Urinary Catecholamines, Metanephrines, and 3-Methoxytyramine in a Single LC/MS/MS Run

Using Agilent Bond Elut Plexa SPE, 1290 Infinity LC, and 6460 Triple Quadrupole LC/MS

Application Note

Clinical Research

Authors

Linda Côté and Christophe Deckers
Agilent Technologies Canada
St-Laurent, Québec, Canada

Abstract

We developed a highly sensitive and specific LC/MS/MS method to quantitate catecholamines (dopamine, epinephrine, and norepinephrine), metanephrine, normetanephrine, and 3-methoxytyramine in urine. A single solid phase extraction procedure was used to simplify sample preparation and clean up some interferences in urine. The analytical method achieved the required functional sensitivity and quantitated analytes over a sufficiently wide dynamic range with a single injection. Reproducibility was excellent for all compounds (CV < 6 %). All calibration curves displayed excellent linearity, with $R^2 > 0.9997$.

Agilent Technologies

Introduction

A single and efficient solid phase extraction (SPE) sample preparation procedure was developed for the simultaneous extraction of dopamine, epinephrine, norepinephrine, metanephrine, normetanephrine, and 3-methoxytyramine (Figure 1) in urine. Calibrators were created by spiking clean urine with various concentrations of each analyte. Liquid chromatography/triple quadrupole mass spectrometry (LC/MS/MS) is ideal for the rapid analysis of multiple analytes such as these. The chromatographic system used an Agilent Pursuit pentafluorophenyl (PFP) column and a mobile phase of methanol and water containing 0.2 % formic acid. We monitored quantifier and qualifier MRM transitions, and used deuterated internal standards for each analyte to ensure accurate and reproducible quantitation.

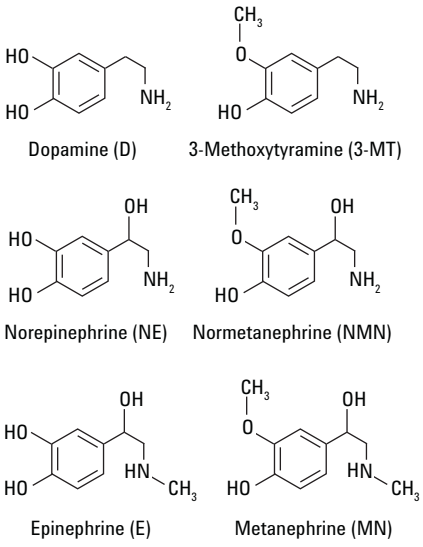

Figure 1. Structures of catecholamines, metanephrines, and 3-methoxytyramine.

Experimental

LC method

The LC system consisted of an Agilent 1290 Infinity LC Binary Pump, well-plate sampler with thermostat, and temperature-controlled column compartment. If an LC system with different delay volume is used, the gradient may need to be adjusted and verified to reproduce the same chromatography.

MS method

The Agilent MS/MS system consisted of an Agilent 6460 Triple Quadruple Mass Spectrometer with Agilent Jet Stream technology and Agilent MassHunter Software B.06.00.

MS method parameters

Parameter	Value
Ion mode	Agilent Jet Stream ESI+
Gas temperature	325 °C
Drying gas (nitrogen)	5 L/min
Nebulizer gas (nitrogen)	35 psi
Sheath gas (nitrogen)	375 °C
Sheath flow	12 L/min
Capillary voltage	3,000 V
Nozzle voltage	0 V
Q1/Q3 resolution	0.7 unit
Dwell time	20 ms
Delta EMV	200 V

LC method parameters

Parameter	Value
Column	Agilent Pursuit PFP, 2 × 150 mm, 3 µm (p/n A3051150X020)
Guard column	Agilent Pursuit PFP MetaGuard, 200Å, 2 mm, 3 µm, 3/pk, (p/n A3051MG2)
Mobile phase	A) 0.2 % Formic acid in water B) Methanol
Column temperature	40 °C
Autosampler temperature	4 °C
Injection volume	20 µL
Needle wash	1:1:1:1 MeOH:ACN:IPA:H_2O + 0.1 % Formic acid (Flush port for 20 seconds)
Flow rate	0.3 mL/min
Gradient	Time (min) %B 0 0 1.0 0 2.5 60 6.0 60 6.1 0 9.0 0

Chemicals and reagents

Calibrators were purchased from Cerilliant, Round Rock, TX, Cambridge Isotopes Laboratories, Tewksbury, MA, and Medical Isotopes, Pelham, NH. DC Mass Spect Gold urine MSG5000 was purchased from Golden West Biologicals, Temecula, CA. Lyphocheck controls 376 and 377 were from Bio-Rad. Burdick & Jackson LC/MS-grade methanol and reagents were from VWR. 2-Aminoethyl diphenylborinate was from Sigma-Aldrich, Corp. St. Louis, MO.

The diphenyl-boronate complexing agent was prepared by combining 200 mL of 2 M NH_4Cl/NH_4OH buffer with 400 mg diphenylboronic acid ethanolamine ester (2-aminoethyl diphenylborinate) and 1 g disodium EDTA. Diphenylboronic acid does not dissolve easily and may require mixing slowly overnight to dissolve completely.

The 2 M NH_4Cl/NH_4OH buffer was prepared by dissolving 107 g of NH_4Cl in 1 L water and adding 30 % NH_4OH to pH 8.5.

The 0.2 M NH_4Cl/NH_4OH wash buffer was prepared by adding 50 mL of the 2 M NH_4Cl/NH_4OH buffer to 450 mL water, followed by 20 mg EDTA and adjustment to pH 8.5 with 30 % NH_4OH.

The 5 % methanol in wash buffer was made by adding 25 mL methanol to 475 mL wash buffer and adjusting to pH 8.5 with 30 % NH_4OH, as before.

All buffers and the complexing agent were stored at 4 °C, and their pH checked before use.

Table 1. MRM transitions.

Compound	Precursor	Product	Frag (V)	CE (V)	CAV (V)
Dopamine*	154.1	137.1	75	8	3
Dopamine	154.1	91.1	75	28	3
Dopamine-D4	158.1	141.1	75	8	3
Norepinephrine*	152.1	107	116	16	5
Norepinephrine	152.1	77.1	116	30	5
Norepinephrine-D6	176.1	158.1	65	4	5
Epinephrine*	184.1	166.1	70	8	5
Epinephrine	184.1	107.1	70	24	5
Epinephrine-D6	190.1	172.1	70	8	5
3-Methoxytyramine*	151.1	91.1	135	20	3
3-Methoxytyramine	151.1	119	135	12	3
3-Methoxytyramine-D4	155.1	95.1	135	24	3
Normetanephrine*	166.1	134	105	16	3
Normetanephrine	166.1	106.1	105	20	3
Normetanephrine-D3	169.1	137.1	105	16	3
Metanephrine*	180.1	165.1	120	16	5
Metanephrine	180.1	148.1	120	16	5
Metanephrine-D3	183.1	168.1	120	16	5

* Quantification transition

Sample preparation

Standard calibrators were prepared by spiking DC Mass Spect Gold urine with each catecholamine and metanephrine analyte. Serial dilutions in DC Mass Spect Gold urine were used to achieve the remaining standard calibrator concentrations. For total measurements, samples (500 µL) were hydrolyzed with 25 µL of 6 NHCl, mixed, incubated at 90 °C for 15 minutes, then cooled at room temperature. For free measurements, samples were used as is. The SPE procedure was as follows:

1. Pretreat sample: To 0.5 mL urine, add 40 µL internal standards mix and 0.8 mL diphenyl-boronate complexing agent; adjust to pH 7.5 to 9.5 with NH4OH.
2. Condition the SPE cartridge (Agilent Bond Elut Plexa, 30 mg, 3 mL, p/n 12109303) with 1 mL methanol and 1 mL aqueous wash buffer (0.2 M NH_4Cl/NH_4OH).
3. Add pretreated samples.
4. Wash with 1 mL of 5 % methanol wash buffer, and then dry at full vacuum for 5 minutes.
5. Elute with 1 mL of 5 % formic acid in water, then apply a 5 inch Hg vacuum for 30 seconds.
6. Transfer to an autosampler vial, and inject into the LC/MS/MS system.

Data analysis

Agilent MassHunter Quantitative Data Analysis Software (B.06.00) was used for data analysis. A 1/x weighting factor was applied during linear regression of the calibration curves. The quantitation using MassHunter Quantitative Software was performed by comparing chromatographic peak area ratio to a known concentration of the internal standards.

Results and Discussion

Chromatographic separation of all analytes (Figure 2) was achieved with an Agilent pentafluorophenyl (PFP) column. The separation of epinephrine and normetanephrine, and metanephrine and 3-methoxytyramine are especially critical since these compounds share common fragments. Without proper separation by retention time, fragmentation of these compounds can cause interferences with one another and lead to inaccurate quantitation.

To study recovery of the SPE extraction procedure, a mix of all six analytes at nine different concentrations was spiked into 0.1 % formic acid in water. This mix also contained the six internal standards. The nine mixes were subjected to the SPE extraction procedure and analyzed. They were also injected as is, without going through SPE. Absolute recoveries for each internal standard were calculated and varied from 53 to 112 % (Table 2). However, for the SPE-extracted solutions, when calculating the concentrations with the internal standards using the peak area ratios corrections (the relative recoveries), very good corrections were evident. These varied between 94 and 104 % (Table 2), confirming that the internal standards corrected for variations in the SPE extraction.

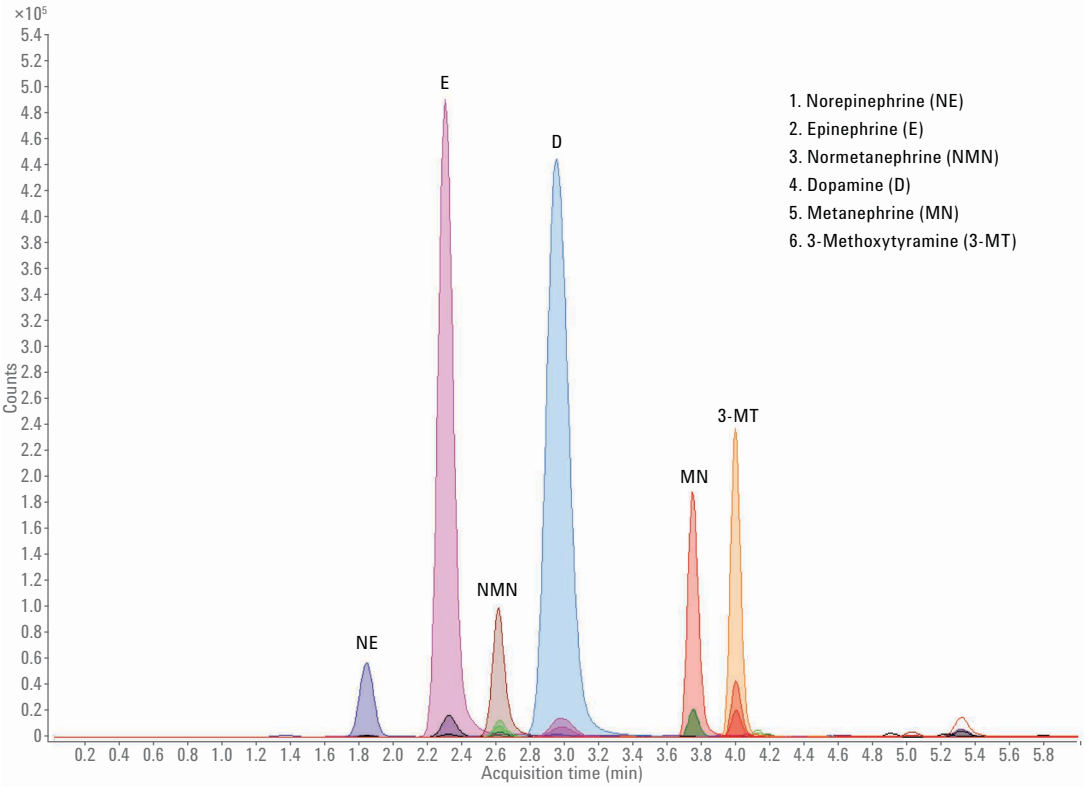

Figure 2. Chromatography of catecholamines, metanephrines, and 3-methoxytyramine.

Table 2. Absolute and relative recoveries of SPE procedure.

	Absolute recovery (%) (n = 9)		Relative recovery (%) with ISTD correction (n = 9)		
Compound	Average	SD	Range	Average	SD
Dopamine	73.5	2.4	95.0-103.4	100.0	2.5
Norepinephrine	112.5	4.9	99.0-102.4	100.0	1.0
Epinephrine	90.3	3.6	94.5-104.4	100.0	2.9
3-Methoxytyramine	53.2	3.6	94.4-102.5	100.0	3.0
Normetanephrine	88.7	7.5	97.3-102.0	100.0	2.0
Metanephrine	93.9	3.6	97.2-103.9	100.0	2.2

Calibration standards and Bio-Rad Lyphocheck controls were extracted over a period of three days, three times a day to establish both inter- and intraday precision and accuracy. All six analytes had inter- and intraday accuracies within 8 %, and coefficient of variation values were less than 6 % for all concentrations within the linear range (Tables 3 to 6). The analytical method had excellent linearity within the measured range of 1.56 to 1,000 ng/mL, with an R^2 value greater than 0.9997 (Figure 3) for the catecholamines. The measured range was 4.69 to 3,000 ng/mL, with an R^2 value greater than 0.9999 for the metanephrines and 3-methoxytyramine (Figure 3).

Conclusions

We developed a robust analytical method for quantifying catecholamines, metanephrines, and 3-methoxytyramine in urine. Offline solid phase extraction for simultaneous extraction of all six analytes from urine is shown with excellent recoveries. Chromatographic separation of the analytes using conditions compatible with LC/MS/MS were also developed. Typical method performance results were well within acceptable criteria.

Table 3. Summary of analyte performance for catecholamines.

Compound	R^2	Concentration (ng/mL)	Concentration (nmol/L)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 5
Dopamine	0.9997	1.56	10.2	107.5	1.0	2.7
		62.5	408.0	99.1	1.7	2.0
		1,000	6,528.3	101.3	0.1	0.3
Norepinephrine	0.9999	1.56	9.2	102.9	0.9	5.4
		62.5	369.4	101.1	3.5	4.0
		1,000	5,910.9	101.1	0.6	0.6
Epinephrine	0.9998	1.56	8.5	101.6	4.3	2.7
		62.5	341.2	100.9	2.5	2.0
		1,000	5,458.4	100.3	0.4	0.3

Table 4. Summary of analyte performance for metanephrines and 3-methoxytyramine.

Compound	R^2	Concentration (ng/mL)	Concentration (nmol/L)	Accuracy (%) n = 3	Intraday CV (%) n = 3	Interday CV (%) n = 5
3-Methoxytyramine	0.9999	4.69	28	95.7	1.1	3.6
		187.5	1,121.4	102.9	0.9	2.0
		3,000	17,942.1	100.0	0.2	0.3
Normetanephrine	0.9999	4.69	25.6	100.1	1.5	3.2
		187.5	1,023.45	102.0	1.1	2.5
		3,000	16,375.2	100.7	0.2	0.2
Metanephrine	0.9999	4.69	23.8	100.5	0.3	2.8
		187.5	950.7	102.0	0.5	2.2
		3,000	15,210.6	100.8	0.1	0.2

Table 5. Results of Bio-Rad QC run by LC/MS/MS (range determined by Bio-Rad using HPLC). All measurements are in ng/mL.

		Level 1			Level 2		
Compound	Free/Total	Range (HPLC)	Measured (ng/mL)	CV (%)	Range (HPLC)	Measured (ng/mL)	CV (%)
Dopamine	Free	44.4–75.0	61.4	3.4	377–629	509	2.8
Norepinephrine	Free	31.3–51.6	38.4	5.8	156–239	192	4.8
Epinephrine	Free	9.62–19.1	14.3	5.3	67.8–104	86.7	2
3-Methoxytyramine	Total	28.6–48.7	44.7	3.8	381–572	557.7	2.2
Normetanephrine	Total	220–366	300.7	2.4	1,084–1,630	1,379.2	2.8
Metanephrine	Total	69.0–116	91.2	2	434–655	612	2.5

Table 6. Results of Bio-Rad QC run by LC/MS/MS (range determined by Bio-Rad using HPLC). All measurements are in nmol/L.

		Level 1			Level 2		
Compound	Free/Total	Range (HPLC)	Measured (nmol/L)	CV (%)	Range (HPLC)	Measured (nmol/L)	CV (%)
Dopamine	Free	290–490	401	3.4	2,465–4,105	3,323	2.8
Norepinephrine	Free	185–305	227	5.8	920–1,410	1,135	4.8
Epinephrine	Free	52.5–104	78	5.3	370–570	473	2
3-Methoxytyramine	Total	171–291	267	3.8	2,280–3,420	3,335	2.2
Normetanephrine	Total	1,200–2,000	1,641	2.4	5,920–8,900	7,528	2.8
Metanephrine	Total	350–590	462	2	2,200–3,320	3,103	2.5

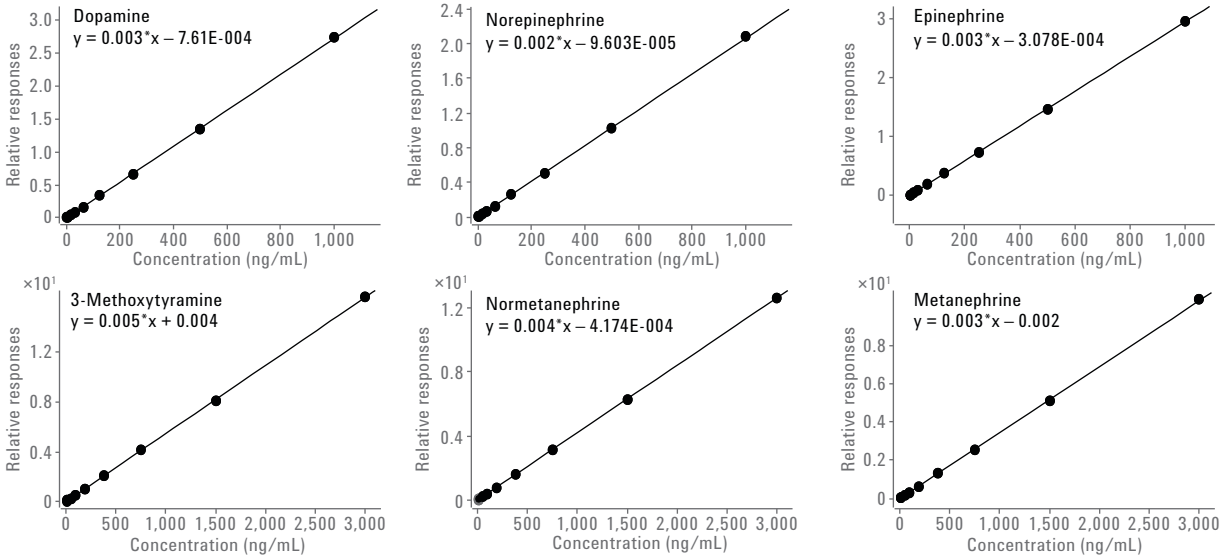

Figure 3. Calibration curves for catecholamines, metanephrines, and 3-methoxytyramine.

References

1. Whiting, M. J. Simultaneous measurement of urinary metanephrines and catecholamines by liquid chromatography with tandem mass spectrometric detection. *Ann. Clin. Biochem*. **2009**, *46*, pp 129-136.

2. Talwar, D.; *et al.* Extraction and separation of urinary catecholamines as their diphenyl boronate complexes using C solid-phase extraction 18 sorbent and high-performance liquid chromatography. *J. Chromatog. B* **2002**, *769*, pp 341-349.

3. Anon. Extraction of Catecholamines from Urine, AN1071A; Dr. Wéber Consulting KFT, Göd-Felsögöd, Hungary. www.weber.hu/PDFs/SPE/AN1071S_CatecholaminesUrine.pdf

www.agilent.com/chem/clinicalresearch

Published in the USA, January 7, 2016
5991-6194EN

Agilent Technologies