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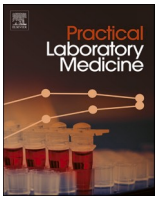

Development and validation of a simple, fast and sensitive liquid chromatography-tandem mass spectrometry method to establish reference intervals for 24-h urinary free normetanephrine, metanephrine and methoxytyramine

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ABSTRACT

Objective: To develop and validate a rapid liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to detect urinary free metanephrines and methoxytyramine, establishing reference intervals.
Methods: Urine samples were diluted with isotope internal standard solution, then analyzed directly using tandem mass spectrometry with multiple reaction monitoring measurement and electrospray ionization source in positive ion mode. Analytical parameters including linearity, lower limit of quantitation, imprecision and accuracy of the method were evaluated. The reference intervals for urinary catecholamine metabolites were established by analyzing 24-h urine samples collected from 81 apparently healthy volunteers.
Results: The analytical times for MN, NMN, and 3-MT were at 2.79, 2.80, and 2.74 min, respectively. The method displayed excellent linearity ($r > 0.99$) in the range of 1-1000 ng/mL, with lower limits of quantification (LLOQ) at 0.50 ng/mL for MN and NMN, and 0.25 ng/mL for 3-MT. The method's intra-day and inter-day imprecisions were less than 8 %. The method recovery ranged from 96.8% to 105.8 % for MN, 89.7%–106.4 % for NMN, and 93.5%–106.2 % for 3-MT. No carry-over was observed during the analysis of all analytes. The LC-MS/MS method was used to establish reference intervals in 24-h urine samples from 81 apparently healthy volunteers. There was no association of sex with urinary free metabolites.
Conclusion: This study established a novel, fast and sensitive LC-MS/MS method for determining urinary free catecholamine metabolites, which could facilitate screening and diagnosis for catecholamine-related tumors more conveniently and quickly.

1. Introduction

Pheochromocytomas and paragangliomas (PPGLs) are rare neuroendocrine tumors arising from chromaffin cells residing in the adrenal medulla and sympathetic or parasympathetic extra-adrenal paraganglia, respectively [1,2]. These tumors are predominantly pheochromocytomas, accounting for 80–85 %, compared to paragangliomas with an incidence of 15–20 % [3]. The prevalence of

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PPGLs in adults with hypertension in general outpatient clinics ranges from 0.2 to 0.6 %, with a lower prevalence in the general population, whereas the prevalence is estimated at 1.7 % in children with hypertension [4,5]. Although most PPGLs are non-malignant and can be cured by early diagnosis and operative treatment [5,6]; approximately 10 % of pheochromocytomas and up to 15–35 % of abdominal paragangliomas are invasive or metastatic [7]. As such, a swift and accurate diagnosis of PPGLs is crucial for disease control.

PPGLs primarily synthesize, store, and secrete excess catecholamines, including epinephrine (E), norepinephrine (NE), and dopamine (DA), which can result in a variety of clinical symptoms including hypertension, headache, sweating, and life-threatening cardiovascular events [8]. Biochemical testing for PPGLs is appropriate for symptomatic patients, those with adrenal incidentalomas, or those with a confirmed genetic predisposition [9]. Given the sporadic secretion and short half-lives of the catecholamines, they may be normal between episodes and their plasma levels are unstable, requiring strict collection and storage procedures [10]. The three catecholamines (E, NE and DA) are metabolized to methoxyepinephrine (metanephrine, MN), methoxynorepinephrine (normetanephrine, NMN), and 3-methoxytyramine (3-MT), respectively, by the catechol-*O*- methyltransferase (COMT) [11]. These metabolites with longer half-lives are much more stable and their plasm levels and excretion in urine are much more constant compared with the native catecholamines [12], which making them better biomarkers for diagnosing PPGLs. MN and NMN are collectively referred to as metanephrines (MNs).

The 'Expert Consensus on the Diagnosis and Treatment of Pheochromocytoma and Paraganglioma (2020 Edition)' recommends liquid chromatography-tandem mass spectrometry for the detection of free plasma or urinary MNs as the primary diagnosis of PPGLs [13]. Whilist, some paragangliomas only produce DA. 3-methoxytyramine, the O-methylated metabolite of dopamine, is a marker for these tumors. Thus, the combination of 3-MT detection can enhance the sensitivity of PPGL diagnosis and provide useful information to estimate the risk of metastasis [14,15]. In addition, levels of catecholamine metabolites in the blood may be affected by patient emotions and posture; collecting blood in a seated rather than supine position could cause false positives [4]. Liquid chromatography with electrochemical detection (LC-ECD) is used for analysis of catecholamine metabolites, but it requires time-consuming sample preparation and is interfered by many substances. Compared with UV, fluorescence and electrochemical detectors, mass spectrometry has higher sensitivity. Gas chromatography mass spectrometry (GC-MS) is increasingly used for the detection of catecholamines and their metabolites, but derivation during detection greatly limits the application of this method. Several studies have utilized the gold standard method of liquid chromatography-tandem mass spectrometry for detecting catecholamine metabolites in urine, employing various sample purification assays. However, the application of LC-MS/MS in clinical analysis is hindered by the cumbersome and time-consuming nature of sample cleanup and preconcentration steps, such as solid-phase extraction (SPE) [16,17] or derivatization [18]. Moreover, the quantification of urine MNs through SPE followed by LC-MS/MS analysis yielded unsatisfactory results in terms of sensitivity (3.5 ng/mL for MN and 3.7 ng/mL for NMN) and recovery (82–107 % for MN and 75–79 % for NMN) [17].

Consequently, we established a simple, time- and cost-effective method of direct dilution combined with liquid chromatography-tandem mass spectrometry that has high sensitivity and specificity for rapidly detecting urinary catecholamine metabolites. We have also evaluated its analytical parameters, establishing the reference intervals for urinary free normetanephrine, metanephrine and methoxytyramine.

2. Materials and methods

2.1. Materials and reagents

The LC–MS/MS analysis utilized a Jasper LC system (Paolo Alto, USA) and an AB Sciex Triple Quad 4500 MD with electrospray ionization. The column used was the Venusil CAs column, 100 mm × 2.1 mm, 5 μm, from Bonna-Agela Technologies, Tianjin, China. Eppendorf Centrifuge 5430R with low-temperature and high-speed was used. MNs (1 mg/mL in methanol, Lot: C-110), 3-MT (1 mg/mL in methanol, Lot: M − 171), (±)-Metanephrine-d3 hydrochloride (100 μg/mL in methanol, Lot: M − 148), (±)-Normetanephrine-d3 hydrochloride (100 μg/mL in methanol, Lot: N-068), and 3-Methoxytyramine-d4 (100 μg/mL in methanol, Lot: M − 172) were purchased from Cerilliant (Texas, USA). HPLC grade methanol and acetonitrile were from Merk, USA. Formic acid was from Thermo Fisher Scientific (Waltham, MA, USA), and ammonium formate was from Sigma (St. Louis, MO, USA). Deionized water was purified using a Millipore Synergy UV water purification system (Billerica, MA, USA). 24-h Urine samples from apparently healthy volunteers were collected in clean containers containing 5 g of boric acid, and 1 mL samples were stored at −80 °C until analysis.

2.2. Calibrator and quality control solutions

10 μL of standard stock solutions of 3-MT and MNs were added to 980 μL of 50 % methanol (containing 0.1 % formic acid), yielding a mixed standard intermediate solution with a concentration of 10 μg/mL. This mixed standard intermediate solution was then diluted with 50 % methanol (containing 0.1 % formic acid) to produce working standard solutions with concentrations of 1,000, 500, 100, 50, 10, 5, and 1 ng/mL for the standard curve. Additionally, solutions with concentrations of 800, 40, and 3 ng/mL were prepared as quality controls.

2.3. Isotope internal standard working solution

The isotope internal standard working solution was prepared by diluting the internal standard stock solution in 50 % methanol (containing 0.1 % formic acid) with a concentration of 25 ng/mL. This solution was stored in a refrigerator at −20 °C for preservation.

2.4. Sample preparation

10 μL of the working standard solution/quality control/sample was mixed with 20 μL of the isotopic internal standard working solution and 220 μL of acetonitrile (containing 2 % formic acid). The mixture was then centrifuged, and 100 μL of the supernatant was taken for analysis.

2.5. Chromatographic conditions

The chromatographic conditions involved a mobile phase consisting of 0.1 % formic acid aqueous solution with 10 mM ammonium formate and 0.1 % formic acid solution in acetonitrile-water (9:1) with 10 mM ammonium formate. The run time for each test was set at 5.5 min, with gradient elution conditions set as follows: 0–0.5 min, 0 % A; 0.5–1.8 min, 0–20 % A; 1.8–3.0 min, 20–25 % A; 3.0–3.1 min, 25–50 % A; 3.1–3.8 min, 50 % A; 3.8–4.0 min, 50–0% A; and 4.0–5.5 min, 0 % A. The flow rate was set at 0.4 mL/min, and the injection volume was 5 μL, with a column temperature of 40 °C.

2.6. Mass spectrometer conditions

Analysis was performed using electrospray ionization (ESI) in positive ion mode and multiple reaction monitoring (MRM). The ion source temperature was set at 550 °C. The settings for nebulizer gas (Gas 1), auxiliary heating gas (Gas 2), curtain gas (Curtain Gas), and collision gas (CAD) were 60, 60, 30, and 9 psi, respectively. The quantitative and qualitative ion pairs, *m/z* ratios, declustering potential (DP), and entrance potential (EP) for MN, NMN, 3-MT, MN-d3, NMN-d3, and 3-MT-d4 are demonstrated (See Supplemental Data Table S1).

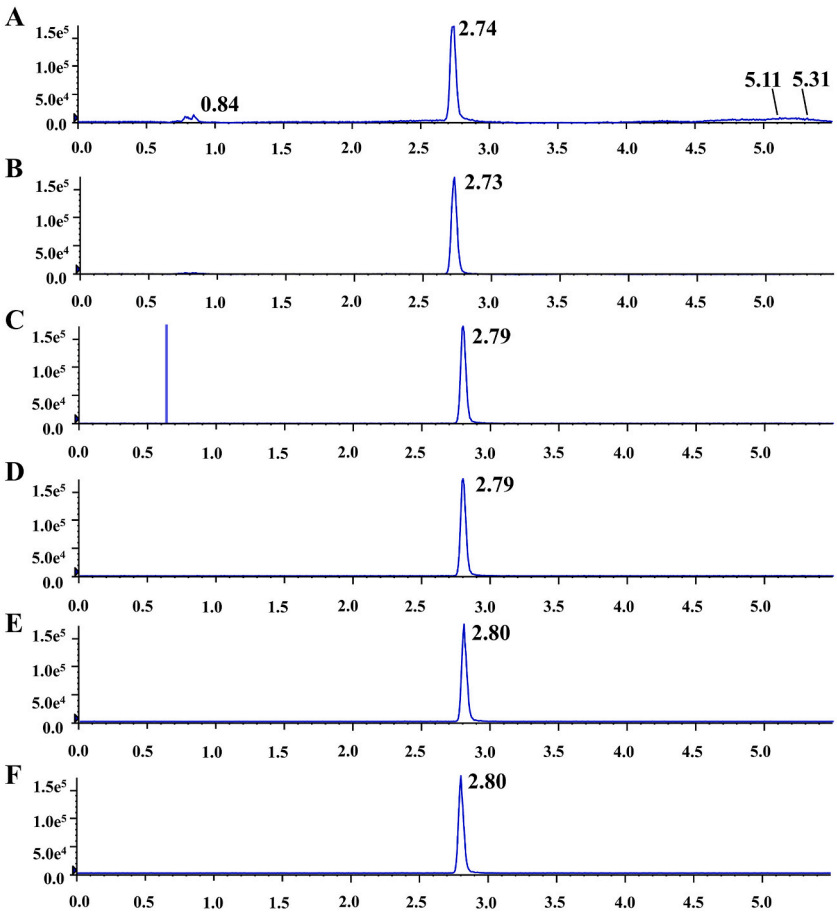

Fig. 1. MRM chromatograms of 3-MT, MN, NMN with their respective internal standards
The x-axis represents time (min) and the y-axis indicates signal intensity; MRM chromatograms of 3-MT, MN and NMN and their respective internal standards. (A) 3-MT elution at 2.74 min for 50 ng/mL of 3-MT standard; (B) 3-MT-d4 elution at 2.73 min; (C) MN elution at 2.79 min 50 ng/mL of MN standard; (D) MN-d3 elution at 2.79 min; (E) NMN elution at 2.80 min 50 ng/mL of NMN standard; (F) NMN-d3 elution at 2.80 min.

Table 1
Inter-day (RSD%) and intra-day (RSD%) imprecision in quality control samples.

Compound	Samples	Mean (ng/ml)	Inter-day imprecision (R.S.D.[d]%)	Intra-day imprecision (R.S.D.[d]%)
3-MT	LQC[a]	3.11	4.55	7.61
	MQC[b]	39.52	3.53	3.3
	HQC[c]	801.4	1.33	4.23
MN	LQC	2.98	4.30	4.70
	MQC	39.55	3.05	4.20
	HQC	785.80	1.63	3.91
NMN	LQC	3.11	3.73	4.20
	MQC	40.15	1.69	3.06
	HQC	790.59	3.24	4.99

Abbreviations: LQC, Low quality control; MQC, Medium quality control; HQC, High quality control; and RSD, relative standard deviation.

2.7. Method validation

We developed and validated a novel LC-MS/MS assay, following the C62-A document, for measuring catecholamine metabolites [19].

2.7.1. Linearity and lower limit of quantitation

To determine linearity and lower limit of quantitation, we conducted a linear regression analysis. The regression equation was derived using a "1/X^2″ weighting, with a correlation coefficient of $r > 0.99$. For the lowest point of the diluted standard, each concentration was tested 20 times with a $\leq$20 % coefficient of variation and a signal-to-noise ratio (S/N) $\geq$ 20. The standard concentration meeting the criteria was established as the lower limit of quantification (LLOQ).

2.7.2. Imprecision and trueness

We tested three levels of quality control solutions repeatedly over five days with three replicates per concentration processed daily to assess both inter-day and intra-day imprecision. The imprecision had to meet a criterion of CV $\leq$ 15 %, and the deviation of each measurement from the theoretical value must be within $\pm$15 %. The trueness of the method was assessed by measuring the addition of pure quality control solutions to different urine specimens. Six urine samples from different patients were collected. Low, medium, and high concentrations of the control solution (3 ng/mL, 40 ng/mL and 800 ng/mL) were added to each urine specimen to create spiked samples, and the spiking recovery was calculated. Each concentration's spiking recovery must fall within the range of 85–115 %.

2.7.3. Matrix effect and carry over

The matrix effect was evaluated using six urine specimens from distinct patients (A). These samples were mixed with high and low quality control solutions (B) in specific proportions: A (100 %), B (100 %), C (50 %:50 %), D (80 %:20 %), E (20 %:80 %). The measured concentration values were compared to the theoretical concentration values to determine acceptability, with an acceptable bias less than 20 %. The standard curve's lowest point was initially sampled repeatedly, followed by alternating sampling between its highest and lowest points. Results of the first injection's low-concentration sample were compared with those of subsequent injections. A difference of less than 20 % between results constitutes lack of significant.

2.8. Reference ranges

81 apparently healthy volunteers were enrolled from Renji Hospital affiliated to School of medicine Shanghai Jiaotong University in our study. The group included 49 females and 32 males, aged 18–76 years (median 45.67). All participants were free of cardiac, hepatic, and renal dysfunction and had no endocrine disorders, diabetes, hypertension, infectious diseases, or recent medication use. We validated reference ranges for 24-h catecholamine metabolites by analyzing the 2.5–97.5 percentile distribution. This study was registered with the Ethics Committee of Renji Hospital affiliated with Shanghai Jiao Tong University School of Medicine (Registration Number: RA-2022-314).

3. Statistical analysis

Statistics were analyzed using SPSS software (version 26). Differences between men and women were evaluated with two-tailed Student's t-tests, using GraphPad Prism software (version 7, GraphPad Prism Software, Inc., San Diego, CA). Effects of sex, body surface area, and age with the concentrations of urinary free catecholamine metabolites assessed by pearson correlation and multiple linear regression analysis. Significant difference was determined as P value < 0.05.

Table 2
Trueness calculated by six human urine samples and expressed as percent recovery.

3-MT					MN				NMN			
	Endogenous (ng/mL)	Spiking (ng/mL)	Found (ng/mL)	Recovery (%)	Endogenous (ng/mL)	Spiking (ng/mL)	Found (ng/mL)	Recovery (%)	Endogenous (ng/mL)	Spiking (ng/mL)	Found (ng/mL)	Recovery (%)
Urine A	36.21	3	40.90	104.52	29.28	3	35.31	109.38	28.67	3	33.23	104.94
Urine B	30.46		31.35	106.20	7.58		10.30	97.32	18.39		21.57	100.83
Urine C	33.27		38.93	111.00	24.25		28.40	104.22	19.81		22.85	100.16
Urine D	35.73		39.37	101.64	12.56		16.88	108.51	8.88		12.17	102.41
Urine E	23.31		27.48	104.43	12.58		14.78	94.84	18.78		21.78	100.00
Urine F	14.06		16.99	99.57	4.66		7.85	102.53	9.39		11.58	93.49
Urine A	36.21	40	71.29	93.54	29.28	40	75.20	99.86	28.67	40	61.63	89.75
Urine B	30.46		63.38	95.27	7.58		47.25	99.29	18.39		57.31	98.15
Urine C	33.27		66.08	91.69	24.25		64.76	100.79	19.81		54.83	91.67
Urine D	35.73		73.51	97.07	12.56		53.32	101.45	8.88		46.31	94.74
Urine E	23.31		56.66	89.50	12.58		50.05	95.18	18.78		54.96	93.50
Urine F	14.06		52.00	96.20	4.66		44.36	99.34	9.39		46.24	93.63
Urine A	36.21	800	838.19	100.24	29.28	800	854.41	103.03	28.67	800	801.37	96.71
Urine B	30.46		773.61	93.60	7.58		845.32	104.67	18.39		870.78	106.40
Urine C	33.27		783.83	94.20	24.25		849.45	103.06	19.81		826.79	100.85
Urine D	35.73		794.94	95.12	12.56		769.19	94.66	8.88		732.55	90.56
Urine E	23.31		760.70	92.40	12.58		773.47	95.19	18.78		742.51	90.68
Urine F	14.06		822.75	101.07	4.66		825.64	102.61	9.39		810.06	100.08

Table 3
Matrix effects of the method.

	3-MT			MN			NMN		
	Theoretical concentration (ng/mL)	Measured concentration (ng/mL)	Bias (%)	Theoretical concentration (ng/mL)	Measured concentration (ng/mL)	Bias (%)	Theoretical concentration (ng/mL)	Measured concentration (ng/mL)	Bias (%)
L 1:1	15.92 ± 4.32	15.70 ± 4.63	−1.42	9.08 ± 4.81	9.05 ± 5.29	−0.21	10.16 ± 3.69	10.46 ± 4.05	2.91
L 4:1	23.67 ± 6.91	24.19 ± 7.81	2.18	12.72 ± 7.70	13.71 ± 9.30	7.76	14.46 ± 5.90	14.42 ± 6.30	−0.26
L 1:4	8.17 ± 1.73	7.98 ± 2.05	−2.28	5.43 ± 1.92	5.22 ± 2.21	−3.78	5.86 ± 1.48	5.82 ± 1.83	−0.69
H 1:1	414.42 ± 4.32	386.48 ± 20.85	−6.74	407.58 ± 4.81	399.10 ± 32.27	−2.08	408.66 ± 3.69	391.32 ± 26.51	−4.24
H 4; 1	183.07 ± 6.91	167.34 ± 22.10	−8.59	172.12 ± 7.70	158.06 ± 16.05	−8.17	173.86 ± 5.90	160.99 ± 12.45	−7.40
H 1:4	645.77 ± 1.73	619.20 ± 26.18	−4.11	643.03 ± 1.92	629.97 ± 26.29	−2.03	643.46 ± 1.48	638.69 ± 15.80	−0.74

Data are expressed as Mean ± SEM.

4. Results

4.1. Typical chromatograms of urine samples

The retention times for 3-MT, MN, and NMN were 2.74, 2.79, and 2.80 min, respectively, while the internal standards had retention times of 2.73, 2.79, and 2.80 min, respectively. The components were well-separated without any interference. Representative chromatograms were displayed (Fig. 1).

4.2. Method validation

MN, NMN, and 3-MT exhibited excellent linearity in the range of 1-1000 ng/mL. Correlation coefficients for each test exceeded 0.99. LLOQs for MN, NMN, and 3-MT were set at 0.50, 0.50, and 0.25 ng/mL, respectively, with bias below 20 % and imprecision less than 15 %.

Intra-day and inter-day imprecision fulfilled acceptance criteria of less than 10 % (Table 1). Trueness was performed using a spiking and recovery experiment with six human urine samples and three spiking QC solutions. Trueness for all compounds met the acceptable limit of 15 %, as shown in Table 2. Matrix effect was not significant, ranging from −8.59 % to 7.76 % (Table 3). The carryover for MN, NMN, and 3-MT were all less than 10 % (Table 4), suggesting the method has no significant carryover.

4.3. Reference intervals of urinary free MN, NMN and 3-MT

This study collected 24-h urine samples from 81 apparently healthy individuals, 32 males and 49 females, aged 45.86 ± 12.80 (See Supplemental Data Table S2). Males had a significantly larger body surface area (BSA) than females. Urinary free MN concentrations were higher in males, while urinary free NMN and 3-MT were comparable in both genders (See Supplemental Data Fig. S1 A-C). After multivariate correction for age and BSA, the effect of sex on urinary free MN disappeared (Table 5). BSA was positively associated with urinary free NMN ($P = 0.028$) and 3-MT ($P = 0.038$). Similar results were observed after multivariate analysis. Age had a significant negative association with urinary free MN ($P = 0.010$) and 3-MT ($P = 0.007$) but not with urinary free NMN (Table 5). After multivariate analysis, age remained significantly associated with urinary free 3-MT ($P = 0.018$) and MN ($P = 0.003$). The 2.5–97.5 % percentiles for urine concentrations of free MN, NMN, and 3-MT were established (Table 5).

5. Discussion

In this study, we developed and validated a novel LC-MS/MS method to concurrently detect free MN, NMN, and 3-MT in 24-h urine specimens. The approach involves direct dilution for sample analysis, and is simplified, fast, economical, and requires minimal operator proficiency. We also established reference intervals for urinary free MN, NMN, and 3-MT in healthy Chinese population.

PPGLs patients' classic triad of headache, sweating and palpitations can aid diagnosis but is present in only 24 % of cases [20,21]. PPGLs can cause hypertension, nausea, myocardial infarction, bradyarrhythmias, tachyarrhythmias and acute heart failure, although at a lower frequency than the triad [20]. All these clinical signs and symptoms are not specific to PPGLs, making accurate diagnosis challenging [22]. Surgical resection is curative for most benign PPGLs [23], emphasizing the need for precision in diagnosis.

Plasma or urinary metanephrines are recommended for screening of PPGLs diagnosis[4]. However, various studies indicate that measuring free metanephrines in plasma may produce false-positive results [24,25]. While, the urinary free MNs rarely increase in non-PPGLs, causing fewer false-positive results [24]. Moreover, blood sample collection for PPGL diagnosis requires patients to maintain a supine position under stress-free conditions, which can be challenging in outpatient settings. Some PPGLs only produce dopamine, and its O-methylated derivative, 3-MT, aids in diagnosing such tumors, especially in malignant PPGLs [26,27]. Some PPGLs only produce dopamine, and 3-MT analysis can assist in diagnosing such tumors, especially malignant ones. To increase PPGLs diagnosis sensitivity and provide meaningful information about tumor malignancy and metastasis, we developed a simple LC-MS/MS method for simultaneous analysis of urinary free MN, NMN, and 3-MT.

Our analysis of linearity, precision, trueness, matrix effect, and carryover yielded excellent results. The method involves a small

Table 4
Carryover of the method for urinary free catecholamine metabolites.

	3-MT			MN			NMN		
Samples		L-L	H-L		L-L	H-L		L-L	H-L
L1	0.88			1.05			1.07		
L2	0.90	0.90		0.91	0.91		1.20	1.20	
L3	0.86	0.86		0.96	0.96		0.98	0.98	
H1	1035.1			956.65			962.63		
H2	1032.48			968.08			992.82		
L4	0.92		0.92	0.98		0.98	1.10		1.10
H3	1062.26			953.57			1002.47		
H4	1021.21			939.81			993.10		
L5	0.88		0.88	0.95		0.95	1.18		1.18
L6	0.91	0.91		1.09	1.09		1.13	1.13	
L7	0.97	0.97		1.07	1.07		1.15	1.15	
L8	0.87	0.87		1.16	1.16		1.05	1.05	
H5	981.93			994.22			1004.99		
H6	1011.337			952.61			952.19		
L9	1.12		1.12	1.03		1.03	1.13		1.13
H7	1031.63			954.55			1039.52		
H8	985.00			979.77			993.19		
L10	0.95		0.95	1.02		1.02	1.19		1.19
H9	1037.60			952.44			979.25		
H10	1008.57			959.51			992.31		
L11	1.01		1.01	1.04		1.04	1.18		1.18
Mean		0.90	0.98		1.04	1.00		1.10	1.16
Carryover (%)	−8.89			3.85			−5.45		

Abbreviations: L, 1 ng/mL for catecholamine metabolite standard; H, 1000 ng/mL for catecholamine metabolite standard; H-L, running two samples in the sequence of H-L; and L-H, running two samples in the sequence of L-H.

Table 5
Multivariate analysis of effects of sex, age and BSA on urinary free catecholamine metabolites and reference intervals.

Metabolites	Sex		Age		BSA		2.5 % ~ 97.5 % percentiles (nmol/24-h)
24-h MN	0.032	0.199	0.010	0.018	0.187	0.801	29.20 ~ 174.97
24-h NMN	0.495	0.074	0.584	0.674	0.028	0.004	37.66 ~ 214.62
24-h 3-MT	0.338	0.362	0.007	0.003	0.038	0.018	63.75 ~ 353.09

Data are demonstrated as *P*-values.

sample volume and a short chromatographic run time, and our one-step direct dilution pretreatment simplifies the process compared to the complicated solid-phase extraction employed in prior studies [28,29]. These advantages reduce operating cost and enable rapid reporting of urine catecholamine metabolite concentrations to clinicians, making PPGLs diagnosis possible.

In addition, we established reference ranges for urinary free MN, NMN, and 3-MT in apparently healthy volunteers from the Chinese population, facilitating the measurement of catecholamine metabolites in this group. The inconvenience of collecting urine for 24-h results in a limited reference population size. We plan to establish reference intervals for urinary free catecholamine metabolites, corrected for creatinine, using random urine samples. Consistent with a previous study, we found no gender-related differences in urinary free MN, NMN, or 3-MT concentrations [30]. We also found a positive association between BSA and urinary free NMN and 3-MT levels. Additionally, Eisenhofer et al. reported a negative effect of age on urinary free MN and 3-MT [31]. Our study also demonstrated a negative relationship of urinary free MN and 3-MT with age, indicating the potential benefits of establishing age-related reference intervals for urinary free catecholamine metabolites in improved PPGLs screening and diagnosis.

6. Conclusion

In conclusion, we have developed a simple and fast LC-MS/MS method for measuring urinary free MN, NMN, and 3-MT, which can aid in the diagnosis and prognosis of PPGLs.

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CRediT authorship contribution statement

Yan Song: Writing – original draft, Validation, Investigation, Funding acquisition. **Runhao Xu:** Methodology. **Dan Liu:** Software.

Jie Zhang: Writing – review & editing, Supervision.

Declaration of competing interest

None of the authors has a conflicting financial interest.

Data availability

Data will be made available on request.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2024.e00358.

References

[1] K. Wang, J. Crona, F. Beuschlein, A.B. Grossman, K. Pacak, S. Nölting, Targeted therapies in pheochromocytoma and paraganglioma, The Journal of Clinical Endocrinology & Metabolism 107 (11) (2022) 2963–2972.
[2] H.P.H. Neumann, D.L. Longo, W.F. Young, C. Eng, Pheochromocytoma and paraganglioma, N. Engl. J. Med. 381 (6) (2019) 552–565.
[3] J.W.M. Lenders, G. Eisenhofer, M. Mannelli, K. Pacak, Phaeochromocytoma, Lancet 366 (9486) (2005) 665–675.
[4] J.W. Lenders, Q.Y. Duh, G. Eisenhofer, A.P. Gimenez-Roqueplo, S.K. Grebe, M.H. Murad, M. Naruse, K. Pacak, W.F. Young Jr., Pheochromocytoma and paraganglioma: an endocrine society clinical practice guideline, The Journal of clinical endocrinology and metabolism 99 (6) (2014) 1915–1942.
[5] J.W.M. Lenders, M.N. Kerstens, L. Amar, A. Prejbisz, M. Robledo, D. Taieb, K. Pacak, J. Crona, T. Zelinka, M. Mannelli, T. Deutschbein, H.J.L.M. Timmers, F. Castinetti, H. Dralle, J. Widimský, A.-P. Gimenez-Roqueplo, G. Eisenhofer, Genetics, diagnosis, management and future directions of research of phaeochromocytoma and paraganglioma: a position statement and consensus of the Working Group on Endocrine Hypertension of the European Society of Hypertension, J. Hypertens. 38 (8) (2020) 1443–1456.
[6] C. Jimenez, G. Xu, J. Varghese, P.H. Graham, M.T. Campbell, Y. Lu, New directions in treatment of metastatic or advanced pheochromocytomas and sympathetic paragangliomas: an American, contemporary, pragmatic approach, Curr. Oncol. Rep. 24 (1) (2022) 89–98.
[7] A. Chrisoulidou, G. Kaltsas, I. Ilias, A.B. Grossman, The diagnosis and management of malignant phaeochromocytoma and paraganglioma, Endocr. Relat. Cancer 14 (3) (2007) 569–585.
[8] L. Canu, G. Parenti, G. De Filpo, M. Mannelli, Pheochromocytomas and paragangliomas as causes of endocrine hypertension, Front. Endocrinol. 10 (2019) 333.
[9] E. Sbardella, Z. Maunsell, C.J.H. May, M. Tadman, T. James, B. Jafar-Mohammadi, A.M. Isidori, A.B. Grossman, B. Shine, Random 'spot' urinary metanephrines compared with 24-h-urinary and plasma results in phaeochromocytomas and paragangliomas, Eur. J. Endocrinol. 183 (2) (2020) 129–139.
[10] Q. Liu, W. Zhou, C. Zhang, Detection status and standardization expectation of catecholamines and their metabolites, Chinese Journal of Laboratory Medicine 43 (3) (2020) 6.
[11] E. Sbardella, Z. Maunsell, C.J.H. May, M. Tadman, T. James, B. Jafar-Mohammadi, A.M. Isidori, A.B. Grossman, B. Shine, Random 'spot' urinary metanephrines compared with 24-h-urinary and plasma results in phaeochromocytomas and paragangliomas, Eur. J. Endocrinol. 183 (2) (2020) 129–139.
[12] J.W.M. Lenders, A. Januszewicz, M. Fassnacht, F. Beuschlein, R. Sinnott, H.J.L.M. Timmers, F. Megerle, T. Deutschbein, S. Fliedner, M. Pęczkowska, E. Tsourdi, K. Langton, N. Rogowski-Lehmann, J. Masjkur, C. Pamporaki, M. Peitzsch, A. Prejbisz, G. Eisenhofer, Biochemical diagnosis of chromaffin cell tumors in patients at high and low risk of disease: plasma versus urinary free or deconjugated O-methylated catecholamine metabolites, Clin. Chem. 64 (11) (2018) 1646–1656.
[13] E.B.o.C.M. Association, Expert consensus on diagnosis and treatment of pheochromocytoma and paraganglioma (2020 edition), Chinese Journal of Endocrinology and Metabolism 36 (9) (2020) 737–750.
[14] P. Gupta, M.L. Khurana, R. Khadgawat, C.S. Bal, G. Kumar, S.C. Sharma, N. Tandon, Plasma free metanephrine, normetanephrine, and 3-methoxytyramine for the diagnosis of pheochromocytoma/paraganglioma, Indian journal of endocrinology and metabolism 19 (5) (2015) 633–638.
[15] R. Garcia-Carbonero, F. Matute Teresa, E. Mercader-Cidoncha, M. Mitjavila-Casanovas, M. Robledo, I. Tena, C. Alvarez-Escola, M. Arístegui, M.R. Bella-Cueto, C. Ferrer-Albiach, F.A. Hanzu, Multidisciplinary practice guidelines for the diagnosis, genetic counseling and treatment of pheochromocytomas and paragangliomas, Clin. Transl. Oncol. 23 (10) (2021) 1995–2019.
[16] K. Wang, X. Gao, H. Cong, W. Zhang, X. Shi, S. Dai, Z. Zhou, Stability and reference intervals of spot urinary fractionated metanephrines and methoxytyramine by tandem mass spectrometry as a screening method for pheochromocytoma and paraganglioma, Endocrine 69 (1) (2020) 188–195.
[17] H.I. Woo, J.S. Yang, H.J. Oh, Y.Y. Cho, J.H. Kim, H.-D. Park, S.-Y. Lee, A simple and rapid analytical method based on solid-phase extraction and liquid chromatography–tandem mass spectrometry for the simultaneous determination of free catecholamines and metanephrines in urine and its application to routine clinical analysis, Clin. Biochem. 49 (7–8) (2016) 573–579.
[18] H. Lu, J. Yu, J. Wang, L. Wu, H. Xiao, R. Gao, Simultaneous quantification of neuroactive dopamine serotonin and kynurenine pathway metabolites in gender-specific youth urine by ultra performance liquid chromatography tandem high resolution mass spectrometry, J. Pharmaceut. Biomed. Anal. 122 (2016) 42–51.
[19] Clinical and Laboratory Standards Institute, Liquid chromatography-mass spectrometry methods, C62-A ، CLSI (2014).
[20] A. Soltani, M. Pourian, B.M. Davani, Does this patient have Pheochromocytoma? a systematic review of clinical signs and symptoms, J. Diabetes Metab. Disord. 15 (1) (2016).
[21] P.-F. Plouin, A.-P. Gimenez-Roqueplo, Initial work-up and long-term follow-up in patients with phaeochromocytomas and paragangliomas, Best Pract. Res. Clin. Endocrinol. Metabol. 20 (3) (2006) 421–434.
[22] A. Cerqueira, T. Seco, A. Costa, M. Tavares, J. Cotter, Pheochromocytoma and paraganglioma: a review of diagnosis, management and treatment of rare causes of hypertension, Cureus (2020).
[23] D. Granberg, C.C. Juhlin, H. Falhammar, Metastatic pheochromocytomas and abdominal paragangliomas, The Journal of Clinical Endocrinology & Metabolism 106 (5) (2021) e1937–e1952.
[24] J.I.L. Hye Jeong Kim, Yoon Young Cho, Soo Youn Lee, Jung Han Kim, Byong Chang Jung, Sun Wook Kim, Jae Hoon Chung, Yong-Ki Min, Myung-Shik Lee, Moon-Kyu Lee, Jae Hyeon Kim, Diagnostic accuracy of plasma free metanephrines in a seated position compared with 24-hour urinary metanephrines in the investigation of pheochromocytoma, Endocr. J. 62 (3) (2015) 243–250.
[25] P.K. Lenders Jw, M.M. Walther, W.M. Linehan, M. Mannelli, P. Friberg, H.R. Keiser, D.S. Goldstein, G. Eisenhofer, Biochemical diagnosis of pheochromocytoma: which test is best? JAMA 287 (11) (2002) 1427–1434.
[26] D. Rao, M. Peitzsch, A. Prejbisz, K. Hanus, M. Fassnacht, F. Beuschlein, C. Brugger, S. Fliedner, K. Langton, C. Pamporaki, V. Gudziol, A. Stell, A. Januszewicz, H. J.L.M. Timmers, J.W.M. Lenders, G. Eisenhofer, Plasma methoxytyramine: clinical utility with metanephrines for diagnosis of pheochromocytoma and paraganglioma, Eur. J. Endocrinol. 177 (2) (2017) 103–113.

[27] N. van Duinen, D. Steenvoorden, I.P. Kema, J.C. Jansen, A.H.J.T. Vriends, J.P. Bayley, J.W.A. Smit, J.A. Romijn, E.P.M. Corssmit, Increased urinary excretion of 3-methoxytyramine in patients with head and neck paragangliomas, The Journal of Clinical Endocrinology & Metabolism 95 (1) (2010) 209–214.
[28] M. Peitzsch, D. Pelzel, S. Glöckner, A. Prejbisz, M. Fassnacht, F. Beuschlein, A. Januszewicz, G. Siegert, G. Eisenhofer, Simultaneous liquid chromatography tandem mass spectrometric determination of urinary free metanephrines and catecholamines, with comparisons of free and deconjugated metabolites, Clin. Chim. Acta 418 (2013) 50–58.
[29] H. Wang, X. Zhang, Q. Zhen, W. Zou, H. Chen, C. Luo, M. Ding, Detection of spot urinary free metanephrines and 3-methoxytyramine with internal reference correction for the diagnosis of pheochromocytomas and paragangliomas, J. Chromatogr. B 1156 (2020).
[30] E. Pussard, M. Neveux, N. Guigueno, Reference intervals for urinary catecholamines and metabolites from birth to adulthood, Clin. Biochem. 42 (6) (2009) 536–539.
[31] G. Eisenhofer, M. Peitzsch, D. Kaden, K. Langton, A. Mangelis, C. Pamporaki, J. Masjkur, A. Geroula, M. Kurlbaum, T. Deutschbein, F. Beuschlein, A. Prejbisz, S. R. Bornstein, J.W.M. Lenders, Reference intervals for LC-MS/MS measurements of plasma free, urinary free and urinary acid-hydrolyzed deconjugated normetanephrine, metanephrine and methoxytyramine, Clin. Chim. Acta 490 (2019) 46–54.