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Pre-analytical and analytical validations and clinical applications of a miniaturized, simple and cost-effective solid phase extraction combined with LC-MS/MS for the simultaneous determination of catecholamines and metanephrines in spot urine samples

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ABSTRACT

It remains a challenge to simultaneously quantify catecholamines and metanephrines in a simple, sensitive and cost-effective manner due to pre-analytical and analytical constraints. Herein, we describe such a method consisting of a miniaturized sample preparation and selective LC-MS/MS detection by the use of second morning spot urine samples. Ten microliters of second morning urine sample were subjected to solid phase extraction on an Oasis HLB microplate upon complexation with phenylboronic acid. The analytes were well-resolved on a Luna PFP column followed by tandem mass spectrometric detection. Full validation and suitability of spot urine sampling and biological variation were investigated. The extraction recovery and matrix effect are 74.1–97.3% and 84.1–119.0%, respectively. The linearity range is 2.5–500, 0.5–500, 2.5–1250, 2.5–1250 and 0.5–1250 ng/mL for norepinephrine, epinephrine, dopamine, normetanephrine and metanephrine, respectively. The intra- and inter-assay imprecisions are $\leq$9.4% for spiked quality control samples, and the respective recoveries are 97.2–112.5% and 95.9–104.0%. The Deming regression slope is 0.90–1.08, and the mean Bland-Altman percentage difference is from −3.29 to 11.85 between a published and proposed method (n=50). A correlation observed for the spot and 24 h urine collections is significant (n=20, $p<0.0001$, r: 0.84–0.95, slope: 0.61–0.98). No statistical differences are found in day-to-day biological variability (n=20). Reference intervals are established for an apparently healthy population (n=88). The developed method, being practical, sensitive, reliable and cost-effective, is expected to set a new stage for routine testing, basic research and clinical applications.

1. Introduction

Catecholamines and metanephrines, including norepinephrine (NE), epinephrine (E), dopamine (DA), normetanephrine (NMN) and metanephrine (MN) (see Supplementary Fig. 1 for their chemical structures), play vital roles in various physiological processes and regulations of the immune system [1,2]. Besides the diagnostic screening for pheochromocytoma and neuroblastoma [3,4], compelling evidence has indicated imbalanced catecholamines and metanephrines are associated with a wide range of neurological and autoimmune disorders, such as Alzheimer's disease, depression, attention-deficit hyperactivity disorder (ADHD), schizophrenia, Parkinson's disease, anxiety and rheumatoid arthritis [2,5–9]. Hence, measurement of these analytes in biological fluids will be of great clinical importance to support disease monitoring and new therapeutic drug development [10–13]. Due to difficult collection of tissues, and supine rest constraint of blood draw for plasma, urine has been recommended as an alternative specimen for the analysis owing to its non-invasive and convenient collection [10,14–16]. Among a wide range of methodologies with different detectors applied for the measurement of urinary catecholamines and metanephrines [6,12,17,18], liquid chromatography-tandem mass spectrometry (LC-MS/MS) has gradually taken the center stage due to its inherent selectivity and sensitivity [19–26].

Although LC-MS/MS offers considerable improvements over

Abbreviations: SPE, solid phase extraction; PBA, phenylboronic acid; NE, norepinephrine; E, epinephrine; DA, dopamine; NMN, normetanephrine; MN, metanephrine; ADHD, attention-deficit hyperactivity disorder; LC-MS/MS, liquid chromatography-tandem mass spectrometry; IS, internal standard; ESI, electrospray ionization; MRM, multiple reaction monitoring; Cr, Creatinine; QC, quality control; LLOQ, lower limit of quantification; NH_4Cl solution, 0.2 M ammonium chloride aqueous solution at pH 8.5; CLSI, Clinical and Laboratory Standards Institute; LOD, lower limit of detection; r, correlation coefficient; SD, standard deviation; CV, coefficient of variation

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other technologies, several pre-analytical and analytical constraints make its use cumbersome and impractical. In the pre-analytical perspective, the predominant sampling method for urinary catecholamine and metanephrine analysis is to collect urine over a 24 h period, which is tedious and may introduce errors by improper collection. Spot urine collection at different times of the day has been proposed as an alternative sampling solution in view of its convenience and practicability [10,27]. However, distinct diurnal excretion of these compounds impedes the application of spot urine collection [22,28,29]. In the analytical point of view, it remains a challenge to simultaneously quantify these analytes in a simple, sensitive, cost-effective and high-throughput manner due to low excretion, high polarity, poor ionizability and instability. An effective sample cleanup for the complex urine matrix prior to analysis is crucial. Liquid-liquid extraction [19,25] and solid phase extraction (SPE) [17,20–24] have become the popular choices among other techniques [12,30]. It is noteworthy that the pH of the pre-treated urine sample was crucial for extraction recovery in the reported assays. A high volume of urine (e.g., 0.5–1.0 mL) generally required and a wide pH variation (i.e., 4.5–8.5) of individual urine samples [31] caused substantial pH differences for the pre-treated urine sample. Hence, to achieve a reliable extraction, it was cumbersome to adjust pH of each sample to a narrow pH range. For example, a liquid-liquid extraction needed a pH of 8.5–9.5 prior to LC-MS/MS analysis [19,25]; SPE using Oasis WCX, the most common cleanup of these analytes, required a narrow pH range of 6–7 [23,26,32].

Additionally, the instability of catechol moiety in strong basic conditions makes the pH adjustment more risky and prone to errors. A sample cleanup with combined SPE on Oasis HLB sorbent and PBA complexation was demonstrated to effectively improve the stability through the instant formation of a stable catecholamine-PBA complex under alkaline condition [12,21,24]. However, pH adjustment for all the samples to 7.5–9.5 remained to obtain a good extraction efficiency. The laborious sample preparation for the available assays adversely affected the speed and throughput of the assay, which has become the bottleneck of routine testing [33]. Furthermore, sample cleanup with regular SPE sorbent required substantial consumption of isotopically-labeled internal standard (IS), reagent and solvent, which can be improved by SPE using a microplate with smaller sorbent (2 mg vs 30 mg regular sorbent).

Although various assays have been reported for the detection of urinary catecholamines or metanephrines, the majority of these methods were designed for either catecholamines [17–19,22,24] or metanephrines [20,34], not both. Simultaneous LC-MS/MS analysis would not only reduce costs and improve throughput for routine testing, but also facilitate clinical interpretation. Available assays either lacked sensitivity to monitor low levels of E [21,26] or a dynamic range to directly measure high concentrations of DA and metanephrines in a single run [23]. Additionally, it was a difficult task to obtain good extraction recovery and minimal matrix effect for all the analytes [12,26,32].

To overcome these challenges, we aimed to develop and validate an analytical method for the simultaneous quantitation of catecholamines and metanephrines by employing a simple, specific and miniaturized SPE of minimal urine sample without the tedious pH adjustment. Another goal of this study was to evaluate the spot collection of the second morning urine sample as an alternative sampling to 24 h urine collection and determine reference intervals. The proposed multiplexing method, being simple, sensitive, cost-effective and high-throughput is expected to be of great interest for basic research, clinical study and routine testing.

2. Materials and methods

2.1. Materials and chemicals

Catecholamine and metanephrine standard solutions were provided by Cerilliant (Round Rock, Texas, USA). Isotopically-labeled internal standards (IS) d_6-NE, d_3-E, d_4-DA, d_3-NMN and d_3-MN were purchased from CDN Isotopes (Pointe-Claire, Quebec, Canada). Endocrine urine controls were obtained from Chromsystems (Gräfelfing, Munich, Germany). Mass Spect Gold® Urine from Golden West Biologicals (Temecula, CA, USA) was used as blank urine containing no detectable analytes. Oasis HLB 96-well μElution plate (2 mg/30 μm) was obtained from Waters (Milford, MA, USA). LC-MS grade of water, water with 0.1% formic acid (HCOOH), methanol (MeOH) and acetic acid (HOAc), analytical grade of 28% ammonium hydroxide solution (NH_4OH), ammonium chloride (NH_4Cl) and 2-aminoethyl diphenyl borate were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. LC-MS/MS conditions

Chromatographic measurement was performed on a Shimadzu Prominent UFLC-XR system (Columbia, MD, USA) equipped with a Luna PFP (2) column (150 mm, 2.1 mm i.d., 3 μm) guarded with a pre-column from Phenomenex (Torrance, CA, USA). Analyte detection was performed by an AB Sciex Triple Quad 5500 mass spectrometer (Foster City, CA, USA) in positive electrospray ionization (ESI) mode with multiple reaction monitoring (MRM). The column oven was kept constant at 30 °C and the auto sampler was set at 4 °C. The analytes were eluted by gradient mobile phases containing 0.01% HCOOH in water (Mobile phase A) and 0.01% HCOOH in MeOH (Mobile phase B). After 5 μL of sample was loaded on the column, the initial LC gradient of 5% B was linearly increased to 35% within 2.5 min. Then, the gradient was quickly ramped to 95% B in 0.1 min and held for 1.2 min; finally the column was equilibrated at 5% B for 1.7 min. The flow rate was 0.45 mL/min and the cycle time was 5.5 min

Only the LC effluent from 0.6 min to 4.0 min was subjected to the mass spectrometer utilizing a diverter valve. Analyst software version 1.6.2 from AB Sciex was used for instrument control, data acquisition and analysis. The ion spray voltage was optimized to 2000 V at 650 °C and curtain gas was maintained at 35 psi. The nebulizer gas was set at 50 psi, while the heater gas was maintained at 55 psi and CAD gas was kept at 8 psi. The MRM transitions and compound parameters were individually optimized and are summarized in Table 1.

Table 1
Optimized MRM parameters.

Analyte	MRM transition (*m/z*)	DP (V)	EP (V)	CE (eV)	CXP (V)
NE (quantifier)	152.0 > 107.0	110	10	23	10
NE (qualifier)	152.0 > 135.0	110	10	23	12
d_6-NE	158.0 > 139.0	101	10	25	10
E (quantifier)	166.0 > 107.0	130	10	27	10
E (qualifier)	166.0 > 135.0	130	10	21	12
d_3-E	169.0 > 107.0	130	10	27	10
DA (quantifier)	154.0 > 91.0	55	10	32	11
DA (qualifier)	154.0 > 119.0	55	10	24	12
d_4-DA	158.0 > 95.0	55	10	32	11
NMN (quantifier)	166.0 > 121.0	120	10	23	15
NMN (qualifier)	166.0 > 106.0	95	10	25	10
d_3-NMN	169.0 > 137.0	95	10	23	12
MN (quantifier)	180.0 > 165.0	121	10	23	16
MN (qualifier)	180.0 > 148.0	121	10	23	15
d_3-MN	183.0 > 151.0	130	10	25	24