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Article

Simultaneous Determination of Catecholamines and Metanephrines in Urine by Liquid Chromatography Electrospray Ionization Tandem Mass Spectrometry: Successful Clinical Application

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Determination of urinary catecholamines and metanephrines is important for diagnosis of pheochromocytoma. Liquid chromatography tandem mass spectrometry (LC-MS/MS) method has been developed for the determination of catecholamines and metanephrines in human urine using deuterated internal standards (IS). In this method, 1000 µL of urine samples were treated by liquid-liquid extraction using ethyl acetate and subjected to LC-MS/MS analysis using positive electrospray ionization (ESI+). A BDS HYPERSIL™ C18 column (125 mm × 3 mm, 3 µm) was used and mobile phase was water:methanol (98:2, v/v) with 0.25% of formic acid at 200 µL min^{-1}. The method has a chromatographic running time of approximately 10 min. The average of recovery was 92.9-106.1% for epinephrine, 94.6-107.7% for norepinephrine, 98.0-108.8% the dopamine, 94.2-105.1% the metanephrine, 97.0-106.0% for normetanephrine. The precision was lower than 7.0% for all analytes. Furthermore, this method has been implemented successfully in routine laboratory due to its easy execution and excellent precision.

Keywords: LC-MS/MS, ESI-MS, catecholamines, metanephrines, pheocromocytoma

Introduction

The quantitation of urinary catecholamines and their metabolites is used in biochemical screening for the diagnosis of pheochromocytoma.[1]

Pheochromocytomas are a tumor arising from chromaffin cells of the adrenal medulla. About 15-20% of such tumors are extra-adrenal in origin and is termed paraganglioma or extra-adrenal pheochromocytoma. Both forms have similar clinical symptoms and are characterized by secretion catecholamines in high quantity.[2]

Hypertension was found in 70-90% of patients diagnosed with pheochromocytoma. Although they might be directly related, the percentage of hypertensive which have pheochromocytoma is small, about 0.1-0.6%.[3,4] In the 1980's, autopsy studies indicate that many tumors are missed, resulting in premature mortality.[4,5] However, in a recent study, it was reported only one undiagnosed pheochromocytoma of each 2031 (0.05%) coronial autopsies. Probably, advances in biochemical diagnosis, combined with other factors, have contributed to rate decrease.[6] A clearer understanding of the role that catecholamines and their metabolites (metanephrines) play in the emergence of certain diseases is important not only for diagnosis purposes, but also for the research and development of new drugs.[7]

There are many works in literature in terms of analytical methodology for analysis of catecholamines and metanephrines. In the last few years, several procedures have been reported for both plasma and urine samples including colorimetric and radiometric enzymatic assay,[8] high performance liquid chromatography (HPLC) with electrochemical detection (ECD),[9-11] gas chromatography-mass spectrometry (GC-MS)[12] and liquid chromatography tandem mass spectrometry (LC-MS/MS),[13-15] but some of these techniques have laborious extraction procedures, poor sensibility or lack selectivity.

LC-MS/MS has become popular in bioanalysis for measuring catecholamines and metanephrines in urine. One of the major advantages of MS detection is its ability to quantify and identify compounds, by associating their retention times with structural information,[7] and can resolve many of the analytical shortcomings that can impact on diagnostic specificity.[16]

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The aim of this work is to develop a simple and rapid LC-MS/MS method for quantitative determination of epinephrine (EPI), norepinephrine (NE), dopamine (DA), metanephrine (MN) and normetanephrine (NMN) in human urine and its application in clinical laboratory routine.

Experimental

Chemicals

Epinephrine hydrochloride, norepinephrine hydrochloride, dopamine hydrochloride, metanephrine, normetanephrine hydrochloride, and 2-aminoethyldiphenylborinate were obtained from Sigma-Aldrich (St. Louis, MO, USA). Deuterium labelled compounds D3-epinephrine, D6-norepinephrine hydrochloride, D4-dopamine hydrochloride, D3-metanephrine hydrochloride and D3-normetanephrine hydrochloride were supplied by CDN Isotopes Inc. (Pointe-Claire, QC, CDN). Hydrochloride acid, formic acid, ammonium hydroxide 25%, ethyl acetate and HPLC-grade methanol were purchased from Merck (Darmstadt, DE).

Internal standards and calibrators

Calibrators stock solution, internal standards and the other intermediate calibrators standards were prepared in hydrochloric acid 0.01 mol L^{-1}. 2-Aminoethyldiphenylborinate solution was prepared in water:methanol (90:10, v/v) with ammonium hydroxide 0.25%. Urine controls were prepared by adding catecholamine and metanephrines standards in human urine pool.

Improve sample preparation

The sample extraction was improved with different solvents and two different pH values (8.5 and 9.5). The solvent and mixtures tested were ethyl acetate, chloroform:isopropyl alcohol (80:20), chloroform:diethyl ether (80:20) and chloroform:isopropyl alcohol (50:50). The solid phase extraction (SPE) protocol described by Whiting[22] were modified and tested with three different SPE cartridges, Bond Elut Plexa, Oasis HLB and Oasis WCX (all 30 mg-1.0 mL). The sample volume used was changed for 1.0 mL and the elution volume for 250 µL of formic acid 1.0% for better sensibility. A standard solution with concentrations EPI 23.0 ng mL^{-1}, NE 90.0 ng mL^{-1}, DA 415.0 ng mL^{-1}, NM 420.0 ng mL^{-1}, NMN 630.0 ng mL^{-1} prepared in solution of hydrochloric acid 0.01 mol L^{-1} were analyzed in triplicate for each solvent and SPE cartridges indicated above.

Final sample preparation protocol

To 1000 µL of urine was added 100 µL deuterated internal standard solution and 1600 µL of 2-aminoethyldiphenylborinate solution. The pH was adjusted to a value of 9.5 with ammonium hydroxide 5% and they were submitted to a vigorous stirring for 60 s using a vortex apparatus. 1500 µL of ethyl acetate was added in this solution and vortexed for more 60 s. This mixture was centrifuged (5 min at 2000 g) and 800 µL of the supernatant was evaporated with a vacuum concentrator. The extract was reconstituted with 200 µL of the mobile phase.

To determinate catecholamines and glucoronide-desconjugated metanephrines (free metanephrines), the extraction procedure are described in previous paragraph. For determination of total metanephrines (conjugated and desconjugated metanephrines) is necessary a sample hydrolysis acid step before the extraction process. So, 1000 µL of urine was acidified with 50 µL of 2.0 mol L^{-1} HCl and hydrolyzed for 40 min in an incubator at 100 °C.

Instrumentation

Chromatography was performed on a Waters Alliance HT (Waters, Milford, MA) equipped with a BDS HYPERSIL™ C18 column (125 mm × 3 mm, 3 µm particle size, Thermo Scientific). The isocratic mobile phase consisting of water:methanol (98:2 v/v) with 0.25% of formic acid pumped at a flow rate of 200 µL min^{-1}. The column was maintained at 30 °C with a column oven. The injection volume was 20 µL. The method has a chromatographic running time of approximately 10 min. Detection was performed on a Quattro Micro tandem mass spectrometer triple quadrupole using a positive electrospray ionization (Waters, Milford, MA).

To tune the mass spectrometer, a separately solution of epinephrine, norepinephrine, dopamine, metanephrine, normetanephrine and their respective internal standards in methanol:water (50:50, v/v) were infused directly into the mass spectrometer at a flow rate of 10.0 µL min^{-1}. The parameters were optimized to maximize the intensity of each precursor ions and their product ion selected.

Quantitative data were obtained by multiple reaction monitoring (MRM) of the protonated precursor ion at the form $[M + H]^+$ or at the form $[M + H - H_2O]^+$. The ions monitored and corresponding fragments voltages are listed in Table 1.

Analyte recovery

Assessment of recovery was performed by analysis of pooled urine adding all biogenic amines. Each level

Table 1. Mass spectrometric conditions optimized for each analyte

Biogenic amine	Precursor / (*m/z*)	Product / (*m/z*)	Dwell time / s	Cone voltage / V	Collision energy / eV
Epinephrine	184	166	0.15	15	10
Epinephrine-d_3	187	169	0.15	15	10
Norepinephrine	152	107	0.15	25	15
Norepinephrine-d_6	158	113	0.15	25	15
Dopamine	154	91	0.15	15	25
Dopamine-d_4	158	95	0.15	15	25
Metanephrine	180	165	0.15	25	15
Metanephrine-d_3	183	168	0.15	25	15
Normetanephrine	166	134	0.15	25	15
Normetanephrine-d_3	169	137	0.15	25	15

was extracted in triplicate. Aliquots of urine pool were spiked with 3.0, 23.0 and 43.0 μg L^{-1} of EPI, 10.0, 90.0 and 170.0 μg L^{-1} of NE, 15.0, 415.0 and 815.0 μg L^{-1} of DA, 5.0, 120.0 and 500.0 μg L^{-1} of MN, 15.0, 120.0 and 1000.0 μg L^{-1} of NMN. Aliquots of urine pool that were not spiked were extracted in parallel for the calculations of relative recoveries. The relative recovery was determined according to equation 1, where C_F is the concentration determined in fortified sample, C_U is the concentration determined in unfortified sample and C_A is the concentration of analyte added.

$$\text{Recovery}(\%) = \frac{(C_F - C_U)}{C_A} \times 100 \quad (1)$$

Method linearity

The linearity was assessed by using six calibration standards prepared in HCl 0.01 mol L^{-1}. The concentration of these calibrators was 3.0, 13.0, 23.0, 33.0, 43.0 and 53.0 μg L^{-1} for epinephrine, 10.0, 50.0, 90.0, 130.0, 170.0 and 210.0 μg L^{-1} for norepinephrine, 15.0, 215.0, 415.0, 615.0, 815.0 and 1015.0 μg L^{-1} for dopamine, 20.0, 220.0, 420.0, 820.0, 1220.0 and 1420.0 μg L^{-1} for metanephrine and 30.0, 330.0, 630.0, 1230.0, 1830.0 and 2130.0 μg L^{-1} for normetanephrine. The six standard levels were extracted in triplicate. The linear regression was evaluated using ANOVA, Jackknife test was used to remove the outliers and Brown-Forsythe test to evaluate homoscedasticity of residuals.

Limits of detection and quantification

The limit of detection (LOD) was determined according to equation 2 and the limit of quantification (LOQ) was determined according to equation 3,[17] where SD is the standard deviation of the intercepts of 5 calibration curves prepared in different days and slope is average slopes of these curves.

$$\text{LOD} = (3 \times \text{SD})/\text{slope} \quad (2)$$

$$\text{LOQ} = (10 \times \text{SD})/\text{slope} \quad (3)$$

Precision

The assays of precision were evaluated by repeatability and intermediate precision using three concentrations levels. Internal quality controls were prepared in urine pools along of the analytical range studied. Repeatability was determined by analysis of each internal quality control extracted in sextuplicate and processed within the same day and by the same analyst. Intermediate precision was assessed by measurements of quality controls above in three different days and different analysts. The coefficient variation (CV) was expressed in percentage.

Carry-over and cross-talk

The carry-over occurs when the analytes present in a first sample are still detected in samples subsequent and is due to any contamination that may occur in the system, from the first injection. The test was prepared and injected in the following sequence: (*i*) sample blank extracted; (*ii*) lower limit of calibration (LLOC) extracted; (*iii*) upper limit of calibration (ULOC) extracted; (*iv*) sample blank extracted; (*v*) ULOC extracted; (*vi*) sample blank extracted; (*vii*) ULOC extracted; (*viii*) sample blank extracted. Each sample was prepared in duplicate.

The selectivity of the detection system was verified by cross-talk test. The cross-talk occurs when there is cross-detection between the analyte and the internal standard.

The samples were injected on system, one by one, and it was possible to detect peaks or interference observed in the other channel. The test was prepared in the following sequence: (*i*) extracted sample blank; (*ii*) LLOC with internal standard; (*iii*) ULOC of analyte extracted without the internal standard; (*iv*) zero sample (sample blank with internal standard) extracted. Each sample was prepared in duplicate.

Results and Discussion

The protonated ions monitored were $[M + H - 18]^+$ and $[M + H]^+$. The precursor ion at the form $[M + H]^+$ is preferred for generating the product ions spectrum. However, some analytes are unstable in the ion source and loose water easily during electrospray ionization.[14] So, to get better sensitivity, it was chosen the ion at the form $[M + H - H_2O]^+$ for the analysis of NE, MN and NMN. For EPI and DA analysis, we monitored the form $[M + H]^+$. To choose the daughters ions we used the most intense fragmentation ion of each analyte. The same conditions were used for deuterated internal standards. The analyte spectra obtained are presented in Figures S1-S5 in the Supplementary Information (SI) section.

The sample extraction was tested with protocol described in the Improve sample preparation section. The results obtained with Bond Elut Plexa and liquid-liquid extraction (LLE) with ethyl acetate were satisfactory. However, we can concentrate the sample many times for LLE and obtain better area. For the SPE cartridges used in this study, it was not possible to concentrate more than 4 times. The LLE using ethyl acetate in pH 9.5 showed better results for simultaneous dosage of catecholamines and metanephrines. The data results for different solvents and SPE tested are shown in the SI section (Table S1) and represented in Figure 1. Furthermore, the LLE developed have a lower cost in comparison with SPE, shorter execution time, practicality and good clean up sample, attested by the low frequency equipment cleaning, approximately twice a year.

The method was linear for all compounds studied within the range of six calibrators used. The linear analytical range was between 3.0 and 53.0 μg L^{-1} for epinephrine, 10.0 and 210.0 μg L^{-1} for norepinephrine, 15.0 and 1015.0 μg L^{-1} for dopamine, 20.0 and 1420.0 μg L^{-1} for metanephrine and 30.0 and 2130.0 μg L^{-1} for normetanephrine. All the curves were homoscedastic in the range of concentration evaluated. The limit of detection, limit of quantification and the regression parameters are listed in Table 2.

The dilution was validated for samples that exceed the curve in 10 times for catecholamines and 2 times for metanephrines. The concentrated samples were analyzed in sextuplicate. Dilution precision was less than 4.7% for EPI, 7.0% for NE, 3.5% for DA, 3.0% for MN and 4.2% for NMN.

No significant effect of carry-over and cross-talk were observed in the retention times of the compounds. Epinephrine and normetanephrine are isobaric and share some equal ion transitions, so it is necessary a good chromatographic separation for the analysis of these compounds by LC-MS/MS and we managed it with the condition mentioned in this work. The chromatograms for the monitored MRM transitions for a human urine sample are presented in Figures 2 and 3 and no interfering peaks were observed in the chromatograms. Sample chromatograms of total ion chromatogram (TIC), MRM chromatograms of calibration point, sample human urine and sample blank are shown in the SI section (Figures S6-S9).

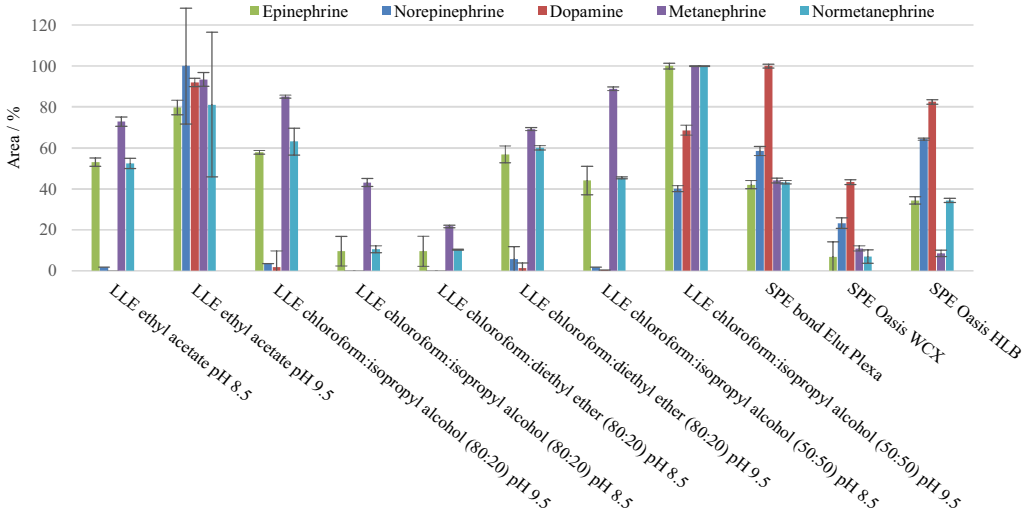

Figure 1. Efficiency of conditions of LLE and SPE procedures tested in the optimization step.

Table 2. Parameters of linearity, LOD and LOQ of all catecholamines and metanephrines studied

Analyte	LOD / (μg L^{-1})	LOQ / (μg L^{-1})	Slope	Intercept	Correlation coefficient	Linear range evaluated / (μg L^{-1})
EPI	0.3	1.0	0.021417	–0.00264423	0.999146	3.0-53.0
NE	1.9	6.0	0.022935	–0.00609044	0.996918	10.0-210.0
DA	3.6	11.1	0.007841	–0.0041276	0.998270	15.0-1015.0
MN	1.3	4.4	0.008592	–0.00493495	0.999757	20.0-1420.0
NMN	2.4	8.0	0.005083	0.00227989	0.999767	30.0-2130.0

LOD: limit of detection; LOQ: limit of quantification; EPI: epinephrine; NE: norepinephrine; DA: dopamine; MN: metanephrine; NMR: normetanephrine.

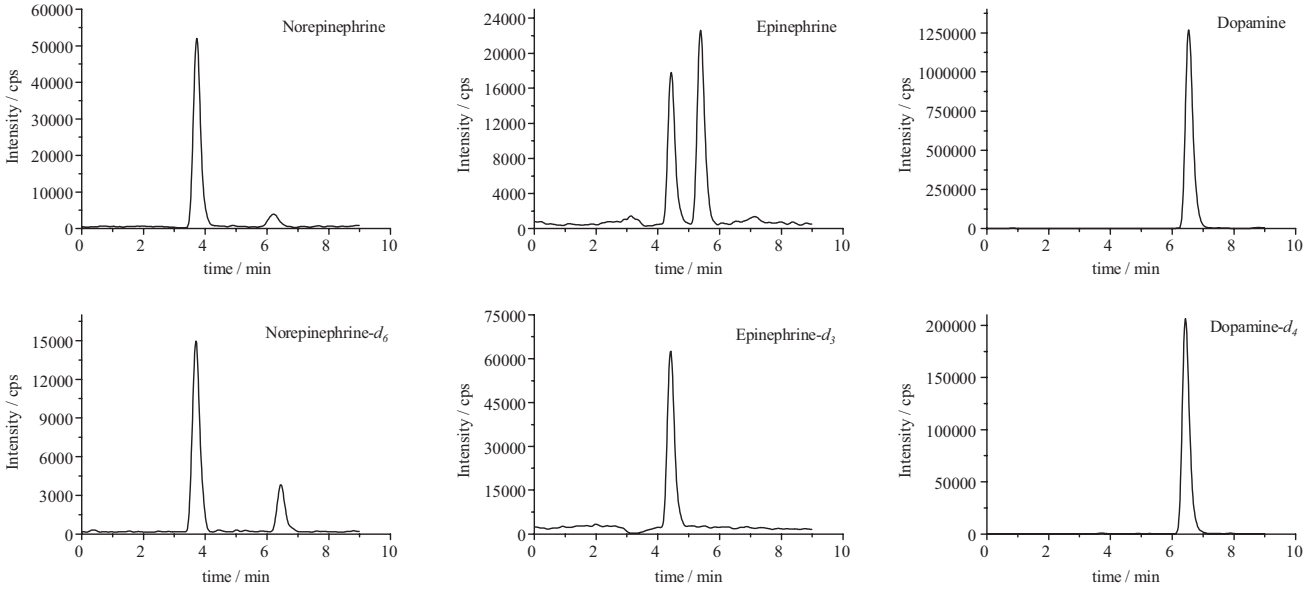

Figure 2. Example of the chromatogram of catecholamines and internal standards in sample human urine.

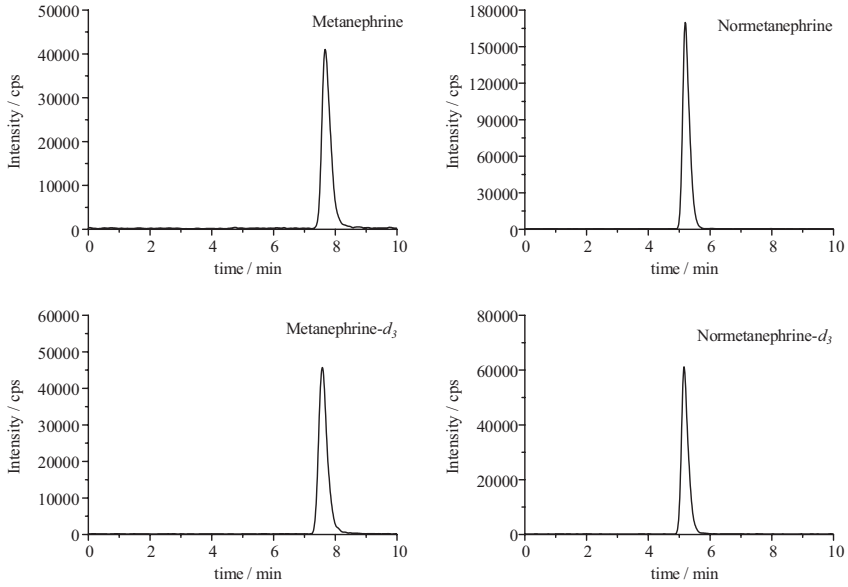

Figure 3. Example of the chromatogram of metanephrines and internal standards in sample human urine.

Data to access repeatability and intermediate precision was processed for three days with the use of three independently prepared urine pools. These results are shown in Table 3.

Experiments to assess extraction recovery were performed with a pool human urine containing catecholamines and metanephrines standards. The medium range of relative recovery for the epinephrine was 92.9-106.1% over an interval of 6.0-47.0 μg L^{-1}, for the norepinephrine, 94.6-107.7% over a range of 40.0-200.0 μg L^{-1}, for the dopamine, 98.0-108.8% over a range of 265.0-1065.0 μg L^{-1}, for the metanephrine, 94.2-105.1% over a range of 40.0-540.0 μg L^{-1} and for the normetanephrine, 97.0-106.0% over a range of 55.0-1050.0 μg L^{-1}.

Table 3. Repeatability and intermediate precision of the LC-MS/MS method for urinary catecholamines and metanephrines

	Intra-assay (n = 6)		Inter-assay (n = 18)	
	Mean / (μg L^{-1})	CV / %	Mean / (μg L^{-1})	CV / %
EPI	6.4	4.0	6.2	4.2
	16.5	2.2	16.7	2.5
	46.6	1.7	46.4	1.5
NE	26.0	3.7	25.7	3.2
	79.6	4.6	75.3	4.9
	193.3	2.3	199.0	5.9
DA	191.9	3.2	197.8	3.1
	425.2	3.4	412.0	3.3
	1021.2	1.8	1045.2	2.8
MN	70.1	3.4	74.6	6.7
	565.8	1.0	566.8	1.5
	1082.6	1.6	1063.5	2.4
NMN	142.6	4.3	155.6	6.9
	1009.5	1.2	1037.0	3.3
	1897.2	1.4	1924.5	2.6

CV: coefficient variation; EPI: epinephrine; NE: norepinephrine; DA: dopamine; MN: metanephrine; NMR: normetanephrine.

It was not found described in literature simultaneously determination of catecholamines and metanephrines in urine by LLE and LC-MS/MS. Analysis using LLE involving 2-aminoethyl-diphenylborinate (DPBA) with a complexating agent is described in Kushnir *et al.*,[13] Hollenback *et al.*,[18] and Forster *et al.*[7,19] However, these works use only catecholamines. Simultaneous determination of catecholamines and metanephrines by LLE was related by Chan *et al.*[20] Although the extraction described by Chan *et al.*[20] be as simple as in this study, the analytes are measured indirectly through the quantification of 9-fluorenylmethyloxycarbonyl (FMOC) derivatives by fluorescence detector, besides the chromatographic running time is very long, more than 40 min.

The use of 2-aminoethyl-diphenylborinate for extraction procedure has several advantages over extraction methods utilizing the alumina, cation-exchange or boronate sorbents that are commonly used for urinary catecholamines.[21] The minimum amount of organic solvent used in analysis chromatographic, the shorter extraction preparation and the chromatographic running time make a rapid and economic method for the determination of catecholamines and metanephrines in sample urine. Although an initial high investment is necessary for the acquisition of the LC-MS/MS system and the high value of maintenance of this equipment, it is easily offset by gains with sensibility, specifity and reproductibility.

Furthermore, LC-MS/MS sensibility and selectivity are superior that conventional HPLC for this bioanalytical analysis.

The method described in this work was established in clinical routine of a great laboratory of Latin America, where they already analyzed more than 18,000 samples. Figure 4 shows the proportion of exams analyzed *per* year.

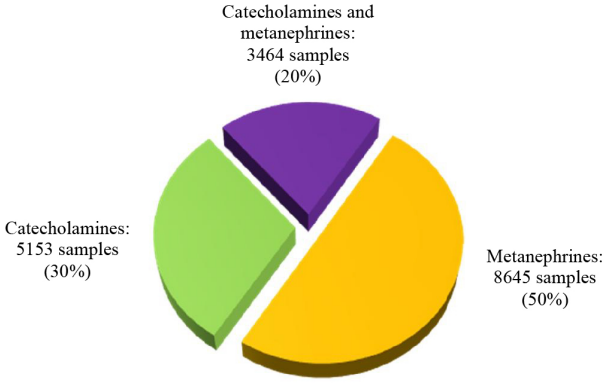

Figure 4. Proportion of exams of catecholamines and/or metanephrines analyzed along one year.

We have been participating of assays interlaboratory College of American Pathologists (CAP) for over a year. The results obtained are into the acceptability limit of the provider and they are according with the mean of other laboratories participants. The Pearson's correlation coefficients obtained were between 0.943 and 0.995 for each analyte and the mean of other laboratories indicating a good correlation of the results. For EPI, NE, DA and NMN there was not statistical difference by paired *t*-test calculated a 95% confidence interval and for MN calculated in a 99% confidence interval. Although there is not a specific group for LC-MS/MS in this program, studies have reported a good correlation between results of LC-MS/MS and HPLC-ECD method.[13,22] The results of last rounds are shown in Table 4.

Table 4. Results of assays interlaboratory (CAP)

	N-B 2013				N-A 2014			
	Sample	Result / (ng mL^{-1})	Mean / (ng mL^{-1})	Limits of acceptability / (ng mL^{-1})	Sample	Result / (ng mL^{-1})	Mean / (ng mL^{-1})	Limits of acceptability / (ng mL^{-1})
	N-07	88.7	74.9	32.6-117.2	N-01	12.4	13.9	10.7-17.1
EPI	N-08	89.2	75.3	29.0-121.6	N-02	81.6	79.5	40.0-119.1
	N-09	15.7	14.2	8.3-20.0	N-03	12.6	14.1	10.1-18.1
	N-07	194.0	193.4	128.6-258.1	N-01	41.9	50.1	41.2-59.1
NE	N-08	201.2	195.2	131.8-258.6	N-02	128.7	193.4	117.1-269.1
	N-09	48.0	50.0	37.7-62.3	N-03	46.1	50.3	35.2-65.4
	N-07	559.6	483.0	284.6-681.3	N-01	101.9	92.2	73.8-110.7
DA	N-08	559.5	486.1	309.4-662.7	N-02	399.2	489.5	355.0-624.0
	N-09	109.7	93.9	46.2-141.6	N-03	103.6	93.3	73.4-113.3
	N-07	603	544.3	310-778	N-01	130	101.8	56-148
MN	N-08	613	534.3	332-737	N-02	678	536.9	334-740
	N-09	112	106.9	63-151	N-03	130	102.5	60-145
	N-07	1333	1295.5	907-1684	N-01	355	329.4	211-448
NMN	N-08	1274	1313.4	939-1687	N-02	1072	1375.4	992-1759
	N-09	320	328.1	229-428	N-03	365	334.4	209-459

EPI: epinephrine; NE: norepinephrine; DA: dopamine; MN: metanephrine; NMR: normetanephrine.

Conclusions

The LC-MS/MS method was designed to quantify epinephrine, norepinephrine, dopamine, metanephrine and normetanephrine in human urine and has been developed successfully for the quantitative analysis in routine using LLE with 2-aminoethyl-diphenylborinate. It showed a good performance in validation parameters as precision with CV less than 4.6% for repeatability, 5.9% for intermediate precision and limits of detection and quantification comparable with other works in literature.[14,15,18] It also presented a satisfactory performance at interlaboratory program promoted by CAP. This method has been applied in a routine of about approximately 1500 samples *per* month. It is a practical, precise and robust procedure for laboratory clinical trial. This method is certainly promising in detection of biogenic amines and has been used routinely in Hermes Pardini Laboratory for over a year.

Supplementary Information

Supplementary information is available free of charge at http://jbcs.sbq.org.br as PDF file.

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