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RESEARCH ARTICLE

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A dilute-and-shoot liquid chromatography–tandem mass spectrometry method for urinary 18-hydroxycortisol quantification and its application in establishing reference intervals

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Abstract

Background: Eighteen-hydroxycortisol (18-OHF) is a potential biomarker for differential diagnosis of the two major primary aldosteronism subtypes, aldosterone-producing adenoma, and idiopathic hyperaldosteronism.

Methods: Urine samples were processed, and the 18-OHF in urine samples were successfully quantified by in-house established dilute-and-shoot liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. Separation was accomplished on a Sigma Ascentis Express C18 column with a gradient mixture of phase (A) 0.2% formic acid in water and phase (B) 0.2% formic acid in methanol at a flow rate of 0.4 ml/min. Mass spectrometric detection was performed in positive electrospray ionization mode via a mass spectrometer.

Results: The linearity of urinary 18-OHF ranged from 4.28 to 8.77×10^3 nmol/L, with a lower limit of quantification at 4.28 nmol/L. The intra- and inter-precision were both below 3%. The range of analytical recovery was 97.8%–109.2%. The validated dilute-and-shoot LC–MS/MS method was compared with the SPE LC–MS/MS method modified from the one reported in 2013. The results by Passing–Bablok regression analysis and Bland–Altman plotting demonstrated a good agreement between the two methods. The presented method was then applied to establish sex-specific reference intervals from 62 males and 62 females, respectively. The calculated 2.5%–97.5% reference intervals for 24-h urinary 18-OHF were 113–703 nmol/day for males and 71.2–450 nmol/day for females.

Conclusion: The presented dilute-and-shoot LC–MS/MS method for 18-OHF quantification showed a good performance in the clinical application. Furthermore, the sex-specific reference intervals for 24-h urinary 18-OHF were first established and quite important for its application in primary aldosteronism subtyping.

Beibei Zhao and Jin Bian are co-first authors

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KEYWORDS
18-Hydroxycortisol, dilute-and-shoot LC–MS/MS, primary Aldosteronism, reference interval

1 | INTRODUCTION

The most common cause of secondary hypertension is primary aldosteronism (PA), which is characterized by excessive aldosterone secretion. Primary aldosteronism patients account for 2%–23% of hypertensive patients in various research cohorts.[1–3] The two dominant subtypes of PA are aldosterone-producing adenoma (APA), accounting for 35%, and idiopathic hyperaldosteronism (IHA), accounting for 60%.[4] Primary aldosteronism subtyping at the present stage is still a major challenge the management of PA. It is crucial to distinguish APA from IHA since IHA could be treated with medications, whereas APA requires adrenalectomy. Because of its high specificity and sensitivity (>90%), adrenal venous sampling (AVS) is currently the gold standard for distinguishing APA from IHA.[5] Nonetheless, AVS is difficult to implement as an invasive procedure due to its stringent requirements for facilities and skills, as well as its high risk of failure (around 20%–30%).[6–8] As a result, the need for reliable biochemical indicators is urgent. Several endogenous biomarkers had been studied to reduce AVS, including urinary 18-hydroxycortisol (18-OHF).[9–13] 18-OHF is a hybrid steroid produced by the adrenal gland, which was discovered in the urine of a PA patient in 1982.[14] Urinary 18-OHF is low in healthy subjects, essential hypertensives, and PA patients with IHA but significantly increased in PA patients with APA and familial hyperaldosteronism.[11,15–17]

Many techniques have been applied in the determination of 18-OHF, quantitation of urinary 18-OHF has been reported using radioimmunoassay,[18] enzyme-linked immunoassay,[19–21] fluoroimmunoassay,[22] high-performance liquid chromatography (HPLC) with an ultraviolet detector,[23] and gas chromatography/mass spectrometry.[24] Liquid chromatography–tandem mass spectrometry (LC–MS/MS) has been recently applied to measure urinary 18-OHF, using solid-phase extraction (SPE) for sample preparation.[25] Solid-phase extraction (SPE) is the most common sample preparation method used to treat blood and urine samples for the quantification of 18-OHF by LC–MS/MS.[21] To the best of our knowledge, all published articles in this field reported their methods with an SPE process. Although SPE is recommended to deal with samples with a complicated matrix and/or a low amount of analyte, it is time-consuming and labor-intensive. So it seems to be unnecessary to apply SPE for sample preparation if a more straightforward scheme is alternative. Dilute and shoot which is simpler and faster has been successfully applied to prepare samples for the measurements by LC–MS/MS. Dilute-and-shoot has been applied to process urine samples for many analytes such as drugs (anti-doping, therapeutic drugs monitoring), nutrients (vitamins), and hormones (steroids and their corresponding metabolites and analogs) to make the analysis efficient and high-throughput, as well as less labor-intensive.[26,27] But it has not been applied to prepare urine samples for 18-OHF quantification, the only single methodology article for 18-OHF measurement by LC–MS/MS applying SPE for sample preparation.[25] In addition, reference intervals for 24-h urinary 18-OHF measured by LC–MS/MS method have not been reported.

Herein, we report the validation of a dilute-and-shoot LC–MS/MS method for the quantitation of urinary 18-OHF in healthy normotensive Chinese subjects and sex-dependent reference intervals.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Eighteen-OHF and its stable-isotope labeled internal standard 18-OHF-9,11,12,12-d_4 (IS) were from IsoSciences (Ambler, PA, USA) and Cambridge Isotope Laboratories (Andover, MA, USA), respectively. SPE 50 mg C18 Hypersep column used to prepare urine samples with SPE LC–MS/MS method was from Thermo scientific (Rockwood, TN, USA). HPLC-grade methanol were from Merck (Darmstadt, Germany). HPLC-grade formic acid was from Sigma-Aldrich (St. Louis, MO, USA). Deionized water was purified with a Milli-Q system (Millipore Corporation, Bedford, MA, USA).

2.2 | Preparation of the IS solution, calibrators, and quality control (QC) samples

The IS was dissolved in methanol to make a working solution at 2.09×10^3 nmol/L. Calibrators were prepared with standard 18-OHF in 35% (v/v) methanol at concentrations of 0.00, 4.28, 8.57, 17.1, 34.3, 68.5, 137, 274, 548, 1.10×10^3, 2.19×10^3, 4.39×10^3, and 8.77×10^3 nmol/L. QC samples were prepared with spiked pooled patient samples at low, medium and high levels of 18-OHF at 298, 660, and 1.15×10^3 nmol/L.

2.3 | Sample preparation

Fifty microlitre of urine samples, calibrators or QC samples, 100 µl of the IS working solution were pipetted into 1.5 ml Eppendorf tubes and diluted with 350 µl water, followed by vortexing (5 min). After centrifugation (10,621*g*, 5 min), 4.0 µl of the final solution was directly injected onto LC–MS/MS system for analysis. For the method correlation study, we modified the SPE sample preparation procedure of Jin et al. method.[25] Each SPE column was conditioned with 2 ml methanol and then was equilibrated with 2 ml H_2O. Samples were first pre-treated as described in the dilute-and-shoot method before loading onto the conditioned and

equilibrated SPE column. In the modified procedure, 18-OHF and IS were eluted with 500 µl methanol into the 96-well plate. After evaporation under a gentle nitrogen flow at 50°C, the analytes were reconstituted in 500 µl 35% (v/v) methanol, and 4.0 µl was injected for analysis.

2.4 | Instrumentation and conditions

The sample analysis was performed on the TRIPLE QUAD 4500 mass spectrometry system (SCIEX, MA, USA) interfaced with a Shimadzu HPLC system containing a LC-20AD XR HPLC pump, a SIL-20AC XR auto-sampler, and a CTO-20A column oven. Separation of 18-OHF from the matrix interferences was carried out on a Sigma Ascentis Express C18 Column (50×2.1 mm, 2.7 µm) with a gradient. The mobile phase consisted of (A) 0.2% formic acid in Milli-Q water and (B) 0.2% formic acid in HPLC-grade methanol. The temperatures of the auto-sampler and column were at 4 and 35°C, respectively. Table 1 shows the mobile-phase gradient programming. Electrospray ionization was in positive mode, and Table 2 lists the multiple reaction monitoring (MRM) transitions and tuning parameters. The analysis of chromatograms, mass spectra, and quantitation was performed on the Analyst® software (SCIEX, version 1.6.2) and MultiQuant (SCIEX, version 3.0.3).

2.5 | Method validation

The method was validated for the evaluation of a surrogate matrix for calibrators, interference from isomeric endogenous steroid (6β-hydroxycortisol, 6β-OHF), LLOQ, linearity, precision, recovery, carryover, and stability according to the US Food and Drug Administration (FDA) guidelines on biological method validation. The matrix effect was evaluated by mixing the alternative matrix of multiple urine samples.[28]

2.5.1 | The evaluation of calibrator's matrix

Thirty-five percent (v/v) methanol (the initial mobile phase) was evaluated as a surrogate matrix due to 18-OHF is an endogenous analyte in urine samples. Six replicates of blank matrix and six replicates of zero calibrators were analyzed to evaluate whether 35% (v/v) methanol was qualified as the surrogate matrix of 24-hour urine for 18-OHF quantification. The blank and zero calibrators should be free of interference at the retention times of 18-OHF and the internal standard.

TABLE 1 Mobile-phase gradient of liquid chromatography

Time (min)	B (%)	Flow rate (ml/min)
0.00	35.0	0.4
1.00	35.0	
3.50	60.0	
3.51	95.0	
4.50	95.0	
4.60	35.0	
5.50	35.0	

Note: B, 0.2% formic acid in methanol; injection-to-injection time, 5.50 min;

2.5.2 | Isomeric interference

Interference study was performed to investigate whether the endogenous isomeric interference, 6β-OHF, would co-elute with the target analyte 18-OHF. Standard 18-OHF and standard 6β-OHF were mixed in surrogate matrix and analyzed with the presented LC–MS/MS method. 18-OHF and 6β-OHF should elute separately with the method.

2.5.3 | Matrix effect

Six pooled urine samples at low, medium, and high concentrations of 18-OHF were mixed with low and high levels of standard 18-OHF in surrogate matrix in 1:1 ratio, respectively, in triplicate. The passing criteria for matrix effect evaluation was the measured response ratio (peak area of analyte/peak area of IS) of each 1:1 mixture being within 20% of the mean response of pooled urine samples and standard 18-OHF in surrogate matrix.

2.5.4 | Linearity and LLOQ

Thirteen samples for the linearity study were prepared by spiking different amounts of 18-OHF standards in the surrogate matrix. These solutions were subjected to LC–MS/MS analysis as previously described. For linear regression analysis, the calibration curve was constructed by plotting the 18-OHF/IS peak area ratio (y) against the 18-OHF concentration (x), at a weighting of $1/X$. The linearity was accepted as recoveries of 85%–115% of the expected linear range with

TABLE 2 Parameters of the mass spectrometer

Parameters		Values
Source temperature		550°C
Curtain gas		35 psi
Collision gas		7.0 psi
IonSpray voltage		5500 V
Nebulizer pressure		60 psi
Declustering potential		70 V
Collision energy		27 V
Dwell time		80 ms
MRM	**Quantification**	**Qualification**
18OHF	379.2–267.2	379.2–285.2
18OHF-d_4	383.3–271.2	383.3–289.2

the coefficient of variations (CVs) < 5%. The LLOQ was determined by six runs in three consecutive days of diluted urine samples at which the signal-to-noise ratio (S/N) was >10 and CV for six suns was <20%.

2.5.5 | Precision and recovery

Intra- and inter-assay precision performance was evaluated using low, medium, and high levels QC samples. Approved as CVs < 15%. Accuracy was determined by recovery testing using pooled urine samples spiked with standard 18-OHF, evaluated as the ratio of the calculated spiked amounts against the actual spiked amount of 18-OHF standard. The acceptance criterion was recoveries between 85% and 115%.

2.5.6 | Carryover

Carryover was assessed by running authentic urine samples in a sequence of low-high-low 18-OHF concentrations samples for triplicate. The acceptable criterion of carryover was that the high concentration samples carried over to the low concentration sample below 20% of the LLOQ.

2.5.7 | Stability

The stability of urine samples was evaluated by comparing the concentrations of 18-OHF in fresh samples with those preserved at room temperature (R.T.), 2–8, −20, and −80°C for 1, 3, 7, 14, and 21 days. The stability of processed urine samples placed in the auto-sampler (8°C) of the LC–MS/MS system (at 8°C) for 0, 24, 48, and 72 h was also evaluated. The recoveries at each level should be within 85% and 115%.

2.6 | Methods comparison

A total of 20 authentic urine samples at 18-OHF concentrations of covering the validated linear range were prepared by the dilute-and-shoot and SPE methods in parallel. Urine 18-OHF was measured by LC–MS/MS on the same instrument using identical parameters.

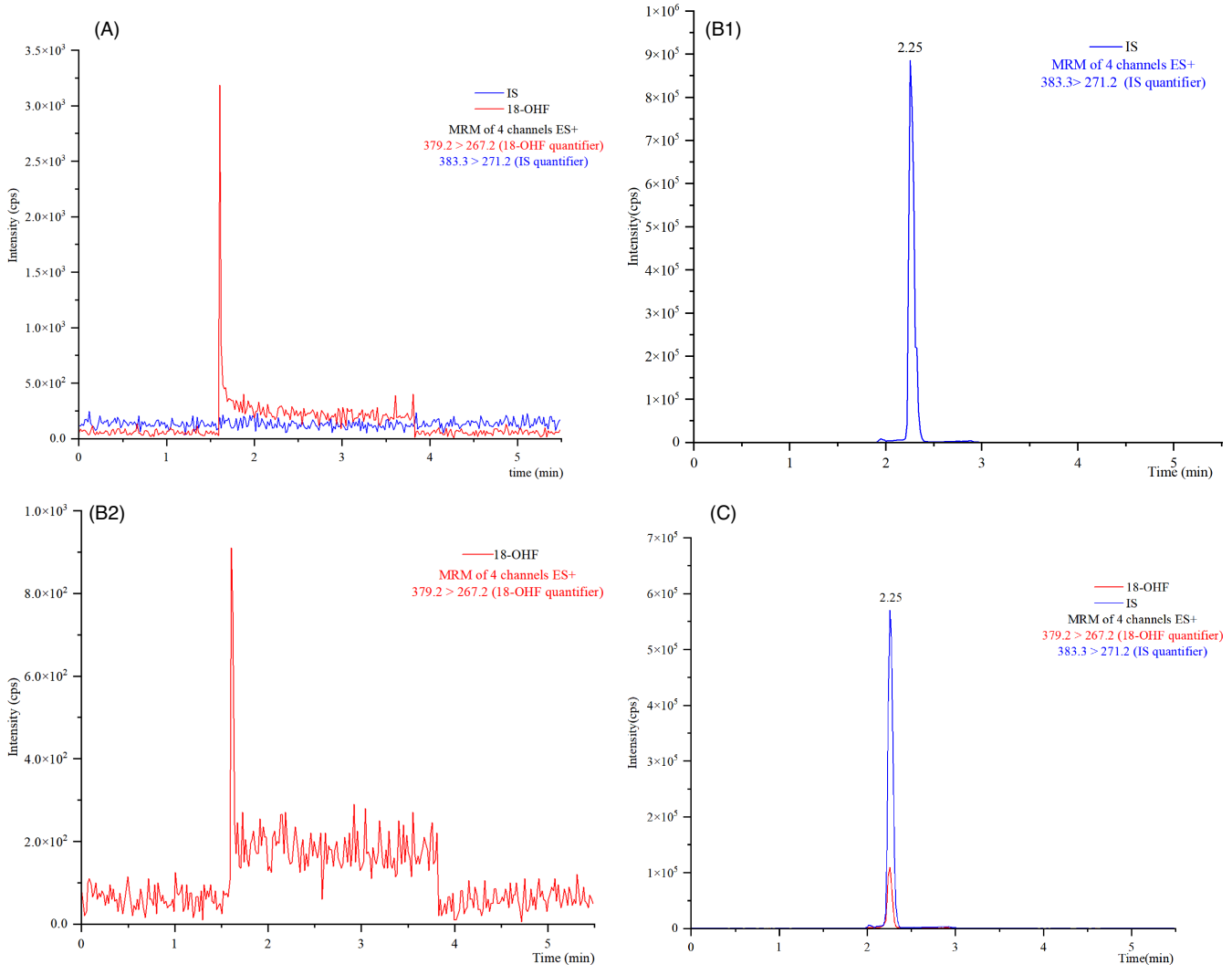

FIGURE 1 0 (A) 18-OHF (in red) and the IS (in blue) in 35% (v/v) methanol, (B1) the IS (in blue) in zero calibrators and (B2) the 18-OHF (in red) in zero calibrators(C) 18-OHF (in red) and the IS (in blue) in a urine sample

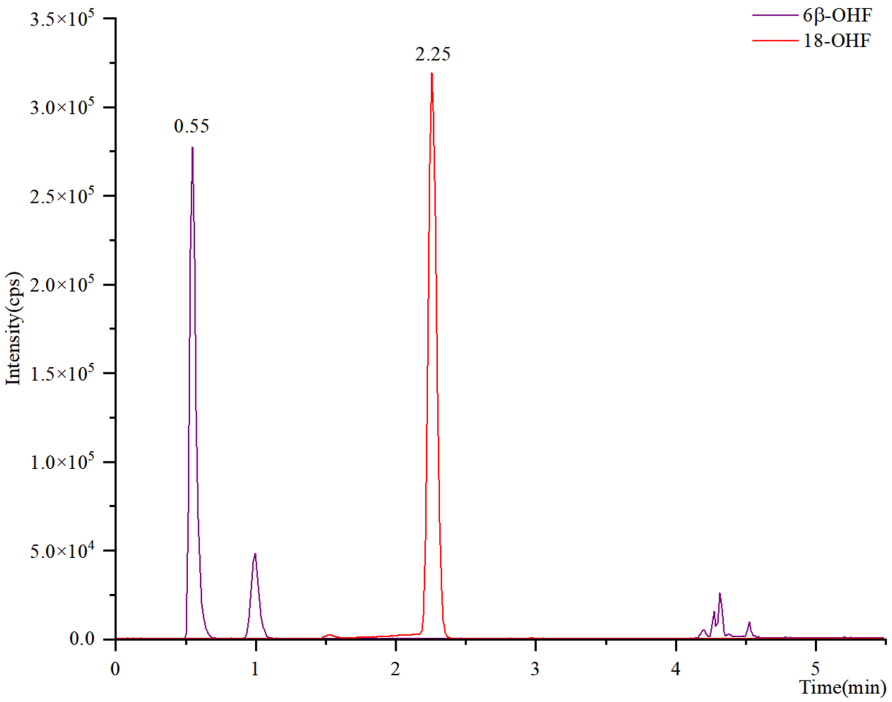

FIGURE 2 The XICs (extracted ion chromatograms) of 6β-OHF and 18-OHF

TABLE 3 Intra- and inter-assay precision of urinary 18OHF

	Intra-assay precision		Inter-assay precision	
Urinary 18OHF level	$\bar{x} \pm s$ (nmol/L)	CV (%)	$\bar{x} \pm s$ (nmol/L)	CV (%)
Low	298 ± 5.10	1.7	291 ± 3.70	1.3
Medium	660 ± 11.1	1.7	649 ± 14.6	2.2
High	$1.15 \times 10^3 \pm 17.3$	1.5	$1.12 \times 10^3 \pm 22.3$	2.0

TABLE 4 Recovery testing of urinary 18OHF

Urinary 18OHF level	Urine sample (nmol/L)	Spiked concentration (nmol/L)	Accuracy (%)
Low	119	70.9	105.6
		109	107.0
		191	105.3
Medium	537	382	102.0
		523	106.5
		927	105.8
High	1607	1090	98.6
		1526	102.9
		2651	101.5

TABLE 5 Carryover evaluation of dilute-and-shoot LC–MS/MS method for urinary 18OHF

Sample	18OHF (nmol/L)	(C3–C1)/C1 (%)	$(C3_{mean}–C1_{mean})/C1_{mean}$
C1	26.2	−11.1	−3.6%
C2	7.83×10^3		
C3	23.3		
C1	24.7	5.3	
C2	7.89×10^3		
C3	26.0		
C1	25.6	−4.7	
C2	7.98×10^3		
C3	24.4		

Correlation between the two methods was evaluated by Passing–Bablok regression analysis and Bland–Altman plotting.

2.7 | Reference subjects

To establish reference intervals of 24-h urinary 18-OHF, 124 reference subjects (50% males, 19–78 years old) were recruited. The inclusion criteria were adults with blood pressure <130/80 mmHg, no medical history on anti-hypertensives or hormonal treatment, and no smoking and/or alcohol habits. Females who were taking oral contraceptives, pregnant, or breastfeeding were excluded. All reference subjects and subjects whose urine samples were used for method comparison signed written informed consents. Each subject was given one plain bottle to collect 24 h urine. Upon receiving each urine collection, the urine volume was measured, and a 5-ml aliquot

was kept frozen at −20°C until the LC–MS/MS analysis. This study approval was by the ethics committees at Fuwai Hospital (Beijing, China).

2.8 | Statistical analysis

Statistical analysis was performed on MedCalc (Ostend, Belgium, version 15.2.2). Significance was at $p < 0.05$. Shapiro–Wilk test was applied in the normal distribution test for the measurement data. The measurement data conforming to the normal distribution were expressed as mean ± standard derivation, and the inter-groups' comparisons were performed by *t* test; While the measurement data not conforming to the normal distribution were expressed as median (interquartile range), and the inter-groups' comparisons were performed by investigated by Kruskal–Wallis testing with post hoc analysis. The counting data were presented as number (percentage), the comparisons between groups were explored with chi-square test (χ^2 test). Reference intervals were established according to CLSI EP28-A3c document for defining, establishing, and verifying reference intervals in the clinical laboratory.

3 | RESULTS

3.1 | Method validation

3.1.1 | The evaluation of calibrator's matrix

Figure 1 showed that no peak of interferences was observed near the retention times of 18-OHF and the IS in 35% (v/v) methanol. The extracted ion chromatograms of 18-OHF in 35% (v/v) methanol with those in urine samples and zero calibrators showed that no interference co-eluted with 18-OHF, demonstrating that 35% (v/v) methanol was qualified as the surrogate matrix of urine for 18-OHF quantification.

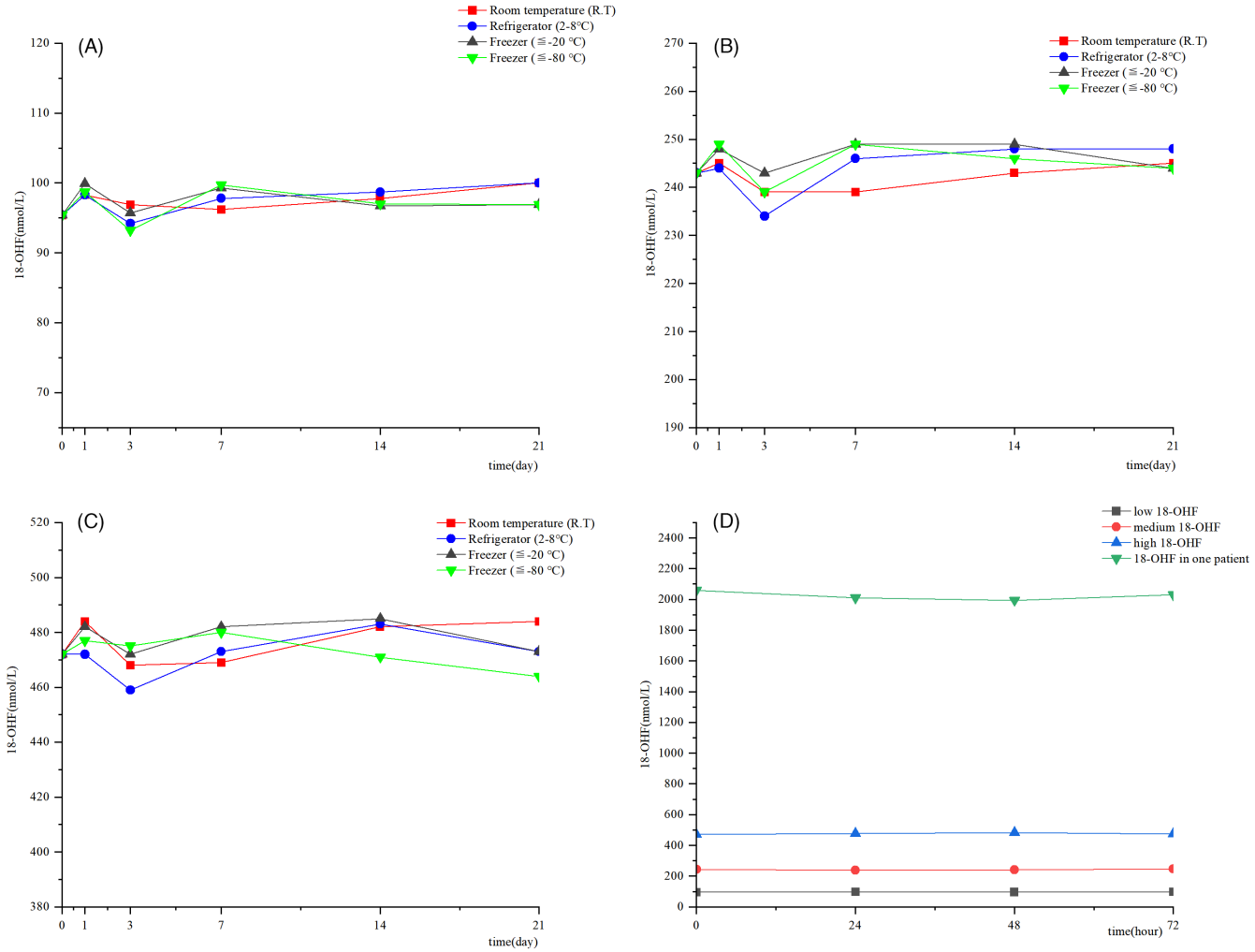

FIGURE 3 The stability of urine samples with a (A) low (B) medium (C) high 18-OHF level under four different temperatures. (D) The stability of processed urine samples with low, medium and high 18-OHF level and that of one patient placed in the auto-sampler (8°C)

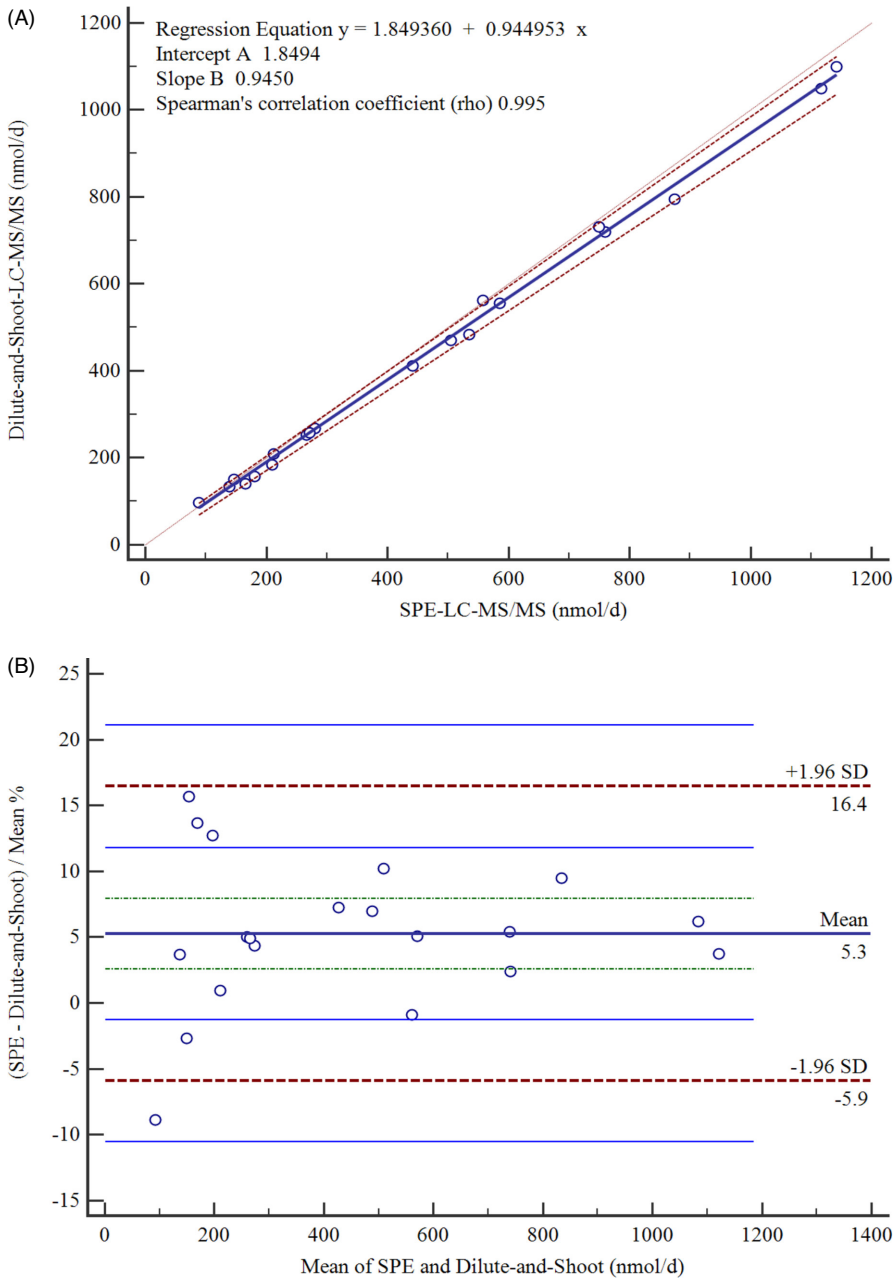

FIGURE 4 Comparison between dilute-and-shoot LC-MS/MS and SPE LC-MS/MS of urinary 18-OHF by (A) Passing-Bablok regressions (B) Bland–Altman plot

3.1.2 | Isomeric interference

Standard 18-OHF in surrogate matrix (2.19×10^3 nmol/L) was used to evaluate the influence of endogenous interference 6β-OHF on the quantification of 18-OHF. The solution was divided to four aliquots, one was reserved for later use, the other three were spiked with low, medium, and high concentrations of 6β-OHF into 35% methanol, respectively, three aliquots in parallel. The samples were measured by LC–MS/MS, and the recovery of 18-OHF were calculated. Shown in Figure 2, the retention time of 6β-OHF was at 0.55 min, which is far away from the peak of 18-OHF at 2.28 min. The recovery of 18-OHF in samples spiked with 6β-OHF was between 85% and 115%. The retention time and intensity of signal of 18-OHF in spiked samples were consistent with the no spiked ones. Therefore, the quantification of 18-OHF was not interfered by the endogenous isomer 6β-OHF.

3.1.3 | Matrix effect

The variability between the signal responses of mixed samples and the mean signal responses of urine samples and standard solutions were confirmed as bias <4.3%.

TABLE 6 Clinical characteristics of 124 healthy participants and their body mass index (BMI) distribution

	S.T.-Female (ST, $n = 14$)	N.W.-Female (NW, $n = 45$)	O.W.-Female (OW, $n = 2$)	Obesity-Female (OB, $n = 1$)	S.T.-Male (ST, $n = 9$)	N.W.-Male (NW, $n = 41$)	O.W.-Male (OW, $n = 10$)	Obesity-Male (OB, $n = 2$)
Age, years (IQR)								
Mean ± SD	25.4 ± 2.1	32.1 ± 12.5	36.5 ± 21.9	22	27.1 ± 14.7	30.5 ± 11.3	28.1 ± 8.3	25 ± 7.1
Min-max	22–30	22–78	21–52	22	21–66	19–73	22–50	20–30
Body mass index, kg/m^2 (IQR)								
Mean ± SD	17.5 ± 0.8	20.8 ± 1.7	27 ± 1.3	36.7	17.7 ± 0.6	21.7 ± 1.9	26.4 ± 1.1	33.3 ± 4.2
Min-max	16.2–18.4	18.5–24.8	26–27.9	36.7	16.5–18.4	18.5–24.8	25.1–28.4	30.3–36.3
24 h 18-OHF, nmol/day								
Mean ± SD	207.0 ± 105	241.4 ± 86	303.5 ± 33.2	234	295 ± 101.1	316.7 ± 130.6	383.9 ± 133.8	484.5 ± 328.8
Min-max	46–442	111–456	280–327	234	151–474	94–693	228–652	252–717

Note: Data are displayed as median (interquartile range) or number (percentage).

Abbreviations: IQR, interquartile range; N.W., normal weight; *n*, number of patients; O.W., overweight; S.T., slightly thin.

3.1.4 | Linearity and LLOQ

The linearity range was from 4.28 to 8.77×10^3 nmol/L, with an LLOQ at 4.28 nmol/L. The linearity within constructed ranges was as good as $r^2 > 0.995$.

3.1.5 | Precision and accuracy

Table 3 shows that intra- and inter-assay precision CV were up to 2.2%. The recovery was 98.6%–107.0% in urine samples (Table 4).

3.1.6 | Carryover

No carryover was observed in the low 18-OHF sample with a concentration at near LLOQ after that with a concentration almost at the highest calibrator, shown in Table 5.

3.1.7 | Stability

Urine samples collected from three subjects were used to evaluate the stability of urine samples preserved in R.T., 2–8, −20 and −80°C for 0, 1, 3, 7, 14, and 21 days as well as the stability of processed urine samples placed in the auto-sampler (8°C). The variations of samples with lowest (95.4 nmol/L), medium (243 nmol/L), and highest (472 nmol/L) of 18-OHF were presented herein to show the changes of 18-OHF level in samples of different concentrations of 18-OHF. As shown in Figure 3 subplot A, B, and C, low, medium, and high concentrations of 18-OHF in urine samples could be preserved, without obvious changes in 18-OHF level, at R.T. for 21 days with CVs of less than 3.0%, at 2–8°C for 21 days with CVs of <2.5%, at −20°C for 21 days with CVs of <3.0%, at −80°C for 21 days with CVs of <4.1%. In a previous article, researchers reported the urinary 18-OHF level in PA patients could be more than 2×10^3 nmol/day,[25] so we supplementarily reported the stability of a processed patient sample which was measured in the clinical application of this verified method. The urinary 18-OHF level of the patient is beyond 2×10^3 nmol/L, and the results showed the processed samples could be placed in the auto-sampler for 72 h with CVs of <4.0%.

3.2 | Comparison between dilute-and-shoot LC–MS/MS and SPE LC–MS/MS

Figure 4, the Spearman's correlation coefficients of urinary 18-OHF varied from 0.988 to 0.998 (all $p < 0.0001$) between the two methods. Passing–Bablok regressions displayed the slope and intercept were 0.9450 and 1.8494, which demonstrated the great accordance between the two methods. The Bland–Altman plot showed only one point (5%) located outside the limits of agreement

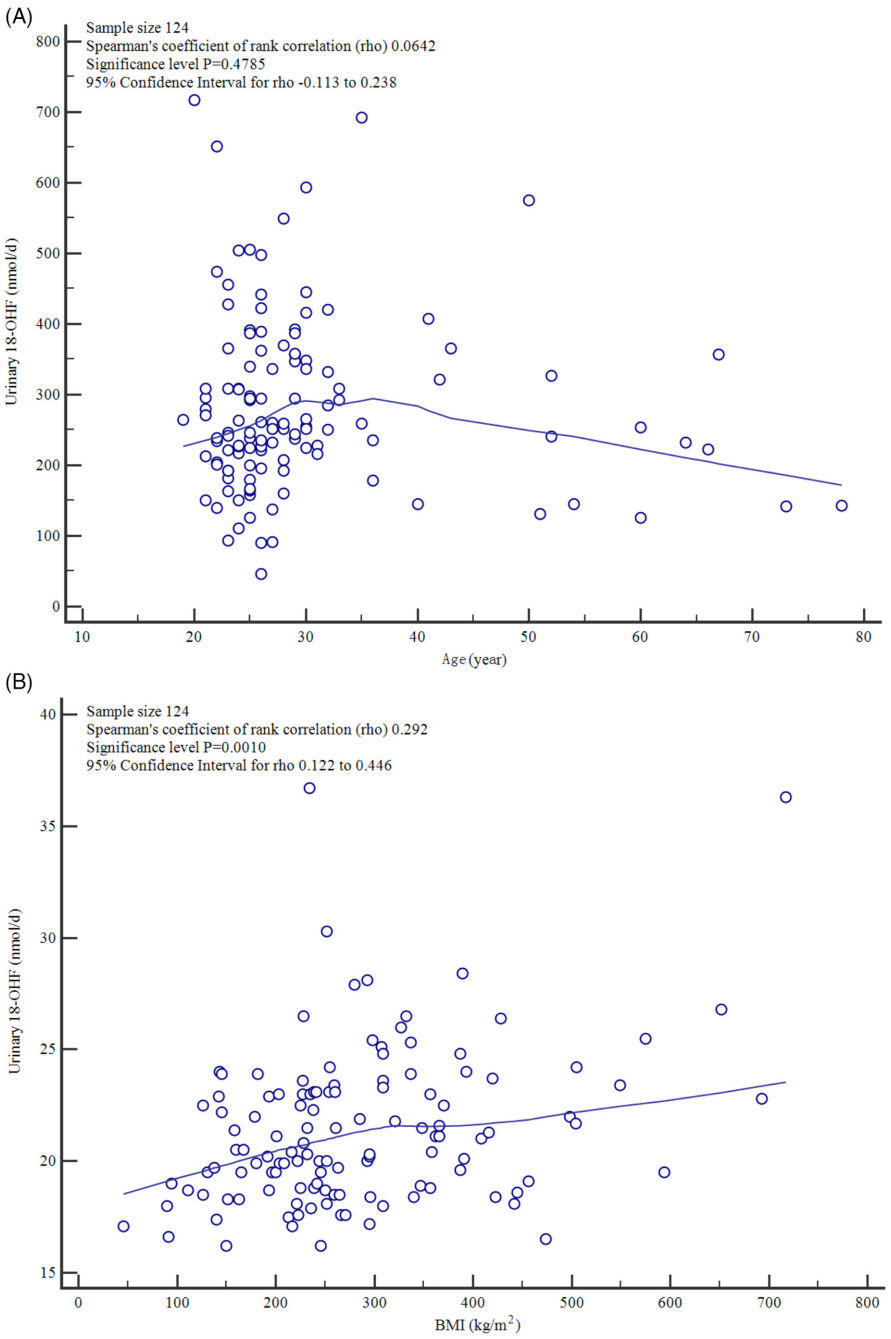

FIGURE 5 Rank correlation between (A) age (year) (B) BMI (kg/m^2) and urinary 18-OHF (nmol/d)

and 95% confidence interval (CI), but still inside the maximum allowed difference. All other points are randomly dispersed along the mean difference line. The arithmetic mean of the difference was 5.3%, and the 95% CI of biases between the two assays were −5.9% to 16.4%, indicating the systematic and concentration-dependent bias between dilute-and-shoot LC–MS/MS and SPE LC–MS/MS is not significant.

3.3 | Reference intervals for 24-h urinary 18-OHF

To establish reference intervals of 24-h urinary 18-OHF, urine samples collected from 124 healthy subjects (62 males and 62 females) were measured by this validated dilute-and-shoot LC–MS/MS method. The clinical characteristics of 124 healthy participants is shown in Table 6. The impacts of age and body mass index (BMI) on the 18-OHF secretion were statistically analyzed by MedCalc. For the establishment of the reference interval of 24-h urinary 18-OHF, the rank correlations between age VS 18-OHF, BMI VS 18-OHF and the difference in 18-OHF levels between males and females were investigated first to confirm whether specific interval references were required. Figure 5, the correlation between age and urinary 18-OHF was not significant ($p > 0.05$) while that between BMI and urinary 18-OHF was significant ($p < 0.05$), but the contribution on urinary 18-OHF by BMI was weak (Spearman's coefficient of rank correlation is 0.292, which is smaller than 0.5). To further investigate

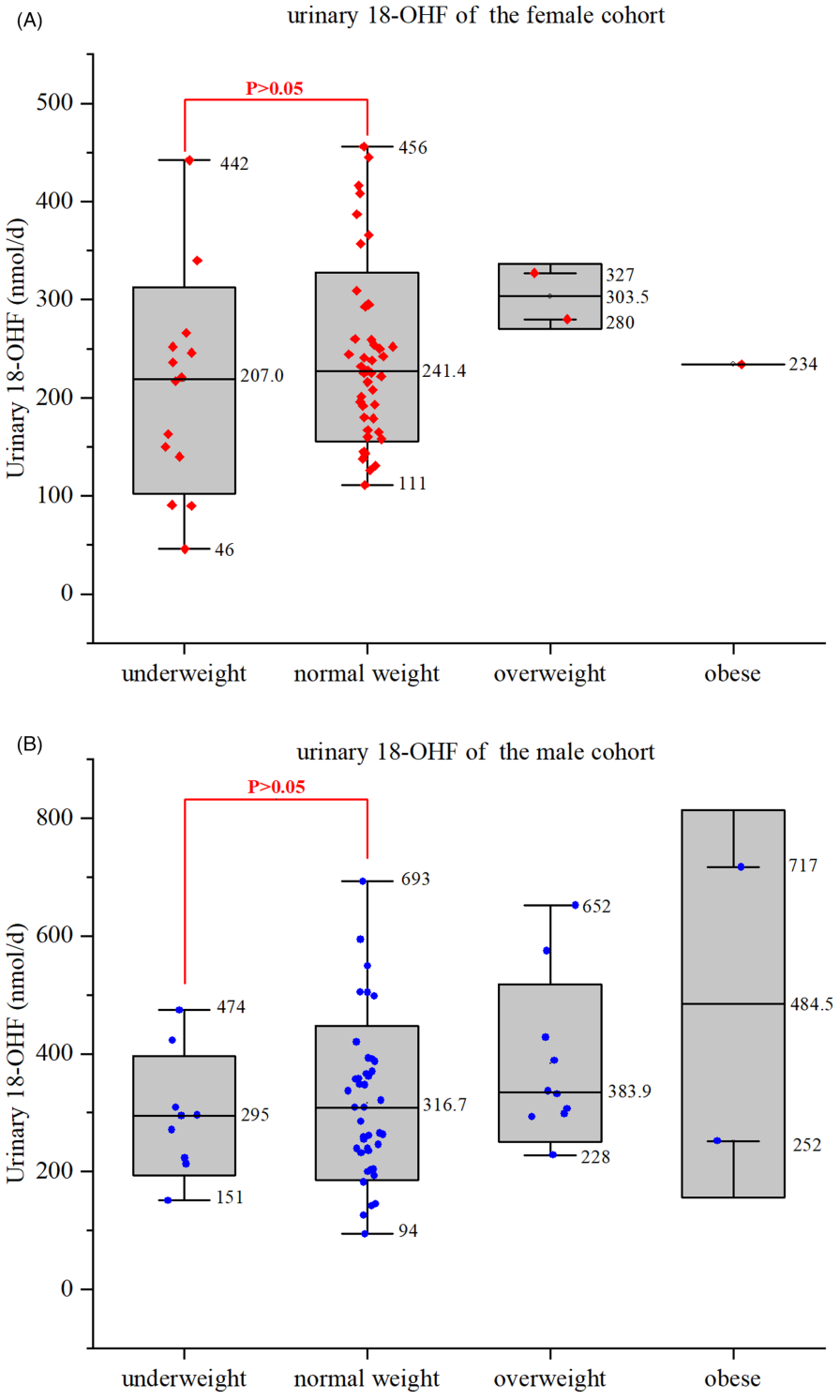

FIGURE 6 The urinary 18-OHF level of the (A) female (B) male cohort. ($p > 0.05$: underweight vs. normal weight (Mann–Whitney *U*-test), the samples sizes of overweight subgroup and obese subgroup are not enough for statistical analysis, hence the data are for display only)

whether BMI is a factor for 18-OHF secretion, the comparisons of various subgroups in males and females according to BMI classifications were conducted, respectively, shown in Figure 6. The results demonstrated that in this research BMI is not an impact factor for the 18-OHF secretion and further contribute to the difference of 18-OHF level in genders. At this stage, the impact of age and BMI on 18-OHF secretion have been confirmed as insignificant, and then the difference in 18-OHF levels between males and females was investigated by Mann–Whitney *U* test. As shown in Figure 7, a significant difference (two-talied probability $p < 0.001$) which was unexpected was observed between males and females in 24-h urinary 18-OHF level, hence sex-specific reference intervals were established from 62 males and 62 females respectively. Lower and upper cutoff values determined as the 2.5 and 97.5 percentiles were 113 to 703 nmol/day for males and 71.2–450 nmol/day for females, respectively.

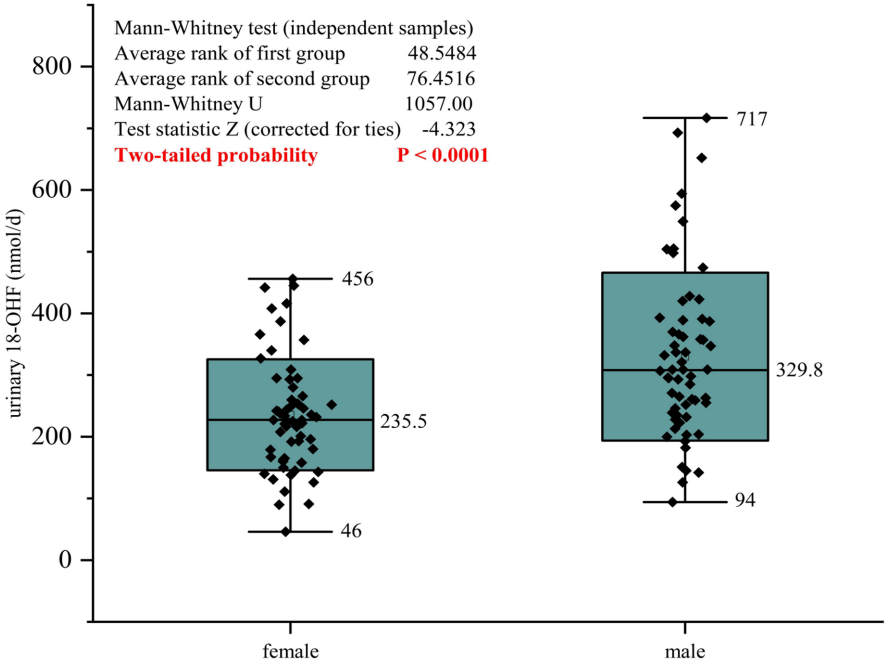

FIGURE 7 Box-whiskers plots for 24-h urinary 18-OHF for 62 healthy females and 62 healthy males.

TABLE 7 Method parameters of SPE LC–MS/MS method and proposed dilute-and-shoot LC–MS/MS method

Method	Sample volume	Sample preparation	Run time	Linearity	Precision	Accuracy	IS
SPE LC–MS/MS[25]	200 μl	SPE	10 min	26.5–6.61 × 10^3 nmol/L	intra-&inter-: <3.4%	98.0%–103.7%	6a-methylprednisolone
Dilute-and-shoot LC–MS/MS	50 μl	Dilution	5.5 min	4.28–8.77 × 10^3 nmol/L	intra-: 1.5%–1.7% inter-: 1.3%–2.2%	97.8%–109.2%	18-hydroxycortisol-d_4

4 | DISCUSSION

Even though AVS is recommended as the gold standard for PA subtyping,[5] it in general is seldom executed in clinics due to its invasive nature, extreme requirements, poor standardization, and innegligible risk of failure. Many techniques have been applied to quantify 18-OHF in urine samples since 18-OHF was identified in 1982.[14] In terms of the techniques applied to quantify the 18-OHF substance, radioimmunoassay and enzyme-linked immunoassay due to inevitable cross-reactions and radioactive contaminations have been unpopular for 18-OHF measurement. Gas chromatography–tandem mass spectrometry has been complained of the tortuous sample preparation using derivatization, while HPLC was low efficient because of the quite long analysis time as 240 min.[23]

LC–MS/MS was applied to determine urinary 18-OHF in 2013 for the first time.[25] In the research, specialists applied solid-phase extraction to process urine samples for urinary 18-OHF quantification by LC–MS/MS. SPE is a universal method in the sample preparation of mass spectrometric detection. It, because of the great performance of purification and analyte-enriching, is usually applied to deal with samples with complicated matrix, for instance, blood and tissue, and samples with low concentration of target analytes.[29] However, the SPE method demands additional consumables (SPE plates or columns), more organic reagents for conditioning, equilibrating, washing, and eluting and also more labors for operations. Dilute-and-shoot method is a one-step sample preparation method, in which the samples were prepared by only dilution. It requires no more materials and reagents and is absolutely simple and fast. Despite previous researches on determination of 18-OHF choose SPE to process the urine and blood samples, it is old-fashioned at current stage for urine sample preparation to quantify 18-OHF, of which the concentration is not extreme low in human urine.

To establish and develop clinical test methods, not only the performance of the method should be taken into consideration but also the clinical application scenarios. Since its isolation and identification, 18-OHF has been determined in various human samples (serum, plasma, urine as well as tissues) and applied to differentiate PA subtypes. The previous study has proved that urinary 18-OHF, as a single biomarker, was the optimal biochemical indicator for the differentiation between APA and IHA;[12] hence, specialists have recommended prioritizing urinary 18-OHF in the diagnosis of PA subtypes, meaning that measuring multiple analytes in one method is not of more value in the case of PA subtyping. On the other hand, the complicated panel method containing multiple

analytes would lead to the loss of accuracy and stability of individual analyte quantification. For instance, in the method of measuring three steroids, cortisol, cortisone, and 6-sulfatoxymelatonin in urine sample simultaneously by dilute-and-shoot LC–MS/MS method,[30] the intra- and inter-precision were 20.83% and 22.5%, respectively. While the proposed method for the individual determination of urinary 18-OHF showed a much better intra- and inter-precision of 1.7% and 2.2%. And less variation of test method contributes to better confidence in the acceptance of PA subtyping using the indicator of urinary 18-OHF.

The validation of specificity of the dilute-and-shoot LC–MS/MS method proved that samples were prepared well by dilution and no interferences coeluting with the analytes and the IS was observed. The baseline of noise is also acceptable as shown in Figure 1. When compared to the SPE LC–MS/MS method reported in 2013,[25] our method was faster to quantify urinary 18-OHF in only 5 min with 4-fold less consumption of urine. The linearity of the validated method was broader as from 4.28–8.77 × 10^3 nmol/L. The validated method was more sensitive to provide an LLOQ at 4.28 nmol/L. The precision (below 3%) and accuracy (97.8%–109.2%) of this method were also better than the SPE LC–MS/MS method as expected, shown in Table 7. In addition, stable-isotope labeling standard 18-OHF (18-OHF-d_4) which has the same physical and chemical properties as the targeted analyte was used in the presented method but not in the Jin et al.'s method to trace the performance of 18-OHF in the whole process of preparation as well as analysis to avoid the potential matrix effect and other errors. The comparison conducted between SPE LC–MS/MS method modified according to Jin et al.'s method[25] and the proposed dilute-and-shoot LC–MS/MS method manifested the quantification of 18-OHF was in a perfect agreement between the two methods as expectation.

To evaluate the clinical utility of urinary 18-OHF as a non-invasive biochemical indicator for PA subtyping, many studies have been carried out to explore the levels of urinary 18-OHF among different groups of subjects such as PA patients, hypertension patients, and healthy subjects.[17,20,24,25,30-36] However, the sex-specific reference intervals of 24-h urinary 18-OHF have never been established by LC–MS/MS method. Even though in previous study gender, age, and BMI have been regarded as factors of steroids' secretion,[37] our further investigation has shown that in the 18-OHF case age and BMI are not factors of its secretion. And as demonstrated in Figure 7, the levels of 24-h urinary 18-OHF in males and females were significantly different, proving that it is essential to establish sex-specific reference intervals for males and females separately. It also indicated that gender should be taken into consideration as a factor influencing the levels of 18-OHF in individuals when using urinary 18-OHF in the differential diagnosis between APA and IHA.

5 | CONCLUSION

In this research, a simple and rapid dilute-and-shoot LC–MS/MS method was developed and validated to quantify 18-OHF in human urine. It is, to the best of our knowledge, the first SPE-free LC–MS/MS method for 18-OHF determination, making the measurement of urinary 18-OHF much more cost-effective and simplified. The dilute-and-shoot LC–MS/MS method was proved to be capable to replace the SPE LC–MS/MS method by the comparison of Passing–Bablok regression analysis and Bland–Altman plotting. The proposed method was successfully applied to establish sex-specific reference intervals from 62 males and 62 females. More healthy subjects from multiple centers should be enrolled in the research to present more representative reference intervals of 24-h urinary 18-OHF in the future.

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CONFLICT OF INTEREST

The authors declare there are no conflicts of interest regarding the publication of this article.

DATA AVAILABILITY STATEMENT

The data used to support the findings of this study are available from the corresponding author upon request.

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