thermo scientific

TECHNICAL NOTE 65131

Determination and quantitation of catecholamines and metanephrines in urine with an efficient sample preparation and LC-MS/MS workflow for clinical research use

Author

Sergio Indelicato,
Thermo Fisher Scientific France,
Villebon sur Yvette, France

Keywords

Catecholamines, metanephrines, metanephrine, normetanephrine, epinephrine, norepinephrine, dopamine, urine, liquid-liquid extraction, LLE, TSQ Endura MS

Application benefits

- Simple sample preparation (LLE using a complexing reagent) reduces need of solid phase extraction
- Robust chromatography separation using mixed-mode column
- Increased confidence in quantitation with robust triple quadrupole MS
- Wide concentration ranges covered

Goal

Implementation of a simple, efficient, and economic non-SPE sample preparation method for an analytical method for the quantification of catecholamines and metanephrines in urine using the Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer for clinical research use

Introduction

Quantitation of catecholamines and metanephrines is essential for biochemical screening of several diseases. There are many reports of LC-MS/MS quantitation of catecholamines and metanephrines for both plasma and urine matrices.[1–3] Liquid chromatography (LC) coupled to triple quadrupole mass spectrometry (MS/MS) is a widely used technology for robust, sensitive quantitation of analytes in biological matrices.

ThermoFisher SCIENTIFIC

Mass spectrometry offers the unique ability of increased specificity and sensitivity to quantify and identify compounds by associating their retention times with structural information.

In this report, an analytical method that allows for analysis and quantification of metanephrine, normetanephrine, epinephrine, norepinephrine, and dopamine in urine, without the need for a solid-phase extraction step, is reported. Stable-isotope-labeled internal standards are used for quantification. The developed liquid-liquid extraction (LLE) sample preparation process is a time-effective and cost-effective solution for the pre-analytical phase. Compounds extracted from urine are then injected onto a Thermo Scientific™ UltiMate™ 3000 LC system. Mass spectrometric detection is performed by selected reaction monitoring (SRM) on a TSQ Endura triple quadrupole mass spectrometer using heated electrospray ionization in positive mode. The method, in which the chromatographic separation is achieved using a mixed-mode column, was analytically evaluated using charcoal stripped urine spiked with the compounds of interest for the lower limit of quantification (LLOQ), linearity range, accuracy, intra- and inter-assay precision, and matrix effect evaluation.

Experimental

Target analytes

Target analytes and corresponding internal standards are reported in Figure 1.

Sample preparation

Sample cleanup is performed using a simple LLE procedure with ethyl acetate as the extraction solvent and 2-aminoethyl diphenylboronate as the complexing reagent. The diphenyl boronate forms a stable, negatively charged complex with cis-hydroxyl groups of catecholamines, which has strong affinity for the apolar solvent, when operating in alkali media.[4]

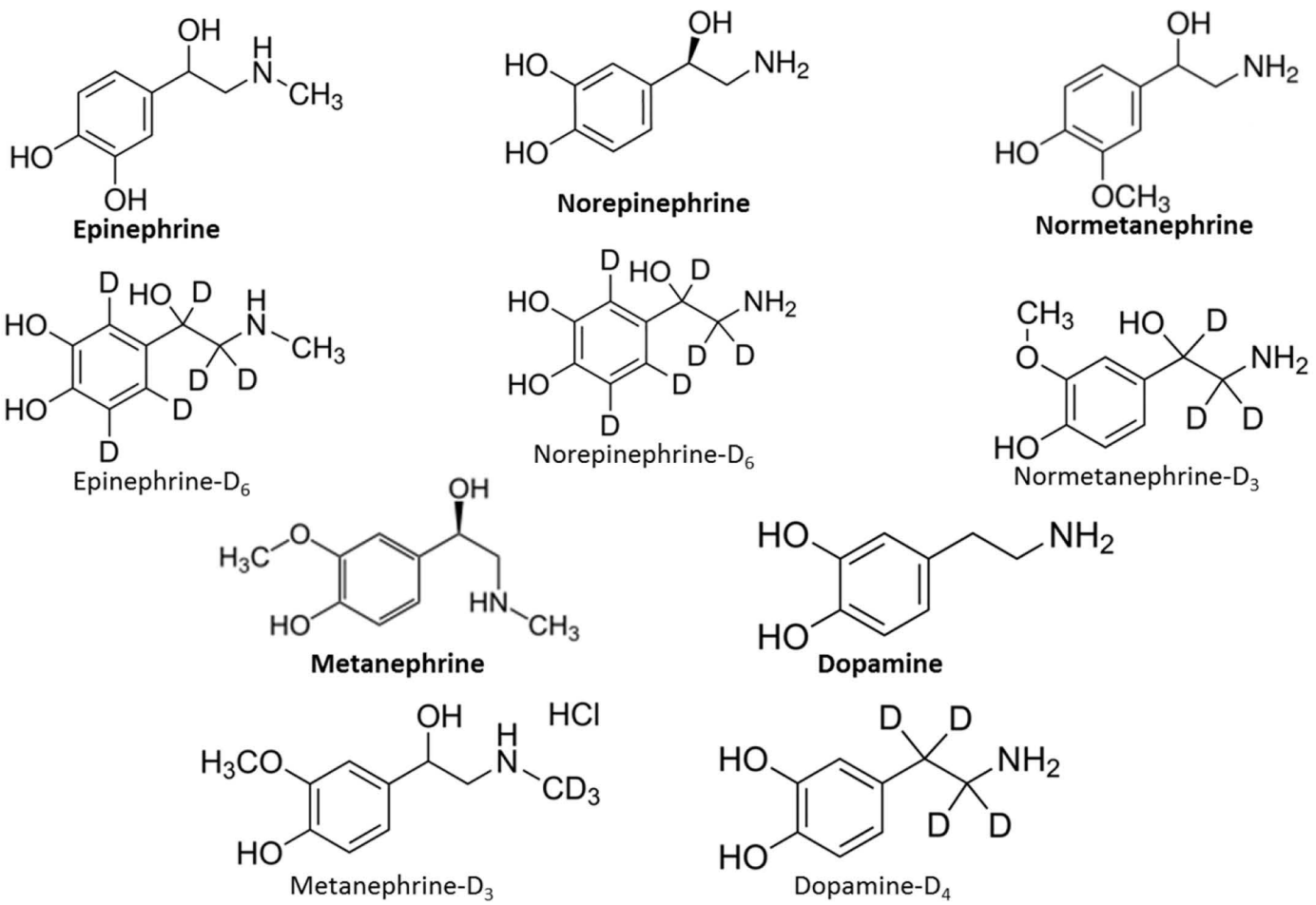

Figure 1. Structures of target compounds and corresponding internal standards.

To prepare the sample, 50 µL of internal standard solution and 1600 µL of complexing reagent solution at the concentration of 2 g/L were added to 1.0 mL of centrifuged urine in a centrifugation tube. The pH was adjusted to 9.5 using acetic acid. The tube was vortex-mixed for 60 seconds and 1.5 mL of ethyl acetate was added. After vortex-mixing and centrifugation, 800 µL of supernatant was recovered, evaporated to dryness using a flow of nitrogen, and finally reconstituted with 200 µL of mobile phase A.

The sample preparation procedure is summarized in Figure 2.

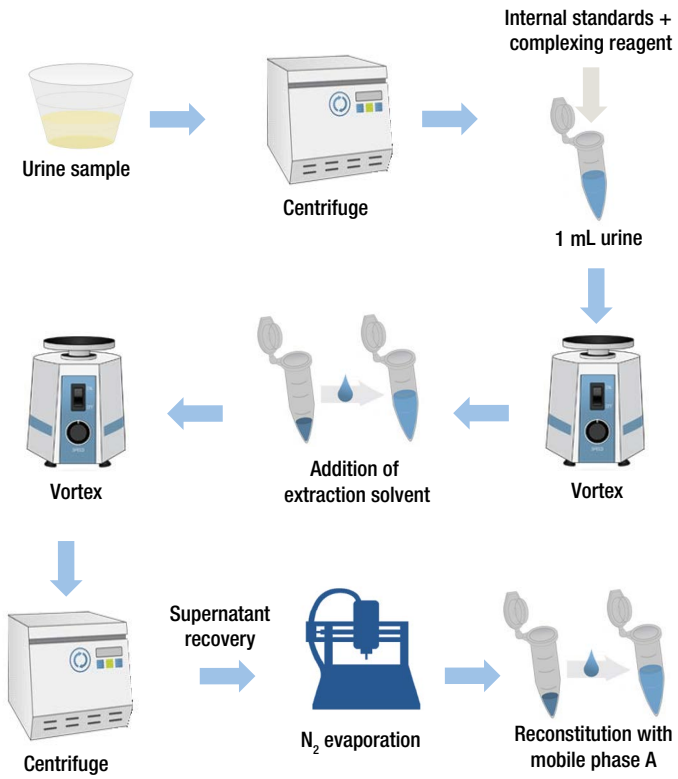

Figure 2. Sample preparation scheme.

Liquid chromatography

A 10.0-minute gradient elution was performed using an UltiMate 3000 RS LC system. Mobile phases consisted of 2 mM ammonium formate in water with 0.1% formic acid and acetonitrile with 0.1% formic acid. Chromatographic separation was achieved using a 2.1 × 150 mm (3 µm) Thermo Scientific™ Acclaim™ Mixed-Mode WCX-1 column at 40 °C. Further details of the chromatographic method are reported in Table 1.

Table 1. HPLC settings.

LC system:	UltiMate 3000 RS Binary Gradient LC
Analytical column:	Acclaim Mixed-Mode WCX-1, 2.1 × 150 mm (3 µm), held at 40 °C
Mobile phases:	A: 2 mM ammonium formate in water + 0.01% formic acid B: acetonitrile + 0.1% formic acid

Gradient profile:

Time (min)	*Flow Rate (mL/min)*	*A (%)*	*B (%)*
0.0	0.40	98	2
3.5	0.40	98	2
5.0	0.40	10	90
5.5	0.50	10	90
7.0	0.50	10	90
7.5	0.40	98	2
10.0	0.40	98	2

Injection volume:	10 µL
Column temp.	40 °C

Mass spectrometry

Target analytes and internal standards were detected by scheduled SRM on a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operated in positive mode. The MS settings are reported in Table 2. A 0.5-minute acquisition time window was used for each analyte and two SRM transitions were included in the acquisition method for quantification and confirmation. Details of the SRM transitions for compounds and internal standards, together with the corresponding source fragmentation, RF lens, and collision energy values, are reported in Table 3.

Table 2. MS settings.

Source type:	Heated electrospray ionization (HESI)
Vaporizer temperature:	400 °C
Capillary temperature:	350 °C
Spray voltage positive:	3500 V
Sheath gas:	50 AU
Sweep gas:	1 AU
Auxiliary gas:	15 AU
Data acquisition mode:	Selected-reaction monitoring (SRM)
Collision gas pressure:	1.5 mTorr
Cycle time:	0.500 s
Q1 mass resolution (FWMH):	0.7
Q3 mass resolution (FWMH):	0.7

Determining LOQ and assay precision

The method performance was evaluated by obtaining limit of quantification (LOQ), linearity range, accuracy, intra- and inter-assay precision, and matrix effect for each analyte. Eight calibration levels containing all analytes were prepared by spiking charcoal stripped urine with the proper volumes of standard solutions of the compounds of interest. Each calibrator was extracted and analyzed in replicates of five to evaluate sensitivity and linearity. A maximum percentage bias between nominal and back-calculated concentration of 15% was set as an acceptance criterion for all the calibrators (20% for the lowest). Controls at four levels containing all the analytes were also extracted and analyzed in triplicate for three consecutive days, to evaluate the precision of the method. A maximum percentage bias between nominal and back-calculated concentration of 20% was set as acceptance criterion for all the control samples.

Table 3. SRM settings for target compounds.

Compound	Precursor Ion (*m/z*)	Source Fragment (V)	RF Lens (V)	Product Ion (*m/z*)	Collision Energy (V)	Ion Type
Dopamine	154.06	0	142	137.05	10	Quan
				91.03	24	Confirming
Dopamine-D4	158.06	0	80	140.99	10	Quan
				94.04	22	Confirming
Epinephrine	166.06	28.6	153	107.05	20	Quan
				135.00	15	Confirming
Epinephrine-D6	172.06	28.6	172	157.07	20	Quan
				111.05	21	Confirming
Metanephrine	180.06	0	116	165.04	17	Quan
				148.04	18	Confirming
Metanephrine-D3	183.06	0	123	151.07	19	Quan
				168.13	18	Confirming
Norepinephrine	152.06	4.1	93	135.00	13	Quan
				107.07	18	Confirming
Norepinephrine-D6	158.06	4	136	111.05	18	Quan
				139.07	14	Confirming
Normetanephrine	166.06	10.2	86	121.07	17	Quan
				149.07	13	Confirming
Normetanephrine-D3	169.06	20.4	133	137.04	17	Quan
				109.11	20	Confirming

Accuracy for the assay was evaluated in terms of trueness of measurement, measuring the percentage bias between nominal and average back-calculated concentration for each calibrator level. Intra-assay precision was evaluated as the percentage RSD (%RSD) using the controls in replicates of three (n=3) analyzed in one batch. Inter-assay precision was evaluated on the same controls in replicates of nine (n=9) prepared and analyzed on three different days.

Matrix effect was measured for each analyte as the percentage ratio between analyte / internal standard area ratio in matrix samples and in water at the same concentration in replicates of three.

Data analysis

Data were acquired and processed using Thermo Scientific™ TraceFinder™ 4.1 software.

Results and discussion

The reported assay is based on a simple and economic LLE protocol followed by LC-MS/MS detection. It is quick and does not require the use of specific accessories or consumables during the pre-analytical phase. The assay proved to be linear in the tested calibration range (0.4–900 ng/mL) for all the analytes of interest, with an LLOQ of 0.4 ng/mL and a correlation factor (R^2) always above 0.998. The concentrations of prepared calibrators and controls and a calibration performance summary for all the target analytes are reported in Tables 4 and 5, respectively. Representative chromatograms for the LLOQ (0.4 ng/mL) for all compounds together with the corresponding calibration curves are reported in Figure 3. The %RSD value for all the controls for each compound for intra- (n=3) and inter-assay (n=9) precision are reported in Tables 6 and 7, respectively. Matrix effect values are reported in Table 8.

Table 4. Concentration of calibrators and controls prepared in charcoal-stripped urine.

Calibrator ID	Concentration (ng/mL)
Cal-0	0
Cal-1	0.4
Cal-2	1.2
Cal-3	3.7
Cal-4	11.1
Cal-5	33.3
Cal-6	100
Cal-7	300
Cal-8	900
Ctrl-1	1
Ctrl-50	50
Ctrl-200	200
Ctrl-800	800

Table 5. Calibration performances summary.

Target Compound	Internal Standard	Retention Time (min)	Curve type	Origin	Weighting	R^2	LOQ (ng/mL)
Metanephrine	Metanephrine-$^2H_3$	3.95	Linear	Ignore	1/X	0.9991	0.4
Normetanephrine	Normetanephrine-$^2H_3$	3.53	Linear	Ignore	1/X	0.9992	0.4
Dopamine	Dopamine-$^2H_4$	3.65	Linear	Ignore	1/X	0.9991	0.4
Epinephrine	Epinephrine-$^2H_6$	3.03	Linear	Ignore	1/X	0.9983	0.4
Norepinephrine	Norepinephrine-$^2H_6$	2.90	Linear	Ignore	1/X	0.9979	0.4

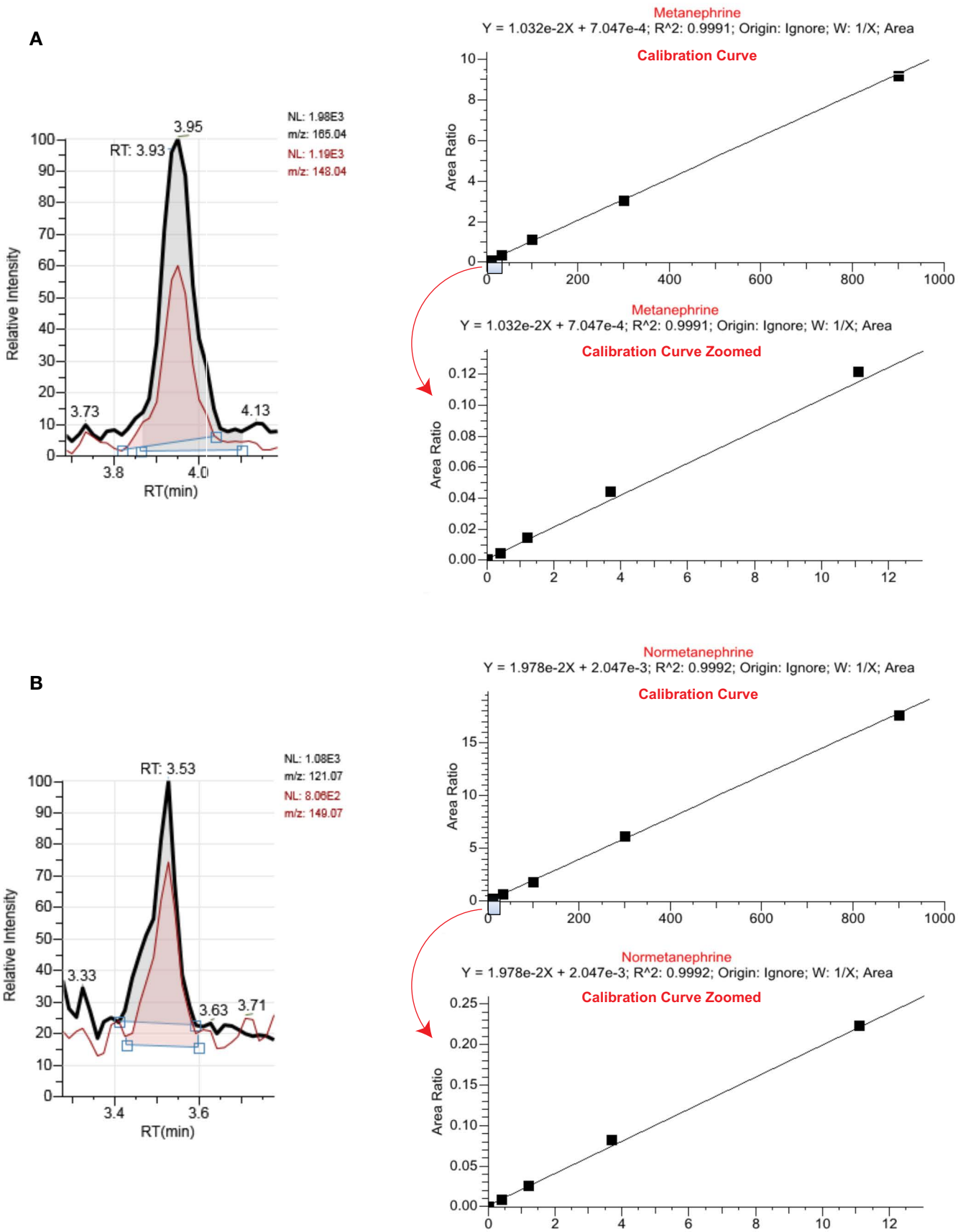

Figure 3-1. Chromatograms at LLOQ level (0.4 ng/mL) and calibration curves for metanephrine (A) and normetanephrine (B).

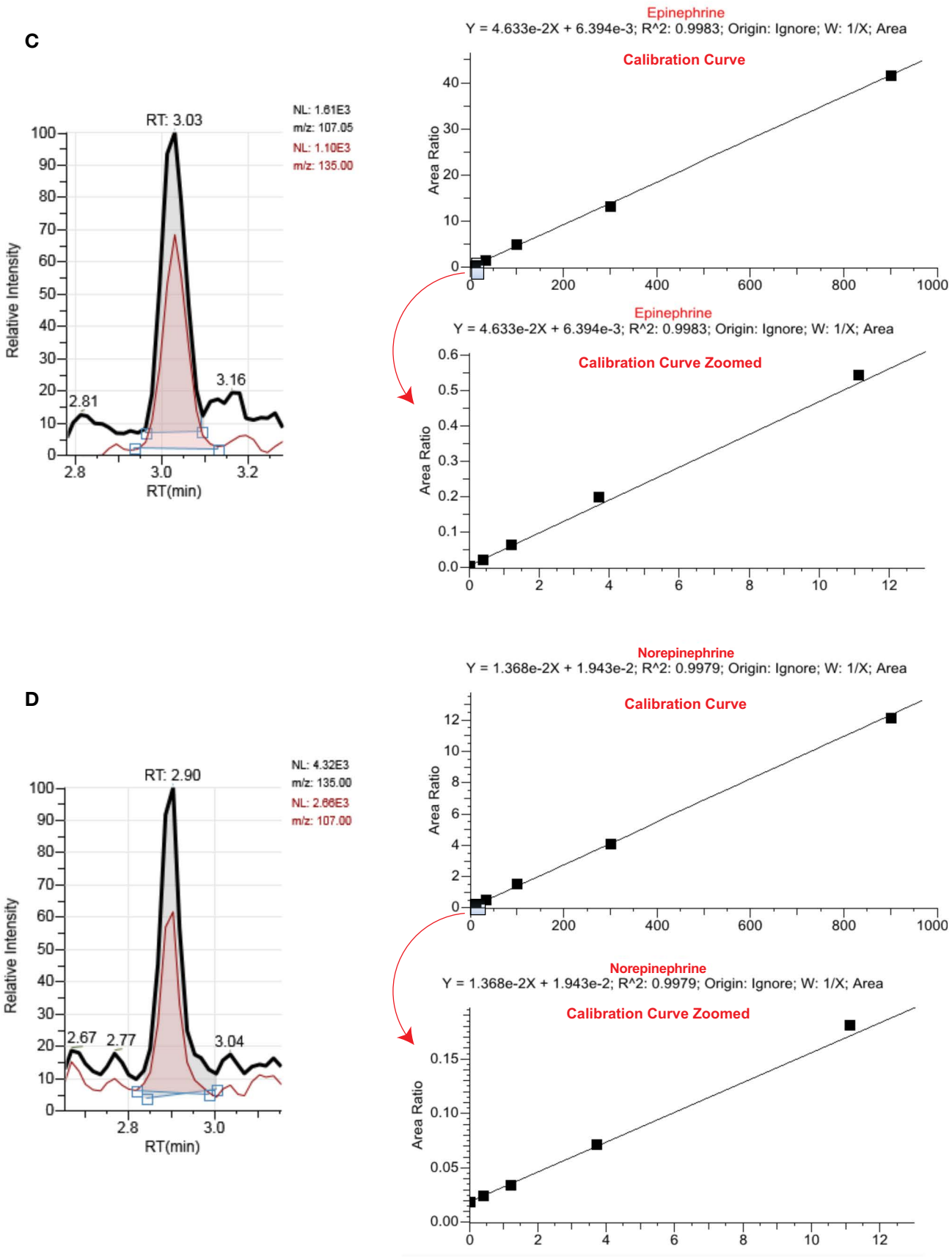

Figure 3-2. Chromatograms at LLOQ level (0.4 ng/mL) and calibration curves for epinephrine (C) and norepinephrine (D).

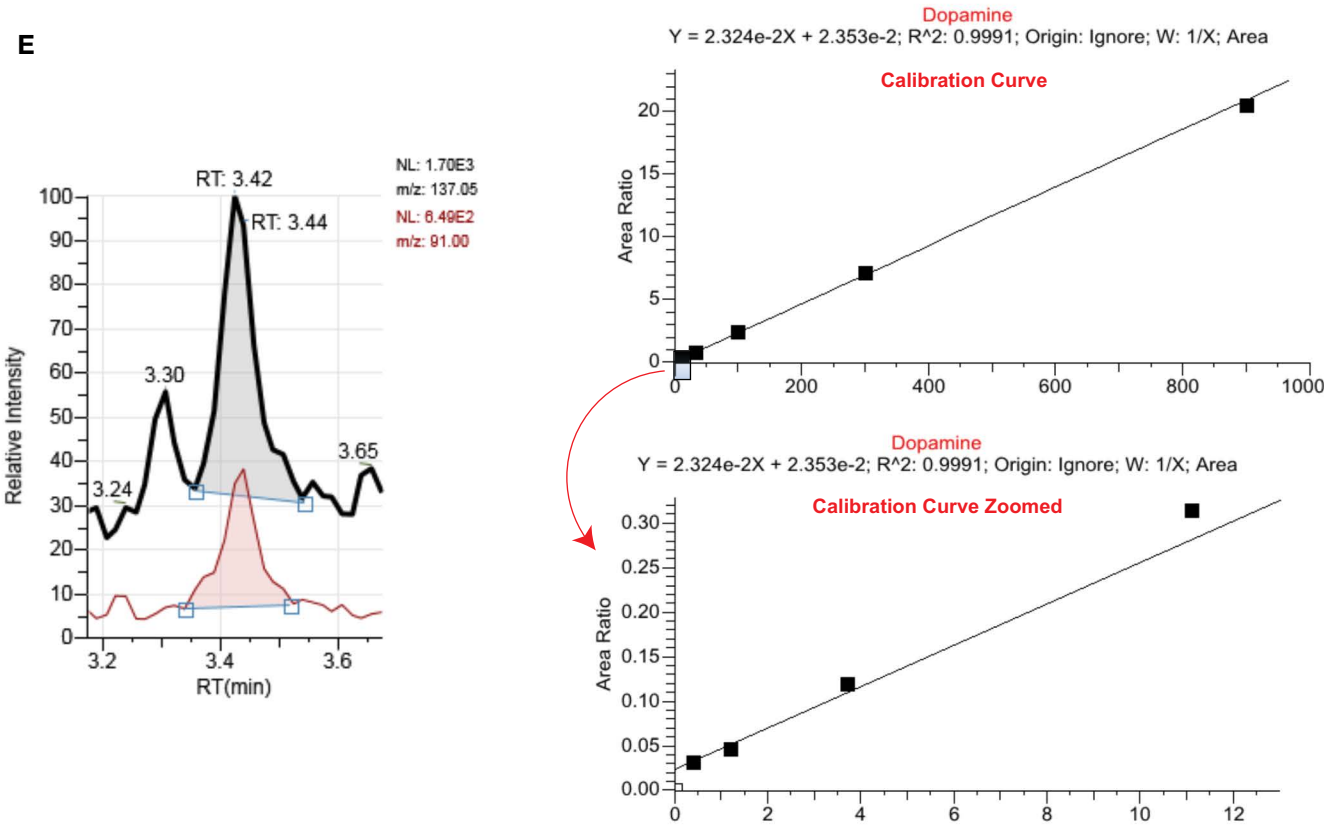

Figure 3-3. Chromatogram at LLOQ level (0.4 ng/mL) and calibration curves for dopamine (E).

Table 6. %RSD for intra-assay (n=3) precision.

Target Compound	Ctrl-1	Ctrl-50	Ctrl-200	Ctrl-800
Metanephrine	8.34	3.12	4.44	2.09
Normetanephrine	3.67	9.89	2.33	0.45
Epinephrine	14.90	7.51	3.69	0.66
Norepinephrine	2.91	10.59	2.09	1.68
Dopamine	12.37	5.60	1.03	3.90

Table 7. %RSD for inter-assay (n=9) precision.

Target Compound	Ctrl-1	Ctrl-50	Ctrl-200	Ctrl-800
Metanephrine	7.82	6.02	3.32	2.89
Normetanephrine	4.82	9.71	2.80	2.45
Epinephrine	12.91	8.75	2.91	2.17
Norepinephrine	11.38	8.10	2.71	2.10
Dopamine	14.55	3.96	3.11	2.98

Table 8. Matrix effect.

Target Compound	Cal 3 Recovery (%)	Cal 4 Recovery (%)	Cal 5 Recovery (%)	Cal 6 Recovery (%)	Cal 7 Recovery (%)
Epinephrine	127.0	90.9	86.5	95.5	105.5
Norepinephrine	115.0	109.5	95.4	93.4	112.3
Metanephrine	130.0	85.4	86.5	88.7	102.8
Normetanephrine	123.6	96.1	85.7	86.3	105.4
Dopamine	187.5	203.5	86.0	83.7	87.0

Conclusions

A liquid chromatography-tandem mass spectrometry method for clinical research for the quantification of metanephrine, normetanephrine, epinephrine, norepinephrine, and dopamine in urine was implemented and analytically evaluated on an UltiMate 3000 RS system connected to a TSQ Endura triple quadrupole mass spectrometer. This analytical method, based on a simple LLE for sample cleanup instead of the classical SPE approach and on a robust chromatographic separation by means of a mixed-mode column, meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy, and intra- and inter-assay precision.

References

1. Kushnir, M.M.; Urry, F.M.; Frank E.L.; Roberts W.L.; Shushan, B. *Clin. Chem.* **2002,** *48,* 323.
2. Taylor, R.L.; Singh, R. J. *Clin. Chem.* **2002,** *48,* 533.
3. Petteys, B.J.; Graham, K.S.; Parnas, M.L.; Holt, C.; Frank, E.L. *Clin. Chem. Acta,* **2012,** *413,* 1459.
4. Talwar, D. et al. *J. Chromatogr. B,* **2002,** *769,* 341–349.

For research use only. Not for use in diagnostic procedures.

Find out more at **thermofisher.com**

ThermoFisher SCIENTIFIC