Quantitative profiling of cortisol metabolites in human urine by high-resolution accurate-mass MS

Yi Wang*,1, Naomi Fujioka2 & Chengguo Xing1

1Department of Medicinal Chemistry, College of Pharmacy, University of Florida, Gainesville, FL 32610, USA
2Division of Hematology, Oncology & Transplantation, University of Minnesota, Minneapolis, MN 55455, USA
*Author for correspondence: chengguoxing@cop.ufl.edu

Aim, materials & methods: Urinary cortisol profile has the potential as a diagnostic biomarker. We therefore developed a stable-isotope dilution ultraperformance chromatography multistage MS-based method to quantify cortisol and 16 metabolites in human urines. **Results & conclusion:** The LOD for cortisol and its metabolites ranges from 0.02 to 5.81 pg/μl urine. The inter- and intraday variations were 3.7–12.9% and 3.5–15.6%, respectively. Among the metabolites analyzed, significant person-to-person heterogeneity was observed, demonstrating the need for comprehensive metabolite profiling in diagnosis. Nevertheless, the glucuronides of dihydrocortisol, dihydrocortisone, tetrahydrocortisol, allo-tetrahydrocortisol and tetrahydrocortisone are the major ones. The sum of the glucuronidated and free forms constitute >93% of the metabolites analyzed, which is termed as total cortisol equivalent. Total cortisol equivalent may serve as a surrogate of cortisol secretion.
  Clinical trial registration number: NCT02500472

Cortisol has the potential to serve as a biomarker to estimate stress level [1–3]. Plasma or salivary cortisol as a biomarker is challenging, because its abundance is subjected to diurnal fluctuation [4]. Urinary measurement, on the other hand, captures cortisol secretion over a period of time and may be more reliable [5]. Since cortisol is extensively metabolized with only about 1% of plasma cortisol excreted into urine in its parent form [6–8], both cortisol and its metabolites need to be quantified, particularly given the potential person-to-person heterogeneity of cortisol metabolism, which has never been quantitatively estimated.

The major metabolic pathways of cortisol in human are summarized in Figure 1 [9]. Various methods have been developed to quantify cortisol and its metabolites in human urines, including ELISA, HPLC, GC–MS and HPLC–MS/MS [10]. GC–MS has been extensively employed to measure cortisol metabolites in human biofluids, which have reported reference values for cortisol metabolites in human urine [11–13]; however, derivatization was typically needed prior to analyses due to the low volatility of cortisol and its metabolites [11,14,15]. Lack of high selectivity is another limit for GC–MS and LC–MS-based methods. Although MS/MS-based methods exhibited superior selectivity [9,10,16], measurement of ion transitions with low-resolution MS, such as multiple reaction monitoring with triple quadrupoles MS, typically suffers from interfering metabolites from matrices, which co-elute with targeted metabolites and exhibit the same transition at the nominal mass. The interference sometimes can cause problems in peak integration and quantification of targeted metabolites. In contrast, high-resolution accurate-mass measurement MS at MS^n scan stage enables us to extract the product ions at a mass tolerance of 5–10 p.p.m. window, which greatly reduces the interfering peaks from a complex matrix and produces cleaner chromatograms for reliable quantification. In addition, it also remains to be determined the relative abundances of free, glucurodinated and sulfate forms of cortisol metabolites in a particular subject and how heterogeneous they are among subjects. The major metabolites may serve as a better surrogate of total cortisol secretion than cortisol
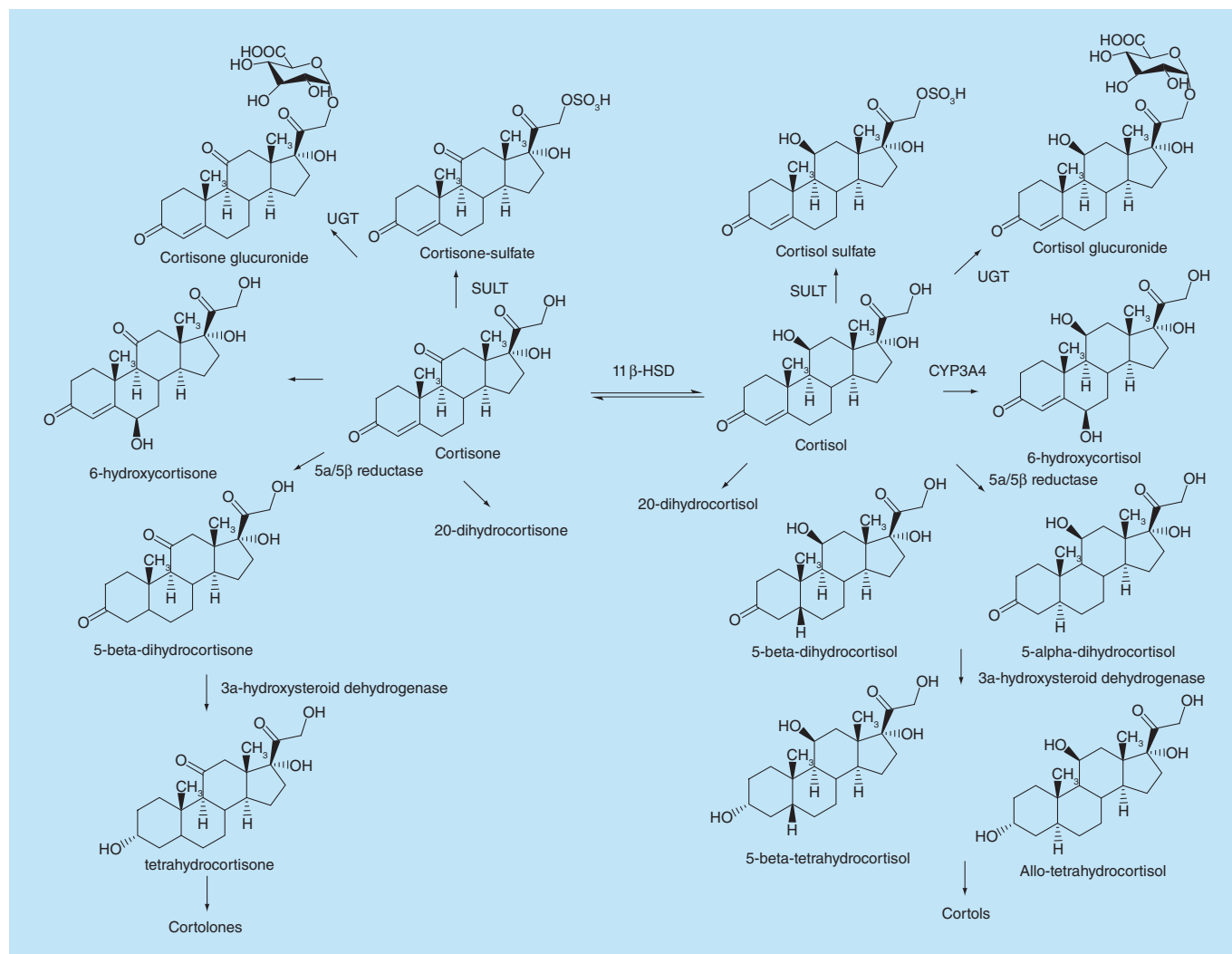
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Figure 1.   Major metabolic pathways of cortisol in human.

itself to estimate stress level. The relative abundance of cortisol metabolites may have diagnostic values since it would reflect the activities of the key enzymes involved in cortisol metabolism [17,18].

In this study, we developed a sensitive stable-isotope dilution ultra-high performance liquid chromatography multistage MS (UHPLC–MS^n)-based method with high-resolution accurate-mass Orbitrap to quantify cortisol and its 16 metabolites (Figure 1) in human urines. We also applied this method to evaluate the impact of tobacco smoking on the urinary profiling of cortisol metabolites in humans. By profiling these metabolites among nonsmokers (n = 14) and smokers (n = 21), we identified the major metabolites and characterized the level of person-to-person heterogeneity. The sum of five cortisol metabolites and their glucuronides accounted for >93% of the total metabolites quantified herein. We defined the sum of free, glucuronidated and sulfate forms of cortisol metabolites as the total cortisol equivalent (TCE). TCE provides a better estimate of total cortisol excretion than cortisol alone. Its level in smokers was found four-times of that in nonsmokers.

Materials & methods
Chemicals & materials
Cortisol, [9,11,12,12-^{2}H$_4$]-cortisol, 5α-dihydrocortisol, 5β-dihydrocortisol, 6α-hydroxycortisol, 6β-hydroxycortisol, [9,11,12,12-^{2}H$_4$]-6β-hydroxycortisol, allo-3α-tetrahydrocortisol (A-THF), 5β-tetrahydrocortisol (THF), [2,2,4,4,21,21-^{2}H$_6$]-allo-3α-tetrahydrocortisol, cortisone, [2,2,4,6,6,9,21,21-^{2}H$_8$]-cortisone, 5β-tetrahydrocortisone (THE) and [2,2,4,4,21,21-^{2}H$_6$]-tetrahydrocortisone were purchased from Toronto Research

Chemicals (TRC, ON, Canada). [9,11,12,12-^{2}H$_4$]-cortisol-21-sulfate was purchased from Sigma–Aldrich (MO, USA). The chemical purity and isotope purity were above 99% as provided by TRC (MO, USA). Identification of those standards was confirmed with high-resolution MS and H-NMR by TRC. Stability tests of isotope-labeled standards were performed biweekly during their storage at -20°C. Water, formic acid, methanol and acetonitrile were Optima LC/MS grade from Fisher Scientific (NJ, USA). β-Glucuronidase was from *Helix pomatia* and purchased from Sigma–Aldrich. Oasis MCX and WAX SPE cartridges (30 mg) were purchased from Waters (MA, USA). StrataX reverse-phase cartridges (33 μm, 10 mg/1 ml) were purchased from Phenomenex (CA, USA). Other chemicals were purchased from Sigma–Aldrich unless stated otherwise. Cortisol- and cortisone-sulfates were synthesized in house (details in Supplementary Data).

Human urine samples

Overnight urine samples of human smokers (n = 21) were obtained from a clinical trial conducted at the University of Minnesota. The study was approved by the Institutional Review Board at the University of Minnesota and all subjects provided informed, written consent. Urine samples of human nonsmokers (n = 14) were purchased from BioreclamationIVT (Bioreclamation, Inc., MD, USA). The details of urine sample collection of nonsmokers were unknown.

Enrichment of cortisol metabolites from urine

For free form of cortisol metabolites, human urine (100 μl) was mixed with H$_2$O (100 μl) and dichloromethane (800 μl). For the sum of free and gluc forms of cortisol metabolites, urine (100 μl) and H$_2$O (100 μl) were first treated with β-glucuronidase (500 units) at 37°C for 6 h followed by the addition of dichloromethane (800 μl). For both types of analyses, [^{2}H$_4$]-cortisol and [^{2}H$_8$]-cortisone were spiked at a level of 2 pg/μl urine; [^{2}H$_4$]-6-hydroxycortisol was spiked at 50 pg/μl urine; [^{2}H$_6$]-A-THF and [^{2}H$_6$]-THE were spiked at 100 pg/μl urine. The dichloromethane fraction was speed vacuumed to dryness, re-suspended in 10% CH$_3$OH (1 ml), applied to a StrataX cartridge, and washed by 10% CH$_3$OH (1 ml) and H$_2$O (4 ml). The StrataX cartridge was preconditioned with CH$_3$OH (1 ml) followed by H$_2$O (2 ml). The metabolites were eluted with CH$_3$OH (1 ml), speed vacuumed to dryness and re-suspended in 10% CH$_3$OH (50 μl) for analysis.

For cortisol- and cortisone-sulfate, human urine (100 μl) was mixed with CH$_3$OH (-20°C, 900 μl) to precipitate the proteins. [^{2}H$_4$]-cortisol-21-sulfate was spiked at a level of 5 pg/μl urine. The mixture was centrifuged at 13,000 × *g* for 20 min. The supernatant was transferred to an Eppendorf tube, acidified with HCO$_2$H (0.5%, final concentration), applied to a WAX cartridge, and washed with 0.5% HCO$_2$H in H$_2$O (2 ml) followed by CH$_3$OH (1 ml). The WAX cartridge was preconditioned with 2.5% NH$_4$OH in CH$_3$OH (1 ml) and 0.5% HCO$_2$H (2 ml). The metabolites were eluted with 2.5% NH$_4$OH in CH$_3$OH (1 ml), speed vacuumed to dryness and re-suspended in 10% CH$_3$OH (50 μl) for analysis.

Measurement of cortisol metabolites by ultraperformance LC ESI multistage MS

Mass spectral data of each cortisol and cortisone metabolites were acquired by direct infusion with a Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer (Thermo Fisher, CA, USA) and a Heated ESI (HESI-II) source (Thermo Fisher). The flow rate of direct infusion was set as 10 μl/min. Instrument tuning parameters were as follows: sheath gas, 5; auxiliary gas, 0; auxiliary gas temperature, 50°C; capillary temperature, 300°C; spray voltage, 4 kV; 1μ scan; maximum injection time, 100 and 200 ms for full MS and MS^n, respectively; in source collision-induced dissociation, 10 eV; and higher-energy collisional dissociation (HCD), 20 for MS^2. Resolution was set as 70,000 and 17,500 at *m/z* 200 for full MS and MS^n, respectively. The isolation width was set at *m/z* 1 for MS^2 scan modes. All analyses were conducted in the positive ionization mode.

The urinary samples following SPE were assayed by targeted UHPLC–MS^2 or pseudo UHPLC–MS^3 employing a Dionex Ultimate 3000 RS (Thermo Fisher) and a Q Exactive Hybrid Quadrupole Orbitrap Mass Spectrometer. The samples were resolved through an Atlantis dc18 column (150 × 2.1 mm, 3 μm particle size, 100 Å; Waters) with a 25-min linear gradient from 99% A (H$_2$O with 1% CH$_3$CN and 0.01% HCO$_2$H) to 99% B (CH$_3$CN with 5% H$_2$O and 0.01% HCO$_2$H) at a flow rate of 150 μl/min. The same UHPLC system was employed for cortisol- and cortisone-sulfate, but employed a 25-min linear gradient from 99% A (5 mM ammonium acetate in 10% CH$_3$CN) to 50% B (5 mM ammonium acetate in CH$_3$CN with 10% H$_2$O) at a flow rate of 150 μl/min. Chromeleon 7.2 Chromatography Data System was used for the UHPLC management. Parallel reaction monitoring was employed to measure the cortisol metabolites. The parameters for HESI-II were set as follows: sheath gas, 15;

auxiliary gas, 5; auxiliary gas temperature, 200°C; capillary temperature, 300°C; spray voltage, 4 kV; 1μ scan; maximum injection time, 200 ms for MS^n; and HCD, 20. In-source CID collision energy was set as 10 eV for pseudo UHPLC–MS^3. Resolution was set as 17,500 at m/z 200 for MS^n. The isolation width was set at m/z 1 for MS^n scan mode. Automated gain control was set at 50,000 for Orbitrap MS^2. The Orbitrap was routinely calibrated in the positive-ion mode using Pierce LTQ Velos ESI Positive Ion Calibration Solution (2 μg/ml caffeine, 1 μg/ml MRFA, 0.001% Ultramark 1621 and 0.00005% n-butylamine; Thermo Fisher).

Calibration curves

The postextraction spike method was employed. Since the levels of endogenous cortisol metabolites in the human urine were well above the LOD, a synthetic urine was used as the blank matrix [19]. Nine-point calibration curves (0–10,000 fg/μl urine) were constructed for cortisol and cortisone. Ten-point calibration curves (0–200 pg/μl urine) were constructed for 6-hydroxycortisol. Ten-point calibration curves (0–1000 and 0–2000 pg/μl urine) were constructed for THF/A-THF and THE, respectively. Ten-point calibration curves (0–10,000 and 0–20,000 pg/μl urine) were constructed for dihydrocortisol and dihydrocortisone, respectively. [^{2}H$_4$]-cortisol, [^{2}H$_4$]-6-hydroxycortisol, [^{2}H$_6$]-A-THF, [^{2}H$_4$]-cortisol sulfate, [^{2}H$_8$]-cortisone and [^{2}H$_6$]-THE were spiked as internal standards at levels of 2, 50, 100, 5, 2 and 200 pg/μl, respectively. LOD and LOQ were estimated by 3.3 σ/s and 10 σ/s above the background level signal of a blank matrix, respectively (σ is the SD of the slope [s] of the calibration curve) [20].

Method validation

Method validation follows the US FDA guideline [21]. Performance of this method was validated by specificity, precision, accuracy, within-day and between-day reproducibility using the pure standards of cortisol metabolites that were spiked into the blank urine matrix at four different concentrations on 3 different days. Matrix effect was evaluated by the ratios of ion signals of cortisol standards that were spiked to the urine matrix after sample preparation to the ion signals of standards that were spiked directly to the solvent. Accuracy was expressed as the percentage of the mean of measured values to theoretical values. Short-term stability of cortisol metabolites in the matrix was evaluated after three freeze and thaw cycles, and 1-, 6- and 24-h incubation in the blank urine at 37°C.

Total nicotine equivalents & urinary creatinine

The smoking status of nonsmokers and smokers was confirmed by the measurement of total nicotine equivalents (TNE) in the urine, which is the sum of nicotine, cotinine, 3-hydroxycotine, their glucuronides and nicotine-N-oxide [22]. Creatinine was measured using a modified Jaffe reaction method [23] for urine volume adjustment. The level of urinary cortisol metabolites was expressed as pmol/mg creatinine.

Statistical analysis

Two-tailed t-test was performed for the analysis of the results between smokers and nonsmokers using GraphPad Prism (v. 7.00). $p < 0.05$ was considered statistically significant.

Results & discussion
Mass spectrometric characterization of cortisol metabolites

Mass spectra of cortisol and its metabolites at MS^2 and pseudo MS^3 [24] scan stages, which were characterized by direct infusion of pure standards with accurate mass measurements by Orbitrap, exhibited excellent mass accuracy. The observed masses of product ions were within 3 p.p.m. window of the calculated m/z values. As an example, product ion mass spectra of cortisol (m/z 363.2162 vs calculated m/z 363.2166, Δ1.1 p.p.m.) and [^{2}H$_4$]-cortisol (m/z 367.2413 vs calculated m/z 367.2417, Δ1.1 p.p.m.) contained ions at m/z 97.0652 (C$_6$H$_9$O, vs calculated m/z 97.0648, Δ4.1 p.p.m.), m/z 121.0649 (C$_8$H$_9$O, vs calculated m/z 121.0648, Δ0.8 p.p.m.), m/z 135.0803 (C$_9$H$_{11}$O, vs calculated m/z 135.0804, Δ0.7 p.p.m.), m/z 267.1739 and 271.1989 ([M+H-C$_2$H$_8$O$_4$]$^+$), m/z 309.1844 and 313.2094 ([M+H-3H$_2$O]$^+$), m/z 327.1950 and 331.2200 ([M+H-2H$_2$O]$^+$) and m/z 345.2054 and 349.2306 ([M+H-H$_2$O]$^+$) (Figures 2A & B).

Production mass spectra of cortisol metabolites and corresponding isotope-labeled standards are shown in Supplementary Figures 1–3. THF and A-THF (m/z 367.2479) tend to undergo in-source fragmentation by ESI in full MS scan, losing one to three molecules of water to produce a base peak at m/z 331.2268. THF/A-THF and [^{2}H$_6$]-A-THF exhibited different relative abundances of product ions at m/z 135.1166, m/z 147.1167,
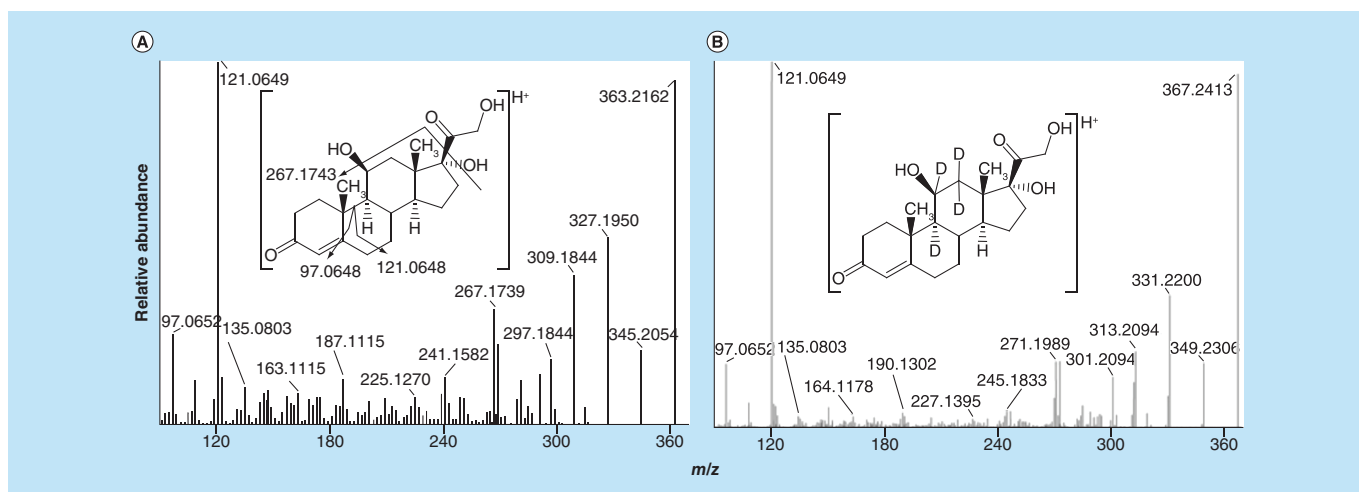
Figure 2.   Product ion mass spectra of (A) cortisol (m/z 363.2 >) and (B) [$^2H_4$]-cortisol (m/z 367.2 >) at MS^2 scan stage.

m/z 139.1415 and m/z 151.1415 (Supplementary Figure 2). After confirmed their identification by H-NMR and UHPLC–MS, we suspected that the positioning of the deuterium atoms might cause the different relative abundance in ionization. In-source fragmentation of parent ions in full MS scan was also observed for THE and dihydrocortisone. Therefore, we employed a pseudo-MS^3, which was to use the in-source fragmentation by applying a 10 eV in-source CID followed by product-ion scanning in the Orbitrap, for quantification of THF, A-THF, THE and dihydrocortisone (Supplementary Figures 2E–G & 3A & B). Atmospheric pressure chemical ionization-based ionization has been employed for cortisol metabolites; however, we aimed to develop a method to simultaneously measure different cortisol metabolites, instead of switching the sources between ESI and atmospheric pressure chemical ionization for different compounds. By employing the cation exchange SPE cartridge, we extracted the cortisol- and cortisone-sulfates from the urine and directly measured them by UHPLC-MS/MS (Supplementary Figures 2C & 3D).

Supplementary Figure 4 shows the UHPLC–MS^2 and UHPLC–MS^3 profiles of the pure standards of cortisol, their metabolites and corresponding isotope-labeled standards. We assayed both α- and β-anomers of 6-hydroxycortisol, 5-tetrahydrocortisol and 5-dihydrocortisol, but did not achieve chromatographic separation of the two anomers under current UHPLC conditions. Therefore, the integration of the peaks of 6-hydroxycortisol, 5-tetrahydrocortisol and 5-dihydrocortisol included both α- and β-anomers. Deuterium isotope effect caused [$^2H_6$]-A-THF to elute 0.5 min earlier than the unlabeled THF and A-THF (Supplementary Figure 4A). Previous studies reported a partial separation of THF and A-THF by using chromatographic condition with 0.1% acetic acid or formic acid (FA) [9,25]. However, we observed considerable ion suppression and lost 40–80% ion signals of cortisol metabolites when the concentration of FA increased from 0.01 to 0.05%, and 0.1% during optimization of chromatographic conditions. No deuterium isotope effects were observed for the isotope-labeled standards of other cortisol metabolites.

Method validation

Because the endogenous levels of urinary cortisol, 6-hydroxycortisol and cortisone are above the LOD, a synthetic urine sample was employed as the blank matrix for calibration curve construction. Human urine samples from nonsmokers were used as blank matrices for the other cortisol metabolites. In the blank urine sample spiked with LLOQ level of cortisol metabolites, six to nine scans were acquired across the full width of the peaks (Supplementary Figure 5). At the high spike level of standards, 14–22 scans were acquired across the full width of the peak (Supplementary Figure 5).

The calibration curves of cortisol metabolites are shown in Supplementary Figure 6. Because dihydrocortisol and dihydrocortisone, which were estimated by [$^2H_6$]-THF and [$^2H_6$]-THE, exhibited different MS/MS fragmentation, slopes of their calibration curves were not close to 1. We estimated LOD and LOQ by 3.3 σ/s and 10 σ/s, respectively (σ is the standard deviation of the slope [s] of the calibration curve) [26], because there was no measurable background signal at the MS^2 and MS^3 scan stage in the blank sample acquired by the high-resolution

Table 1. Estimates of LOD and LOQ for cortisol metabolites in human urines.		
Metabolites	LOD (pg/μl urine)	LOQ (pg/μl urine)
Cortisol	0.05	0.16
6-Hydroxycortisol	1.20	3.63
A-THF/THF	0.79	2.39
Dihydrocortisol	1.56	4.74
Cortisol sulfate	0.23	0.77
Cortisone	0.02	0.08
THE	1.72	5.21
Dihydrocortisone	5.81	17.61
Cortisone-sulfate	0.28	0.84

[$^2H_4$]-cortisol, [$^2H_4$]-6β-hydroxycortisol, [$^2H_6$]-A-THF, [$^2H_8$]-cortisone, [$^2H_6$]-THE and [$^2H_4$]-cortisol sulfate were employed as internal standards.
A-THF: Allo-3α-tetrahydrocortisol; THE: 5β-tetrahydrocortisone; THF: 5β-tetrahydrocortisol.

Orbitrap. LOD and LOQ values of cortisol metabolites were summarized in Table 1. Sensitivities were achieved for cortisol and cortisone in the urine with LOD as low as 0.05 and 0.02 pg/μl urine, respectively, 10–75-times more sensitive than the methods in previous reports [9,25]. LOD and LOQ estimates were higher for A-THF/THF, 6-hydroxycortisol, dihydrocortisol, THE and dihydrocortisone, likely because these compounds were labile under ESI and underwent in-source fragmentation to lose H_2O molecules in the full MS scan, which caused a drop of ion signals measured at MS^2 and MS^3 scan stages. Previous studies also reported LOD values to be 0.2–1 pg/μl for A-THF, THF and THE using low-resolution MS [9]. In our study, A-THF, THF and THE were not detectable at a mass tolerance of 5 p.p.m. when spiked at 0.2 pg/μl urine. However, when the mass tolerance increased to 500 p.p.m., interfering peaks from the matrix were observed that co-eluted with A-THF/THF and THE (Supplementary Figure 7).

The accuracy, precision and reproducibility of our methods were summarized in Supplementary Tables 1 & 2. The within day and between day variations were 2.8–12.9% and 3.2–15.6%, respectively. Recovery rates ranged 50–92% after sample workup, depending on each metabolite. The matrix effect for cortisol metabolites at LLOQ and high range is summarized in Supplementary Table 3. Matrix effect values, which were calculated as the ratios of peak areas of pure standards spiked to the blank urine after sample preparation to the peak areas of the standards spiked to the pure solvents, ranged from 82 to 142% and 95 to 120%, respectively (Supplementary Table 3). These data suggest the sample preparation by tandem liquid–liquid extraction with dichloromethane and offline SPE greatly reduced the ion suppression from the urine. Freeze-and-thaw stability and short-term in-matrix stability of cortisol metabolites were also examined and summarized in Supplementary Table 4. Freeze-thaw caused relatively small loss of signals except for A-THF/THF at the LLOQ level and dihydrocortisone at the high range. Short-term in-matrix stability experiment showed that the signals of cortisol metabolites at the high spiked level after a 24-h test were 71–114% of the control with dihydrocortisone being the lowest one while there was no appreciable loss within 1 h for any analyte. At the LLOQ- spiked level, signals of most metabolites decreased significantly after the 24-h test except for the two sulfates. It should be noted that the endogenous level of the free and gluc forms of these metabolites in the human urine are in the range of the high spiked level, thus the stability at the high spike level is more clinically relevant.

UHPLC–MS^n analysis of cortisol metabolites in human urine

We then employed this targeted UHPLC–MS^n method to quantify cortisol metabolites in the urine samples of 14 nonsmokers and 21 smokers. Their smoking status was confirmed by quantifying the urinary TNE. With the comprehensive understanding of nicotine metabolism, TNE (the sum of nicotine, cotinine, 3-hydroxycotinine, their glucuronide conjugates and nicotine N-oxide, which captures 80–90% of nicotine metabolites) has been demonstrated as an excellent surrogate to estimate human smoking status [22,27–29]. The average TNE values in smokers and nonsmokers are 158 ± 85 (40–423) and 4 ± 2 (2–8) nmol/mg creatinine, respectively (Supplementary Figure 8). Figure 3 shows examples of the reconstructed ion chromatograms of cortisol metabolites in the urine of a nonsmoker and a smoker. Elution time and mass spectra of these metabolites in the urine sample were in agreement with the data acquired by the pure standards. High-resolution accurate-mass measurement of Orbitrap at MS^n scan stage enabled us to extract the product ions at a mass tolerance of 5 p.p.m. window, which greatly
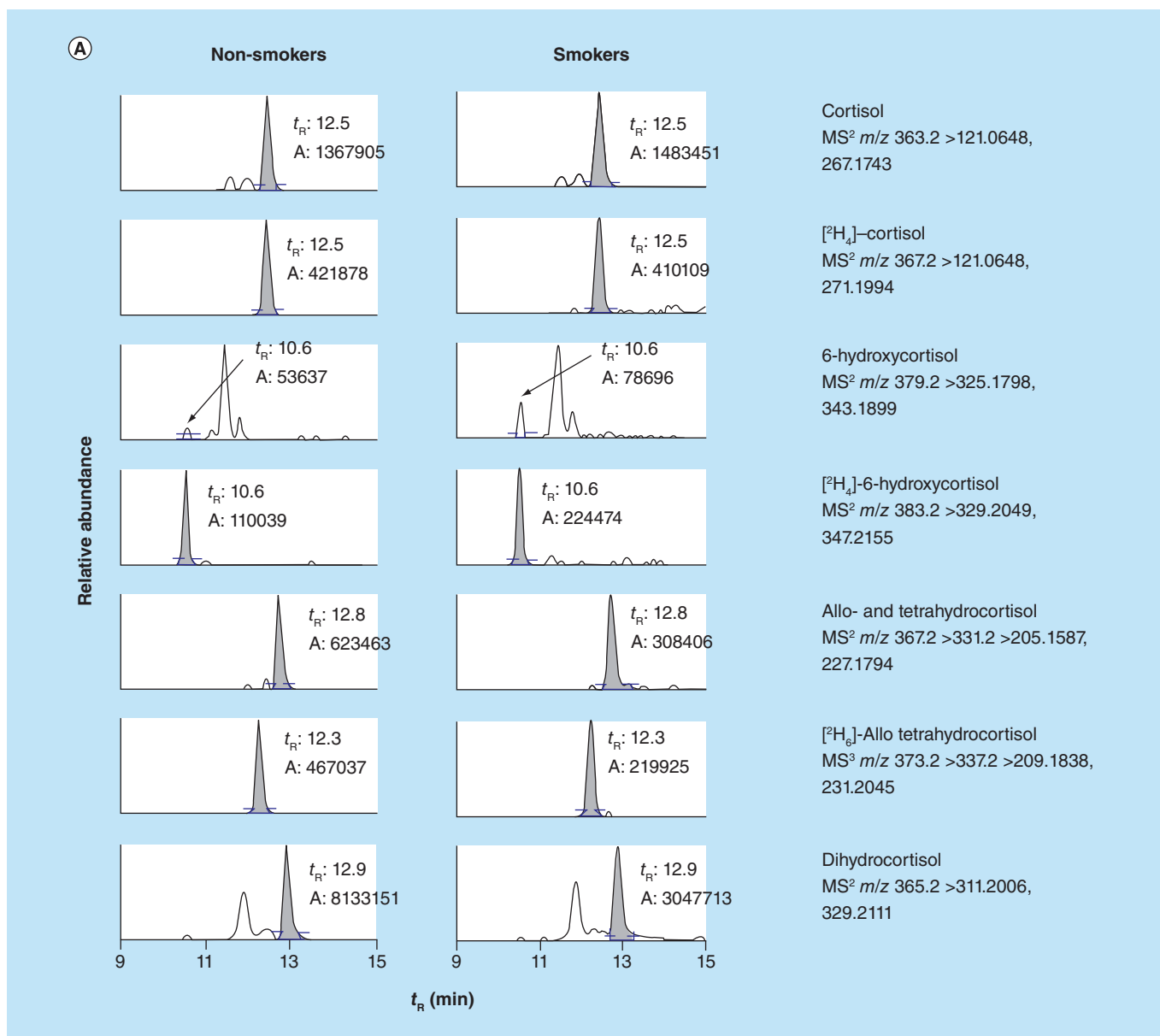
Figure 3. Reconstructed ion chromatograms of (A) cortisol and (B) cortisone metabolites in the urine samples of human nonsmokers and smokers. The mass extraction window was ±5 p.p.m.

reduced the interfering peaks from the matrix and produced cleaner ion chromatograms for reliable quantification in comparison to the low-resolution MS-based method (Supplementary Figure 7). Although other peaks were seen on the chromatograms of 6-hydroxycortisol, dihydrocortisol, THE and dihydrocortisone even at a mass tolerance of 5 p.p.m., those peaks did not co-elute with targeted metabolites (Figure 3). We also observed the level of 6-hydroxycortisol decreased by 14–30% after β-glucuronidase treatment (data not shown), which suggested that 6-hydroxycortisol was not stable, consistent with the result of its stability test (Supplementary Table 4). Therefore, we only quantified the free form of 6-hydroxycortisol.

The estimates of cortisol and its metabolites in the urine of 14 nonsmokers and 21 smokers upon creatinine correction were summarized in Table 2. The levels of urinary total cortisol and cortisone were comparable to the values reported in a previous study (12–80 μg/24 h for cortisol; 25–341 μg/24 h for cortisone) [16,25]; however, the abundance of total dihydrocortisol, A-THF/THF, dihydrocortisone and THE determined in our study was 10–100 fold higher [16]. The level of those metabolites may be underestimated in the previous study,
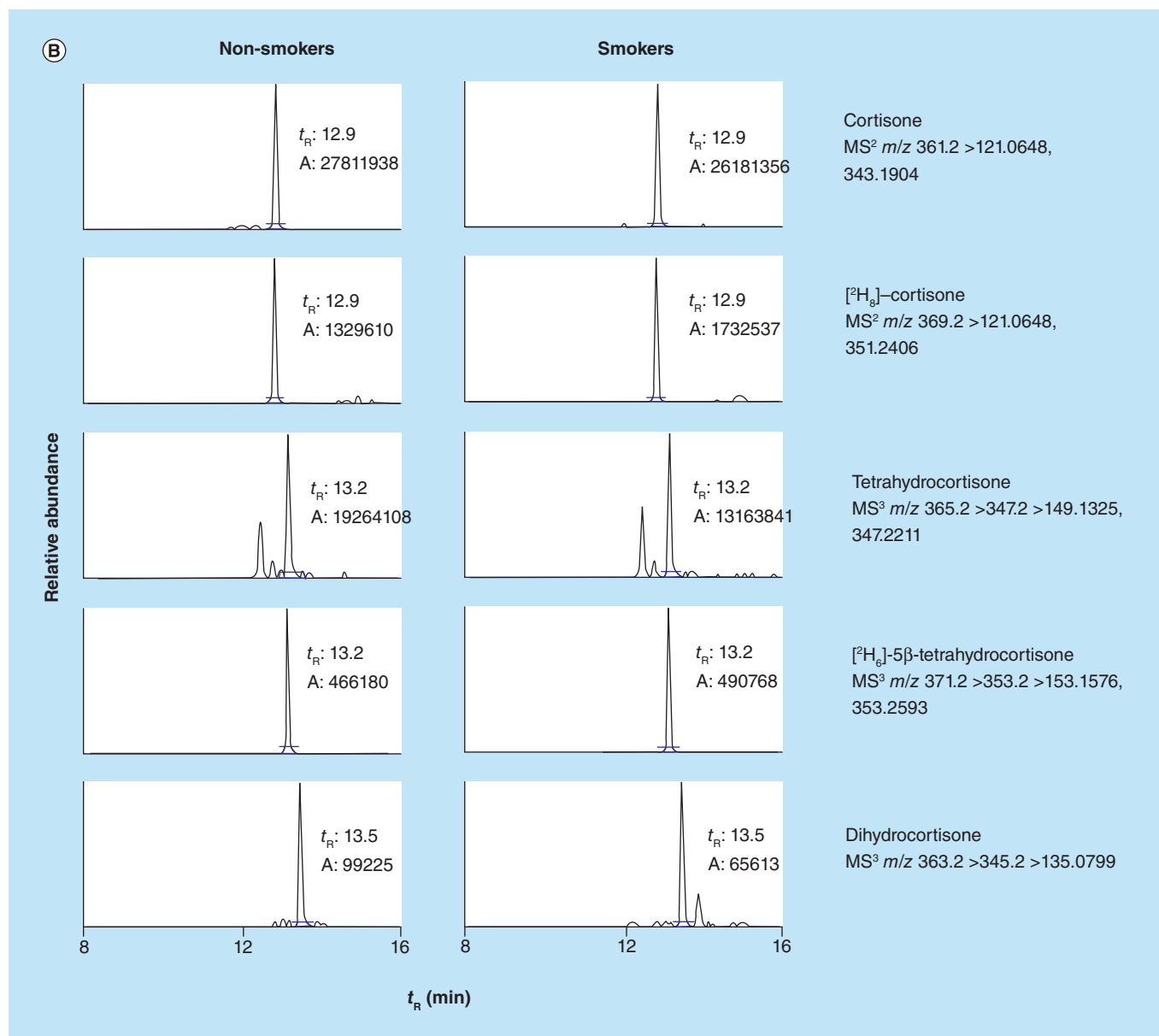
Figure 3.   Reconstructed ion chromatograms of (A) cortisol and (B) cortisone metabolites in the urine samples of human nonsmokers and smokers (cont.). The mass extraction window was ±5 p.p.m.

because they were estimated based on $[^2H_4]$-cortisol and $[^2H_4]$-hydroxycortisol being the internal standards. As demonstrated in Table 1, dihydrocortisol, A-THF/THF, dihydrocortisone and THE were labile and underwent in-source fragmentation under ESI, which caused their signals to decrease by 15–130-fold compared with cortisol and cortisone. The free forms of dihydrocortisol, THE and dihydrocortisone in some subjects were below LOD. In all the samples, cortisol and cortisol gluc were the low abundant metabolites, accounting for not >0.1% of the metabolites quantified, raising the question of them as the surrogate of cortisol secretion. On the other hand, the sum of the free and glucuronide forms of dihydrocortione, dihydrocortisol, A-THF/THF and THE accounted for >92% of the cortisol metabolites in all 35 urines was analyzed. These data suggest that the sum of these five metabolites and their glucuronides, which can be obtained from a single analysis with β-glucuronidase treatment, offers a close approximation of the total amount of cortisol and metabolites in urine, which is termed as urinary TCE. There is a difference in the values of total 5-dihydrocortisol measured in our study and those reported values in the literature [30]. However, most, if not all, of previous reports only quantified either the free form or the total

Table 2. Quantities of cortisol metabolites in the urine of 14 nonsmokers and 21 smokers.

Metabolites	Smokers		Nonsmokers	
	Level (pmol/mg creatinine)	Relative abundance	Level (pmol/mg creatinine)	Relative abundance
Cortisol	3–58	<0.07%	0.4–61[†]	<0.04%
Cortisol gluc	8–565	<0.02%	1–17	<0.03%
THF/A-THF	1–71	<0.03%	30–1635	<0.94%
THF/A-THF gluc	612–9139	0.14–9.50%	352–11,584	0.29–5.38%
Dihydrocortisol	4–3628	<0.01–2.52%	0.4–56[†]	<0.18%
Dihydrocortisol gluc	14,542–280,266	2.88–83.40%	14,064–408,434	13.72–92.34%
Cortisol sulfate	28–179	<0.11%	4–148	<0.03%
6-hydroxycortisol	5–616	<0.33%	1–1871[†]	<0.01–6.08%
Cortisone	18–184	<0.17%	11–231	<0.18%
Cortisone gluc	18–2132	<0.01–2.34%	12–562	<0.31%
THE	1–243[†]	<0.17%	0.5–217[†]	<0.08%
THE gluc	2559–34,770	0.57–9.55%	988–41,734	2.35–14.85%
Dihydrocortisone	14–5213	<0.01–5.15%	2–2405[†]	<0.01–1.38%
Dihydrocortisone gluc	19,447–1,868,017	9.39–95.39%	4460–196,412	1.41–69.85%
Cortisone sulfate	1–380[†]	<0.14%	1–217[†]	<0.08%
Total dihydrocortisol + dihydrocortisone + THF/A-THF + THE		97.04–99.96%		92.91–99.82%

[†] When the measured levels were below the LOD, half of the LOD values of the metabolites were reported herein.
A-THF: Allo-3α-tetrahydrocortisol; THE: 5β-tetrahydrocortisone; THF: 5β-tetrahydrocortisol.

form. In addition, they rarely performed the quantitative analysis to each individual subject. Of course, our results remain to be validated in samples from larger populations.

More importantly, there was significant abundance heterogeneity of these metabolites among individuals. For instance, the abundance of dihydrocortisol gluc varied between 2.9 and 83.4% among smokers, and 13.7 and 92.3% among nonsmokers. Likewise, the abundance of dihydrocortisone gluc varied between 9.4 and 95.4% among smokers, and 1.4 and 69.9% among nonsmokers. Similar extent of variation was observed among other metabolites. These variations may be due to the biological and physiological differences among the participants, particularly the UDP-glucuronosyltransferase activity, which have significant human heterogeneity [31]. The large variations were not likely due to differential reaction kinetics, because smoker urine collection was well controlled and similar degrees of variation were observed with the nonsmoker urines. Further investigations are warranted to determine the source for such variations and whether these variations are of clinical diagnosis values.

Cigarette smoking & urinary total cortisol equivalent

It is well known that cigarette smoking helps smokers cope with stress [32,33]. However, nicotine addiction could lead to increased long-term stress [34]. Indeed, cigarette smoking has been reported to increase cortisol level in human plasma and saliva [35,36]. A positive correlation was observed as well between the content of nicotine in different cigarettes and the magnitude of increase in plasma cortisol [37]. In this study, we therefore explored the association between urinary TCE and smoking status. The average TCE in smokers was 512 ± 101 nmol/mg creatinine, which is fourfold that in nonsmokers (128 ± 31 nmol/mg creatinine; p = 0.0046) (Figure 4). Consistent with previous plasma and saliva observations [35–37], smoking appeared to increase the cortisol secretion in human urine.

Conclusion

We developed a UHPLC–MS^n method using high-resolution accurate-mass Orbitrap. The method can detect and quantify cortisol and 16 of its metabolite in human urine with a sensitivity as low as 55 amol (20 fg) cortisol metabolite per μl urine in 100 μl urine. We also used this method to profile cortisol metabolites in human nonsmokers and smoker urines. The sum of the free and glucuronide forms of dihydrocortisol, A-THF, THF, dihydrocortisone and THE, termed TCE, offers an approximation of the total cortisol metabolites. Interestingly the TCE values are fourfold in the urines of smokers to nonsmokers, consistent with the previous reports that tobacco use may increase cortisol secretion. Besides stress, urinary cortisol metabolite profiles may be useful for
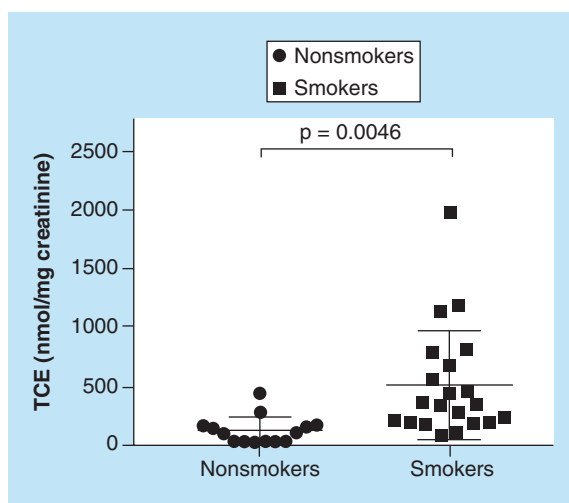
Figure 4. Estimates of total cortisol equivalent in the urines of nonsmokers and smokers. Unpaired two-tailed t-test.
TCE: Total cortisol equivalent.

disease diagnostics. For instance, the ratio of cortisol to cortisone, an estimate of 11β-hydroxysteroid dehydrogenase activity, has been proposed to evaluate children with adrenal diseases and Cushing syndrome [17,18]. Previous clinical studies also reported the ratio of allo-tetrahydrocortisol and THF to tetrahydrocortisone as an indicator of hypertension and depression [38–40]. Our method, which exhibits excellent sensitivity and selectivity to measure free and total forms of cortisol, cortisone, A-THF, THF and THE, has the potential in disease diagnosis clinically.

There are also some limitations of our method. We did not quantify 6-hydroxycortisone, 20-dihydrocortisol, 20-dihydrocortisone, cortols and cortolones (Figure 1), which were estimated to be at the comparable levels of cortisol and cortisone by GC–MS in urine [13]. Efforts are needed in the future to quantify these metabolites and their conjugates. In addition, we used in-source fragmentation coupled with MS/MS in Orbitrap to quantify THF, A-THF, THE and dihydrocortisone; however, there is a possibility that the ions from in-source fragmentation may come from other steroids, which happen to co-elute with our targeted compounds on the chromatogram, due to their structural and physicochemical similarities. We also did not have the detailed information on the commercially purchased urine samples of nonsmokers, such as collection time, subject health conditions, which may introduce effects to the TCE quantified. Thus, a more systematic study is needed to confirm the impact of tobacco smoking on the quantitative profiling of cortisol metabolites in human urine in the future.

Future perspective

Because of the advancement of mass spectrometers and commercially available isotope-labeled standards of cortisol metabolites, the LC–MS/MS-based method with high-resolution accurate-mass mass spectrometers provides better accuracy, selectivity and sensitivity in quantification of cortisol metabolites in human biofluids. Urine can be a great matrix for identification of biomarkers for disease diagnosis because of the noninvasive collection, large volumes and cumulative information of a period of time. Our method can be adapted to diagnostic laboratories and clinics for disease diagnosis.

Summary points

Method sensitivity & validation
- The method is sensitive to detect attomole cortisol metabolites per microliters human urine samples.
- First report to directly assay cortisol- and cortisone-sulfates by SPE following online UHPLC–MS/MS.

Application of the method
- First study to report the free, glucuronide and sulfate forms of major cortisol metabolites and the relative abundance of cortisol metabolites in human urines.
- Urinary cortisol metabolites level in smokers is 4× greater than nonsmokers.
- Our method can be applied for disease diagnosis.

Supplementary data

Data sharing statement

Deidentified individual data that underlie the results reported in this article (text, tables, figures and appendices), along with the study protocol, will be available indefinitely for anyone who wants access to them.

Acknowledgements

Financial & competing interests disclosure

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