thermo Scientific Hypercarb column (3 x 50 mm, 3 µm particle size)

Mobile Phases

Loading Pump

Mobile Phase A: 0.1% Formic Acid (aq)

Mobile Phase B: 5% Ammonium Hydroxide in Acetonitrile

Mobile Phase C: 1:1:1 Acetone: Acetonitrile: Isopropanol

Mobile Phase D: 50 mM Ammonium Formate with 1% Formic Acid

Elution Pump

Mobile Phase A: 50 mM Ammonium Formate with 1% Formic Acid

Mobile Phase B: 0.1% Formic Acid in Acetonitrile

FIGURE 1. Chemical structure of metanephrine and normetanephrine

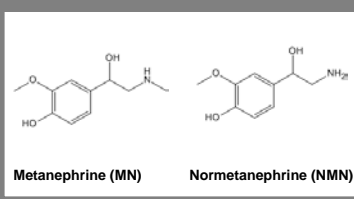


FIGURE 2. Calibrators preparation method

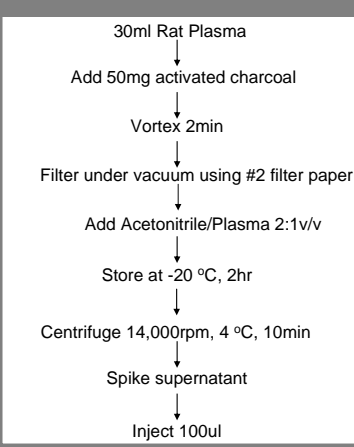


Table 1. Positive selective reaction mode (+SRM) transitions and other MS parameters for test compounds

Compound	Parent Ion	Fragment Ion	Collision Energy	S-Lens Offset
Metanephrine	180.100	148.165 (qualifier) 165.185	17 15	67 67
Normetanephrin	166.086	134.153	16	55
Metanephrine-d ₃	183.118	151.146 168.210	21 18	69 69
Normetanephrine-d ₃	169.104	137.197	19	58

Mass Spectrometer Parameters

MS analysis was carried out on a TSQ Vantage triple stage quadrupole mass spectrometer.

The Major MS parameters were as follows:

Ion Polarity:	Positive ion mode
Spray Voltage (V):	3500
Vaporizer Temperature (°C):	480
Capillary Temperature (°C):	235
Sheath Gas Pressure (N ₂):	60 units
Auxiliary Gas Pressure (N ₂):	25 units
Scan Type:	Selective Reaction Monitoring (SRM)
Collision Gas Pressure (mTorr):	0.9
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7

Positive selective reaction mode (+SRM) transitions and other MS parameters for target compounds are shown in Table 1. The entire experiment was controlled by Aria operating software 1.6.2 and the data was processed using Thermo Scientific LCQuan 2.5.6 quantitative software after subtracting background using Thermo Scientific Xcalibur 2.0.7 SP1 data system software.

Results

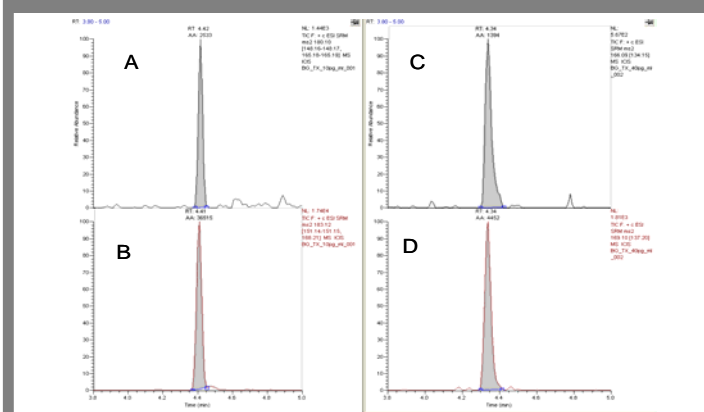
The quantitation of metanephrines is analytically challenging for clinical researchers due to its extremely low concentrations in biological matrices. It is well documented that TurboFlow methods are able to remove endogenous compounds from biological fluid effectively without time-consuming offline sample preparation, thus reducing ion suppression effects and increasing detection limits significantly¹⁻³.

Figure 3 shows a representative chromatogram for the assay at the low end of the curve. Figure 4 shows a representative chromatogram for the assay at the high end of the curve. Figure 5 shows the linear calibration curves for both MN and NMN.

The excellent linearity fits over the range of 10-500 pg/mL for MN and 40-500 pg/mL for NMN, which was at least four-times more sensitive than other published online sample extraction methods⁴. The limit of detection (LOD) levels for each compound was 10 pg/mL. The CV values showed less than 10% deviation for the lower limit of quantitation (LLOQ) of both curves and were in the range of 1%-7% and 3%-8% deviation for all the other points on the calibration curve of MN and NMN, respectively. Carryover was determined to be less than 20% of LLOQ. A minimum of 90% recovery was achieved. The variability was determined by processing and analyzing five replicates of each of four QC samples (50, 75, 100, 200 pg/mL). The results showed that the % RSDs were 7.0 and 7.5 for MN-d₃ and NMN-d₃, respectively, which were well below the validation guideline of 15%⁵.

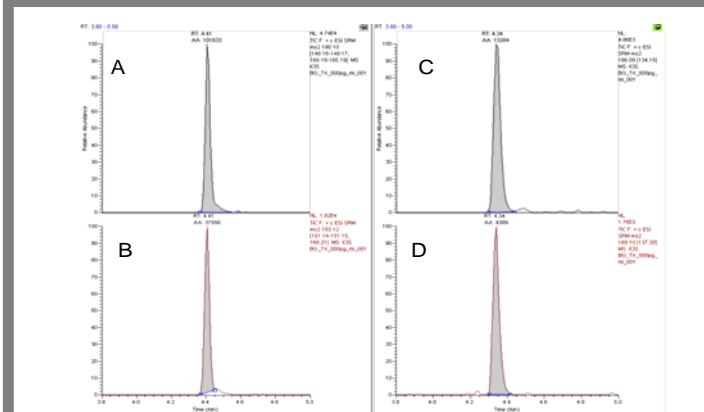
After further mass spectrometer optimization, the quantitation limits for both compounds using this method could be as low as 2 pg/mL (data not shown). The traditional criterion for a positive result is a normetanephrine level no less than 164 pg/mL or a metanephrine level no less than 98 pg/mL⁶. Currently, major clinical laboratories publish their LLOQs of the normetanephrine and metanephrine fractions are in the range of 39-148 pg/mL and 36-57 pg/mL respectively⁷⁻⁸. Any numbers below 39 pg/mL and 36 pg/mL for NMN and MN, respectively are reported as below the limit⁶. Therefore, this clinical research method offers the benefit of online sample extraction and currently achieves equal or better detection limits than the major laboratories in the field.

FIGURE 3. The representative chromatogram for the assay at the low end of the calibration curve



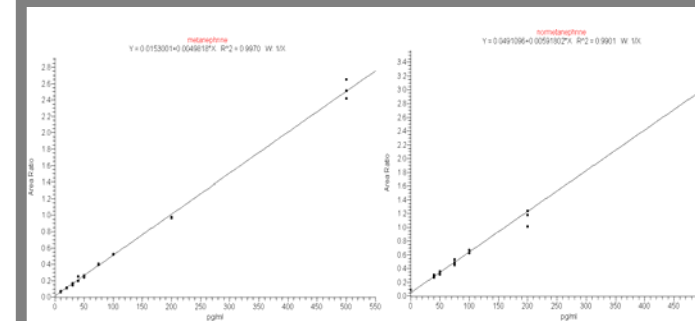
A. MN (10 pg/mL); B. MN-d₃ (200 pg/mL); C. NMN (40 pg/mL); D. NMN-d₃ (200 pg/mL);

FIGURE 4. The representative chromatogram for the assay at the high end of the calibration curve



A. MN (500 pg/mL); B. MN-d₃ (200 pg/mL); C. NMN (500 pg/mL); D. NMN-d₃ (200 pg/mL);

FIGURE 5. Linear calibration curves for both test compounds



Conclusions

No	Performance Specifications	MN	NMN	Comment
1	LOD (pg/mL)	2	2	Verify
2	LOQ (pg/mL)	10	10	Verify
3	Recovery	100+/-20%	100+/-20%	
4	Assay linearity pg/mL	10-500	40-500	
5	Precision, %CV	1 to 7	3 to 8	10 at LLOQ
6	Carryover at LLOQ, %	<20	<20	
7	Total analysis time, min		10	
8	Sample prep time, min		-	Crashed plasma
9	Sample volume µL		100	
10	No of samples/hr using TLX-1		5	20 with TLX-4
11	Interference due to Dopamine, Epinephrine, NorEpiDrug, desipramine, ephedrine sulfate, and chlorpromazine, acetaminophen		To be verified	

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