Plasma Free Metanephrines Quantitation with Automated Online Sample Preparation and Liquid Chromatography—Tandem Mass Spectrometry

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Key Words
TSQ Vantage, Clinical Research, TurboFlow Technology, Metanephrine, MN, Normetanephrine, NMN, Pmets, Pheochromocytoma

Goal
To develop an automated method to quantitate plasma free metanephrines reducing method time while maintaining analytical performance compared to the original offline SPE method.

Introduction
Plasma free metanephrine (MN) and normetanephrine (NMN), collectively known as Pmets, are preferred biomarkers for pheochromocytoma for clinical research. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has become widely used to measure Pmets because of its high analytical specificity.

Recently, we reported an LC-MS/MS method for measuring Pmets using ion-pairing solid phase extraction (IP-SPE) and porous graphitic carbon (PGC) column chromatography[1,2]. Although the method is fast and analytically sensitive, it can be further improved by automating the offline sample preparation with online sample preparation technology, which is more time- and cost-effective.

Thermo Scientific TurboFlow technology is an automated online sample preparation technology that has been coupled to LC-MS/MS for the quantitative analysis of a variety of biological samples.

To date, its use has been reported in clinical research, pharmaceutical analysis, bioanalysis, environmental testing, food safety, and forensic toxicology.

Methods
Sample Preparation
The 0.5-mL samples of human plasma and of charcoal stripped serum (CSS) were spiked with internal standards (IS) and then mixed with 0.25 mL of 10% tricholoacetic acid (w/v) in water. The mixtures were vortexed and stored at –30 °C for 30 minutes. Then, the mixtures were centrifuged at 16,000 g for 10 minutes, and 100 µL of the supernatants were injected for LC-MS/MS analysis.

LC-MS/MS Conditions
LC-MS/MS analysis was performed on a Thermo Scientific TSQ Vantage triple stage quadrupole mass spectrometer coupled with a Thermo Scientific Transcend TLX-1 system. The TurboFlow™ method with automated online sample preparation was performed with a TurboFlow Cyclone MCX-2 column. Perfluoroheptanoic acid (PFHA) was used as the ion-pair during the sample preparation.

| | | | | Loading | | | | | | Eluting | | | | | |
Start	Sec	Flow	Grad	%A	%B	%C	%D	Tee	Loop	Flow	Grad	%A	%B	%C	%D
00:00	30	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-
00:30	30	2.00	Step	90.0	10.0	-	-	====	out	0.50	Step	90.0	-	-	10.0
01:00	90	0.10	Step	90.0	10.0	-	-	T	in	1.00	Step	90.0	-	-	10.0
02:30	1	0.10	Step	90.0	10.0	-	-	====	in	0.50	Step	98.0	2.0	-	-
02:31	300	0.50	Step	-	-	-	100.0	====	in	0.50	Ramp	60.0	40.0	-	-
07:31	60	2.00	Step	-	-	100.0	-	====	in	1.00	Step	-	100.0	-	-
08:31	60	2.00	Step	70.0	30.0	-	-	====	in	1.50	Step	-	-	100.0	-
09:31	150	2.00	Step	100.0	-	-	-	====	out	1.00	Step	98.0	2.0	-	-

Figure 1. TurboFlow and LC method

Loading:
A: 0.1% PFHA in water
B: 60% ACN in water
C: Mixture of isopropanol, ACN and acetone (1:1:1 v/v/v) with 0.3% formic acid
D: 5 mM NH_4Ac and 50% ACN in water

Eluting:
A: 50 mM NH_4FA and 1% formic acid in water
B: 0.1% formic acid in ACN
C: Mixture of isopropanol, ACN and acetone (9:9:2 v/v/v)
D: 0.1% PFHA in water.

Eluting LC column temperature: 70 °C

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Analytical separation was carried out on a Thermo Scientific Hypercarb column (50×3 mm, 5.0-µm particle size) at 70 °C. The total LC runtime was 12 minutes (Figure 1). The mass spectrometer was operated with a heated electrospray ionization (HESI-II) source in positive ionization mode. Data was acquired in selected-reaction monitoring (SRM) mode.

Validation

The validation procedure included tests for 1) recovery; 2) lower limit of quantitation (LLOQ), dynamic range, accuracy; 3) precision; 4) ion suppression; 5) carryover; and 6) interferences.

Results and Discussion

Charcoal stripped serum (CSS) was first evaluated by comparing it to human plasma using a generally adopted mixing study[3]. It was determined that CSS is an appropriate matrix to conduct the validation experiments.

Recovery

The extraction recovery was assessed by comparing the direct injection to the TurboFlow method injection of MN, NMN, MN-d3 and NMN-d3 spiked in mobile phase (n=2). The absolute recovery of MN, NMN and their IS ranged from 56.4% to 62.4%, and the relative recovery of MN and NMN was 90.9% and 97.8%, respectively (Table 1).

Determination of LLOQ, Linearity and Accuracy

CSS was spiked with MN and NMN to achieve final concentrations of 500 and 1000 pg/mL, respectively. A serial two-fold dilution with CSS was performed to make eight levels of linearity samples with concentration ranges of 500 to 3.9 pg/mL and 1000 to 7.8 pg/mL for MN and NMN, respectively. Linearity samples were analyzed in triplicate along with one set of calibrators. The calibration curve was constructed by plotting the analyte:IS peak area ratio vs. analyte concentration.

The linearity was determined to be 6.3 to 455.4 pg/mL for MN and 12.6 to 954.5 pg/mL for NMN. Within the linear range, the accuracy ranged from 80.6% to 93.5% for MN, and from 80.9% to 101.7% for NMN. The CV (n=3) from all linearity levels ranged from 3.1% to 13.7% for MN, and from 1.6% to 10.7% for NMN (Table 1 and Figures 2 and 3). The determined LLOQ was 6.3 pg/mL for MN and 12.6 pg/mL for NMN (Table 2).

Table 1. Recovery

	Online Extraction (mean ± CV)[b]	Direct Injection (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (500 pg/mL)[a]	60281 ± 2.7%	106866 ± 10.5%	56.4	90.9
NMN (250 pg/mL)[a]	32186 ± 5.6%	51878 ± 9.4%	62.0	97.8
MN-d3 (500 pg/mL)[a]	40716 ± 1.1%	66790 ± 11.4%	61.0	N/A
NMN-d3 (500 pg/mL)[a]	28983 ± 3.7%	46482 ± 11.8%	62.4	N/A

[a] MN, NMN, MN-d3 and NMN-d3 were spiked to mobile phase at specified concentration levels.

[b] Measured peak area with CV (n=2)

Table 2. LLOQ, dynamic range and accuracy

Dilution factor	MN				NMN			
	Expected (pg/mL)	Measured (pg/mL)	CV of triplicates (%)	Accuracy (%)	Expected (pg/mL)	Measured (pg/mL)	CV of triplicates (%)	Accuracy (%)
128	3.91	5.5	17.2	71.1	7.8	7.4	35.3	94.9
64	7.81	6.3	13.7	80.6	15.6	12.6	10.7	80.9
32	15.6	13.9	7.2	88.8	31.3	30.8	1.6	98.7
16	31.3	27.5	4.9	88.0	62.5	61.0	6.0	98.1
8	62.5	56.6	10.3	90.6	125.0	121.2	9.2	96.9
4	125.0	112.2	4.0	89.8	250.0	254.2	9.4	101.7
2	250.0	233.7	3.1	93.5	500.0	496.9	2.7	99.4
1	500.0	455.4	4.0	91.1	1000.0	954.5	3.3	95.5
Mean (%)				88.9				95.9
Stdev (%)				4.1				6.9

Precision

Precision was assessed with spiked CSS. Inter- and intra-assay CV values at low and high quality control concentrations of both analytes varied between 2.0% and 10.5% (Table 3).

Table 3. Precision data

Charcoal Stripped Serum	MN		NMN	
	31.3 pg/mL	250.0 pg/mL	62.5 pg/mL	500.0 pg/mL
Intra 1 (%) n=5	6.7	4.2	4.5	5.4
Intra 2 (%) n=5	4.9	3.0	10.5	4.2
Intra 3 (%) n=5	7.3	4.7	10.0	2.0
Inter-assay (%) n=15	8.4	7.7	8.9	4.8
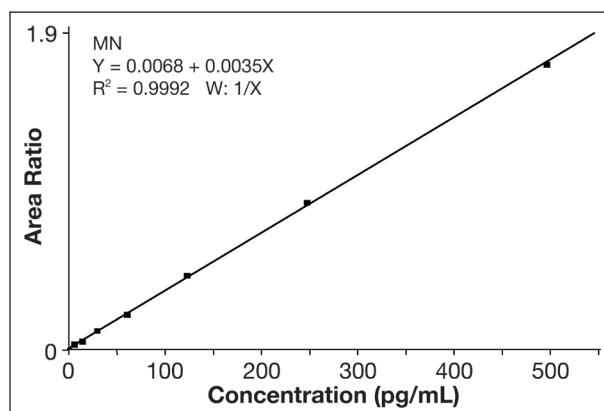
Figure 2. Calibration curve of MN in CSS

MN
$Y = 0.0068 + 0.0035X$
$R^2 = 0.9992$ W: 1/X
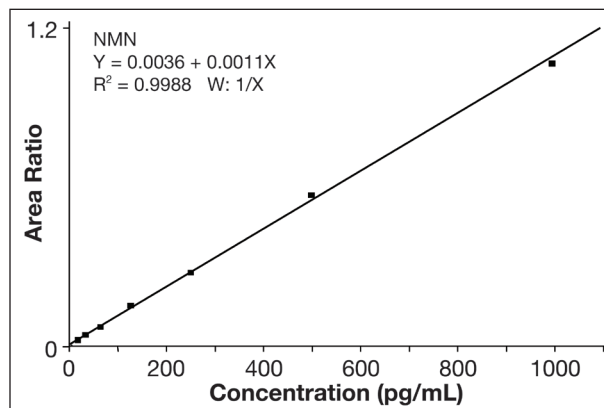
Figure 3. Calibration curve of NMN in CSS

NMN
$Y = 0.0036 + 0.0011X$
$R^2 = 0.9988$ W: 1/X

Ion Suppression

The MS responses of MN-d3 and NMN-d3 in solvent (n=4) and individual human plasma samples (n=4) at the same concentrations (400 pg/mL for both MN-d3 and NMN-d3) were measured with LC-MS/MS analysis. The average MS responses (integrated area) of MN-d3 and NMN-d3 from solvent and real human plasma samples were calculated. The intensity ratios with standard deviations between human plasma (n=4) and solvent (n=4) were 113.3% ± 18.4% and 126.4% ± 18.0% for MN-d3 and NMN-d3, respectively. This indicated that this method has no obvious ionization suppression or enhancement.

Carryover

No carryover was observed.

Interferences

Epinephrine (EPI) and NMN share the same SRM transitions and could not be differentiated just by MS/MS analysis. Using the Hypercarb™ analytical column, the EPI peak was baseline resolved from the NMN peak (0.3 min apart, data not shown).

Data Examples of Clinical Research Samples

Figure 4 shows the SRM chromatograms of MN and NMN in an individual plasma sample. Figure 5 shows the SRM chromatograms of MN and NMN in a CSS sample.
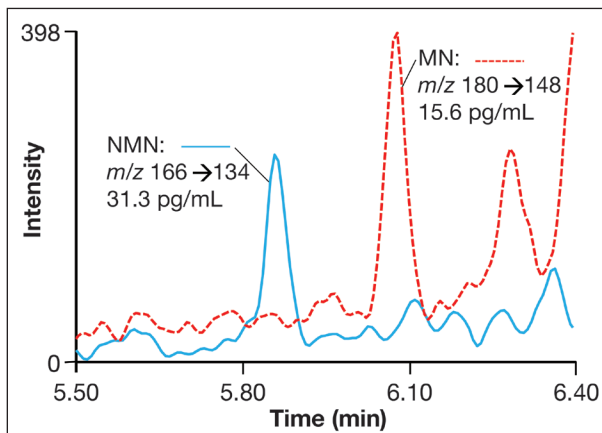

Figure 4. SRM chromatograms of MN and NMN in human plasma sample
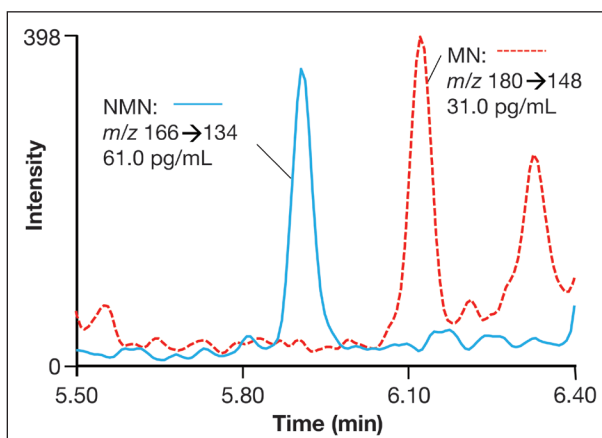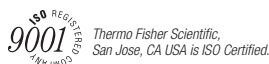

Figure 5. Representative SRM chromatograms of MN (31.0 pg/mL) and NMN (61.0 pg/mL) in CSS sample

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Conclusion

A fast, automated and analytically sensitive LC-MS/MS method was developed to quantify plasma metanephrines for clinical research purposes[4]. By using TurboFlow technology, the sample preparation procedure was significantly simplified compared to a previously reported offline IP-SPE method. The presence of PFHA during the online sample preparation was critical to the success of this method. A PGC column was used for chromatographic separation of metanephrines. The total online extraction and analytical LC runtime was 12 minutes. This method was linear from 6.3 to 455.4 pg/mL for metanephrine and 12.6 to 954.5 pg/mL for normetanephrine, with an accuracy of 80.6% to 93.5% and 80.9% to 101.7%, respectively. The lower limit of quantitation was 6.3 pg/mL for metanephrine and 12.6 pg/mL for normetanephrine. Inter-assay and intra-assay precision for metanephrine and normetane-phrine at low and high concentration level ranged from 2.0% to 10.5%.

Overall, the analytical performance achieved with this automated online TurboFlow method is consistent with the previously reported offline SPE method[2]. More importantly, the online method significantly saved sample preparation time by more than 50% and eliminated the expense of SPE cartridges with an offline approach.

References

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