

# Plasma-free Metanephrine and Normetanephrine Quantitation Using Online Sample Extraction Coupled with Tandem Mass Spectrometry

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## Key Words

- Aria TLX-1
- TurboFlow Technology
- TSQ Vantage MS
- Metanephrines

## 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 quadrupole mass spectrometer. The results demonstrate the method's suitability for clinical researchers to measure MN and NMN.

## Experimental Method

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™ 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-d<sub>3</sub> and normetanephrine-d<sub>3</sub>.

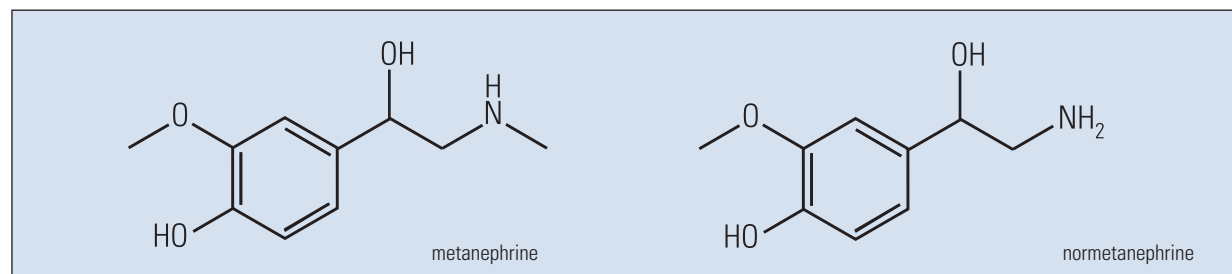


Figure 1: Chemical structure of metanephrine and normetanephrine

## Goal

To develop a quantitative, sensitive, automated LC-MS/MS method optimizing TurboFlow technology for the analysis of metanephrine and normetanephrine in plasma for clinical research laboratories.

## Experimental Conditions

### Sample Preparation

A standard stock solution of 1 µg/mL MN and NMN in methanol was prepared. The rat plasma was first filtered using activated charcoal, followed by a 1:2 crash using cold acetonitrile. The resulting plasma was then centrifuged at 13,000 rpm for 10 minutes. Calibrators were prepared in the supernatant. In this study, all the results are based on the obtained final concentrations. The final concentration for each internal standard was 200 pg/mL. The injection volumes were 0.10 mL.

## Thermo Scientific Aria TLX-1 System Parameters

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

### MS Parameters

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

#### The MS conditions were as follows:

Ion Polarity:	Positive ion mode
Spray Voltage (V):	3500
Vaporizer Temperature (°C):	480
Capillary Temperature (°C):	235
Sheath Gas Pressure (N <sub>2</sub> ):	60 units
Auxiliary Gas Pressure (N <sub>2</sub> ):	25 units
Scan Type:	Selective Reaction Monitoring (SRM)
Chrom Filter Peak Width (s):	6.0
Collision Gas Pressure (mTorr):	0.9
Declustering Voltage (V):	0
Scan Width (m/z):	0.002
Scan Time (s):	0.100
Q1 (FWHM):	0.7
Q3 (FWHM):	0.7

Positive single reaction mode (+SRM) transitions and other MS parameters for target compounds are shown in Table 1.

Compound	Parent Ion	Fragment Ion	Collision Energy	S-Lens Offset
Metanephine	180.100	148.165 (qualifier)	17	67
		165.185	15	67
Normetanephin	166.086	134.153	16	55
Metanephine-d <sub>3</sub>	183.118	151.146	21	69
		168.210	18	69
Normetanephine-d <sub>3</sub>	169.104	137.197	19	58

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

The entire experiment was controlled by Aria operating software 1.6.2. 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 and Discussion

The quantitation of metanephines is analytically challenging for clinical researchers due to the extremely low concentrations of these substances 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 significantly.<sup>1-3</sup>

Figure 2 shows a representative chromatogram for the assay at the low end of the curve. Figure 3 shows a representative chromatogram for the assay at the high end of the curve. Figure 4 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 methods.<sup>4</sup> The limit of detection (LOD) level 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-d<sub>3</sub> and NMN-d<sub>3</sub>, respectively, which were well below the validation guideline of 15%.<sup>5</sup>

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 normetanephine level no less than 164 pg/mL or a metanephine level no less than 98 pg/mL.<sup>6</sup> Currently, major clinical laboratories publish their LLOQs of the normetanephine and metanephine fractions that are in

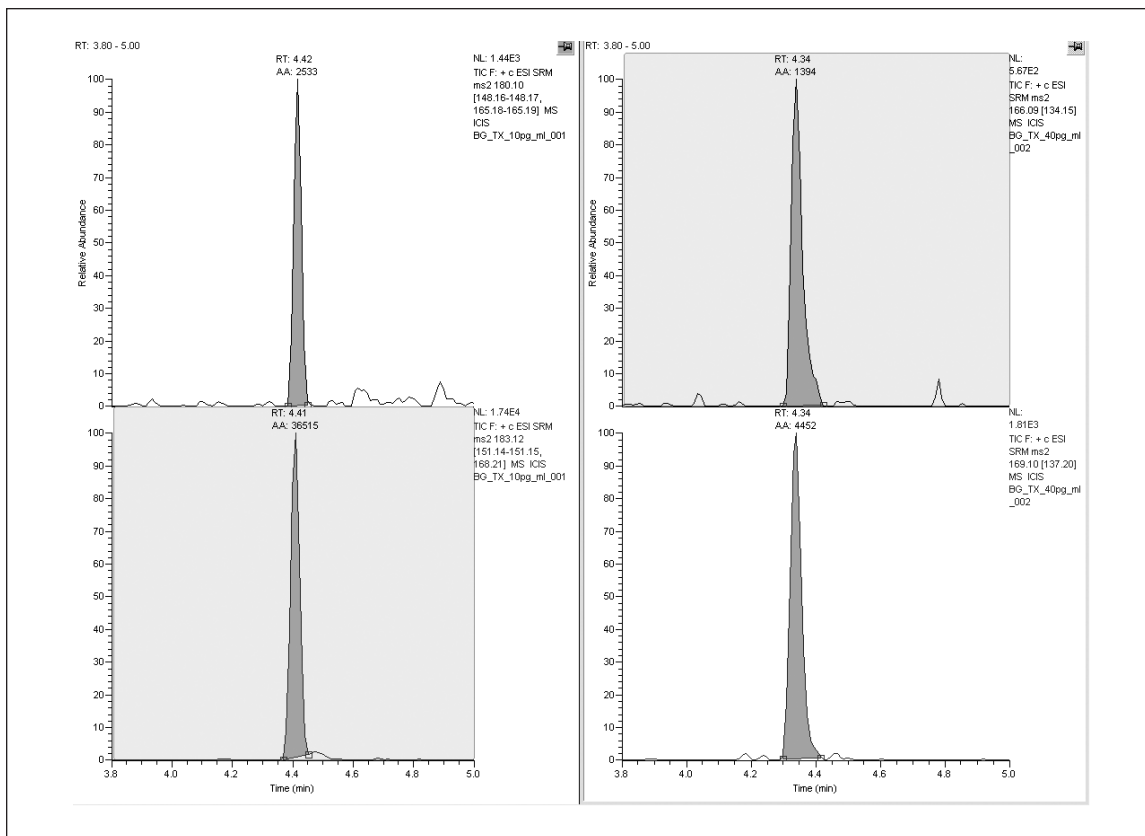


Figure 2: Representative chromatogram for the assay at the *low* end of the calibration curve

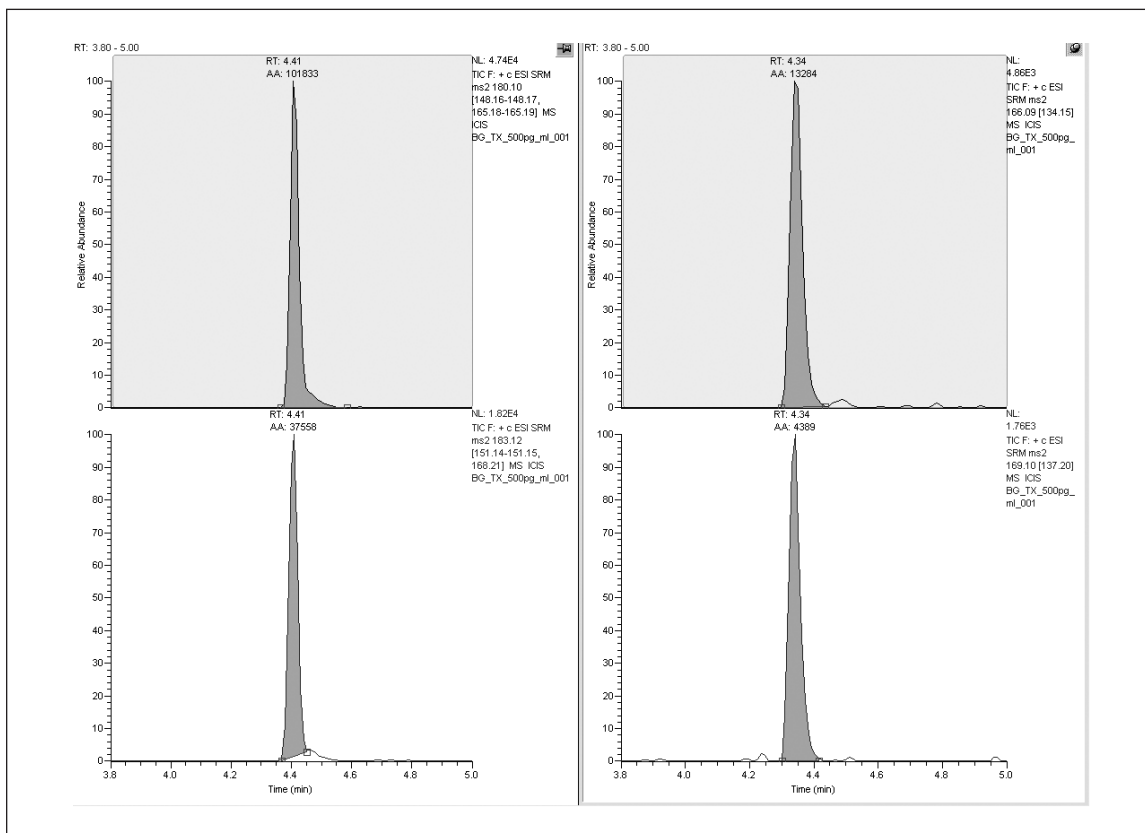


Figure 3: Representative chromatogram for the assay at the *high* end of the calibration curve

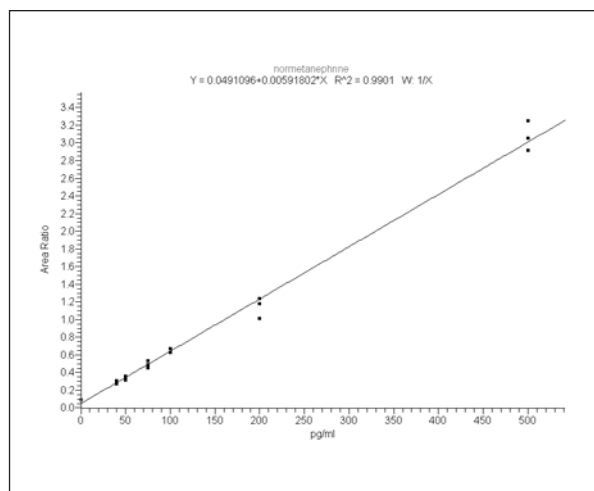
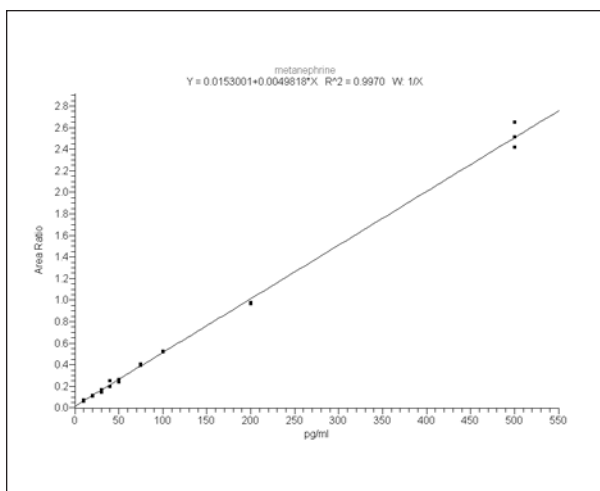


Figure 4: Linear calibration curves for both test compounds.

the range of 39-148 pg/mL and 36-57 pg/mL respectively.<sup>7-8</sup> Any numbers below 39 pg/mL and 36 pg/mL for NMN and MN, respectively, are reported as below the limit.<sup>7</sup> 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.

## Conclusion

LC-MS/MS with TurboFlow technology is a powerful technique for direct analysis of drugs in biological fluids without an extensive number of sample preparation steps. In this study, the use of an Aria TLX-1 LC system in front of a TSQ Vantage mass spectrometer allows for very low levels of detection of each compound investigated in rat plasma and yields results in less than 11 minutes per sample. It should be noted that with the Aria TLX-4 multiplexed system, the results will be available to clinical researchers about every 3 minutes, or 20 per hour, with the use of only one mass spectrometer.

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