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Quantification of Catecholamines and Metanephrines in Urine Using the Thermo Scientific™ TSQ Endura™ Mass Spectrometer for Research Use

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ABSTRACT

Purpose: A rapid analytical method for the quantification of adrenaline, noradrenaline, metanephrine, normetanephrine and dopamine in urine, involving a simple sample preparation procedure, has been developed and analytically validated.

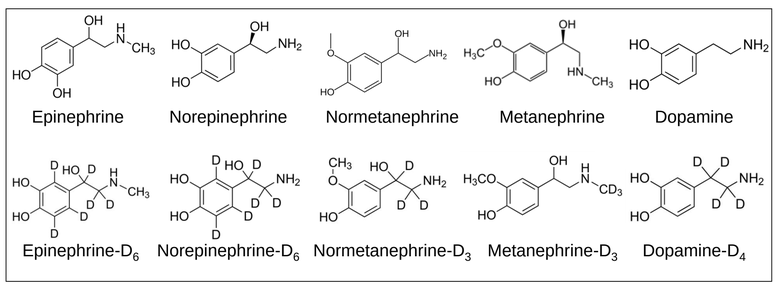
Methods: The method involves a liquid liquid extraction (LLE) of compounds of interest from urine followed by injection onto a Thermo Scientific™ UltiMate™ 3000 system; mass spectrometric detection is performed by Single Reaction Monitoring (SRM) on a Thermo Scientific™ TSQ Endura™ triple quadrupole mass spectrometer using heated electrospray ionization in positive mode.

Results: The method was analytically validated using charcoal stripped urine spiked with the compounds of interest for lower limit of quantification, linearity range, accuracy, intra- and inter-assay precision and matrix effect evaluation.

INTRODUCTION

Adrenaline (also called epinephrine), noradrenaline (also called norepinephrine) and dopamine are generally called catecholamines. Catecholamines are produced by enzymatic decarboxylation of the amino acid tyrosine in the organism, which is derived from dietary sources as well as synthesis from phenylalanine. They act as neuromodulators in the central nervous system being messengers and play a key role in the impulses transmission. Moreover norepinephrine and dopamine act as hormones in the blood circulation. Catecholamines are produced mainly by the chromaffin cells of the adrenal medulla and the postganglionic fibers of the sympathetic nervous system. Metanephrine and normetanephrine are produced by the degradation of catecholamines. They are eliminated in urine as free and conjugated metabolites.
Metabolism of catecholamines is a two steps process: they are first methylated by catecholamine-O-methyltransferase (COMT) producing the metanephrines and afterwards the deamination by monoamine oxidase (MAO) produces the homovanilic acid (HVA) and the vanillylmandelic acid (VMA). While COMT is not present in sympathetic cells but is present in adrenal medulla cells, metanephrines are specific markers of catecholamines in chromaffin cells and their tumors.
Liquid chromatography coupled with tandem mass spectrometry is the gold standard technique for the determination and quantification of these neurotransmitters in urine, due to its analytical sensitivity. The use of labeled internal standard increases the accuracy of the quantification. To reduce the matrix effect, methods presented in literature usually involve an extraction step such as SPE (solid phase extraction) which is complex to set-up. A simple liquid-liquid extraction procedure, using a complexing reagent, has been developed in the reported analytical method for research use.
Target analytes and corresponding internal standards are reported in Figure 1.

Figure 1. Structures of the analyzed catecholamines and metanephrines and the corresponding internal standards.

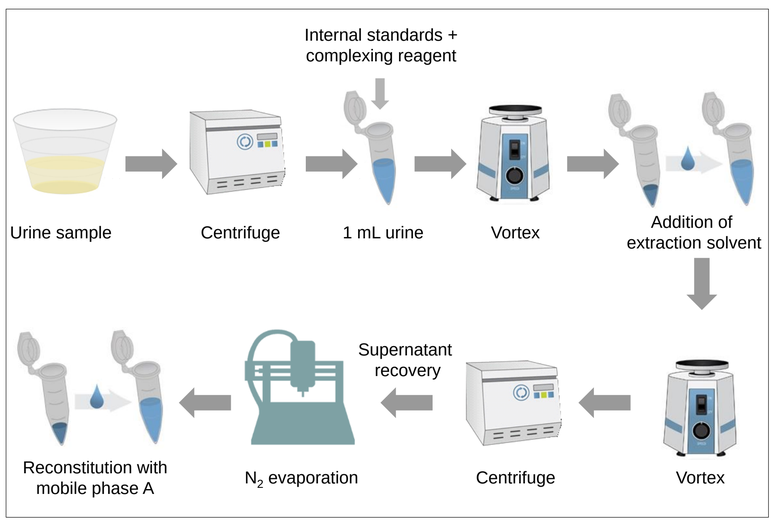


MATERIALS AND METHODS

Sample Preparation

Sample clean-up is performed using a simple LLE procedure with ethyl acetate as the extraction solvent and 2-aminoethyl diphenylboronate as complexing reagent. The diphenyl boronate forms a stable negatively charged complex with cis-hydroxyl groups of catecholamines, that shows a strong affinity for a polar solvent, when operating in alkali media[1].
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. 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.
Sample preparation procedure is summarized in Figure 2.

Figure 2. Sample preparation scheme



Liquid chromatography

A 10.0-minute gradient elution was performed using a Thermo Scientific™ UltiMate™ 3000RS LC system. Mobile phases consisted of 2 mM ammonium formate in water with 0.1% formic acid and acetonitrile with 0.1% formic acid. The chromatographic separation was achieved using a 2.1 × 150 mm (3µ) Thermo Scientific™ Acclaim™ Mixed Mode WCX-1 column at 40° C. Injection volume was10 µL. Further details of the chromatographic method are reported in Table 1.

Table 1. HPLC settings

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

Mass spectrometry

Target analytes and internal standards were detected by scheduled SRM on a TSQ Endura triple quadrupole mass spectrometer with heated electrospray ionization operating 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 conditions.

Source type	Heated electrosprayization (HESI)
Spray voltage	3500 V positive mode
Vaporizer temp	400^{0}C
Ion transfer tube temp	350^{0}C
Sheath gas	50 AU
Sweep gas	1 AU
Auxiliary gas	15 AU
Collision gas pressure	1.5 mTorr
Cycle time	0.500 s
Q1 (FWMH) & Q3 (FWMH)	0.7

Table 3. SRM settings for target compounds.

Compound	Precursor Ion (m/z)	Source Fragm. (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

Method evaluation

The analytical method performance was evaluated by defining the following parameters: limit of quantification, 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 5 replicates to evaluate sensitivity and linearity. A maximum bias percentage between nominal and back-calculated concentration of 15% was set as acceptance criterion for all the calibrators (20% for the lowest). Controls at 4 levels containing all the analytes were also extracted and analyzed in triplicate for three consecutive days, in order 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. Accuracy for the assay was evaluated in terms of trueness of measurement, measuring the bias percentage 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 for three replicates.

Data Analysis

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

RESULTS

The assay proved to be linear in the tested calibration range (0.4 – 900 ng/mL) for all the analytes of interest, with a lower limit of quantification (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 Table 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 values for all the controls for each compound for intra- (n=3) and inter-assay (n=9) precision are reported in Table 6 and 7 respectively. Matrix effect values are reported in Table 8.

Table 4. Concentration of calibrators and controls 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	Curve type	Origin	Weighting	R^2	LOQ (ng/mL)
Metanephrine	Metanephrine-$^2H_3$	Linear	Ignore	1/X	0.9991	0.4
Normetanephrine	Normetanephrine-$^2H_3$	Linear	Ignore	1/X	0.9992	0.4
Dopamine	Dopamine-$^2H_4$	Linear	Ignore	1/X	0.9991	0.4
Epinephrine	Epinephrine-$^2H_6$	Linear	Ignore	1/X	0.9983	0.4
Norepinephrine	Norepinephrine-$^2H_6$	Linear	Ignore	1/X	0.9979	0.4

Figure 3. Chromatograms at LLOQ level (0.4 ng/mL) and calibration curves for metanephrine (a), normetanephrine (b), epinephrine (C), norepinephrine (d) and dopamine (e)

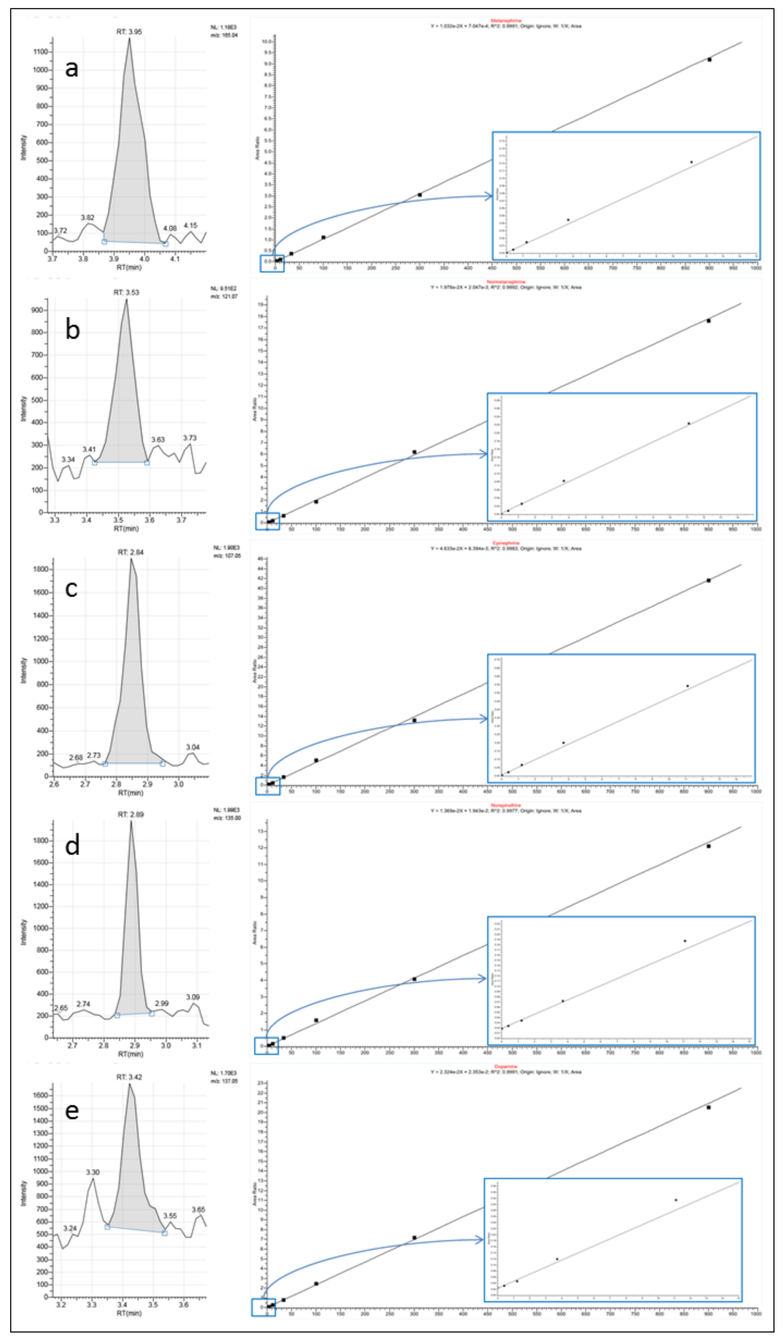


Table 6. %RSD for intra-assay (n=3) precison

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) precison

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 (%)	Cal 4 (%)	Cal 5 (%)	Cal 6 (%)	Cal 7 (%)
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 used for clinical research was developed for the quantification of metanephrine, normetanephrine, epinephrine, norepinephrine and dopamine in urine and analytically validated on a Thermo Scientific UltiMate 3000 system connected to a TSQ Endura triple quadrupole mass spectrometer. This analytical method, based on a simple LLE for sample clean-up, meets research laboratory requirements in terms of sensitivity, linearity of response, accuracy and intra- and inter-assay precision.

For Research Use Only. Not for use in diagnostic procedures.

REFERENCES

1. D. Talwar et al. / J. Chromatogr. B 769 (2002) 341–349

TRADEMARKS/LICENSING

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