Metanephrines in urine by liquid chromatography tandem mass spectrometry

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Neuroendocrine tumors represent a clinically and etiologically diverse group of disorders. These tumors exhibit excessive catecholamines production. Free metanephrines in plasma are prioritized for differential diagnosis of pheochromocytoma due to their highest diagnostic sensitivity and specificity but in routine practice physicians often require parallel determination of urinary metabolites, metanephrine (MN) and normetanephrine (NMN). The aim of this work was development and implementation of method for determination of metanephrines in urine by the liquid chromatography tandem mass spectrometry (LC-MS/MS) suitable for routine practice in diagnostic laboratory which overcomes inconveniences related with classical liquid chromatography approaches, while the main aspects are: simple sample preparation, short runtimes and required analytical parameters.
Keywords: urinary metanephrines, pheochromocytoma, LC-MS/MS

Stanovení metanefrinů v moči metodou kapalinové chromatografie ve spojení s hmotnostní detekcí
Neuroendokrinní tumory představují heterogenní skupinu onemocnění s různou klinickou symptomatologií a etiopatogenezí. Tyto tumory vykazují nadměrnou produkci katecholaminů. Volné metanefriny v plazmě jsou preferovány pro diferenciální diagnostiku feochromocytomu vzhledem k jejich vysoké diagnostické senzitivitě a specificitě, nicméně v rutinní praxi lékaři často vyžadují paralelní stanovení metabolitů v moči, metanefrinu (MN) a normetanefrinu (NMN). Cílem této práce byl vývoj a implementace metody stanovení metanefrinů v moči pomocí kapalinové chromatografie ve spojení s hmotnostní spektrometrií, která je vhodná pro rutinní použití v diagnostické laboratoři. Prezentovaná metoda eliminuje nevýhody klasických chromatografických technik, přičemž zásadními aspekty jsou: jednoduchá příprava vzorku, krátká doba analýzy a požadované analytické parametry.
Klíčová slova: metanefriny v moči, feochromocytom, LC-MS/MS

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Introduction

Neuroendocrine tumors (NETs), such as pheochromocytoma or paraganglioma, which arise from chromaffin cells of the adrenal medulla or from sympathetic ganglia represent a diverse group of disorders with various clinical symptomatology and etiopathogenesis. Patients with NETs are mostly suffering from sustained or episodic hypertension, as a result of elevated catecholamine levels. Such hypertensive crises are life-threatening for the patient and may lead to stroke, heart attack or renal failure[5,8]. Early diagnosis based on biochemical proof of elevated levels of catecholamines and their metabolites is of significant importance. When diagnosis is precisely and early set there is a chance for successful treatment in 90% of cases[2]. And even though the first step in the diagnosis of NETs is based on measurement of metanephrines in plasma in routine practice physicians also often require parallel determination of urinary metabolites, metanephrine (MN) and normetanephrine (NMN)[1,3].

Conventional methods for measurement of urinary metanephrines such as HPLC (liquid chromatography) with UV, fluorescent or electrochemical (ECD) detection or GC (gas chromatography) or immunoassays have a lot of restrictions and inconveniences, e. g. multi-step sample preparation and long runtime for HPLC, time-consuming step of derivatization for GC methods and cross-reactivity, nonspecific binding or interferences for immunoassays[1,4]. Presented LC-MS/MS method overcomes above mentioned inconveniences.

Materials and methods

Sampling

Prior to 24h urine collection, special dietary precautions had to be taken. Diet rich in tyrosine had to be discarded and, after consultation with the physician, some drugs had to be discontinued[6,7]. Urine was collected into the special 2,5 l polypropylene container containing 15 ml of hydrochloric acid to preserve samples for the measurements as described in pre-analytical manual[7]. The content of the container was mixed, using indicator strip pH value was checked and adjusted if needed to value pH = 1-2 and two acidified aliquots of urine were stored at -20 °C for measurement. Prior to analysis, the urine samples were tempered to the room temperature and centrifuged for 5 min at 4000 g to remove the sediment.

Materials

Lyophylised control materials ClinChek®Urine Controls for biogenic Amines (RECIPE Chemicals, Germany); Catecholamine Mix 2 solution, Metanephrine-d3 solution, Normetanephrine-d3 solution (Sigma-Aldrich, USA); Ph. Eur. grade hydrochloric acid 37% (Vitrum VWR, Czech Republic), solvents – LC-MS grade acetonitrile, methanol and water (Vitrum VWR, Czech Republic); Indicator strips (Lach-ner, s.r.o, Czech Republic).

Instruments

An Agilent 6490 Triple Quadrupole LC/MS system with 1290 Infinity LC system was used for LC-MS/MS measurements of metanephrines in urine. Chromatographic data were processed with MassHunter Workstationsoftware version B 06. 00. Separation was performed using SeQuant®ZIC®HILIC HPLC 3,5 mm, 100Å, PEEK 100 x 2,1 mm metal-free HPLC Column (Merck). A Coulochem III HPLC System, ESA Biosciences Inc. was used for HPLC measurements of metanephrines in urine. Chromatographic data were processed with Clarity™ Chromatography station version 4. 0. 2.784.

Sample preparation for LC-MS/MS method

Urine samples, calibrator and control samples for measurement were processed as follows: 15 µl of internal standard (containing 15 ng MN-d3 and 15 ng NMN-d3) was added into 150 µl of sample (calibrator, control, urine) and mixed shortly. pH value was checked whether it is in the range pH ≤ 1. If necessary, the pH value was adjusted using conc. HCl. For acidic hydrolysis samples were incubated for 30 minutes at 95 °C. To 40 µl of incubated material tempered to the room temperature 360 µl of 80% ACN was added. Samples were mixed for 30 seconds on a vortex and centrifuged for 5 minutes at 10000 g.

LC-MS/MS analysis

After centrifugation, 0,5 µl of the solution was injected to chromatographic system. Chromatographic separation was performed on SeQuant®ZIC®HILIC HPLC 3,5 mm, 100Å, PEEK 100 x 2,1 mm metal-free HPLC Column with gradient elution of mobile phases A (50mM NH_4COOH in water) and B (acetonitrile) with the flowrate 0,3 ml/min and temperature 45 °C with MS detection (ion source parameters and MRM transitions – ***Table 1***). The runtime was 6 min with retention times 2,36min for MN and 2,57min for NMN ***(Figure 1)***.

Commercially available IVD diagnostic Kit for HPLC with ECD detection

For commercial method sample preparation and chromatographic evaluation were processed as described in Clin-REP®HPLC Complete Kit Metanephrines in Urine Instruction Manual[8].

Results

To define analytical characteristics of LC-MS/MS method, both lyophilized commercially available materials and patient samples were used. The analytical performance of the new developed LC-MS/MS method was satisfactory. Calibration range for MN was 0,020 – 10,140 µmol/l with limit of quantification 0,020 µmol/l (CV = 2,6%). Calibration range for NMN was 0,021 – 10,890 µmol/l with limit of quantification 0,021 µmol/l (CV = 1,5%). The intra-assay and inter-assay coefficients of variation (CV) were below 10%. Recoveries determined by standard addition ranged from 85 to 101%. This method fulfilled requirements for validation parameters for both analytes ***(Table 2)***.

To assess the suitability of the developed method for routine use in clinical diagnostic laboratory the comparison of developed LC-MS/MS method with reference HPLC method was performed. For this purpose 10 patient samples, 2 control samples and 2 EQAS samples were prepared

Table 2. *Analytical parameters – interassay and intraassay precision*

Precision	Unit	Intraassay (n = 10)		Interassay (n = 10)			
		MN	NMN	MN		NMN	
Cteor	µmol/l	–	–	0,842	5,160	1,780	8,560
MEAN	µmol/l	0,905	1,657	0,887	5,322	1,734	8,227
SD	µmol/l	0,034	0,075	0,036	0,306	0,102	0,729
CV	%	3,8	4,5	4,1	5,8	5,9	8,9

Figure 1. *Chromatogram of MN and NMN*

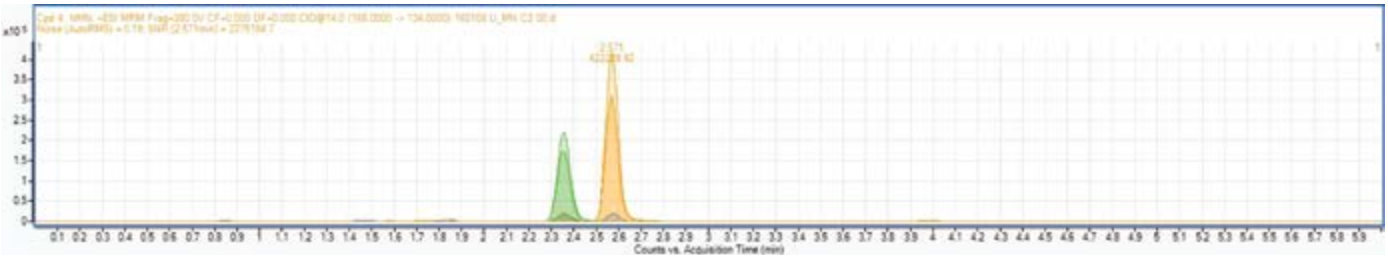


Table 1. *Mass spectrometry and mass transition conditions*

Ion source parameters				Precursor (m/z)	Product (m/z)	Frag	CE
AJS ESI	positive		MN*	180	165	380	11
Gas temp.	135	°C	MN	180	148	380	11
Gas flow	12	l/min	MN-d3*	183	151	380	11
Nebulizer	45	Psi	MN-d3	183	168	380	11
Sheath gas temp.	400	°C	NMN*	166	134	380	14
Sheath gas flow	10	l/min	NMN	166	106	380	18
Capillary	4000	V	NMN-d3*	169	137	380	14
Nozzle voltage	0	V	NMN-d3	169	109	380	18

**quantifier*

and parallelly measured using those two methods. Statistical analysis was performed using the MedCalc 16.1 (Medcalc Software bvba, Belgium) and results are shown in ***Table 3***. Normal distribution of dataset was tested using D'Agostino-Pearson test with the result „reject Normality" so nonparametric Wilcoxon test was used for further comparison. For methods comparison Passing-Bablok nonparametric regression ***(Figure 2)*** and Bland-Altman plot were applied ***(Figure 3)***. For graphic presentation of dataset box-and-whisker graphs were used ***(Figure 4)***.

Table 3. *The parameters of statistical analysis for HPLC and LC-MS/MS methods*

	MN (n = 14)		NMN (n = 14)	
	HPLC	LC-MS/MS	HPLC	LC-MS/MS
Mean µmol/l	0,720	0,737	1,630	1,555
Median µmol/l	0,185	0,197	0,684	0,862
Lowest value µmol/l	0,044	0,075	0,144	0,145
Highest value µmol/l	5,230	5,185	8,797	8,095
p (Wilcoxon test)	0,41		0,50	
Passing- Bablok regression				
Intercept A	-0,012		-0,016	
95% CI	(-0,035 – 0,011)		(-0,133 – 0,066)	
Slope B	1,010		1,091	
95% CI	(0,949 – 1,061)		(0,831 – 1,153)	

Comparison of two methods – reference HPLC method and developed LC-MS/MS method

As the reference method the commercially available HPLC Kit dedicated for HPLC with ECD detection was used. Results of Passing-Bablok regression are shown in ***Table 3***. For the intercept A, for MN and NMN, the 95% confidence interval contains the value 0, this fact excludes the presence of a systematic error of the measurement system throughout the measurement range. For the slope B, for MN and NMN, the 95% confidence interval contains the value 1, this fact excludes the presence of a proportional error of measurement. The Bland-Altman difference plot was used to assess differences between obtained results. The plot has shown equal distribution of the values, there is no trend that would indicate the presence of a systematic error (one outlying point for MN, one for NMN). 95% confidence interval of the mean difference contains value 0, pointing that there is no statistical difference between methods. Using Wilcoxon paired test we reached the same conclusion (p = 0,41 for MN, p = 0,50 for NMN). Graphic presentation of dataset as box-and-whisker graphs supports the results of above described statistical analysis ***(Figure 4)***.

Figure 2. *Passing-Bablok regression – comparison of HPLC and LC-MS/MS methods*

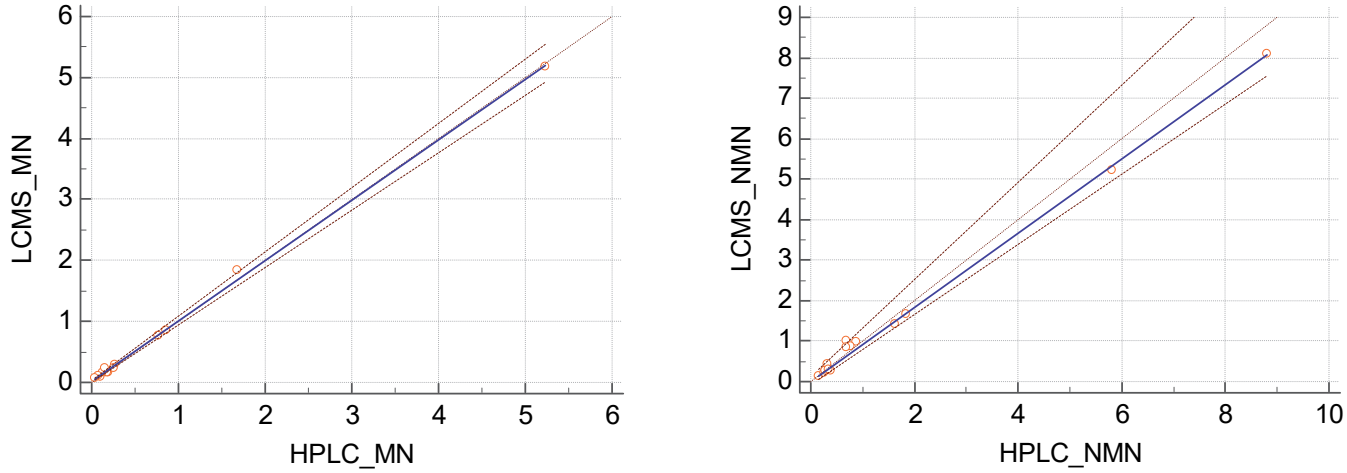


Figure 3. *Blandt-Altman difference graph for HPLC and LC-MS/MS methods*

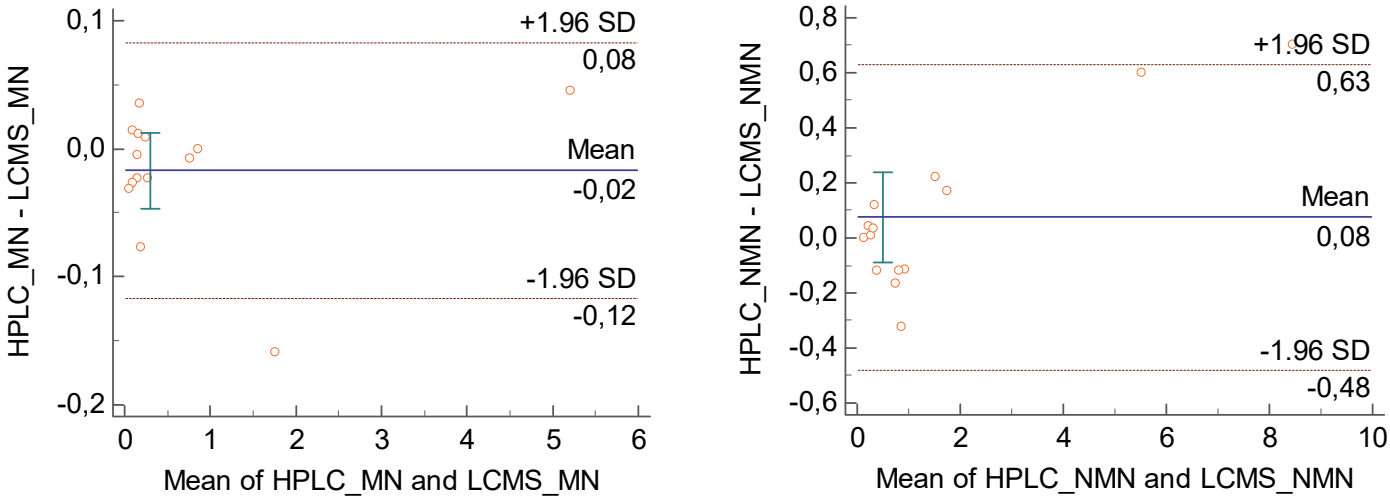
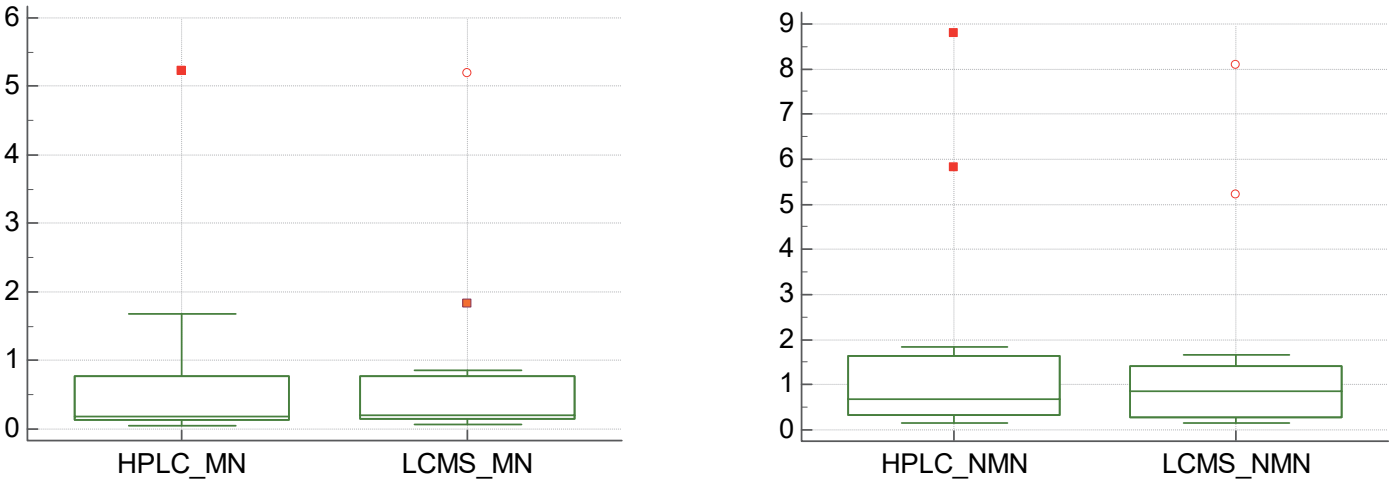


Figure 4. Box-and-whisker graphs for HPLC and LC-MS/MS methods



Discussion

The early settled diagnosis of pheochromocytoma is crucial for treatment and for chirurgical intervention. Diagnostic algorithm for NETs is based on several aspects: clinical symptomatology, laboratory diagnostics and as the last step the exact localisation of the tumor using imaging techniques.

Excessive production of catecholamines and their metabolites is considered a hallmark in the biochemical diagnosis of catecholamine-producing tumors. The measurement of catecholamine levels has a lot of limitations: elevated catecholamine levels may not be specific for pheochromocytoma (sympathetic nervous system activity), the presence of „silent pheochromocytoma" or episodic secretion of catecholamines. Regarding those limitations plasma concentrations of free metanephrines are better indices than other manifestations of catecholamine excess but measurements of metanephrines in urine are still commonly used for the diagnosis of pheochromocytoma[2,4,5].

The aim of the presented work was to develop an analytical method and to assess the suitability of the developed method for use in clinical diagnostic laboratory. As advantages of presented method we can highlight undemanding sample preparation, short runtimes and required analytical parameters. Statistical analysis performed by using the Passing-Bablok regression and Bland-Altman diagram confirmed the suitability of this approach for the measurement of metanephrines in urine in routine diagnostic laboratory. This approach eliminates inconveniences related with previously used classical HPLC method with ECD detection, such as extensive sample preparation and long runtimes (20 min).

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8. Instruction Manual ClinREP®HPLC Complete Kit Metanephrines in Urine

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