Application Note: 539

Quantitative Measurement of Plasma Free Metanephrines by Ion-Pairing Solid Phase Extraction and LC-MS/MS with Porous Graphitic Carbon Column

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Key Words

- TSQ Vantage
- Hypercarb HPLC column
- Clinical Research
- LC-MS/MS

Introduction

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

Because Pmets are very polar, special solid phase extraction (SPE) and chromatographic methods have been developed for their analysis. Ion-paring (IP)-SPE, which has been used to purify a wide range of polar compounds, is well suited for the purification of Pmets.

Goal

To develop an LC-MS/MS method for measuring Pmets using IP-SPE and porous graphitic carbon (PGC) column chromatography.

Methods

Sample Preparation

Thermo Scientific HyperSep C-18 cartridges (1 mL) were preconditioned with acetonitrile and 0.1% perfluoroheptanoic acid (PFHA) before samples were loaded. After sample loading, cartridges were washed with 0.1% PFHA and eluted with 60% acetonitrile. The eluate was dried and reconstituted 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 Accela UHPLC system. A Thermo Scientific Hypercarb column (50 × 2.1 mm, 5 µm particle size) was used. This PGC-based column is highly durable and ideal for retaining and resolving very polar and hydrophilic molecules. The column temperature was maintained at 70 °C. Mobile phases were 1% formic acid in water with ammonium formate, and 0.1% formic acid in acetonitrile. The LC gradient was 7 minutes long.[1]

The mass spectrometer was equipped with a heated electrospray ionization probe (HESI-II) and operated in the positive electrospray ionization mode. MN-d3 and NMN-d3 were used as the internal standards for MN and NMN.

Validation

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

Results and Discussion

1. Interference

Epinephrine (EPI) and NMN share the same selected reaction monitoring (SRM) transitions and could not be differentiated by MS/MS analysis alone. With Hypercarb™ column chromatography, the EPI-d3 peak was baseline resolved from the NMN-d3 peak (Figure 1).

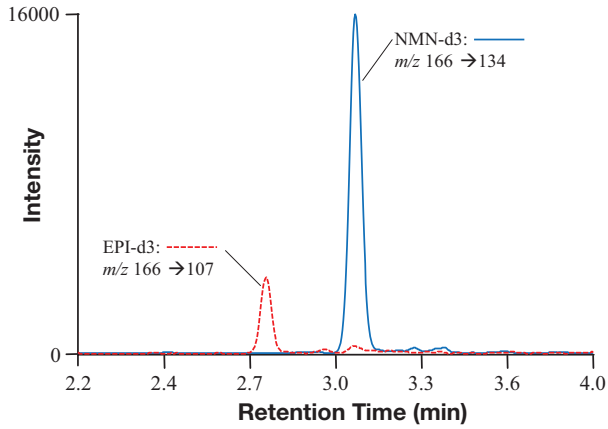

Figure 1. SRM chromatograms of EPI-d3 and NMN-d3 in a processed CSS sample

2. SPE Recovery

Extraction efficiency was assessed in charcoal stripped serum (CSS, n=3). Absolute recovery of Pmets and IS ranged from 86.4% to 97.5%, and the relative recovery of MN and NMN was 97.7% and 113.5%, respectively (Table 1).

Table 1. SPE Recovery

In Charcoal Stripped Serum	Spiked before SPE[a] (mean ± CV)	Spiked after SPE[b] (mean ± CV)	Absolute Recovery (%)	Relative Recovery (%)
MN (n=3)	22865 ± 13.9%	25265 ± 9.3%	90.5	97.7
NMN (n=3)	11165 ± 11.1%	11453 ± 12.5%	97.5	113.5
MN-d3 (n=3)	27809 ± 7.2%	30140 ± 12.9%	92.3	n/a
NMN-d3 (n=3)	22627 ± 9.2%	26192 ± 4.5%	86.4	n/a

[a] *Measured peak area of charcoal stripped serum spiked with 100, 400, 400, and 1600 pg/mL of MN, NMN, MN-d3, and NMN-d3, respectively, before SPE*

[b] *Measured peak area when equivalent amounts of above compounds were spiked after SPE*

3. Ion Suppression

Results from the post-column infusion experiments are shown in Figure 2. Compared to injections of blanks, no obvious ion suppression was detected in the SRM chromatograms of MN-d3 and NMN-d3 using processed human plasma samples.

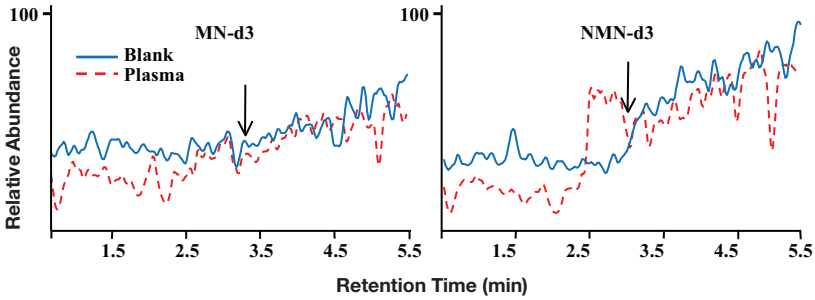

Figure 2. Representative SRM chromatograms of post-column infusion of 100 ng/mL MN-d3 (left) and NMN-d3 (right) after injections of buffer blanks (solid lines) and processed human plasma samples (dashed lines). No internal standards were added to human plasma samples. Arrows indicate retention times of MN and NMN.

4. LLOQ, Linearity and Accuracy

It was determined that CSS is a suitable matrix to conduct this part of validation (mixing study, data not shown). CSS samples with progressively lower concentrations of MN and NMN were prepared in triplicate along with one set of CSS calibrators.

The linearity range was determined to be 7.2 - 486.8 pg/mL for MN and 18.0 - 989.1 pg/mL for NMN (Figure 3). Accuracy ranged from 92.2% to 118.0% for MN, and from 92.1% to 115.0% for NMN. The determined LLOQ was 7.2 pg/mL for MN and 18.0 pg/mL for NMN.

Figures 3 and 4 show the calibration curves for MN and NMN. Figure 5 shows the representative SRM chromatograms of MN and NMN at their LLOQ in CSS.

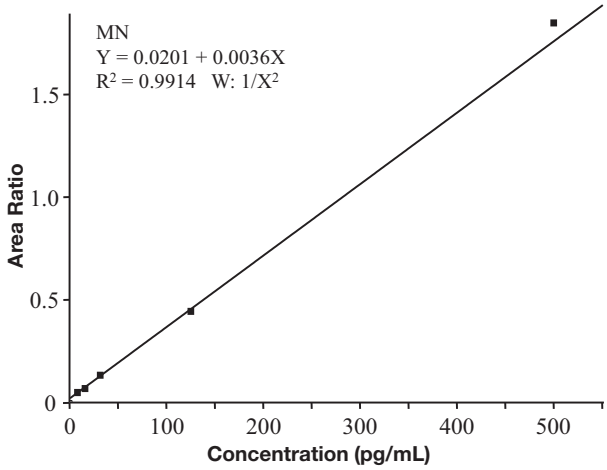

Figure 3. Calibration curve of MN in CSS

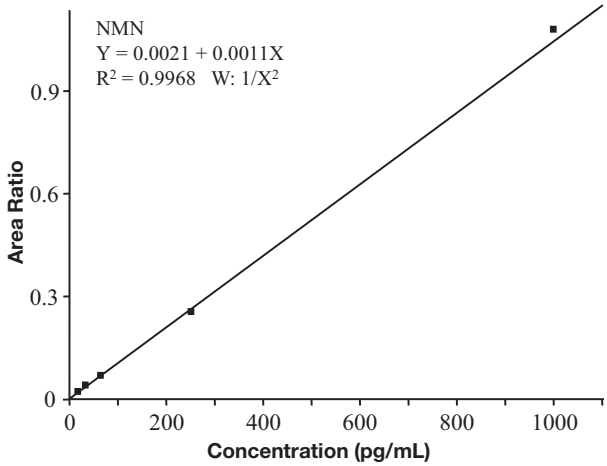

Figure 4. Calibration curve of NMN in CSS

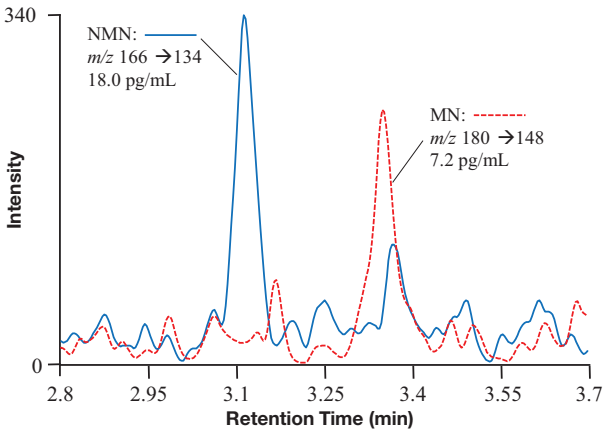

Figure 5. Representative SRM chromatograms of MN and NMN at their LLOQ in a spiked CSS sample.

5. Precision

Precision results are summarized in Table 2.

A) CSS samples: Precision was first assessed with spiked CSS at two concentration levels (25 and 250 pg/mL for MN, and 50 and 500 pg/mL for NMN). Inter- (n=15) and intra-batch (n=5) CV values ranged from 2.1% to 10.9%.

B) Pooled human plasma samples: Precision was also assessed with a spiked human plasma pool (35.6 pg/mL of MN and 53.1 pg/mL of NMN, n=5). The determined intra-assay CV (n=5) was 6.3% and 7.8% for MN and NMN, respectively.

Table 2. Precision Data in Spiked CSS

	MN		NMN	
	25 pg/mL	250 pg/mL	50 pg/mL	500 pg/mL
Intra-assay Precision (%) n=5	10.9	4.6	9.6	2.1
Accuracy (%)	98.9	96.9	110.2	90.9
Inter-assay Precision (%) n=15	10.3	6.5	10.6	5.6
Accuracy (%)	100.6	102.7	108.7	97.4

Figure 6 shows representative SRM chromatograms of MN and NMN using a processed human plasma sample.

6. Carryover

No carryover was observed up to 500 and 1000 ng/mL for MN and NMN, respectively.

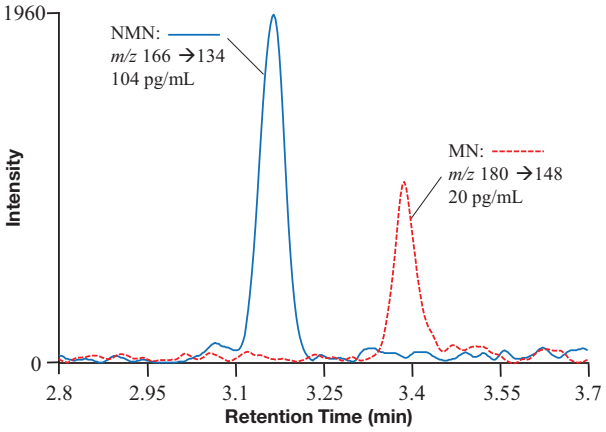

Figure 6. Representative SRM chromatograms of MN and NMN using a processed human plasma sample

Conclusion

A sensitive LC-MS/MS method was developed to quantify plasma free metanephrines in clinical research laboratories. This method has an LLOQ of 7.2 and 18.0 pg/mL for metanephrine and normetanephrine, respectively. Method precision ranged from 2.0% to 10.9%. Ion-pairing SPE was used for sample preparation, and a Hypercarb column was used for chromatographic separation of metanephrines.

Reference

1. He, X.; Gabler, J.; Yuan, C.; Wang, S.; Shi, Y.; Kozak, M. Quantitative Measurement of Plasma Free Metanephrines by Ion-pairing Solid Phase Extraction and Liquid Chromatography-Tandem Mass Spectrometry with Porous Graphitic Carbon Column *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2011**, 879(23), 2355-2359.

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