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Quantitation of Urinary 6 β-Hydroxycortisol and Free Cortisol by Ultra-Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry

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Abstract

A new UPLC-MS method that combined 0.01% formic acid/isopropanol as the mobile phase and an ESI-QTOF instrument has been developed for reliable quantitation of urinary 6β-hydroxycortisol (6βHC) and free cortisol (FC). The use of 0.01% formic acid/isopropanol for binary gradient elution on a reversed-phase C18 column resulted in 3.1 and 5.0 times the peak areas for 6βHC and FC, respectively, as compared to 0.1% formic acid/acetonitrile. Liquid-liquid extraction with ethyl acetate at pH 5 was superior to solid-phase extraction for sample preparation. A mass window of 50 ppm was used for quantitative monitoring of 6βHC (m/z 379.212) and FC (m/z 363.217) using full-mass detection, with the on-column limits of detection as 4.0 and 1.4 fmol, and the lower limits of quantitation as 13.6 and 6.9 fmol for 6βHC and FC. The accuracy of quantitation ranged from 93.3% to 102.3% recoveries at three spiking levels, with the maximum intraday and interday %CV being 3.7 and 5.3 for both analytes. This LC-MS method was then applied to the quantitation of 6βHC and FC in the urine pairs collected during the follicular and premenstrual phases of menstrual cycle from each of sixty-one premenopausal women — forty-one with ovulatory cycles and twenty with subclinical anovulatory cycles. Paired two-tailed T-tests showed no significant difference ($p>0.05$) between the metabolic ratios of urinary 6βHC/FC during the follicular and premenstrual phases in both ovulatory and anovulatory subjects, indicating no correlation of the urinary 6βHC/FC ratios between the two physiological phases of menstrual cycle in these subjects.

Keywords: UPLC; QTOF MS; Free Cortisol; 6β-Hydroxycortisol; Urine; Follicular Phase; Premenstrual Phase; Premenopausal Women.

Abbreviations: **UPLC:** Ultra-high Performance Liquid Chromatography; **MS:** Mass Spectrometry; **6βHC:** 6β-Hydroxycortisol; **FC:** Free Cortisol; **CYP:** Cytochrome P450; **LC-UV:** Liquid Chromatography with UV detection; **GC-MS:** Gas Chromatography - Mass Spectrometry; **MS/MS:** Tandem Mass Spectrometry; **MRM:** Multiple Reaction Monitoring; **QTOF:** Quadrupole Time-of-Flight; **HRMS:** High-Resolution Mass Spectrometry; **IS:** Internal Standard; **ESI:** Electrospray Ionization; **CV:** Coefficient of Variation; **LLE:** Liquid-Liquid Extraction; **SPE:** Solid-Phase Extraction; **EIC:** Extracted Ion Chromatogram; **Ai:** Peak Area of Internal standard; **As:** Peak Area of analyte standard; **LOD**: Limit of Detection; **LOQ**: Limit of Quantitation

Introduction

Accurate quantitation of endogenous metabolites is indispensable for improving our understanding of many pathophysiological processes. This is especially important for clinical studies where large biological variability exists among human subjects [1,2].

Cytochrome P450 (CYP) 3A4 is the most abundant isoform in the CYP-metabolizing enzyme family, and is responsible for catalyzing the C-6β hydroxylation of unconjugated free cortisol (FC), forming the 6β-hydroxycortisol (6βHC) metabolite that is excreted in the urine [3]. This observation led to the hypothesis that urinary 6βHC could be a noninvasive indicator of CYP 3A4 activity within the human body [4,5], and the eventual validation of the urinary 6βHC/FC ratio as an index of CYP 3A4 enzyme activity [3]. A significant controversy still exists as to the effectiveness of the urinary 6βHC/FC ratio for phenotyping CYP 3A4 activity in human subjects [6,7], but accurate quantitation of urinary 6βHC and FC is a prerequisite for calculating the true urinary 6βHC/FC ratio. Historically, the assay of 6βHC and FC in urine has been carried out using immunoassay-based methods [8], but these methods are susceptible to cross-reactions with cortisone and other steroids. More recently, the determination of urinary 6βHC and FC in clinical practice has been performed using LC-UV [9-10], gas chromatography - mass spectrometry (GC-MC) [11], or tandem mass spectrometry (MS/MS) with multiple reaction monitoring (MRM) [12].

LC, particularly ultra-high performance liquid chromatography (UPLC), coupled to high-resolution mass spectrometry (HRMS) is the most commonly-used analytical technique for the untargeted and high-throughput global profiling of endogenous metabolites in biological systems [13]. HRMS provides the resolving power to separate an analyte from its co-eluting isobaric species in complex matrix background, and the accuracy and precision of exact mass measurements offer the possibility of identification and quantification in a single LC-MS acquisition with full-scan detection [14,15]. This technique has been used for reliable quantitation of small-molecule compounds in a variety of applications [16-20]. In this work, a sensitive UPLC-MS method using a quadrupole time-of-flight (QTOF) instrument was developed for the precise and accurate quantitation of 6βHC and FC in urine, in order to determine the metabolic ratios of 6βHC/FC in a cohort of premenstrual women during different phases of their menstrual cycles.

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Materials and Methods

Reagents and materials

Methanol, acetonitrile, isopropanol, ethyl acetate, water, ammonium formate, and formic acid were LC-MS grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA). Free cortisol (FC), 6β-hydroxycortisol (6βHC), and cortisol-d4 were purchased from Steraloids Ltd. (Newport, RI, USA).

Standard solution and calibration curve

The 6βHC and FC were dissolved in methanol at concentrations of 0.2 and 1 mg/mL, respectively, and were used as the stock standard solutions. Dilutions were made from the two stock solutions with 10% methanol to produce a series of working standard solutions that contained a mixture of 6βHC and FC, with concentrations in the range of 2 to 400 ng/mL and 2.5 to 500 ng/mL, respectively. Cortisol-d4 was used as the internal standard (IS) and was dissolved in methanol at a concentration of 100ng/mL. For preparing the internal calibration standard curves, 100-μL aliquots of the IS solution were added to individual 400-μL glass inserts and then dried in an SPD1010 centrifugal speed-vacuum concentrator (Thermo Electron Corp., San Jose, CA, USA). Next, 200-μL aliquots of the working standard solutions described above were added to individual inserts. After a short vortex-mixing, these solutions were used as calibrators for the LC-MS quantitation. Linear regression analysis of the peak area ratios of 6βHC (m/z 379.212) and FC (m/z 363.217) to the peak area of the IS (m/z 367.242) *versus* the concentrations of the individual calibrators (n=8) was used to make the standard calibration curves.

Urine collection and storage

A total of sixty-one premenopausal women at 20-40 years of age, including forty-one women with ovulatory cycles and twenty women with subclinical anovulatory cycles (*i.e.*, without any egg release or rise in luteal phase progesterone levels during the menstrual cycle), were recruited for this study. These subjects were all free from hormonal contraceptive use for at least three months prior to participation, and informed consent was obtained from all subjects participating in this study. Two urine samples from each woman were collected at the Centre for Menstrual Cycle and Ovulation Research, Division of Endocrinology in the Department of Medicine, University of British Columbia, Vancouver, BC, Canada. All of the subjects claimed to have regular menstrual cycles. The two urine samples from each subject were collected on selected days of their menstrual cycles, but at two different physiological phases, *i.e.*, follicular phase *versus* premenstrual phase for the subjects with ovulation and without ovulation. To minimize known circadian hormone variation, all the samples were collected as the first voided morning urine and, once collected, were aliquoted and stored at -70°C.

Sample preparation

Liquid-liquid extraction (LLE): A 100-μL aliquot of the IS solution was added to a 3-mL glass test tube, and dried in the same speed-vacuum concentrator described above. A sample consisting of 200 μL urine was then added to each tube, and mixed with 200 μL of 0.5 M ammonium formate buffer (pH adjusted to 5 with formic acid). One mL of ethyl acetate was then added, mixed with the sample, and the tube was capped. Following vigorous vortex mixing, and centrifugation at 2,000 rpm and 20 °C for 5 min in an R-22 centrifuge (Beckman Coulter Inc. Brea, CA, USA), the upper ethyl-acetate layer was transferred to a 5-mL glass test tube. Care was taken not to disturb the boundary of the two phases during this step. The lower (aqueous) phase was extracted with 1 mL of ethyl acetate two more times in the same manner. All of the ethyl acetate extracts were pooled and filtered through a thin layer of anhydrous sodium sulfate, which was placed on top of a thin layer of glass fiber in a 5-cm glass separatory funnel. After washing the sodium sulfate and the funnel with another 1mL of ethyl acetate, the combined ethyl acetate solution was dried under a gentle nitrogen flow. The residue was dissolved in 200 μL of 10% methanol and subjected to LC-MS.

Solid-phase extraction (SPE): A 100-μL aliquot of urine was mixed with 100 μL of the IS solution and 1800 μL water, and then loaded onto a 100-mg Strata-X polymeric reversed-phase SPE cartridge (Phenomenex Inc., Torrance, CA, USA) which had been pre-wetted with 3 bed volumes of methanol and equilibrated with 3 x 1 mL of 5% methanol in water. Following washes with 3 x 1 mL of 5% methanol, the analytes were eluted with 3 x 1 mL of 80% methanol and the eluate was dried in the same way as described above. The residue was dissolved in 200 μL of 10% methanol and subjected to LC-MS.

UPLC-MS

LC-MS was carried out on an Acquity UPLC system (Waters Corp., Milford, MA, USA) coupled to a Synapt HDMS quadrupole time-of-flight mass spectrometer (Waters Corp.), which was equipped with an atmospheric pressure nebulizer-assisted electrospray ionization (ESI) source operating in the positive-ion mode.

Chromatography was performed on a BEH C18 column (2.1 mm I.D. x 50 mm, 1.7 μm particle size; Waters Corp. The column flow rate was 0.15 mL/min and the column temperature was maintained at 40 °C. Different concentrations of formic acid in water at 0.004%, 0.01%, 0.04%, and 0.1% were used as the mobile phase A, and three organic solvents (methanol, acetonitrile, and isopropanol) containing the same concentrations of formic acid were used as the mobile phase B. The analytical sensitivities of 6βHC and FC were compared using the same linear gradient (3% to 80% B within 20 min) but with different mobile phase combinations. For urine sample measurement, a water/isopropanol solution containing 0.01% formic acid was used as the mobile phase, but with the following optimized linear gradient: 3% to 30% B (0-12 min); 30% to 100% B (12-12.1 min), and 100% B (1 min) followed by a 4-min column equilibrium at 3% B. The injection volume was 5 μL, and was performed using the partial loop injection mode with an overfill factor of 4.

The mass spectrometer was tuned for the highest sensitivity of a lock-mass spray solution (50 pg/μL leucine enkephalin in 50% isopropanol, 10 μL/min) and calibrated using sodiated acetate cluster ions from a 2.5-mM sodium acetate solution in 50% isopropanol. The ESI-MS operation parameters included: spray voltage, 3.0 kV;, desolvation gas (N2) flow and temperature, 700 L/h and 350 °C; drying gas (N2) flow and temperature, 40 L/h and 120 °C; sampling cone voltage, 35V; extraction cone voltage, 4 V; and data acquisition rate, 0.3 s per scan. The full-mass detection range was m/z 100-1000. The background gas (Ar) in the collision cell was held at 0.5 mL/min in order to reduce fragmentation as much as possible while maintaining sensitivity. The lock-mass spray was used to ensure the mass accuracy throughout the LC-MS runs by switching between the sample spray and the lock-mass spray every 40 s. Each lock-mass spectrum was averaged from 2 continuous acquisitions at a scan time of 0.1 s.

Limits of detection (LODs) and limits of quantitation (LOQs)

Standard stock solutions of 6βHC and FC were diluted stepwise with 10% methanol in water, and were used to determine the LODs and the lower and upper LOQs for the two analytes. The LODs were defined as a signal-to-noise ratio (S/N) of 3. The lower LOQs were defined as a S/N of 10, according to the guidelines for bioanalytical method validation [21].

Matrix effect

The matrix effect — electrospray ionization suppression or enhancement was qualitatively evaluated by post-column infusion [22,23]. To do this, a 500 ng/mL of IS solution was prepared and delivered at 0.0167 mL/min using an auxiliary HPLC pump, and added to the LC effluent *via* a mixing tee located between the LC column and the ESI source. Five randomly-selected urine samples were prepared using the same LLE protocol as described in the Section 2.4.1. Liquid-liquid extraction, but without the spiked-in IS. These urine extracts were chromatographed by LC-MS and effective ionization suppression or enhancement of the sample matrix on the IS throughout LC-MS runs was evaluated based on the extracted ion current (EIC) chromatogram of m/z 367.242.

Precision and Accuracy of quantitation

The precision of quantitation was measured as intraday and interday coefficients of variation (CVs) by injecting 5-μL aliquots of different analytical replicates prepared by LLE with ethyl acetate from a pooled urine sample. For the intraday test, 8 replicates were prepared, and analyzed every 3 hours on a same day. For the interday test, 6 replicates from the same urine were prepared on each day and analyzed continuously for 6 days.

The accuracy of quantitation was assessed by performing spiked-in recovery tests on a urine pool. To do this, three levels of 6βHC and FC were spiked into 100-μL aliquots of urine. The spiked-in levels corresponded to 50, 100, or 150 ng/mL of 6βHC, and 100, 200, or 300 ng/mL of FC in the urine. These urine samples were then prepared by LLE, followed by LC-MS analysis, and the percent recoveries were calculated as [(mean observed concentration) / (spiked concentration] x 100%.

Statistical analysis

Paired two-tailed T-tests were performed using the SYSTAT software, version 12 (Systat Inc., Chicago, IL, USA).

Results and Discussion

Optimization of LC-MS conditions

The analytical sensitivity of ESI-MS is dependent on a multitude of instrumental and non-instrumental factors, in addition to the inherent chemical and physical properties of an analyte. The non-instrumental factors include the characteristics of solvents and additives in the ESI spray medium. These factors include surface tension, conductivity, dielectric constant, viscosity, electrolyte concentration, and pH, among others [24]. During the development of this method, we observed that different solvents and different concentrations of formic acid in the mobile phase could significantly affect the analytical sensitivities of 6βHC and FC by LC-ESI-MS. To achieve the highest possible analytical sensitivity for the quantitation of FC and 6βHC, we compared the three most commonly-used organic solvents (methanol, acetonitrile, and isopropanol), in combination with different concentrations of formic acid, for the detection of these two analytes, using the same set of LC-MS conditions and the same linear gradient (3% to 80% B in 20 min). Figure 1A shows the influence of these three organic solvents (with 0.01% formic acid in both solvents A and B) on the peak areas of 6βHC (m/z 379.212) and FC (m/z 363.217). With the injections of a same standard solution containing 6βHC and FC, methanol as solvent B produced relative peak areas of 190% and 130% for 6βHC and FC, respectively, compared to acetonitrile, while isopropanol showed a relative peak area of 280% compared to acetonitrile for both analytes. This comparison shows that isopropanol in the aqueous spray medium significantly improves the ionization efficiencies of both 6βHC and FC, compared to the use of either the aprotic solvent acetonitrile, or the weaker protic solvent methanol. It should be noted that under these conditions, both compounds actually eluted from the LC column at lower percentages of isopropanol than when acetonitrile or methanol were used, due to the stronger elution strength of isopropanol in reversed-phase LC.

Next, the mobile phases using isopropanol, containing different concentrations of formic acid in both solvents A and B, were used to compare the detection sensitivity of 6βHC and FC with the same linear gradient. As shown in Figure 1B, 0.01% formic acid resulted in the highest detection sensitivity of the different formic acid concentrations tested, producing relative peak areas of 170% and 230% for 6βHC and FC, respectively, compared to those achieved with 0.1% formic acid.

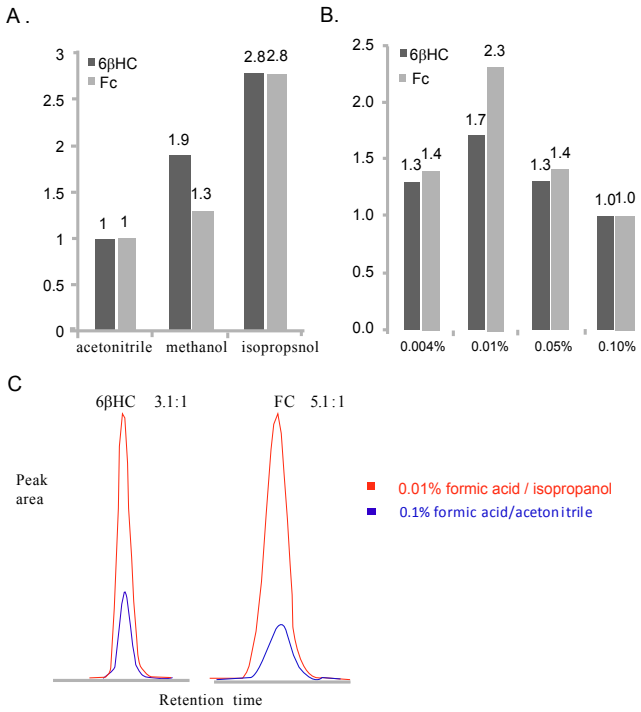

Figure 1: Effect of organic solvents and formic acid concentration on analytical sensitivities of 6β-hydroxycortisol (6βHC) and free cortisol (FC) by gradient-elution UPLC-ESI-QTOF MS. (**A**) Peak area comparison using methanol, acetonitrile, and isopropanol as the eluting solvents. (**B**) Peak area comparison with different percents of formic acid in the mobile phases, and with isopropanol as the eluting solvent. (**C**) Comparison of 6βHC and FC peak areas obtained with isopropanol/0.01% formic acid and acetonitrile/0.1% formic acid as the mobile phases, respectively, using the same linear gradient.

Interestingly, the mobile phases containing 0.004% and 0.04% formic acid showed same enhanced detection sensitivities for both analytes (130% and 140%) compared to 0.1% formic acid.

As shown in Figure 1C, this combination of 0.01% formic acid and isopropanol resulted in peak areas that were 3.1 and 5.1 times the original peak areas for 6βHC and FC, respectively, as compared to the water/acetonitrile/0.1% formic acid mobile phase. The sensitivity enhancement resulting from the use of 0.01% formic acid, instead of higher or lower concentrations, is not well understood, although similar observations have been reported in the analysis of anabolic steroids [25] and flavonoids [26] by LC-ESI-MS. In these analyses, the use of 0.01% acetic acid resulted in the highest analytical sensitivities compared to the other concentrations tested. This might be attributable to a good balance between the ionization suppression from formic acid as an ionic additive in the spray medium and the improved protonation by formic acid as a proton donor through gas-phase ion-molecule proton transfer reaction during ESI.

Based on these observations, a combination of water/isopropanol/0.01% formic acid was selected as the mobile phase for the analysis of the subjects' urine samples in this study.

Sample preparation and matrix effect

Considering the weakly-acidic nature of the hydroxyl groups in 6βHC and FC, a weakly-acidic pH was expected to provide better extraction of 6βHC and FC by LLE with organic solvents. Our preliminary experiments showed that pH 4 to 6 was optimal for the extraction of 6βHC and FC from urine, with reduced urinary matrix effect compared to a lower or higher pH values. A pH of 5 was chosen for LLE of the subjects' urine samples analyzed in this study. In addition, we also compared solid-phase extraction (SPE) using a polymeric RP resin (Strata-X) and LLE with ethyl acetate for sample cleanup. At pH 5, the LLE method produced much cleaner MS spectra throughout the LC runs than did the SPE method (data not shown), and resulted in significantly higher peak responses for both analytes as shown in Figure 2, indicating reduced suppression of the 6βHC and FC ion signals during LC-MS. This may be explained by the fact that SPE and the LC use a similar mechanism, *i.e.*, reversed-phase retention, for sample cleanup and LC separation. In contrast, LLE uses a partially orthogonal mechanism for sample cleanup, *i.e.,* solute-solvent partitioning based on the solute's solubility in two phases *versus* reversed-phase retention, and thus provided a different selectivity which lowered the chemical background during LC-ESI-MS. LLE with ethyl acetate was therefore used for cleanup of the urine samples in this study.

Using an optimized LC gradient with 0.01% formic acid/isopropanol as the mobile phase, the post-column infusion experiment showed that there was no significant ionization suppression at the retention times of 6βHC (4.17 min) and FC (10.80 min) in the LC-MS profiles of the 5 urine samples. Quantitation of the two analytes by UPLC-QTOF MS was based on the EICs for m/z 379.212 and 363.217 (the $(M+H)^+$ values for 6βHC and FC). The appropriate mass window that provided good specificity for quantitation using the full-mass detection mode of the MS instrument was determined, and a minimum mass width of +/- 25 ppm around these m/z values was shown to produce precise peak extraction of the two analytes. Figure 3 shows a typical base-peak chromatogram for a representative urine sample and the EICs for 6βHC, FC, and the IS, indicating that baseline separation was achieved for all of these compounds.

Due to the lack of a commercial source for the isotopically-labeled internal standard for 6βHC, cortisol-d4 was used as a single IS for quantitation of both of the target analytes in this study. It is worth noting that due to the deuterium effect, cortisol-d4 consistently showed a slightly shorter retention time (10.77 min *versus* 10.80 min for FC) in the UPLC-MS runs.

LODs, LOQs and linearity

Under the optimized LC-MS and sample preparation conditions, and using an S/N of 3 as the cutoff, the LODs were determined to be 0.3 and 0.1 ng/mL, equivalent to 4.0 and 1.4 fmol on-column, for 6βHC and FC, respectively. Likewise, with an S/N of 10 as the cutoff, the lower LOQs were determined to be 1.0 and 0.5 ng/mL, equivalent to 13.6 and 6.9 fmol on-column, for 6βHC and FC. The lower LOQs achieved here are close to those measured using an online SPE LC-MRM method, as reported in an earlier publication which showed the lower LOQs of 1.0 ng/mL for 6βHC and 0.2 ng/mL for FC [27]. Within the concentration range of 2 to 400 ng/mL, the regression equation for 6βHC was As/Ai = 0.0077C - 0.0027 with a correlation coefficient of 0.9996 , where As and Ai are the EIC peak areas for the standard compound and the IS, and C is the analyte concentration in ng/mL. Within the concentration range of 2.5 to 500 ng/mL, the regression equation for FC was As/Ai = 0.011C + 0.0486 with a correlation coefficient of 0.9998. . The measured concentrations from individual urine samples involved in this study were within the linear ranges for the two analytes.

Precision and accuracy

The precision was expressed as the intraday reproducibility and the interday reproducibility of the quantitation, which were calculated as the CVs of the 6βHC and FC concentrations measured from replicate analyses of a urine pool, which was prepared from an equal volume of all of the individual urine samples involved in this study. The results

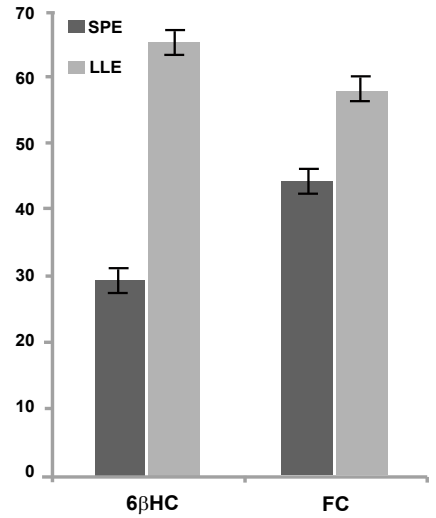

Figure 2: Comparison of UPLC-QTOF MS peak areas for urinary 6βHC and FC following LLE and SPE, respectively.

Compound	Intraday %CV (n=8)	Interday %CV (n=6)
6βHC	2.5	4.8
FC	3.7	5.3

Table 1: Interday and intraday variations of quantitation.

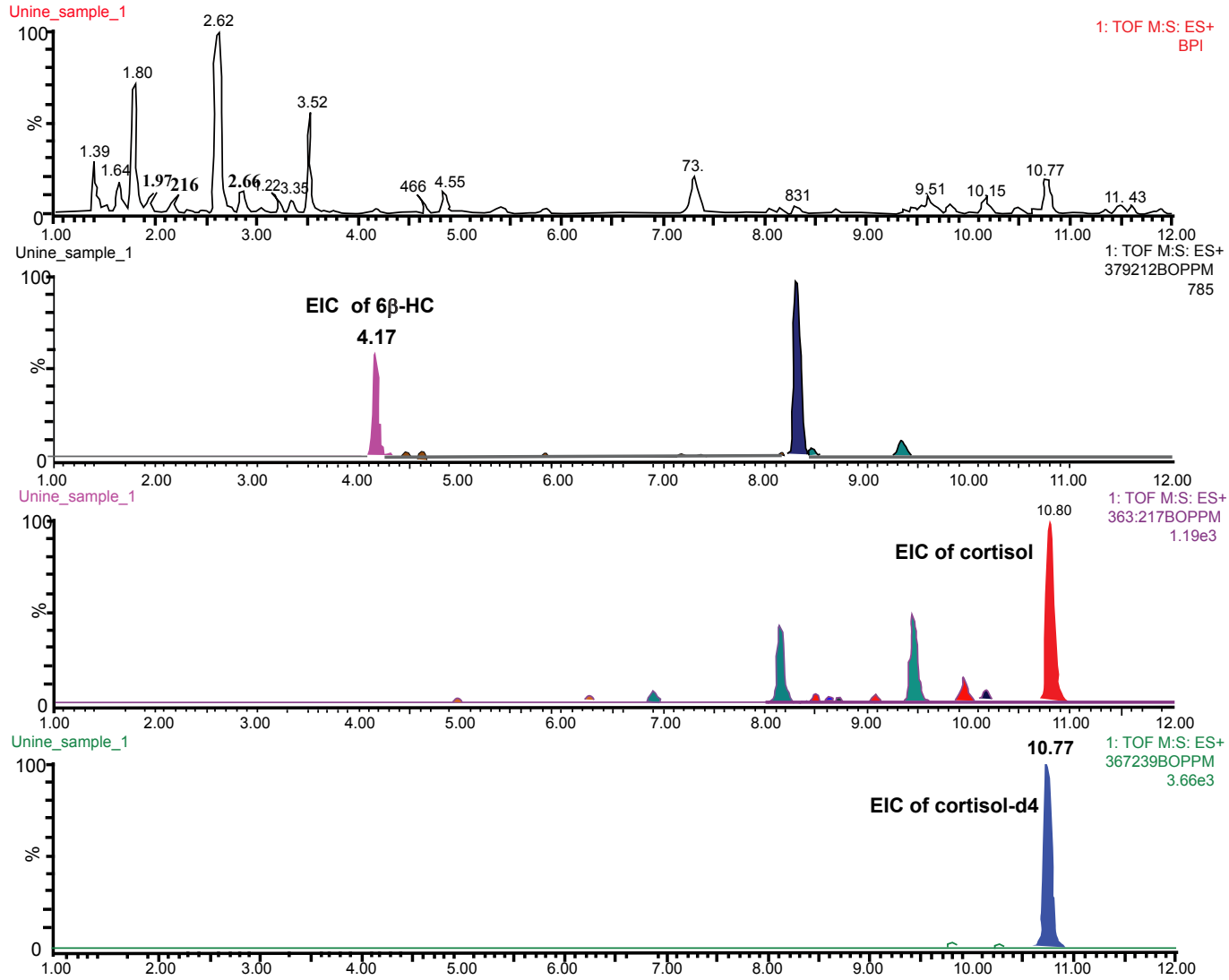

Figure 3: UPLC-MS base peak chromatogram of a urine extract and the extracted ion chromatograms (XIC) of 6βHC, FC, and cortisol-d4 (IS) following LLE with ethyl acetate at pH 5.

Compound	Spiking level (ng/mL)	% Recovery	%CV (n=3)
6βHC	50	102.3	7.0
	100	97.5	7.7
	150	96.5	2.3
FC	100	98.9	2.1
	200	95.8	1.4
	300	93.3	1.9

Table 2: Accuracy of quantitation.

shown in Table 1 indicate that the maximum intraday CV for both analytes was 3.7%, and the maximum interday CVs was 5.3%, which are well below the 10% guideline for bioanalytical method validation [24].

The accuracy was verified by calculating the recoveries of the spiked-in and measured analyte concentrations. The measured recoveries, obtained at three spiking levels, are presented in Table 2, and are within the acceptable range according to the guideline for bioanalytical method validation [21]. As demonstrated, by choosing and optimizing the sample preparation approach using LLE, comparable accuracies for 6βHC and for FC were achieved, even though only a single isotopically-labeled internal standard (cortisol-d4) was used.

Method application

Finally, the developed UPLC-MS method was applied to the assay of 6βHC and FC in the subjects' samples, in order to calculate the metabolic 6βHC/FC ratios in 122 paired urine samples collected from a cohort of sixty-one women with regular and normal-length menstrual

Ovulatory status	6βHC (nmol/mL)	FC(nmol/mL)	βHC/FC	6βHC (nmol/mL)	FC (nmol/mL)	6βHC/FC	P value*
Ovulatory (n=41)	**Follicular**			**Premenstrual**			**0.52**
Range	**15.34- 752.89**	**11.93 – 327.79**	**0.50-10.11**	**17.51-858.81**	**18.70-235.51**	**0.86-17.20**	
Mean	**286.70**	**80.30**	**4.29**	**256.29**	**66.56**	**3.85**	
CV	**63.6%**	**90.5%**	**51.3%**	**66.3%**	**73.63%**	**83.9%**	
Anovulatory (n=20)	**Follicular**			**Premenstrual**			**0.42**
Range	**22.93-884.70**	**15.69-254.64**	**0.81-8.39**	**32.15-533.19**	**8.26-612.40**	**1.43-11.92**	
Mean	**260.38**	**82.00**	**3.93**	**212.28**	**60.27**	**4.45**	
CV	**87.8%**	**95.8%**	**58.3%**	**62.5%**	**105.6%**	**64.7%**	

Table 3: The measured concentration range, mean, and coefficient of variation (CV) of urinary 6β-hydroxycortisol(6βHC), free cortisol (FC), and the 6βHC/FC ratio in ovulatory and anovulatory women, by menstrual cycle phase.

cycles, forty-one of whom had ovulation cycles and twenty of whom had subclinical anovulation cycles. The assay results are shown in Table 3. As can be seen from this table, these subjects showed large variability in the urinary concentrations of 6βHC and FC, with CVs for the measured 6βHC and FC concentrations, and the 6βHC/FC ratios in each of the four subgroups (*i.e.*, follicular *versus* premenstrual phases in subjects with either ovulatory or anovulatory cycles) being >50%. Statistics using paired two-tailed T-tests shows that there are no significant differences ($p>0.05$) in the metabolic 6βHC/FC ratios between the follicular phase and the premenstrual phase of the menstrual cycle in either the ovulatory or the subclinical anovulatory group. These results indicate that considerable inter-individual variability in the metabolic 6βHC/FC ratios exists among these subjects, and there is no correlation of the urinary 6βHC/FC ratios with two different physiological phases of the menstrual cycle, either the ovulatory or the subclinical anovulatory groups. Our results may reflect large differences in the *in vivo* CYP 3A4 activities in these subjects, though the cohort size for these two groups (41 and 20), is relatively small.

Conclusions

A new UPLC-MS method was developed for the precise and accurate quantitation of 6βHC and FC in human urine, using high-resolution ESI-QTOF MS with full-mass detection. With the optimized LC mobile phase and the sample preparation procedure, femto-molar sensitivities on column were achieved, and good precision and accuracy were demonstrated. The method has been successfully applied to the determination of the metabolic 6βHC/FC ratios on a cohort of premenopausal women. This proposed method provides an alternative approach to LC-MRM-MS for reliable quantitation of urinary 6βHC and FC.

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