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A new approach for urinary vanillylmandelic acid determination using eVol microextraction by packed sorbent coupled to liquid chromatography-tandem mass spectrometry

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Abstract

Vanillylmandelic acid (VMA) is one of the most important catecholamine metabolites, and it is usually used to aid in diagnosis of pheochromocytoma and paraganglioma. A new digital control microextraction by packed sorbent (MEPS) procedure coupled to liquid chromatography-mass spectrometry (LC-MS/MS) method has been developed to determine VMA in human urine. We evaluated important parameters influencing MEPS efficiency, including stationary phase, extracting cycles, and sample dilution. In optimized MEPS conditions, Only 10 μL of sample volume and 3 min preparation time for one sample were needed. Chromatographic separation was achieved with a hydrophilic interaction liquid chromatography (HILIC) column using gradient elution. VMA was detected using multiple reaction monitoring (MRM) with an electrospray source operating in negative ion mode. The method was validated for linearity, limit of quantification, accuracy, imprecision, matrix effect, and interference. Linearity was 0.5–100 μg/mL for VMA. Intra-assay, inter-assay, and total imprecision were less than 9.6%. Interferences precluding quantitation of VMA in dilute-and-shoot approach were reduced significantly using a MEPS approach. Method comparison of LC-MS/MS and homogeneous enzyme immunoassay was performed, and the reference interval was established. The developed MEPS-LC-MS/MS method certainly contributes to method robustness and makes it suitable for measurement of urinary VMA in routine clinical biochemistry laboratories.

Keywords: Microextraction by packed sorbent, Vanillylmandelic acid, Liquid chromatography-tandem mass spectrometry, Human urine samples, Pheochromocytoma and paraganglioma

Introduction

Pheochromocytoma and paraganglioma (PPGL) are rare tumors originating from adrenal and extra-adrenal chromaffin cells that are characterized by excessive secretion of large amount of catecholamines (Monteleone et al. 2013). PPGL could lead to various cardiovascular or cerebrovascular diseases and even death if they are not diagnosed and treated in time. Measurement of urinary vanillylmandelic acid (VMA) is frequently used in both the clinical diagnosis and pathological study of this disease because of non-invasive and easy urine specimen collection (Lionetto et al. 2008).

Biochemical tests for VMA typically are performed using high performance liquid chromatography (HPLC), microcolumn chromatography, gas chromatography-mass spectrometry (GC-MS), or immunoassay techniques (Davidson 1989; Tran et al. 2014; Taran et al.

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1997; Tsunoda 2006). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) offers several advantages for polar small molecule metabolites. LC-MS/MS can simplify sample preparation, decrease analysis time, and improve selectivity and sensitivity. In recent years, LC-MS/MS has been developed to determine VMA as a single analyte (Magera et al. 2003; Shen et al. 2015) or in panels with other biomarkers related to neuroendocrine tumors (Lionetto et al. 2008; Clark et al. 2017; Fang et al. 2012; Konieczna et al. 2016; Shen et al. 2019; Grouzmann et al. 2018). Solid phase extraction (SPE) (Magera et al. 2003) and dispersive liquid-liquid microextraction (DLLME) (Konieczna et al. 2016) that were reported for sample cleanup, but dilute-and-shoot method was opted for sample preparation as it was easy and fast (Shen et al. 2015; Clark et al. 2017; Shen et al. 2019; Grouzmann et al. 2018). Although the use of stable isotope-labeled internal standards and designing LC gradients to exclude analytes from suppression zones that can minimize the matrix effects for a quantitative high-throughput clinical assay when using a dilute-and-shoot method, these options still provide insufficient robustness. Clark et al. (2017) had observed matrix interferences in 3% of the specimens, and an alternate LC gradient was designed to resolve the interferences. Recently, microextraction by packed sorbent (MEPS) has evolved a simple, rapid, and environmentally friendly method for extracting a wide range of analytes from different matrices, such as biological fluids (plasma, blood, and urine) (Abdel-Rehim 2011). MEPS is a miniaturized form of SPE technique that approximately 1 mg of the solid phase is built into syringe needle. Several sorbents including silica (C8, C18), ion exchange material, or molecular imprinted polymers (MIPs) can be employed to extract variety of analytes (Abdel-Rehim 2014; Moein et al. 2014; Chaves et al. 2010; Altun et al. 2004; Saracino et al. 2015). MEPS can be used more than 100 times for plasma or urine samples, but a conventional SPE column is used only once. Furthermore, a digital analytical device named eVol® syringe can couple with MEPS syringe to control the speed and volume of the extraction. The semi-automation of sample processing is compatible with various analytical instruments, including LC-MS equipment. This technique, eVol®-MEPS, can meet the requirements of clinical laboratory for the simplicity, rapidity, miniaturization, and small sample volumes of sample preparation and maintain sufficient selectivity, precision, and accuracy.

The aim of the study was to develop and validate a fast, simple, and reliable eVol®—MEPS procedure combined with the LC-MS/MS method to increase throughput, improve analytical specificity for VMA in urine, and further establish reference interval of VMA to assist in diagnosis of PPGL.

Materials and methods

Chemicals and materials

VMA and its stable isotopes internal standard, 4-hydroxy-3-methoxy-d3-mandelic acid (VMA-d3), were from Sigma-Aldrich (Saint-Louis, Missouri, USA). Lyphocheck® Quantitative Controls Levels I and II were purchased from Bio-Rad Clinical Division (Hercules, California, USA). Synthetic urine was prepared by dissolving 0.93 g of urea, 0.19 g of NaCl, and 0.07 g of creatinine in 100 mL of ultra-pure water; the pH of the mixture was adjusted to 5.6 (Saracino et al. 2015). All chemicals and reagents were HPLC grade or analytical grade. HPLC-grade acetonitrile and formic acid were from Fisher Scientific (Fair Lawn, New Jersey, USA), and acetic acid, ammonium acetate, and ammonium formate were commercially obtained from Dikma (Lake Forest, USA). Ultra-pure water was obtained using a Milli-Q system (Waters Millipore, Milford, Massachusetts, USA).

Calibration standards and quality controls

Stock VMA solution was prepared in water at 1000 μg/mL. Calibration standards at concentration of 0.5, 1, 2, 5, 20, 50, and 100 μg/mL were prepared by dilution of the stock solution with synthetic urine. Internal standard (VMA-d3) solution was prepared at a concentration of 20 μg/mL in water.

For the VMA method validation, quality controls (QCs) were prepared at three concentrations: a low QC (3.50 μg/mL), a mid-range QC (10.0 μg/mL), and a high QC (80.0 μg/mL) were made by spiking the stock solutions in healthy specimen pools. Aliquots of stock, calibration, and QCs were stored at − 80 °C.

Sample collection

Urine samples, collected without preservative over 24 h, were selected from samples submitted for clinical testing. De-identified samples were utilised, and patients were enrolled into the study were approval from the Institutional Review Board of Peking University Third Hospital. The patients kindly consented to do the analysis. Total volumes were measured, and 2 mL aliquots were stored at − 80 °C until analysis.

Microextraction by packed sorbent procedure using eVol® syringe

Microextraction in packed sorbent was performed using an eVol® semi-automatic syringe from Thermo Fisher Scientific, consisting of 500 μL volume syringe coupled with a barrel insert and needle (BIN) assemblies packed with anion exchange (AX) sorbent materials. Before using for the first time, a BIN was conditioned with 3 × 100 μL of methanol and 3 × 100 μL of water. Ten microliters of human urine was diluted with 90 μL of water. After that, 7 × 100 μL of diluted human urine

was drawn up and down through the syringe without discarding it. It was important that urine samples were drawn slowly (20 μL/s) to obtain sufficient interaction between the analytes and the sorbent. Then, the sorbent was washed with 100 μL of 5% ammonium hydroxide in water and 5% ammonium hydroxide in ACN, respectively, to remove biological interferences. The analyte was then eluted by 2 × 50 μL water/ACN 1:1 (v/v) (2% formic acid), and the elution was further diluted with 150 μL water containing 0.7% ammonium hydroxide. Aliquot of 100 μL eluate was transferred into auto-sampler vials for injection. After each extraction, the BIN was rinsed with 4 × 100 μL of water/ACN 1:1 (v/v) (2% formic acid) followed by 100 μL of water to prepare it for the following sample. The same AX sorbent could be used for about 150 extractions.

Chromatography and mass spectrometry

Analysis was performed on a Qtrap 5500 triple quadrupole/linearity ion trap mass spectrometer (Applied Biosystems, Foster City, USA) with an electrospray ionization (ESI) probe in negative mode, which interfaced with a Shimadzu LC-20A HPLC system (Tokyo, Japan) including a degrasser, an autosampler, a binary pump, and a column oven. Data were acquired in multiple reaction monitoring (MRM) mode.

Chromatographic separation was achieved on a ZIC-HILIC analytical column (2.1 × 100 mm, 3.5 μm particles; Merck, Damstadt, Germany) maintained at 40 °C. The flow rate was 0.3 mL/min. Mobile phase was composed of acetonitrile (phase A) and 20 mM ammonium formate (phase B) using the following linear gradient program: 0–3.0 min, linear gradient 2–22% B; 3.0–4.5 min, 30% B; 4.5–8.0 min, column equilibration. Total run time was 8 min. The injection volume was 1 μL.

MRM transitions used for quantification and qualification of VMA were m/z 197.0 > 137.1 and 197.0 > 151.1, respectively. The transitions of VMA-d3 were m/z 200.0 > 140.1 (quantifier) and 200.0 > 154.1 (qualifier). The optimized MS parameters were as follows: ion source temperature (TEM), 550 °C; curtain gas (CUR), 50 psi; collisionally activated dissociation (CAD), media; ion source gas 1, (GS1) 55 psi; ion source gas 2 (GS2), 55 psi; ion-spray voltage (IS), − 3000 V; collision energy (CE), − 25 V for VMA (quantifier), − 15 V for VMA (qualifier), − 28 V for VMA-d3 (quantifier), and − 16 V for VMA-d3 (qualifier); entrance potential (EP), − 12 V for VMA and − 8 V for VMA-d3; declustering potential (DP), − 37 V for VMA and − 74 V for VMA-d3; collision cell exit potential (CXP), − 11 V for VMA and − 18 V for VMA-d3.

A qualitative ion ratio (QIR) was used to confirm identify of the analytes. The QIRs were calculated as ratios of the peak area for the quantifier transition to the peak area for the qualifier transition for both the analytes and the internal standards. Patient specimen QIR acceptability range was established as ± 30% of the mean value of the QIRs obtained from the calibration standards.

Method validation

Linearity and analytical sensitivity

Linearity was evaluated by analyzing extracted VMA standards spiked in synthetic urine at 0.5, 1, 2, 5, 20, 50, and 100 μg/mL. Triplicates at each of the seven concentration of VMA were analyzed. Lower limit of quantification (LLOQ) was determined as the lowest measured concentrations with a signal-to-noise (S/N) ratio greater than 10 with the acceptable criteria of precision of a coefficient variation (CV) below 20% and accuracy within 80–120% of expected value.

Imprecision and accuracy

Three control materials at concentration of 3.5, 10.0, and 80.0 μg/mL were used to estimate imprecision and accuracy on three consecutive days in 5 replicates per run, 1 run per day (total of 15 measurements of each level of control).

Extraction recovery

To investigate the MEPS recovery of VMA and internal standard in urine samples, two spiked samples using pooled urine were prepared: pre-spiked and post-spiked. The pre-spiked sample was prepared by spiking VMA and internal standard into pooled urine before MEPS, whereas the post-spiked sample was prepared by spiking VMA and internal standard at equivalent concentrations to the extract obtained after MEPS of pooled urine. The percent extraction recovery was calculated as the percent response ratio of VMA and internal standard in the pre-spiked sample to those in the post-spiked sample.

Carryover

A set of high (83.4 μg/mL) and low (1.0 μg/mL) concentration samples were injected in a sequence that allows for determination of a mean and standard deviation (SD) for a low concentration sample, and a low concentration sample following a high concentration sample. The injection sequence was low, low, high, and low.

Matrix effect

Two methods were performed to evaluate the matrix effect (ME). Firstly, the ME was evaluated by the post-column infusion study. Urine samples prepared without internal standard were injected into the LC column, and 4 μg/mL internal standard solutions were co-infused at 5 μL/min post-column as described by Annesley (2003). Secondly, the ME was also evaluated by comparing the peak area of VMA-d3 instead of VMA spiked in the

solution mobile phase with the post-spiked urine samples from six individual sources at the same concentration (20 μg/mL).

Analytical specificity

Assay interference was tested by analyzing samples spiked with known concentrations of endogenous and exogenous compounds. Recommendations from CLSI EP7-A2, Interference Testing in Clinical Chemistry, were used to guide prepared concentrations of interferences. The potential interferences were spiked into the Lyphocheck® Quantitative Controls at two levels (normal and pathological ranges) before extraction.

Stability

A pool of urine samples spiked with VMA stock solutions was used to assess analyte stability without acid stabilization. The autosampler stability was assessed by keeping the processed QC samples at room temperature for 48 h. The freeze-thaw stability was evaluated by analyzing the QC samples after 3 cycles from − 80 °C to room temperature. The urine samples were analyzed for bench-top stability after storage at room temperature for 42 h. Aliquots of QC samples were stored at − 80 °C for 2 months. All the stability studies were conducted at 3 concentration levels with 3 replicates. The stability of the stock solutions was tested and established under refrigeration (~ 4 °C) for 200 days.

Method comparison and reference interval

Urine specimens from 39 patients were assayed by the new MEPS-LC-MS/MS method and the previous developed LC-MS/MS using "dilute-and-shoot" sample preparation method in our laboratory which referred to the published method (Clark et al. 2017). Internal standard working solution (10 μL) and 0.05% (v/v) formic acid in water (160 μL) were added to urine samples (20 μL) and thoroughly mixed; then, 1 μL was injected for determination.

De-identified 24 h urine specimens submitted for routine testing of VMA by homogeneous enzyme immunoassay (HEI) were also assayed by the new LC-MS/MS method, and the results were compared. Reference interval was evaluated according to the method of Hoffmann (Katayev et al. 2010). Outlier values exceeding the third quartile plus three times the interquartile range were removed. Reference limits were calculated as the central 95% based on extrapolation of the linear portion of the data.

Data analysis

The analyst 1.5.2 software (Applied Biosystems, Forster City, USA) and GraphPad Prism 5.0 (GraphPad Software, San Diego California, USA) were applied to statistical evaluation of the data.

Results and discussions

Optimization of chromatographic conditions

VMA is a low molecular weight and polar compound with poor retention time in regular reversed-phase liquid chromatography (RP-LC). In order to achieve satisfactory retention and to avoid interference from the background, the alternative LC-MS method based on hydrophilic interaction liquid chromatography (HILIC) was developed for successful determination of polar compounds (Kumar et al. 2011). ZIC-HILIC column with packing zwitterionic stationary phase was selected to retain VMA. The composition of mobile phase, buffer type, and pH value was investigated for appropriate resolution, good peak shape, and response of VMA. As the percentage of the organic solvent in the mobile phase increases, the retention factor increases in HILIC mode, and high ratio of ACN was used. The effect of buffer concentration in the mobile phase was investigated. Thus, ammonium acetate and ammonium formate were tested due to their solubility in high amount of organic modifier. The buffer concentration varied from 1 to 40 mM. Although the retention of VMA increased with buffer concentration increased, higher concentrations of salt additives can result in signal suppression for mass spectrometric detection as we all know. Therefore, the best retention and significantly better ionization was obtained when 20 mM ammonium formate buffer was used. To evaluate pH influence on retention time of VMA, ammonium formate buffers containing different amount of formic acid (0.0–0.5%) were tested. Higher acidic concentration resulted in decreased retention time, so optimal retention time for VMA, was noticed under without formic acid additive. Example chromatograms of VMA calibration standard and of healthy and abnormal patient specimens are shown in Fig. 1.

Optimization of eVol® MEPS procedure

In this study, several parameters related to MEPS were optimized using 1 μg/mL of VMA of standard solutions. Optimized parameters included sorbent type, sample volume, and elution conditions which strongly affect the extraction efficiency of MEPS. Firstly, four commercially available MEPS sorbents (C8, C18, SIL, and R-AX) were investigated. Sufficient retention of VMA on the R-AX sorbent was obtained due to anionic exchange interaction.

Retention of VMA on R-AX sorbent was affected by the number of extraction cycles. The different number of cycles increases the extraction efficiency (Fig. 2a). The results obtained indicated that the response was the strongest for the load 7 times. Higher number of

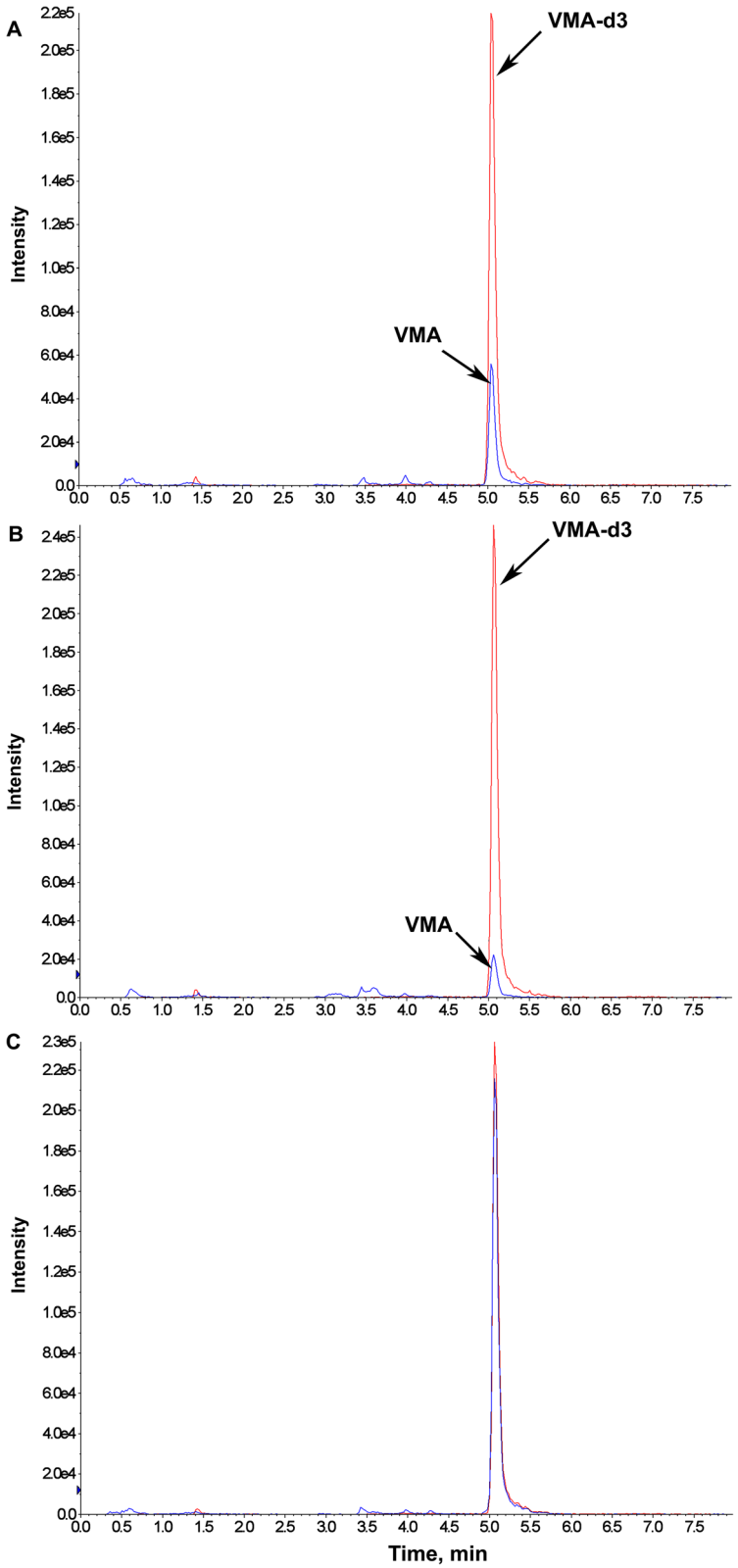

Fig. 1 LC-MS/MS chromatograms of **a** 1 μg/mL calibration standard, **b** healthy patient urine, and **c** abnormal patient urine: vanillylmandelic acid (VMA), vanillylmandelic acid-d3 (VMA-d3)

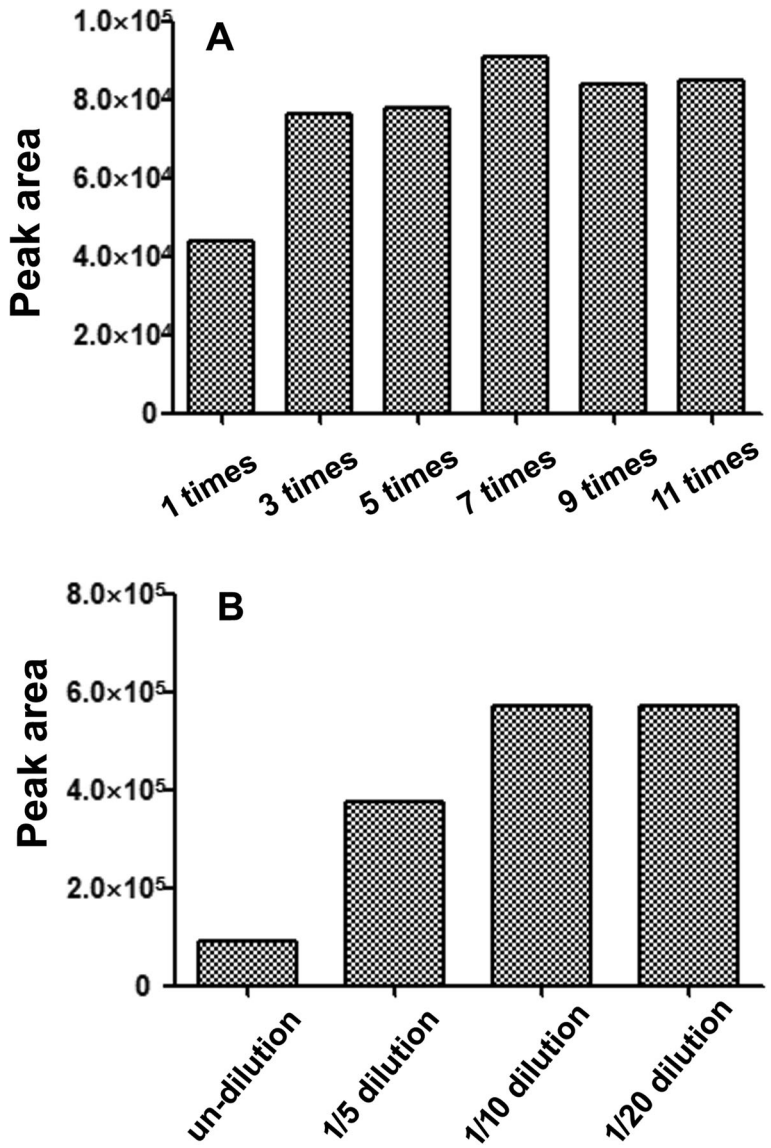

Fig. 2 Influence of different parameters on MEPS efficiency. **a** Effect of the number of extraction cycles on the VMA response. **b** Effect of the urine dilution folds on the VMA response

Table 1 Intra-assay, inter-assay accuracy, and imprecision for VMA in human urine

Analytes	Concentration (μg/mL)	Intra-assay			Inter-assay			Total (%CV)
		Mean ± SD (μg/mL)	Accuracy (%)	CV (%)	Mean ± SD (μg/mL)	Accuracy (%)	CV (%)	
VMA	0.500	0.550 ± 0.040	110.0	7.3	0.532 ± 0.033	106.4	6.3	9.6
	3.50	3.60 ± 0.18	102.9	4.9	3.40 ± 0.22	97.1	6.4	8.1
	10.0	11.1 ± 0.5	111.0	3.3	10.7 ± 0.6	107.0	3.9	5.1
	80.0	83.6 ± 4.0	104.5	4.6	80.6 ± 4.8	100.8	5.7	7.3

extraction cycles did not significantly improve the extraction efficiency and might shorten the lifetime of the MEPS sorbent as complex matrix could saturate the stationary phase with interferences.

The key steps for optimal extraction were using basic washing solvent to convert the VMA into ionized molecule in order to make it retained on R-AX sorbent and secondly, acidic elution solvent to convert it into neutral molecule and to elute it from the sorbent. Therefore, 5% ammonium hydroxide in water followed by 5% ammonium hydroxide in ACN was employed as the most appropriate washing solvents. For the elution step, 2 × 50 μL of 2% formic acid in ACN:water (1:1, v/v) was chosen.

Then, the developed procedure was applied on urine specimens spiked with VMA. A significant leak of the analytes from the MEPS sorbent was observed after the sample loaded. It was probably caused by a strong competition for the sorbent active sites between the analytes and other ions presented in urine. The use of buffer with different pH did not prevent the leakage of analytes. Therefore, dilution of urine was subsequently tested in order to eliminate the competition. From Fig. 2b, we could see that the leakage did not occur when the urine was diluted 10 times with water.

Method validation results

Linearity and analytical sensitivity

Linearity of the developed method was established with the use of seven calibration curves analyzed in triplicate in the nominal concentration range from 0.5–100 μg/mL in synthetic urine samples. From the calibration curve, the linear equation was $y = 0.066\ x + 0.000754$ ($r^2 = 0.9984$), which demonstrated good linearity for quantification. The LLOQ for VMA was 0.5 μg/mL with inter-assay CV of 6.3%, which was sufficient for accurate measurement of samples.

Imprecision and accuracy

Imprecision calculated as the CV for three control materials at low, medium, and high concentration levels was assayed by analyzing five replicates for each day over 3 days. The intra-assay, inter-assay, and total imprecision for VMA were ranged from 1.9 to 9.6%, which demonstrated excellent precision of the method for quantification. Specific data are listed in Table 1.

Accuracy was assessed five replicates at three concentration levels over 3 days by spiking known amounts of VMA into urine samples. The intra-assay, inter-assay, and total accuracy for 3 QC concentrations were in the range of 98.8 to 110.0% (shown in Table 1), which indicated excellent accuracy of this method for clinical application.

Extraction recovery

The extraction recovery of the MEPS method was evaluated by spiking a pool urine with known amounts of VMA at three concentration levels (1, 10, and 80 μg/mL). The absolute recoveries determined for three different concentrations of VMA were ranged from 57.5–70.5%.

Carryover

The carryover was investigated by injecting low, low, high, and low concentration samples in sequence. The calculated carryover values for VMA were 0.007 μg/mL which was less than the error limits (0.027 μg/mL), defined as three

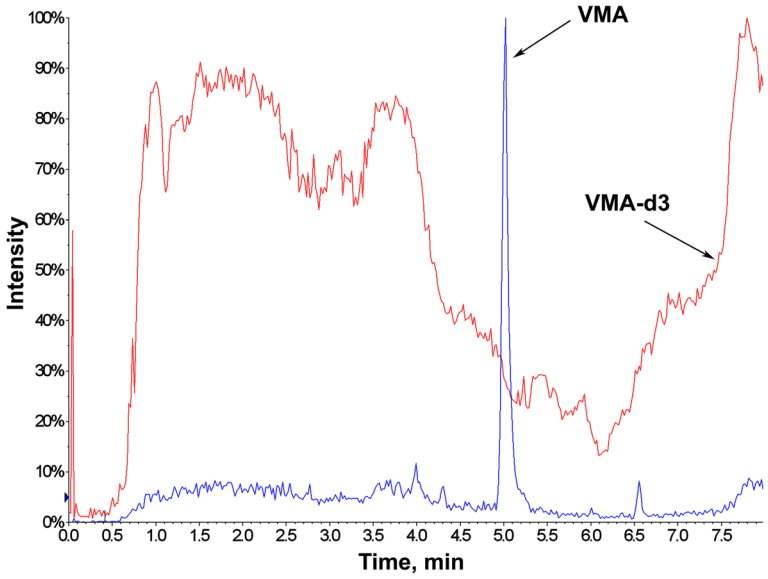

Fig. 3 Post-column infusion experiment

Table 2 Deviations from baseline concentrations of VMA for exogenous potential interferences

Substances	Test concentration(μg/mL)	VMA mean(μg/mL)	VMA determined (μg/mL)	VMA deviation (%)
Theobromine	100	2.93	2.87	− 2.0
		15.8	15.1	− 4.4
Acetaminophen	200	2.93	3.05	4.1
		15.8	16.2	2.5
Labetalol	100	2.93	2.76	− 5.8
		15.8	16	1.3
Amitriptyline	10	2.93	2.7	− 7.9
		15.8	14.9	− 5.7
Pseudoephedrine	10	2.93	2.79	− 4.8
		15.8	15.6	− 1.3
Diphenhydramine	10	2.93	2.96	1.0
		15.8	15.4	− 2.5
Theophylline	100	2.93	2.9	− 1.0
		15.8	17	7.6
Carbidopa	100	2.93	2.84	− 3.1
		15.8	17.4	10.1
Levodopa	100	2.93	2.89	− 1.4
		15.8	16	1.3
Salicylic acid	1000	2.93	3.18	8.5
		15.8	16	1.3
Clonidine	10	2.93	3.13	6.8
		15.8	17.3	9.5
Isoetharine	100	2.93	2.85	− 2.7
		15.8	17.8	12.7
Isoproterenol	100	2.93	2.92	− 0.3
		15.8	15.3	− 3.2
3-O-methyl-dopa	10	2.93	3.11	6.1
		15.8	16.7	5.7
Methyldopa	100	2.93	2.89	− 1.4
		15.8	16.1	1.9
MDA[1]	100	2.93	3.37	15.0
		15.8	17.1	8.2
HMMA[2]	100	2.93	3.13	6.8
		15.8	16.5	4.4
PMMA[3]	100	2.93	2.73	− 6.8
		15.8	15	− 5.1
(phentermine,MDEA[5],amphetamine,MDMA[6],MA[7]) mixture	100	2.93	2.85	− 2.7
		15.8	16.2	2.5
(ibuprofen, naproxen, cotinine, nicotine, caffeine) mixture	1000	2.93	2.86	− 2.4
		15.8	16.1	1.9
Carbamazepine	1000	2.93	2.76	− 5.8
		15.8	16.1	1.9

[1]3,4-Methylenedioxyamphetamine
[2]4-Hydroxy-3-methoxymethamphetamine
[3]4-Methoxymethamphetamine
[5]3,4-Methylenedioxyethylamphetamine
[6]3,4-Methylenedioxymethpheamphetamine
[7]Methamphetamine

Table 3 Deviations from baseline concentrations of VMA for endogenous potential interferences

Substances	Test concentration(μg/mL)	VMA mean(μg/mL)	VMA determined (μg/mL)	VMA deviation (%)
Tryptophan	200	2.93	2.8	− 4.4
		15.8	16.1	1.9
Phenylalanine	200	15.8	17	7.6
		15.8	17.2	8.9
Tyrosine	1000	2.93	2.81	− 4.1
		15.8	16.8	6.3
Melatonin	165	2.93	2.8	− 4.4
		15.8	14.6	− 7.6
Uric acid	500	2.93	2.7	− 7.9
		15.8	15.8	0.0
Ascorbic acid	200	2.93	2.98	1.7
		15.8	16.5	4.4
Urea	2000	2.93	2.82	− 3.8
		15.8	15.5	− 1.9
Glucose	2000	2.93	3.06	4.4
		15.8	16.3	3.2
Riboflavin	108	2.93	3.21	9.6
		15.8	15.5	− 1.9
4-OH-3-methoxyphenyl glycol	1000	2.93	3.02	3.1
		15.8	15	− 5.1
5-Hydroxytryptophol	100	2.93	3.27	11.6
		15.8	16.5	4.4
Nicotinamide	500	2.93	2.7	− 7.9
		15.8	15.5	− 1.9
Pyridoxal 5-phosphate	500	2.93	3.04	3.8
		15.8	15.4	− 2.5
Thiamine	200	2.93	2.87	− 2.1
		15.8	16.2	2.5
Nicotinurirc acid	200	2.93	2.85	− 2.7
		15.8	14.6	− 7.6
Nicotinic acid	200	2.93	2.82	− 3.8
		15.8	15.7	− 0.6
Pyridoxal	200	2.93	2.84	− 3.1
		15.8	15.3	− 3.2
5-Hydroxyindoleacetic acid	100	2.93	2.87	− 2.1
		15.8	15	− 5.1
Metanephrine	100	2.93	3	2.4
		15.8	15.9	0.6
3-Methoxytyramine	200	2.93	2.92	− 0.3
		15.8	14.8	− 6.3
Norepinephrine	100	2.93	2.86	− 2.4
		15.8	15.2	− 3.8
Normetanephrine	100	2.93	2.9	− 1.0
		15.8	15.4	− 2.5
3,4-Dihydroxymandelic acid	1000	2.93	3.25	10.9
		15.8	14.8	− 6.3
3,4-Dihydroxyphenylacetic acid	1300	2.93	3.12	6.5
		15.8	16.2	2.5

Table 4 Urine stability experiment (n = 3)

Concentration (μg/mL)	Room temperature (42 h)		3 freeze-thaw cycle (− 80 °C)		Long-term storage (− 80 °C, 56 days)	
	Deviation (%)	CV (%)	Deviation (%)	CV (%)	Deviation (%)	CV (%)
3.50	− 6.6	3.2	− 2.3	5.0	2.3	3.0
10.0	2.3	2.9	13.3	2.4	0.3	2.8
80.0	1.1	2.2	10.0	2.8	− 4.3	4.5

times the SD of 2 consecutive low results. In consideration of the upper limits of reference interval, the carryover did not influence the diagnosis of PPGL. Therefore, the carryover was not observed in this LC-MS/MS method.

Matrix effect

Complex biological samples are exposed to potential ion suppression, especially with electrospray ionization mass spectrometry. The ME was assessed by a post-column infusion using a large number of specimens (n = 105). All of the co-infusion tests showed similar slightly suppression zones at the retention times of the analytes (Fig. 3). Furthermore, the deuterated internal standards elute very closely with their non-labeled analogs that could compensate well for the signal suppression unless it was severe. Moreover, the absolute ME of VMA-d3 is calculated as Ai/Ai′ × 100% (Ai and Ai′ represent the peak areas of VMA-d3 within and without matrix). The results showed that the mean absolute ME was 73.0% with CV of 11.0%.

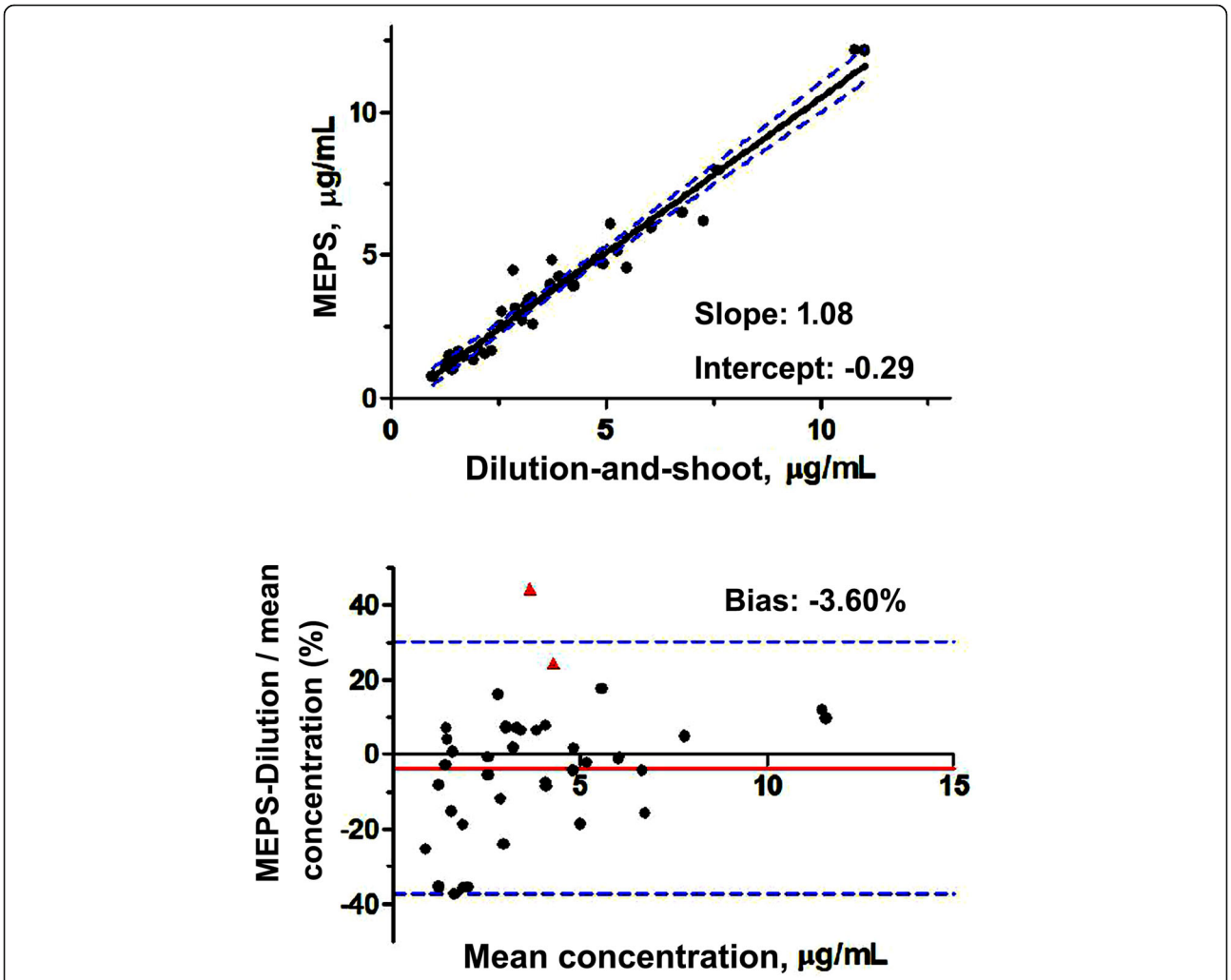

Fig. 4 Comparison between the MEPS-LC-MS/MS and dilute-and-shoot LC-MS/MS assays. Upper plots: weighed Deming fits (solid black lines); lower plots: Bland-Altman analyses (solid black lines). The dotted red lines represent the 95% limit of agreement

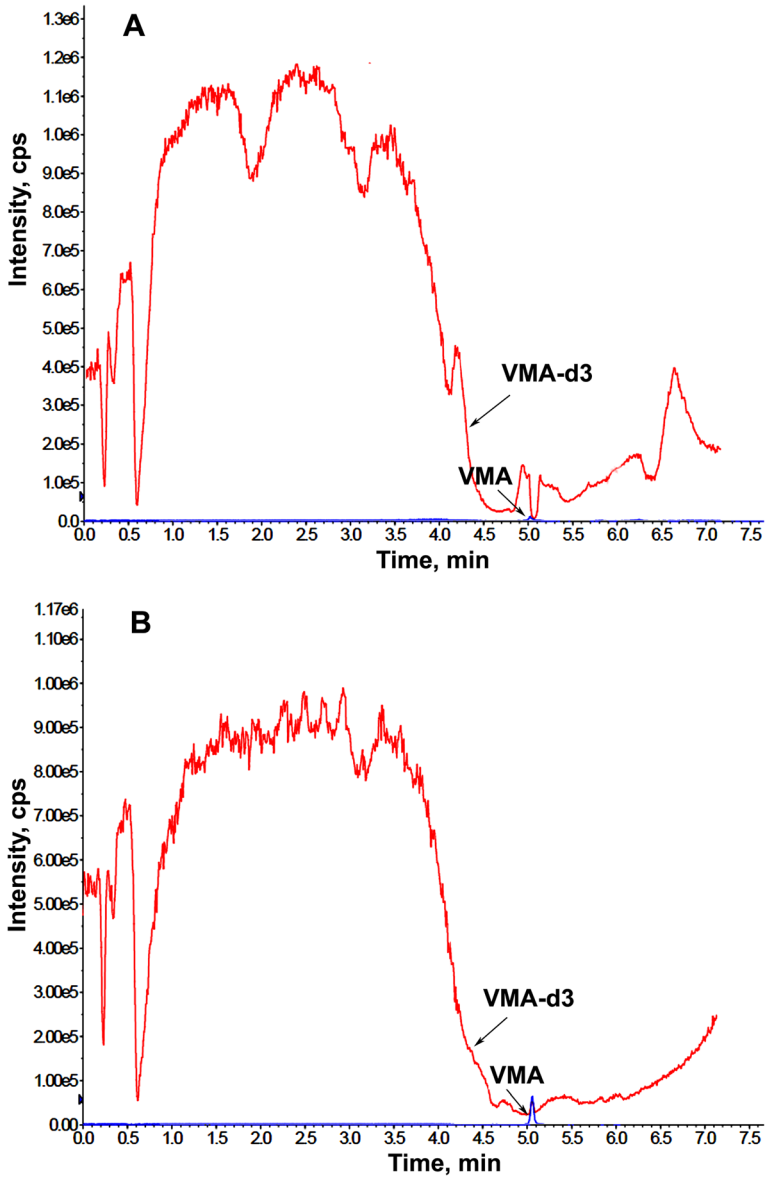

Fig. 5 Mitigation of VMA signal suppression by the MEPS approach. **a** Dilute-and-shoot approach. **b** MEPS approach. VMA-d3 was infusion post-column

Therefore, it demonstrated that the determination of VMA was not significantly influenced by the matrix effect. In comparison to dilute-and-shoot sample preparation, the developed MEPS technique can considerably reduce biological matrix influence as clean as SPE procedure. Furthermore, MEPS procedure is highly selective, reproducible, and involves a shorter sample preparation time: a reduced number of reagents and sample volume to comparison to SPE.

Analytical specificity

Exogenous and endogenous compounds evaluated as potential interferences are listed in Tables 2 and 3, respectively. Percent bias, calculated as a ratio of analyte concentrations in the spiked vs. baseline sample, was used as a measure of interference. The bias for all tested compounds was between − 7.9% and 15.0% for VMA, indicating no interference from any of the compounds tested.

Stability

To evaluate the stability of non-acidified urine samples, urine QC samples spiked with VMA stock solutions were analyzed at different conditions. The stability of urinary VMA was evaluated at room temperature for 42 h, after three freeze-thaw cycles

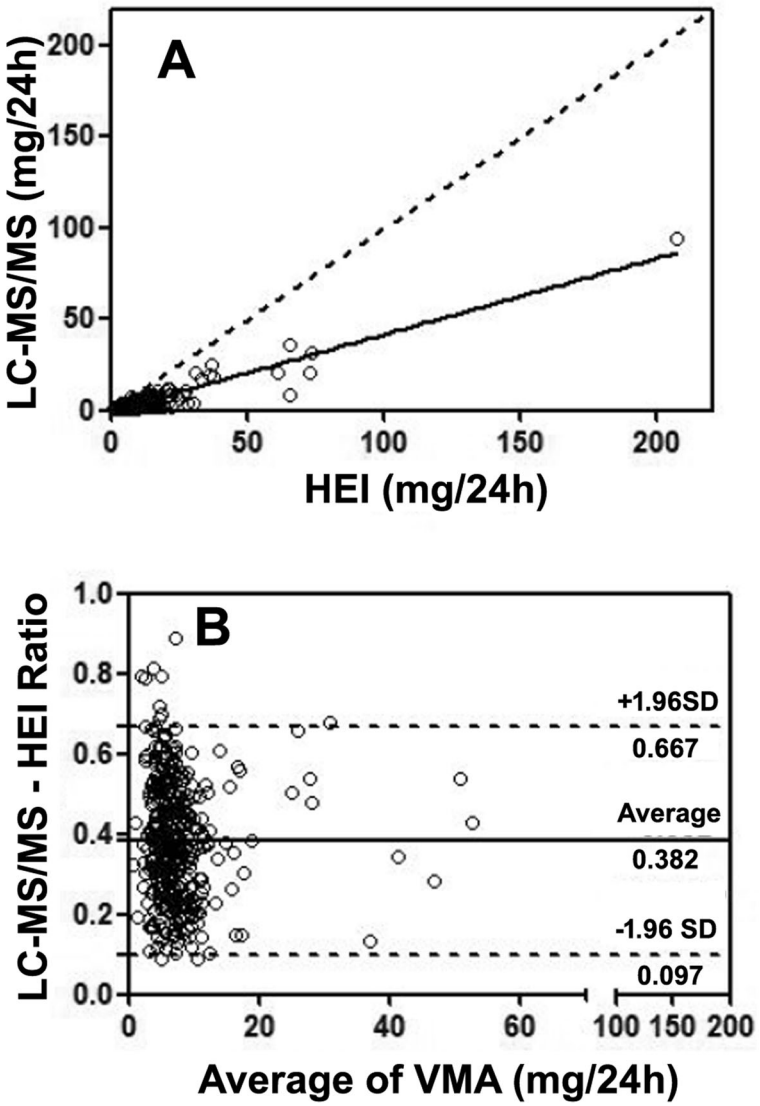

Fig. 6 Method comparison of LC-MS/MS and HEI method for the analysis of VMA. **a** Scatter plots of VMA results by LC-MS/MS (*y* axes) vs by the HEI method (*x* axes). **b** Bland-Altman plots of ratio (*y* axes) vs average values (*x* axes) for VMA results by the LC-MS/MS and HEI method

and long-term storage for 56 days at − 80 °C. The results are listed in Table 4, indicating that the analytes remained considerably stable under the above conditions. The good stability of VMA simplified the precautions needed for laboratory manipulations during the assay procedures. In addition, stock solution of VMA was shown to be stable at 4 °C for 200 days.

Method comparison and reference interval

The concentrations of VMA in urine samples from patients (n = 39) were measured by the new MEPS-LC-MS/MS method and the previous developed LC-MS/MS using "dilute-and-shoot" sample preparation method (Clark et al. 2017) and evaluated with a Bland-Altman analysis and a Deming fit (Fig. 4). The Bland-Altman analysis highlighted no significant systematic bias between the two methods with a mean difference of − 3.60% for the determined concentrations of VMA. The corresponding Deming fit revealed good agreement between both assays with a regression slope of 1.08 for VMA.

However, 2 specimens of VMA results in the tested samples could not be reported due to interference when using "dilute-and-shoot" sample preparation (the red triangles in Fig. 4). The majority of the VMA interferences produced internal standard peak areas below the lower acceptance limit (50% of the batch internal standard peak area median) due to signal suppression by matrix components at the retention

time of VMA. In order to compare the 2 specimens with interference using MEPS and dilute-and-shoot approaches, post-column infusion of a VMA-d3 solution was used as a tool to observe the position of suppression zones in the 2 specimens (see Fig. 5). Reanalysis of 2 specimens showed that interference produced VMA peak areas approximately 10-fold higher when using the MEPS approach instead of dilute-and-shoot approach, indicating that the MEPS approach could significantly mitigate signal suppression experience by VMA and reduced the incidence of non-reportable results.

Reference interval for VMA by the LC-MS/MS method was verified using statistical analysis (the central 95% of the rank-ordered data) and was based on the analysis of urine samples, collected from the control group of 215 patients (F, 104, M, 111) in whom the diagnosis of PPGL had been excluded. The established reference interval for VMA was 0–6.21 mg/24 h (0–31.36 μmol/24 h) which are within the parameters of Mayo Clinic reference interval (Magera et al. 2003) (VMA = 0.0–43.37 μmol/24 h). These results showed the excellent application of this method in clinical laboratories.

In addition, 420 urine specimens successfully analyzed by the HEI method were retested using this new method. The Deming regression shown in Fig. 6a, for these two methods, yielded slopes of 0.421 (95% CI, 0.404 to 0.437) and *y*-intercepts of − 0.606 (95% CI, − 0.886 to − 0.326), indicating a significantly difference between LC-MS/MS and HEI methods. Using Bland-Altman plots (Fig. 6b), the mean difference for LC-MS/MS results was approximately 62% lower compared to the HEI results. More than 97% of compared values differed by less than 2 SD. On the basis of these correlation parameters, the LC-MS/MS method will improve specificity, sensitivity, and repeatability.

Conclusions

A novel MEPS technique with semi-automated eVol® syringe followed by the LC-MS/MS method was developed for the analysis of urinary VMA. This method utilizes eVol® MEPS sample preparation which is efficient decoupling of analytes from the matrix, reduced matrix interference, solvent consumption, and sample volume. R-AX sorbent was used as the stationary phase for sample preparation without loss of selectivity with the re-use of MEPS packing material for about 150 extractions. The developed method is simple, rapid, specify, and robust which has a significant potential as a clinical tool and is suitable for routine clinical laboratory.

Abbreviations

PPGL: Pheochromocytoma and paragangliomas; CAs: Catecholamines; VMA: Vanillylmandelic acid; DLLME: Dispersive liquid-liquid microextraction; SPE: Solid phase extraction; MEPS: Microextraction by packed sorbent; MIPs: Molecular imprinted polymers; LLOQ: Lower limit of quantification; ESI: Electrospray ionization; MRM: Multiple reaction monitoring; HILIC: Hydrophilic interaction liquid chromatography; QIR: Qualitative ion ratio; ME: Matrix effect; SD: Standard deviation

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Not applicable in this section.

Authors' contributions

XX designed the study. Clinical specimens were collected by XX and ZYY. The LC-MS/MS analysis work was executed by ZYY and XX, and XX finished the draft of the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no conflict of interest.

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