

Simultaneous extraction and determination of monoamine neurotransmitters in human urine for clinical routine testing based on a dual functional solid phase extraction assisted by phenylboronic acid coupled with liquid chromatography-tandem mass spectrometry

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Received: 3 November 2016 / Revised: 19 January 2017 / Accepted: 26 January 2017 / Published online: 10 February 2017
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Abstract The major monoamine neurotransmitters, serotonin (5-HT) and catecholamines (i.e., norepinephrine (NE), epinephrine (E), and dopamine (DA)), are critical to the nervous system function, and imbalances of the neurotransmitters have been connected to a variety of diseases, making their measurement useful in a clinical setting. A simple, rapid, robust, sensitive, and specific LC-MS/MS method has been developed and validated for the simultaneous quantitation of urinary serotonin and catecholamines with low cost, which is ideal for routine clinical applications. A simple extraction from complex urine was accomplished using tailored solid phase extraction incorporating phenylboronic acid complexation on a 96-well HLB microplate for the sample extraction and resulted in significantly improved throughput, selectivity, and extraction recovery. Compared to 1–10 mL of urine typically used, this method required only 10 μ L. A rapid chromatographic elution with a total cycle time of 6 min per sample compared to reported run times of 19–75 min was achieved on a PFP column. The sensitivity of 1 and 2 ng mL⁻¹ for the detection of low abundant E and NE combined with the high coverage of 1024 ng mL⁻¹ for DA enabled the multi-analyte detection of these biogenic amines in a single run. Good linearity (2.0–512, 1.0–512, 4.0–1024, and 4.0–1024 ng mL⁻¹ for NE, E,

DA, and 5-HT, respectively), accuracy (87.6–104.0%), precision (\leq 8.0%), extraction recovery (69.6–103.7%), and matrix effect (87.1–113.1% for catecholamines and 63.6–71.4% for 5-HT) were obtained. No autosampler carryover was observed. The analytes were stable for 5 days at 20 °C, 14 days at 4 °C, and 30 days at -20 °C and five freeze-thaw cycles. The easy sample preparation, rapid LC, and multi-analyte MS detection allow two 96-well plates of samples to be extracted within 2 h and analyzed on an LC-MS/MS system within 24 h. The applicability and reliability of the assay were demonstrated by assessment of the reference interval for authentic urine specimens from 90 healthy individuals.

Keywords Monoamine neurotransmitter · Catecholamine · Serotonin · Phenylboronic acid-based solid phase extraction · Liquid chromatography-tandem mass spectrometry · Urine

Introduction

Monoamine neurotransmitters are neurotransmitters and neuromodulators that contain a characteristic amine group connected to an aromatic ring with a two-carbon chain. The major monoamine neurotransmitters consist of serotonin (5-HT) and catecholamines including norepinephrine (NE), epinephrine (E), and dopamine (DA). Their chemical structures are illustrated in Electronic Supplementary Material (ESM) Fig. S1. These biogenic amines play crucial roles in the control and regulation of diverse physiological processes both in the central and peripheral nervous systems. Disruption in neurotransmitter levels may result in various neurological, psychiatric, and endocrine diseases [1–4]. For example, highly elevated catecholamine levels are utilized in clinical diagnosis

Electronic supplementary material The online version of this article (doi:10.1007/s00216-017-0231-z) contains supplementary material, which is available to authorized users.

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of pheochromocytoma and neuroblastoma [5], and differences in catecholamine levels compared to healthy control subjects have been linked to Alzheimer's disease, attention deficit hyperactivity disorder, depression, anxiety, Parkinson's disease, and schizophrenia [6–8]. 5-HT is recognized as an important neurotransmitter in the regulation of mood, sleep, appetite, blood pressure, and sexuality. Dysfunction of the serotonergic system is implicated in numerous disorders, such as carcinoid syndrome, depression, hypertension, bipolar disorder, and migraine [2, 3, 9, 10]. In addition to the conventional roles, emerging research has suggested these biogenic amines play a vital role in the neural-immune communication between the nervous and immune systems [11, 12] and act as crucial modulators in the gastrointestinal system [13].

Considering the great clinical importance of the monoamine neurotransmitters and their value as biochemical biomarkers, the ability to accurately and efficiently measure their concentrations in biological samples would provide a meaningful tool for disease diagnosis and new therapeutic drug development. Various analytical methods with different detection techniques have been reported for the determination of catecholamines and 5-HT in biological fluids such as urine, plasma, peripheral blood mononuclear cells, and cerebrospinal fluid. However, these assays detected either catecholamines [14–20] or 5-HT [9, 21], not both. Several methods have been developed for utilizing microdialysate, a relatively clean matrix, that requires little or no protein precipitation prior to analysis [22–24].

Although a variety of methods and specimen types have been employed for the analysis of monoamine neurotransmitters, none have resulted in an efficient and effective tool for clinical utility. Urine has been increasingly utilized and preferred as a biological matrix in clinical routine testing due to its low cost, easy access, and noninvasive collection, which are additional important considerations for routine analysis [2, 25, 26]. In sharp contrast to the microdialysate, sample pretreatment for quantitative analysis in complex urine samples is demanding [27]. The marked differences between the chemical structure of 5-HT and catecholamines (ESM Fig. S1) pose great challenges for the simultaneous sample preparation, chromatographic separation, and detection of the biogenic amines. Additionally, the instability of the neurotransmitters limits choices for sample preparation. Furthermore, substantial endogenous concentration differences of the biogenic amines in human urine samples not only require a lower limit of quantitation (LLOQ) for detection of trace levels of E but also need a wide linear range to cover high levels of DA in a single analysis [15, 28].

To circumvent the difficulties, different sample preparation strategies and a wide range of detection methods have been explored for multi-analyte detection utilizing urine (Table 1). Gas chromatography (GC)-based mass spectrometry (MS) [29] and tandem mass spectrometry (MS/MS) detection [30]

required time-consuming and cumbersome sample preparation involving derivatization and solid phase extraction (SPE) or solid phase microextraction (SPME). Liquid chromatography (LC) coupled with electrochemical detection (ECD) [31], MS [33], and MS/MS with an atmospheric pressure chemical ionization (APCI) assay [27] lacked adequate sensitivity for monitoring low abundant E and NE in urine specimen. Non-chromatographic methods such as capillary electrophoresis (CE) have been coupled with different detectors for the rapid analysis of monoamine neurotransmitters [34, 35]; however, the drawbacks of requirement of relatively high urine sample (e.g., 2 mL) and insufficient sensitivity limited their widespread application in clinical laboratory. On the other hand, an online micro-solid phase extraction based on a boronate affinity monolithic column followed by LC-fluorescent detection offered a high sensitivity for NE and E [32], but the linear range of 2–50 ng mL⁻¹ was too narrow for detection of high levels of urinary DA, often resulting in reanalysis after sample dilution. In addition, an enrichment factor of only 17 for 5-HT compared to the enrichment capability of 223–243 for catecholamines reveals a poor extraction recovery of 5-HT [32].

Due to the low analyte concentration, a relatively high volume of urine specimen (0.6–2.0 mL) was typically required for analysis. Even a 10-mL urine sample was used to maximize the sensitivity [32]. This high sample volume hampered the utility of urine in routine testing. Additionally, to obtain a good recovery, the notable pH variability [36] of high-volume urine samples required labor-intensive and tedious pH adjustments as part of sample preparation. Furthermore, a fast analytical turnaround time is necessary to achieve a good throughput for routine analysis. An extended analytical run time per sample in the range of 19–75 min was commonly reported (Table 1), which substantially decreased the overall sample analysis speed. What's more, validation was either not performed or largely neglected in the literature for extraction recovery, matrix effect, interference, and reference interval. Last, but not least, method applicability was demonstrated in a very small population of authentic samples ($n \leq 6$). Taken together, despite advances in sample preparation and detection of urinary catecholamines and 5-HT, laborious sample preparation, low extraction recovery for 5-HT, insufficient sensitivity for E and NE, narrow linear range for DA, and extended analytical run time have often been associated with existing assays. Hence, there is demand to develop a simple and effective sample preparation combined with a sensitive and specific detection for the low abundant neurotransmitters in complex human urine.

Boronate affinity-based SPE techniques have been widely employed as a convenient means for the selective extraction of *cis*-diol-containing compounds such as saccharides, nucleosides, glycans, and glycoproteins from different complex matrices. The principle relied on the pH-controlled reversible

Table 1 Overview of the analytical performance of existing methods and comparison with the proposed method for the determination of catecholamines and serotonin in human urine

Sample preparation	Detection technique	Analyte	Linearity (ng mL ⁻¹)	LLOQ (ng mL ⁻¹)	Urine (μL)	Run time (min)	Reference
SPE (MCX cartridge) + Der	GC-MS	NE	10–1000	5.5	2000	19	[29]
		E	20–2500	17.8			
		DA	10–1000	6.3			
		5-HT	10–1000	2.3			
		NE	21–1000	21.3			
Der + SPME	GC-MS/MS	DA	10–1000	0.8	600	23	[30]
		5-HT	10–1000	0.7			
		NE	15–2000	15			
LPME	LC-ECD	E	15–2000	15	1000	75	[31]
		DA	15–2000	15			
		5-HT	20–2000	20			
		NE	3.0–50	0.8 ^a			
Filtration + online micro-SPE	LC-FL	E	2.0–50	0.5 ^a	10,000	21	[32]
		DA	2.0–50	0.5 ^a			
		5-HT	0.2–50	0.1 ^a			
		NE	20–2000	20			
DLLME	LC-MS	E	10–2000	10	1000	20	[33]
		DA	20–2000	20			
		5-HT	10–2000	10			
		NE	21.9–1000	21.9			
PPT + filtration + dilution	LC-MS/MS (APCI)	E	10.7–1000	10.7	NA	20	[27]
		DA	5.0–1000	5.0			
		5-HT	2.2–1000	2.2			
		NE	2.0–512	2.0			
SPE (PBA + HLB microplate)	LC-MS/MS (ESI)	E	1.0–512	1.0	10	6	This work
		DA	4.0–1024	4.0			
		5-HT	4.0–1024	4.0			

Der derivatization, SPME solid phase microextraction, LPME liquid phase microextraction, FL fluorescent detection, DLLME dispersive liquid–liquid microextraction, NA not available, PPT protein precipitation

^a LOD was used since the LLOQ was not reported

covalent interaction between the *cis*-diol-containing compound and boronic acid. We and others have previously demonstrated phenylboronic acid (PBA)-assisted SPE as an effective method for the biological sample extraction of catecholamine [14, 17, 20, 28, 37]. However, application of the previous SPE conditions for catecholamines resulted in poor extraction recovery of 5-HT, which lacked the essential *cis*-diol functional group for PBA complexation. This further revealed the difficulty of simultaneous analysis of these neurotransmitters, and a new optimization of SPE conditions was found necessary.

The objective of this study was to develop and validate an LC-MS/MS multi-analyte detection for catecholamines and 5-HT in human urine specimens incorporating a simple and effective sample extraction with minimal sample volume for clinical routine testing. To the best of our knowledge, an LC-MS/MS-based methodology has rarely been reported for this purpose, though this technology has been recognized as

superior to other common detection techniques in terms of sensitivity and specificity [25, 26]. An additional goal was to demonstrate the applicability and reliability of the method to a larger sample population in a clinical setting, which has scarcely been explored.

Experimental

Materials and chemicals

Catecholamine and serotonin standards were purchased from Cerilliant (Round Rock, TX, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively. Deuterium-labeled internal standards *d*₆-NE, *d*₃-E, *d*₄-DA, and *d*₄-5-HT were provided by CDN Isotopes (Pointe-Claire, Quebec, Canada). Blank urine for the preparation of calibrators and quality control (QC) samples was supplied by Golden West Biologicals

(Temecula, CA, USA). Endocrine urine controls at normal and pathological levels were purchased from Chromsystems (Gräfelfing, Munich, Germany). Water, water with 0.1% formic acid (HCOOH), methanol (MeOH), and acetic acid (HOAc) of LC-MS grade, 28% ammonium hydroxide solution (NH₄OH), ammonium chloride (NH₄Cl), and 2-aminoethyl diphenyl borate of analytical grade were provided by Sigma-Aldrich. Oasis HLB 96-well μ Elution plate (2 mg/30 μ m) from Waters (Milford, MA, USA) was employed for SPE.

LC-MS/MS apparatus and conditions

Liquid chromatography was conducted on an ultrafast liquid chromatography (UFLC-XR) system from Shimadzu Corporation (Columbia, MD, USA). The analytes of interest were separated on a Luna pentafluorophenyl (PFP) column (150-mm, 2.1-mm i.d., 3 μ m) with a guard column from Phenomenex (Torrance, CA, USA) operated at 30 °C. The temperature of the autosampler was maintained at 4 °C and the injection volume was 5 μ L. A gradient mobile phase with a flow rate of 0.45 mL min⁻¹ started with 95% mobile phase A (0.01% HCOOH in water) and 5% mobile phase B (0.01% HCOOH in MeOH). After sample injection, the percentage of mobile phase B was linearly increased from the initial 5 to 25% in 2.0 min. Subsequently, the percentage of mobile phase B was steeply increased to 95% within 0.1 min and held for 2.2 min. Finally, mobile phase B was rapidly reversed to the initial composition of 5% in 0.1 min and then maintained for 1.5 min for equilibration of the column. The total cycle time of a single run was 6.0 min. To prevent contamination of the MS detector, the effluents from the column before 0.6 min and after 4.5 min were diverted to waste.

MS/MS detection was on an AB Sciex Triple Quad 5500 mass spectrometer (Foster City, CA, USA) equipped with a turbo V ion spray source. The analyte quantification was conducted in positive ionization mode with two multiple reaction monitoring (MRM) transitions, including a quantification

transition and a confirmation transition. Analyst software version 1.6.2 from AB Sciex was employed for instrument operation, data acquisition, and calculation. Table 2 summarizes the optimal MRM transitions and instrument parameters.

Urine sample collection and clinical study population

Second morning urine sample was collected into a 5-mL tube containing 0.15 M HCl as preservative to avoid oxidation according to an in-house urine collection protocol [28, 38]. On collection day, the first urine was discarded and the second morning urine was collected 2 h after wake-up with overnight fasting. Urine samples were kept at -20 °C until analysis. Creatinine (Cr) concentration was measured by Roche COBAS INTEGRA 400 plus (CA, USA).

To determine the reference ranges for the monoamine neurotransmitters, second morning urine samples were collected from a cohort of an obviously healthy population ($n = 90$). This included 68 women and 22 men, ranging in age from 18 to 70 years with a median age of 45 years. The individuals provided informed consent and patient history forms. The clinical studies were approved by an internal clinical ethics committee.

Calibration standard and QC sample preparation

A mixed stock solution including 10 μ g mL⁻¹ of all four neurotransmitter calibrators was prepared in 0.2 M HOAc. The highest calibration standard concentration of 1024 ng mL⁻¹ was generated daily by spiking the mixed stock solution into 0.15 M HCl acidified blank urine, which was confirmed with no detectable analytes of interest, as shown in ESM Fig. S2. Subsequently, the calibration curves were established ranging from 0.25 to 1024 ng mL⁻¹ by serial dilution. A combined internal standard (IS) working solution containing *d*₆-NE (10 ng mL⁻¹), *d*₃-E (5 ng mL⁻¹), *d*₄-DA (20 ng mL⁻¹), and *d*₄-5-HT (20 ng mL⁻¹) was prepared by ten times dilution of a mixed IS stock in 0.2 M HOAc. The QC

Table 2 Optimal MRM transitions and instrument parameters

Analyte	Quantification transition (m/z)	Confirmation transition (m/z)	DP (V)	EP (V)	CE (eV)	CXP (V)
NE	152.0 > 107.0	152.0 > 135.0	110	10	23/23	10/12
E	166.0 > 107.0	166.0 > 135.0	130	10	27/21	10/12
DA	154.0 > 91.0	154.0 > 119.0	55	10	23/24	11/12
5-HT	177.0 > 160.0	177.0 > 115.0	46	10	31/35	12/12
<i>d</i> ₆ -NE	158.0 > 139.0	–	100	10	25	10
<i>d</i> ₃ -E	169.0 > 107.0	–	130	10	27	10
<i>d</i> ₄ -DA	158.0 > 95.0	–	55	10	32	11
<i>d</i> ₄ -5-HT	181.0 > 164.0	–	46	10	15	12

Electrospray ion voltage, 2000 V; temperature, 650 °C; nebulizer gas, 50 psi; heater gas, 55 psi; curtain gas, 35 psi; CAD gas, 8 psi

samples used for analytical validation containing LLOQ, low (LQC), medium (MQC), and high QC samples (HQC) were independently prepared by dilution with the acidified blank urine. The normal and pathological levels of endocrine urine controls from Chromsystems were constructed by addition of 8 mL water according to the manufacturer's guidance. All the stock solutions were stored at $-20\text{ }^{\circ}\text{C}$ until use.

SPE protocol

An Oasis HLB 96-well μ Elution extraction plate was activated by 200 μL MeOH followed by equilibration with 200 μL 0.2 M ammonium chloride aqueous solution at pH 8.5 (NH_4Cl solution, pH was adjusted to 8.5 with 28% NH_4OH). Ten microliters of urine sample, calibrator, or QC sample was mixed with 100 μL IS working solution, 50 μL 0.2% PBA solution at pH 8.5 (PBA solution), and 100 μL 1% NH_4OH solution. The sample mixture was slowly loaded to the pretreated extraction plate with mild positive pressure. After sample adsorption, three washes of 200 μL NH_4Cl solution were applied. The plate was completely dried before the addition of 100 μL 5% MeOH in 0.2 M HOAc solution to release the remaining analytes and ISs from the SPE cartridges. The eluate was subjected to LC-MS/MS analysis.

Assay validation

The proposed assay was validated under the guideline of LC-MS method validation approved by the Clinical and Laboratory Standards Institute (CLSI) [39].

To evaluate the linearity of the developed assay, 13 levels of non-zero calibrators ranging from 0.25 to 1024 ng mL^{-1} along with a blank (i.e., without analyte and IS) and a zero sample (i.e., without analyte but with IS) in acidified blank urine were freshly prepared, extracted, and analyzed by LC-MS/MS on 20 different days. The calibration curves were generated by plotting the peak area ratios of the analytes with respective ISs against the nominal concentrations of the calibrators. A linear regression of $y = ax + b$ with a weighting factor of $1/x$ was employed. The LLOQ was determined in the extracted blank urine as the lowest measured concentration with accuracy (recovery) within $\pm 20\%$ of the true value and precision expressed as coefficient of variation (CV) within $\pm 20\%$ and signal-to-noise ratio > 10 . The limit of detection (LOD) was defined as the lowest concentration with signal-to-noise ratio > 3 .

The intra- and inter-day assay precision and accuracy were assessed for the QC samples prepared in the acidified blank urine at LLOQ, low, medium, and high concentrations. Six replicates of the QC samples at four levels were freshly prepared, extracted, and analyzed by LC-MS/MS on three different days. Furthermore, analytical recovery studies were performed in six different urine samples spiked with three levels

of calibrators to support accuracy and precision. Due to the endogenous contribution of these neurotransmitters, analytical recovery was defined as a percentage of [(final concentration – endogenous concentration)/added concentration]. Finally, to assess short-term and long-term assay precision, a pooled urine sample and commercial urine controls from Chromsystems at normal and pathological levels were extracted and analyzed in six replicates on three different days and in duplicate on 20 different days, respectively. Accuracy was calculated as the percentage of the obtained results divided by the values provided by the supplier.

Statistical analyses

Statistical analyses were performed using GraphPad Prism 6 from GraphPad Software Inc. Reference intervals were assessed by EP Evaluator® Release 10 from Data Innovations LLC.

Results and discussion

Optimization of SPE protocol

It remains a challenging task to find an effective sample preparation suitable for both catecholamines and 5-HT in a single extraction due to the distinct chemical structure, as illustrated in ESM Fig. S1. A major limitation of the previously reported assays was the relatively low extraction efficiency [22, 25, 26, 32] and/or substantial matrix effect for some analytes [27, 30]. PBA-based SPE has been demonstrated to deliver a superior specificity than a variety of sample extraction approaches for catecholamines [14, 20, 28]. The principle of this procedure is based on the specific formation of a reversible covalent binding between the *cis*-diol functional group of catecholamines and PBA under a weak alkaline condition, which is strongly retained on the SPE sorbent. This allows effective removal of interfering substances in complex matrices by washing with an alkaline solution containing a substantial proportion of MeOH or acetonitrile. The complex is disrupted by an acid elution to release the free analytes for detection. However, poor recovery ($< 5\%$) for 5-HT was observed in a pilot study with the previously established SPE protocol for catecholamines (Fig. 1A) [37], revealing the difficulty of the simultaneous extraction and the necessity to re-optimize the SPE condition. To overcome these challenges, a few important parameters affecting SPE were studied in detail on a 96-well HLB μ Elution plate, including the washing solution, pH of the loading mixture, urine sample pH, and elution solution. To represent authentic urine samples, pooled urine samples were employed to optimize the SPE conditions. The peak area of IS instead of the analyte was used for evaluation due to the inherent endogenous contribution in urine samples. All the

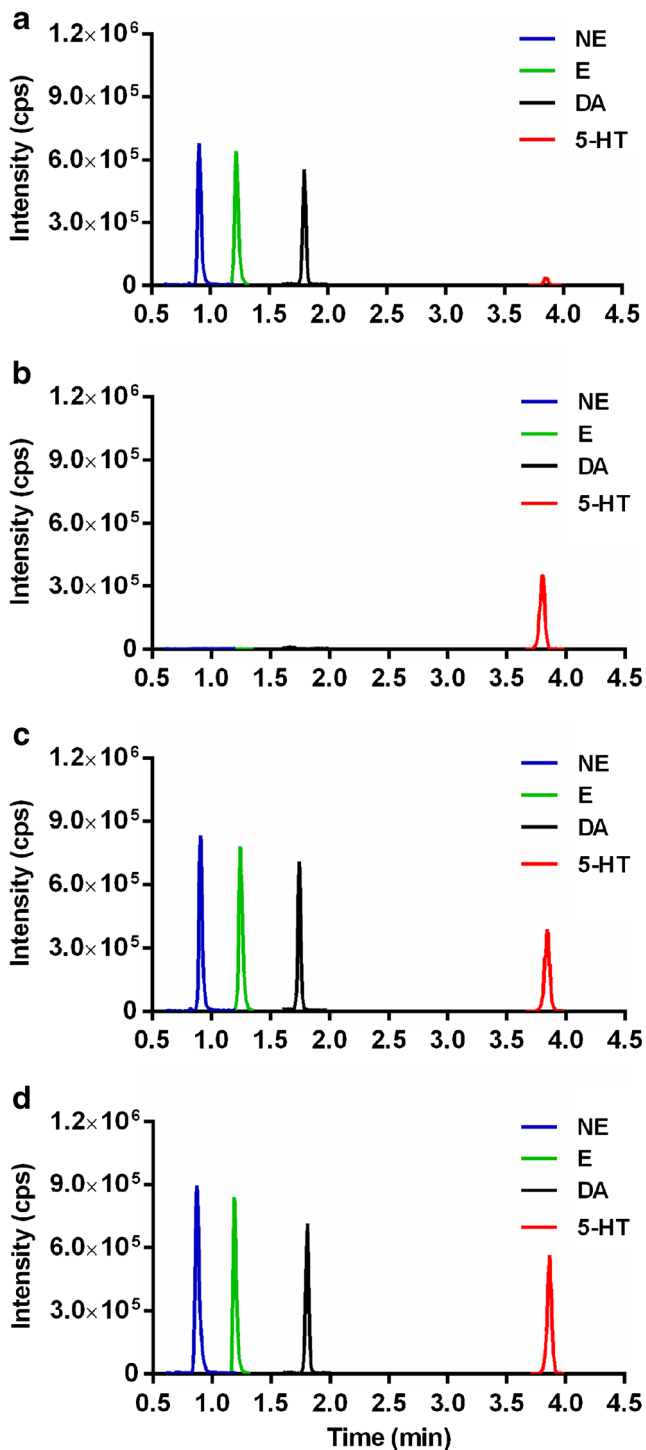


Fig. 1 MRM chromatograms of a standard solution at 100 ng mL^{-1} for each analyte under different conditions. **A** SPE with PBA complexation using 20% MeOH washes according to a previous work [37]. **B** SPE without PBA complexation using 0% MeOH washes. **C** Optimal SPE conditions of this work with PBA complexation using 0% MeOH washes. **D** Direct injection of the neat standard solution

optimization experiments were subjected to extraction and LC-MS/MS analysis in duplicate, unless specified.

Effect of washing solution

Preliminary monitoring of all the SPE fractions indicated that major 5-HT loss occurred during the washing step (data not shown). We hypothesized that 5-HT had poor retention with the HLB sorbent via weaker interactions compared to catecholamine with the formation of a viable PBA complex; thus, 5-HT was easily washed away with high content of MeOH washes. To improve the extraction efficiency of 5-HT, four washing solutions containing varied MeOH contents ranging from 20 to 0% were assessed. As illustrated in Fig. 2A, the decrease of MeOH composition from 20 to 0% yields a 64.1% rise of the mean extraction recovery of 5-HT from 6.6 to 70.7%, representing a profound effect of the selection of the washing solution. Substantial increased recovery has also been observed for NE and E by using a weaker washing solution (Fig. 2A), resulting in recoveries of 104.9 and 101.4% for NE and E compared to 78.4 and 89.9% with 20% MeOH washing, respectively. The organic content in the washing solution has little influence on the recovery of DA. According to these results, aqueous NH_4Cl solution without MeOH was selected as the washing solution for further optimization. To confirm the hypothesis, a comparison SPE experiment was performed under identical conditions, with the exception that the PBA solution was replaced with 50 μL of the alkaline NH_4Cl solution; thus, the PBA complex was not formed. As illustrated in Fig. 1B, C, comparable 5-HT is retained on the sorbent, whereas catecholamines almost flowed out rapidly without PBA complexation. This confirmation reveals that the established SPE protocol involves two different SPE mechanisms, that is, PBA-assisted strong covalent binding for catecholamines and non-PBA-associated weak interactions with the HLB sorbent for 5-HT.

Effect of sample pH

The pH value of the sample plays a pivotal role in the extraction efficiency of the sample pretreatment. A narrow pH range was typically required in the literature to obtain a good extraction recovery [14, 24, 28, 31]. Due to the considerable pH variability in individual urine samples [36] and the high urine volume needed for sample pretreatment, a tedious and time-consuming pH check and adjustment by acid/base to the required range was necessary. Figure 2B shows that a pH range from 7.0 to 9.5 is required to obtain a good extraction recovery of catecholamines, whereas a pH range of 8.5–9.5 is the most suitable for achieving high recovery for 5-HT. Therefore, pH in the narrow range of 8.5–9.5 was optimal to ensure satisfactory extraction efficiency. Nonetheless, the resulting pH of the sample mixture for loading remains constant at 8.9 despite urine samples covering a wide pH range from 4.5 to 8.5 (Fig. 2C). The results indicate that pH adjustment is not necessary for the proposed SPE procedure, thereby significantly shortening the SPE time and improving the overall sample

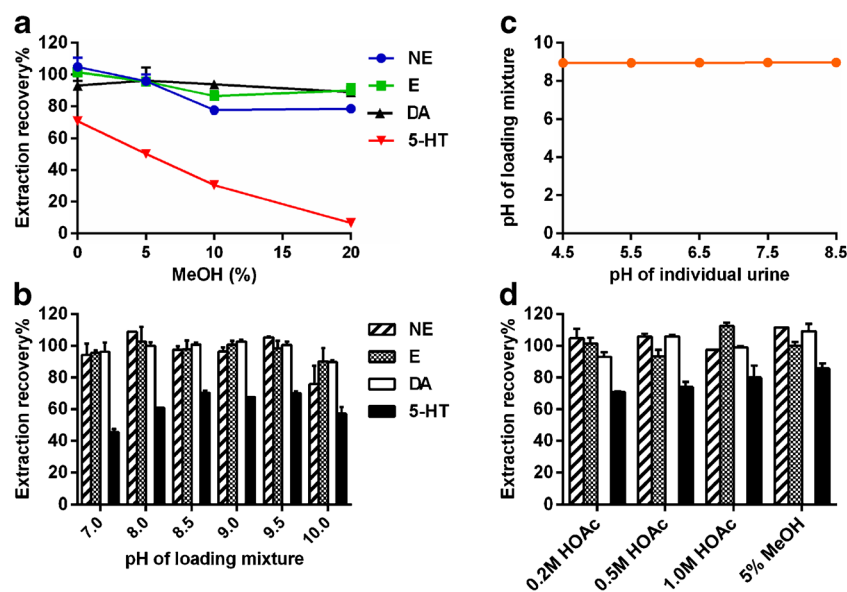


Fig. 2 Optimization of SPE parameters. **A** Effect of washing solution on the extraction recovery, three washes of 200 μ L NH_4Cl solution with 0–20% MeOH, sample mixture pH = 9.0, and elution with 100 μ L of 0.2 M HOAc solution. **B** Effect of pH value of the sample loading mixture on the extraction recovery, three washes of 200 μ L NH_4Cl solution, sample

mixture pH ranging from 7.0 to 10.0, and elution with 100 μ L of 0.2 M HOAc solution. **C** Effect of individual urine pH variability on the pH value of the loading mixture. **D** Effect of elution solution on the extraction recovery, three washes of 200 μ L NH_4Cl solution, sample mixture pH = 9.0, and elution with 100 μ L of 5% MeOH in 0.2 M HOAc

analysis speed. This is attributed to the drastic reduction in urine sample volume from typically over 1000 μ L to 10 μ L owing to the excellent sensitivity of the assay, thus minimizing the effect of pH variation derived from urine samples.

Effect of elution solution

To further enhance the extraction efficiency, the elution solution was explored through the use of a different concentration of HOAc and the addition of MeOH. Figure 2D exhibits the concentration of HOAc having negligible effect on the recovery of these neurotransmitters. However, the addition of 5% MeOH yields a substantial rise in the recovery of 5-HT while maintaining a good recovery of the catecholamines. Hence, 5% MeOH in 0.2 M HOAc was chosen as the optimal elution solution.

The optimal procedure has significantly enhanced the extraction efficiency of 5-HT, allowing the simultaneous extraction of catecholamines and 5-HT in complex biological matrices such as urine (Fig. 1). Additionally, the first-time use of 100% aqueous solution, rather than the common organic washes containing 20–50% MeOH or acetonitrile cited in the literature [14, 17, 20, 28], was an effective washing solution in PBA-associated SPE. This protocol substantially reduced the use of organic solvent and made it environment-friendly.

LC-MS/MS condition optimization

The analytical difficulty of accurately detecting catecholamines and 5-HT in a single analysis was due to the high sensitivity required for the detection of trace amounts of E

and NE, a wide linear range for DA, and a good selectivity for 5-HT. In this work, MS parameters were optimized for each compound and IS separately by infusion experiments, including precursor and product ions and different gas settings, source temperatures, and ion spray voltage. To achieve maximum signal intensity of E and NE, the water loss of protonated ion $[\text{M}+\text{H}-\text{H}_2\text{O}]^+$ was selected as the precursor instead of the commonly used protonated ion $[\text{M}+\text{H}]^+$. On the other hand, the popular $[\text{M}+\text{H}]^+$ was used as the precursor ion for DA and 5-HT, and the optimal CE value was decreased to cover a wide linear range up to 1024 ng mL^{-1} . To add another layer of selectivity for the assay, the ion ratios of quantification transition and the corresponding confirmation transition for each analyte should be less than 30%. It is worthy to point out that the selection of MRM transitions was critical for 5-HT. For example, ratios of 0.60 and 0.50 were observed in an authentic urine sample using MRM transitions of m/z 177.0 > 132.0 and m/z 177.0 > 117.0 as confirmation transitions for 5-HT, indicating a selectivity problem. ESM Figure S3 reveals that 5-HT quantitation is severely interfered with using these two confirmation MRM transitions. To overcome the difficulty, another MRM transition of m/z 177.0 > 115.0 was selected for confirmation and found to be interference-free (ESM Fig. S3), with an ion ratio of 0.80, consequently enhancing the reliability of quantification of 5-HT. The optimized precursor and product ions along with instrument settings are summarized in Table 2.

The technical difficulty of the chromatographic separation of these biogenic amines was derived from the poor retention of NE, relatively strong retention of 5-HT on a regular reversed-phase

column due to their distinct polarity, and the essential chromatographic separation of E from its isomer normetanephrine. This made it crucial to select an appropriate LC condition to maximize the retention of NE and elute 5-HT in a short time. In addition to using a HILIC column [17, 33], ion pair reagents, or laborious derivatization [16], a PFP column has been considered as an effective tool to enhance the chromatographic retention of the high polar catecholamines [15, 18, 20, 28]. However, it has not been applied to the simultaneous separation of catecholamines and 5-HT, to the best of our knowledge. Among several different PFP columns evaluated, including Luna PFP, F5, and Kinetex from Phenomenex, ultra PFPP from Restek, and Hypersil PFP from Thermo Scientific, the Luna PFP was the column of choice in terms of the retention of NE and 5-HT and signal intensity (data not shown). A gradient elution at 0.45 mL min⁻¹ flow with mobile phases containing 0.01% HCOOH in water and 0.01% HCOOH in MeOH was found to be the best elution condition. Under the optimal conditions described in “LC-MS/MS apparatus and conditions,” NE demonstrated reasonably good retention at 0.95 min, and 5-HT was eluted within 4.0 min, enabling a short run of 6.0 min including an equilibration (Fig. 1D).

Method validation

Extraction recovery and IS-normalized recovery

Extraction recovery is not only a vital parameter affecting the sensitivity of a quantitative assay, but it also strongly influences assay reliability, which is of paramount importance in establishing a robust assay for routine testing. It remains a difficult task to achieve satisfactory extraction recovery for the multi-analyte detection of catecholamines and 5-HT in

complex biological samples due to their distinct physicochemical properties [22, 25, 26, 32].

To assess the extraction efficiency, six random human urine samples were spiked with the mixed calibrators at low, medium, and high concentration levels before and after extraction. Non-spiked samples were analyzed in the same batch to determine the endogenous concentration. The extraction recovery in percentage was calculated using the following equation: Extraction recovery (%) = [(Peak area of pre-extraction spiked sample – Peak area of endogenous sample)/(Peak area of post-extraction spiked sample – Peak area of endogenous sample)] × 100. Table 3 exhibits the good recovery values achieved for catecholamines, with mean recoveries in the range of 85.2–103.7% covering the low, medium, and high concentrations. Of note is that the 69.6–72.4% extraction recovery for 5-HT is obtained under the optimal conditions, representing a significant improvement compared to the low recovery (e.g., 7%) [32] previously reported [22, 25, 26]. Additionally, the IS-normalized extraction recovery is compensated by the corresponding IS, as indicated by the notably enhanced mean recovery ranging from 93.9 to 106.0% for 5-HT and from 96.6 to 110.5% for catecholamines.

Matrix effect and IS-normalized matrix effect

Matrix effect, another important aspect influencing the reliability of quantitative LC-MS analysis in complex biological samples, has been largely neglected or not quantitatively assessed in the analysis of catecholamines and 5-HT in existing assays [29–33]. In this work, the matrix effect of the proposed method was evaluated at three concentration levels using the post-extraction method described by Matuszewski et al. [40]. Briefly, extracts of six random human urine

Table 3 Extraction recovery (peak area), IS-normalized recovery, and analytical recovery in urine samples (*n* = 6)

Analyte	Added (ng mL ⁻¹)	Extraction recovery			IS-normalized recovery			Accuracy		
		Mean (%)	SD	CV (%)	Mean (%)	SD	CV (%)	Mean (%)	SD	CV (%)
NE	10	94.6	13.5	14.2	106.4	13.7	12.9	92.6	8.3	9.0
	40	86.0	7.1	8.3	97.1	6.8	7.0	97.3	6.6	6.7
	400	87.3	6.7	7.7	96.6	2.1	2.2	96.2	4.6	4.8
E	10	89.0	12.2	13.7	107.4	11.0	10.2	104.6	5.8	5.5
	40	85.2	14.4	16.9	101.8	8.8	8.6	102.5	4.5	4.4
	400	93.7	15.4	16.4	102.8	9.8	9.6	100.9	5.7	5.6
DA	40	103.7	19.2	18.5	110.5	18.4	16.7	103.0	11.6	11.2
	400	95.8	14.8	15.4	108.3	16.3	15.0	100.9	4.3	4.2
	800	88.7	9.8	11.0	106.9	14.3	13.4	99.9	2.1	2.1
5-HT	40	69.6	6.6	9.5	93.9	6.8	7.3	90.0	7.7	8.6
	400	71.7	5.2	7.2	103.0	9.0	8.7	102.8	4.3	4.2
	800	72.4	8.0	11.0	106.0	9.6	9.0	105.1	6.7	6.3

Table 4 Matrix effect and IS-normalized matrix effect assessed in random urine samples ($n = 6$)

Analyte	Added (ng mL^{-1})	Matrix effect			IS-normalized matrix effect		
		Mean (%)	SD	CV (%)	Mean (%)	SD	CV (%)
NE	10	90.7	4.5	5.0	108.4	7.1	6.5
	40	90.4	4.4	4.9	105.8	5.9	5.6
	400	87.1	6.5	7.5	110.9	4.8	4.4
E	10	106.3	9.4	8.9	106.2	4.1	3.9
	40	106.7	4.9	4.6	104.6	2.6	2.5
	400	91.7	5.1	5.5	104.5	3.6	3.4
DA	40	113.1	8.3	7.4	113.9	3.9	3.4
	400	103.7	2.5	2.5	105.1	2.7	2.6
	800	101.2	11.6	11.5	106.8	1.0	1.0
5-HT	40	67.5	6.1	9.1	107.0	6.3	5.9
	400	63.6	7.9	12.4	100.3	3.9	3.9
	800	71.4	11.7	16.4	103.0	5.8	5.6

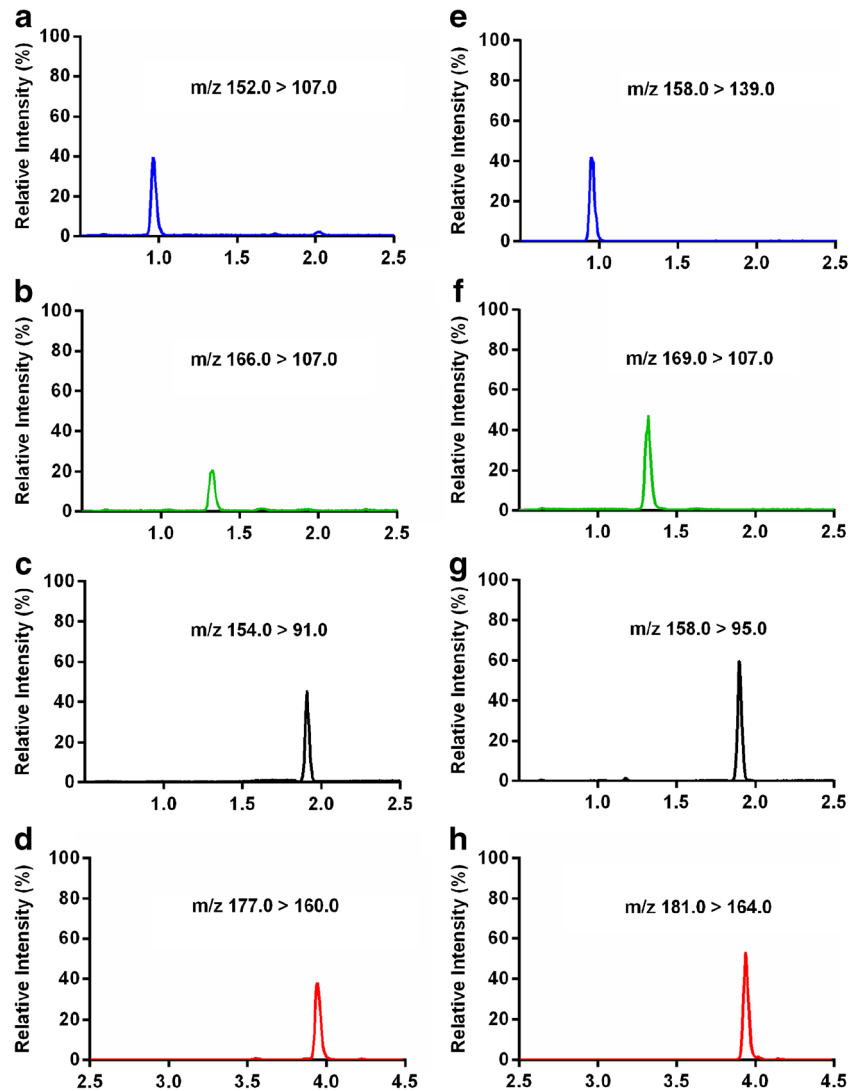
Fig. 3 MRM chromatograms of the monoamine neurotransmitters (left panels) and ISs (right panels) in a real urine sample from a healthy individual. **A** NE quantifier m/z 152.0 > 107.0, 14.7 ng mL^{-1} . **B** E quantifier m/z 166.0 > 107.0, 5.4 ng mL^{-1} . **C** DA quantifier m/z 154.0 > 91.0, 123.0 ng mL^{-1} . **D** 5-HT quantifier m/z 177.0 > 160.0, 54.3 ng mL^{-1} . **E** d_6 -NE. **F** d_3 -E. **G** d_4 -DA. **H** d_4 -5-HT

Table 5 Linearity, LOD, LLOQ, and calibration curve characteristics ($n = 20$)

Analyte	Linearity (ng mL ⁻¹)	LOD (ng mL ⁻¹)	LLOQ (ng mL ⁻¹)	Slope			Intercept Mean	r Mean
				Mean	SD	CV (%)		
NE	2.0–512	1.0	2.0	0.0612	0.0029	4.7	0.0288	0.9993
E	1.0–512	0.25	1.0	0.0229	0.0008	3.5	0.0019	0.9997
DA	4.0–1024	2.0	4.0	0.0500	0.0002	3.6	-0.0029	0.9994
5-HT	4.0–1024	2.0	4.0	0.0015	0.0001	3.4	0.0024	0.9992

samples were spiked with the mixed calibrators at low, medium, and high concentration levels along with ISs. Six neat solutions per level were prepared at the same three concentrations. The matrix effect was expressed as percentage using the following calculation: Matrix effect (%) = [(Peak area of post-extraction spiked sample - Peak area of endogenous sample) / Peak area of standard solution] × 100. As can be seen in Table 4, the mean matrix effects for NE, E, and DA are from 87.1 to 113.1% at the three levels, suggesting negligible matrix effect for the catecholamine measurement. However, a matrix effect of 63.6–71.4% for 5-HT indicated some degree of ion suppression. To further assess whether the matrix effect can be corrected by the use of deuterated ISs, IS-normalized matrix effect was determined by normalizing the corresponding IS peak area. Table 4 illustrates the drastically reduced matrix effect for 5-HT, as revealed by the relative mean matrix effect of 100.3–107.0%. This indicates that the ion suppression is well compensated by the use of IS; thereby, the quantitation is not affected. This is a unique feature of the MS/MS technology over other bioanalytical detections. This is the first

report in which a vicinal *cis*-hydroxyl group was deemed essential, that urinary catecholamines and 5-HT can be purified by the PBA-assisted SPE in a simple extraction with high extraction recovery and mild matrix effect. The significantly improved SPE performance is partially attributed to the ten times dilution of the small urine sample volume (i.e., loading only 10 µL urine and eluting in 100 µL solution) compared to high enrichment of a large volume of urine to maximize the concentration [29, 32, 34].

Specificity

Numerous drugs and metabolites, including labetalol, acetaminophen, and methyl dopa, have been claimed to interfere with the analysis of catecholamine and 5-HT by traditional assays (e.g., immunoassay, fluorescent, and ECD) [10, 14, 20, 26]. However, investigation into interference has been largely neglected or limited to a few potentially interfering compounds [27, 29–33]. An interference study was conducted to evaluate a wide coverage of 47 substances, including major

Table 6 Intra- ($n = 6$) and inter-day ($n = 3$) assay precision and accuracy for spiked QC samples

Analyte	Sample	Added (ng mL ⁻¹)	Intra-day				Inter-day			
			Mean (ng mL ⁻¹)	SD	CV (%)	Accuracy (%)	Mean (ng mL ⁻¹)	SD	CV (%)	Accuracy (%)
NE	LLOQ	2.0	1.9	0.2	8.0	95.8	1.9	0.1	5.6	93.3
	LQC	4.0	4.0	0.2	3.8	100.7	4.0	0.0	1.2	100.1
	MQC	20	20.8	0.6	3.0	104.0	20.5	0.4	1.7	102.6
	HQC	400	404.8	11.0	2.7	101.2	412.8	18.3	4.4	103.2
E	LLOQ	1.0	1.0	0.0	2.3	96.0	1.0	0.0	1.4	94.9
	LQC	4.0	3.9	0.1	2.2	98.1	4.0	0.1	2.2	100.6
	MQC	20	20.1	0.4	2.0	100.3	20.1	0.3	1.2	100.4
	HQC	400	398.7	10.0	2.5	99.7	400.2	4.7	1.2	100.1
DA	LLOQ	4.0	3.8	0.2	4.2	95.2	3.9	0.1	3.8	97.9
	LQC	20	18.9	0.6	3.1	94.7	19.4	0.5	2.4	96.8
	MQC	400	406.0	9.4	2.3	101.5	405.6	2.8	0.7	101.4
	HQC	800	781.0	16.1	2.1	97.6	787.1	5.6	0.7	98.4
5-HT	LLOQ	4.0	3.6	0.1	3.8	89.5	3.5	0.2	4.9	87.6
	LQC	20	20.7	0.7	3.5	103.3	19.9	1.0	5.2	99.7
	MQC	400	414.3	15.5	3.7	103.6	401.4	12.8	3.2	100.3
	HQC	800	791.5	13.7	1.7	98.9	786.6	6.9	0.9	98.3

urinary components, structurally related metabolites, and common drugs or supplements at high clinical concentrations (ESM Table S1) according to CLSI Protocol EP7-A2. Pooled urine samples were spiked with potentially interfering substances at their specific levels and were extracted and analyzed by LC-MS/MS together with the non-spiked pooled urine samples. The acceptance criterion of percent bias was set at $\pm 15\%$, which was calculated as the difference of the concentrations measured in the spiked samples vs. non-spiked urine samples divided by the non-spiked urine samples. The Bias% was $\leq 12.4\%$ for all the tested interfering substances at the described concentrations, revealing that the proposed assay was not substantially affected by the wide range of interfering compounds. Additionally, representative MRM chromatograms of a urine specimen from a healthy individual are demonstrated in Fig. 3. As can be seen, there are no interfering peaks at the retention times of the analytes and ISs, suggesting the high degree of specificity of the developed assay.

Linearity, LOD, and LLOQ

The proposed assay exhibits good linearity ($n = 20$) for the four monoamine neurotransmitters in their respective concentration ranges, as shown in Table 5. Greater than 0.9992

correlation coefficient (r) and a standard deviation (SD) less than 0.0029 with CV less than 4.7% are obtained for all the slopes of calibration curves. The LODs for NE, E, DA, and 5-HT are 1.0, 0.25, 2.0, and 2.0 ng mL^{-1} with corresponding LLOQs of 2.0, 1.0, 4.0, and 4.0 ng mL^{-1} , respectively. The proposed assay enables multi-analyte detection of the panel of urinary neurotransmitters in a single run owing to the high sensitivity for E and NE and the wide linearity range for DA and 5-HT of 4.0–1024 ng mL^{-1} , which have rarely been reported [27, 29–33].

Precision and accuracy

Table 6 illustrates the intra- and inter-day assay precisions of ≤ 8.0 and $\leq 5.6\%$, respectively, for the spiked QC samples based on the analysis of six replicates at LLOQ, low, medium, and high levels on 3 days. The intra- and inter-day assay accuracy, assessed by analytical recovery, ranged from 89.5 to 104.0% and from 87.6 to 103.2%, respectively. In addition, the precision and accuracy of the assay are supported by mean analytical recoveries of 90.0–105.1% with a mean precision $\leq 11.2\%$ in the spike experiment of six random urine samples (Table 3).

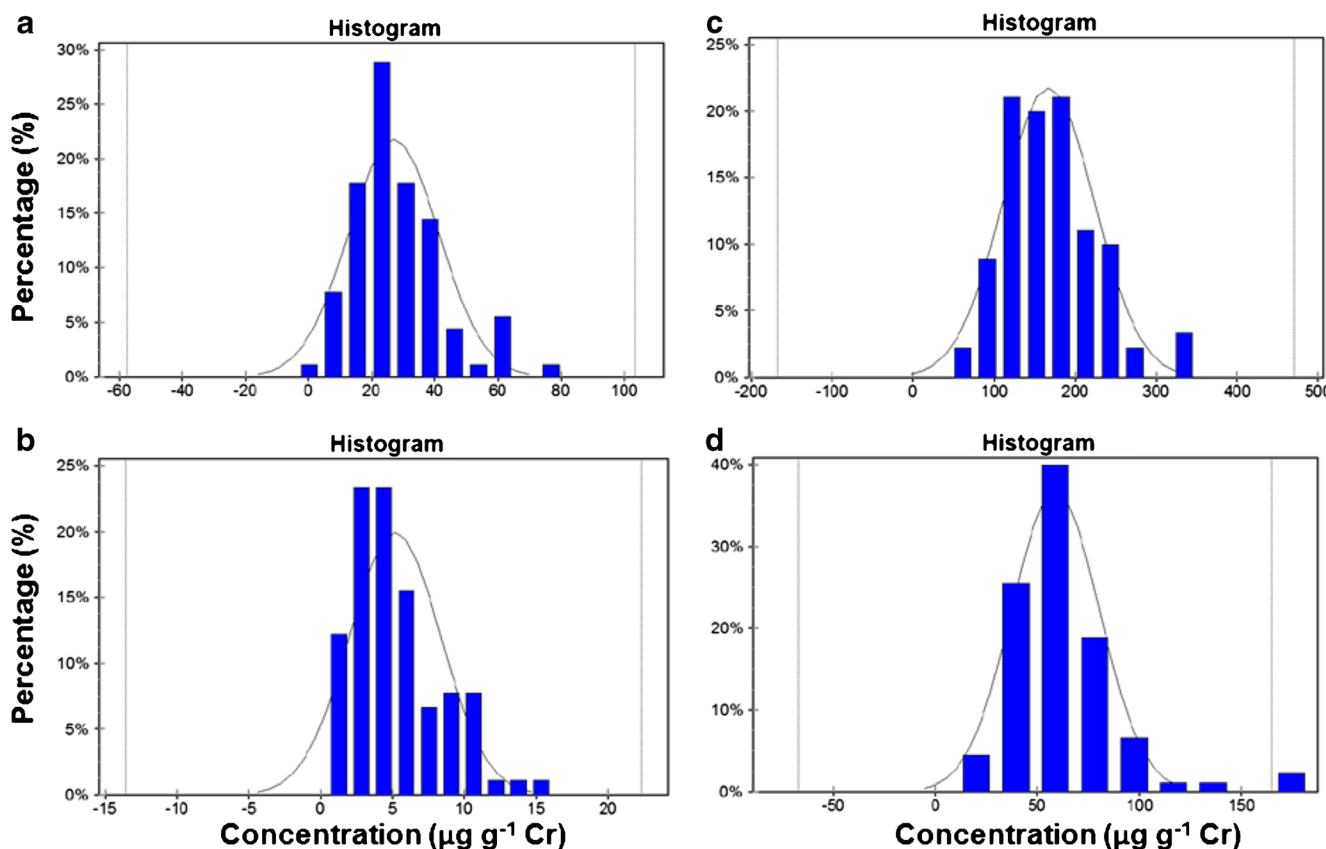


Fig. 4 Distributions of the urinary concentrations of catecholamines and serotonin in reference samples (95th percentile) of a healthy population ($n = 90$). The concentration distributions of individual monoamine

neurotransmitters are illustrated in histograms, with mean values of 27.8 $\mu\text{g g}^{-1}$ Cr for NE (A), 5.3 $\mu\text{g g}^{-1}$ Cr for E (B), 169.2 $\mu\text{g g}^{-1}$ Cr for DA (C), and 63.2 $\mu\text{g g}^{-1}$ Cr for 5-HT (D)

ESM Table S2 exhibits the corresponding short-term intra- and inter-day assay precisions for the pooled urine and Chromsystems normal and pathological urine controls determined in six replicates on 3 days of ≤ 6.0 and $\leq 5.5\%$, respectively. Moreover, a long-term precision $\leq 9.7\%$ is obtained for the three QC samples and an accuracy of 80.1–105.5% is achieved for the Chromsystems QC samples on 20 different days compared to the values provided by the manufacturer (ESM Table S3). These data provide additional support for the precision, accuracy, and robustness of the assay, which are critical for routine testing.

Autosampler carryover

No autosampler carryover effect was observed when sequential injections of an extracted highest standard urine sample were followed by a blank urine sample.

Analyte stability

The stability of the neurotransmitters in urine samples was assessed after storage under different temperatures, including 5 days at 20 °C, 14 days at 4 °C, and 30 days at –20 °C. Two levels (i.e., low and high) of fresh urine samples were aliquoted and analyzed in duplicate after different storage conditions using the proposed assay. Results were compared against the freshly prepared samples. Freeze-thaw stability was also evaluated after five cycles of freezing and thawing. Additionally, stability of post-extraction in 4 °C autosampler was investigated 48 h after the first analysis. As can be seen in ESM Table S4, a change of $\leq 14.9\%$ obtained for all the analytes indicates that the analytes are stable under these storage conditions.

Application of the method to real biological samples

To evaluate the applicability and reliability of the proposed method in real urine samples, the concentrations of four monoamine neurotransmitters were measured in a cohort of healthy population ($n = 90$). Figure 4 exhibits the distributions of the urinary concentrations of monoamine neurotransmitters in the 90 healthy individuals as normal. The parametric reference intervals (95th percentile) were found to be 7.3–64.2 $\mu\text{g g}^{-1}$ Cr (4.3–102.6 ng mL^{-1}) with a mean of 27.8 $\mu\text{g g}^{-1}$ Cr for NE, 1.1–13.3 $\mu\text{g g}^{-1}$ Cr (0.8–17.9 ng mL^{-1}) with a mean of 5.3 $\mu\text{g g}^{-1}$ Cr for E, 80.0–296.7 $\mu\text{g g}^{-1}$ Cr (54.1–565.1 ng mL^{-1}) with a mean of 169.2 $\mu\text{g g}^{-1}$ Cr for DA, and 25.0–132.1 $\mu\text{g g}^{-1}$ Cr (13.4–207.2 ng mL^{-1}) with a mean of 63.2 $\mu\text{g g}^{-1}$ Cr for 5-HT. Of note is that these values agree with those previously reported [9, 15, 28], supporting the reliability of the developed assay despite the markedly reduced sample volume and increased throughput.

Conclusions

The present study describes the development and validation of a simple, fast, green, robust, sensitive, and specific LC-MS/MS method for the simultaneous quantitation of serotonin and catecholamines in human urine with low cost. Compared to the existing methods, the novel aspects of the developed assay include a dramatically reduced sample volume (10 vs. 1000+ μL), easy sample preparation, rapid LC (6 vs. 19–75 min), and multi-analyte MS detection of four monoamine neurotransmitters in a single analysis without the use of derivatization, evaporation, and reconstitution, ion-pairing reagents, and HILIC column. The proposed assay allows two 96-well plates of samples to be extracted within 2 h and analyzed on an LC-MS/MS system within 24 h. The solid validation results together with the successful application of the proposed assay in 90 authentic urine specimens indicate the applicability and reliability of the assay for clinical routine testing. Considering the noninvasive and inexpensive urine sampling, the markedly improved method provides a new strategy for the analysis of neurotransmitters in a clinical laboratory for basic neuroscience research and drug development.

Acknowledgements The authors acknowledge Pheng Yang for providing technical assistance and Melissa Ahrens for critical reading of the manuscript.

Compliance with ethical standards

Ethics approval All clinical studies involving human urine samples were conducted in adherence to the procedure approved by Pharmasan/Neuroscience Institutional Review Board (IRB).

Conflict of interest The authors declare that they have no conflict of interest.

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