GC-MS–Based Quantitative Signatures of Cytochrome P450–Mediated Steroid Oxidation Induced by Rifampicin

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Background: Drug-induced cytochrome P450 (CYP) activity affects endocrine function and drug clearance rates, leading to the development of unpredictable pathologic and toxicologic risks.

Methods: Urinary steroid profiling based on gas chromatography–mass spectrometry (GC-MS) was used for simultaneous quantification of CYP-mediated regioselective hydroxysteroids and their substrates, including 26 androgens, 9 estrogens, 5 progestins, and 7 corticoids. The quantitative data were visualized using a hierarchically clustered heat map to allow identification of CYP-mediated steroid signatures. Twelve healthy subjects were orally administered 600 mg of rifampicin a day for 7 days, and their CYP enzyme activity was evaluated.

Results: Using GC-MS, all 47 steroids were well separated with good peak shapes. This assay had good linearity ($r^2 > 0.994$) in a dynamic range, and the interassay imprecision (% CV) and inaccuracy (% bias) were 3.0%–15.6% and 98.0%–109.2%, respectively. Administration of the CYP3A4 inducer rifampicin produced distinct differences in CYP3A4 and CYP11B1, CYP19A1, HSD11B, and HSD17B, which were indicated by their heat map–visualized steroid signatures.

Conclusions: This CYP-mediated steroid signature profile allows simultaneous assessment of CYP1A, CYP1B, CYP2C, CYP3A, CYP11B, CYP17A, CYP19A, and CYP21A in urine samples. This method could therefore be a useful tool for assessing drug efficacy.

Key Words: cytochrome P450, steroid hydroxylation, GC-MS, steroid signatures, rifampicin

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INTRODUCTION

Human cytochrome P450 (CYP) enzymes, which comprise a super family of hemoproteins, catalyze the oxidation of organic substances, including endogenous and xenobiotic compounds such as steroids and drugs.[1,2] CYP enzyme function can be affected by drugs via activation of transcriptional receptors, which regulate the rates of drug activation or inactivation, leading to the development of unpredictable pathologic and toxicologic risks.[3] In addition, the expression of CYP enzymes depends on genetic variation, environmental factors, or individual conditions, resulting in variability in drug response.[4] Specific drug probes have been investigated to evaluate the phenotypes derived from CYP enzymes that are perturbed by drug interactions; however, these assays have the drawback of requiring the administration of a test compound, a process that could induce adverse reactions.[5,6]

Many CYP assays have been performed with radioimmunoassay,[7] enzyme-linked immunosorbent assay,[8] and liquid chromatography.[9,10] However, immunochemical methods have limited applicability due to the overestimation produced by cross-reaction with other related steroids and the fact that only 1 enzyme can be measured at a time.[10] Colorimetric and fluorometric liquid chromatography methods also have limited efficiency and capacity due to various sources of interference in biologic samples.[10,11] In contrast, mass spectrometry–based techniques have better quantitative reproducibility[12,13] and can be applied to large-scale clinical studies for physiologic monitoring, toxicologic evaluation, and clinical diagnosis.[14–16] Gas chromatography–mass spectrometry (GC-MS) profiling is a powerful technique that is widely used for multitargeted steroid analysis in biologic samples.[15–20] However, current methods are limited to the assessment of only CYP11A, CYP11B, CYP17A, CYP19, and CYP21[16,18–20] and not CYP3A4, which is the most active drug/steroid-metabolizing enzyme expressed in the human liver.[2]

Steroid hormones are biologically active endogenous compounds that regulate various physiologic and pharmacologic processes.[21,22] In biosynthetic pathways, they are produced from cholesterol via CYP enzymes (CYP11A, CYP11B, CYP17A, CYP19A, and CYP21A isoforms) aided by 3β-, 11β-, and 17β-hydroxysteroid dehydrogenases.[21,22] In addition, these CYP enzymes are primarily involved in the nicotinamide adenine dinucleotide phosphate–dependent hydroxylation of androgens, estrogens, progestins, and corticoids. In addition, CYP3A4 accounts for approximately 60% of the total CYP content in the human liver.[2] It is considered a versatile enzyme that can metabolize not only most marketed drugs

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but also various steroid substrates, including testosterone, androstenedione, dehydroepiandrosterone, progesterone, and cortisol, which precedes the hydroxylation reaction that in humans occurs predominantly at the C-6β and -16α positions.[23–26] However, these previously reported findings have been obtained using specific assays for a single steroid metabolized by one or more CYP isoforms, both in vitro and in vivo,[9,11,23–29] and thus might not elucidate the mechanisms of global steroid metabolism. To our knowledge, there are few reports on the simultaneous analysis of multiple CYP enzyme activities on steroid metabolism affected by drugs in human biologic samples.

Because genetic polymorphisms in the CYP family can be evaluated via their metabolic activities on endogenous steroids (**Table, Supplemental Digital Content 1**, http://links.lww.com/TDM/A48), the use of simple and noninvasive CYP assays has been proposed.[9,30] Here, we present a GC-MS–based profiling method for regioselective hydroxysteroids (26 androgens, 9 estrogens, 5 progestins, and 7 corticoids) in human urine mediated by CYP enzymes, followed by a multivariate data analysis to evaluate their metabolic signatures. The aim of this study was to validate a novel approach to GC-MS–based steroid profiling and to provide a method that allows for the detection of quantitative signatures of CYP-mediated steroid oxidation affected by drugs. This method was therefore applied to a human study involving the administration of rifampicin, a known CYP3A4 inducer.[31] This study shows that drug-induced CYP activation affects overall steroid metabolism and exhibits metabolic signatures of CYP activity, which may be a useful tool for assessing both drug efficacy and cross-validation of pharmacogenomics-based CYP biomarkers.

MATERIALS AND METHODS

Chemicals and Reagents

Reference calibrators of the 47 steroids examined in this study (Table 1) were obtained from Sigma (St Louis, MO) or Steraloids (Newport, RI). The internal standards (ISs) 16,16,17-d_3-testosterone for the 26 androgens, 2,4,16,16-d_4-17β-estradiol for the 9 estrogens, 9,11,12,12-d_4-cortisol for the 7 corticoids, and 2,2,4,6,6,17α,21,21,21-d_9-progesterone and 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone for the 5 progestins were purchased from NARL (Pumble, Australia) and C/D/N Isotopes (Pointe-Claire, Quebec, Canada). For solid-phase extraction, an Oasis HLB cartridge (3 mL, 60 mg; Waters, Milford, MA) was preconditioned with 3 mL of methanol followed by 3 mL of deionized water. Sodium acetate (reagent grade), acetic acid (glacial, 99.99%), and L-ascorbic acid (reagent grade) were obtained from Sigma. A 50% glycerol solution of β-glucuronidase extracted from *Escherichia coli* (140 U/mL) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The trimethylsilylating (TMS) agents, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma. All organic solvents used were of analytical or high-performance liquid chromatography grade and were purchased from Burdick & Jackson (Muskegan, MI). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA).

Preparation of Stock and Working Solutions

Each stock solution of the reference calibrator was prepared at a concentration of 1 mg/mL in methanol and working solutions were prepared using methanol at various concentrations ranging from 0.01 to 10 μg/mL. L-Ascorbic acid (1 mg/mL) was used to prevent oxidation of the catechol estrogens, including 2-OH-E1, 2-OH-E2, 4-OH-E1, and 4-OH-E2. All solutions were stored at −20°C until needed and were stable for a minimum of 3 months.

Sample Preparation

Quantitative steroid profiling was performed based on previous reports.[18,32] Briefly, the urine samples (2 mL) were spiked with 20 μL of the 6 IS mixtures (d_4-androsterone, 40 μg/mL; d_3-testosterone, 4 μg/mL; d_4-17β-estradiol, 2 μg/mL; d_8-17α-hydroxyprogesterone, 5 μg/mL; and d_9-progesterone and d_4-cortisol, 10 μg/mL), and they were then extracted with Oasis HLB SPE cartridges at a low flow rate (<1 mL/min). After each sample was loaded onto the cartridge, it was washed with 2 mL of water and eluted twice with 2 mL of methanol. The combined eluate was evaporated under a nitrogen stream and then added to 1 mL of 0.2 mol/L phosphate buffer (pH 7.2), 100 μL of 0.2% ascorbic acid, and 50 μL of β-glucuronidase solution. After incubation at 55°C for 1 hour, the solution was extracted twice with 2.5 mL of methyl *t*-butyl ether (MTBE). The organic solvent was evaporated in an N_2 evaporator at 40°C and further dried in a vacuum desiccator over P_2O_5-KOH for at least 30 minutes. Finally, the dried residue was derivatized with MSTFA/NH_4I/DTE (40 μL; 500:4:2, v/w/w) at 60°C for 20 minutes, and 2 μL of the resulting mixture was subjected to GC-MS in the selected-ion monitoring mode. The extraction solvent was selected to maximize the extraction efficiency with 5 different organic solvents: ethyl acetate: *n*-hexane (2:3, v/v), ethyl acetate: *n*-hexane (3:2, v/v), ethyl acetate: *n*-hexane (4:1, v/v), MTBE, and *n*-hexane.

Instrument Conditions

GC-MS was performed using an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 MSD (Agilent Technologies, Palo Alto, CA). The electron energy was 70 eV and the ion source temperature was 230°C. Each sample (2 μL) was injected in split mode (10:1) at an injector temperature of 280°C and separated through an Ultra-1 capillary column (25 m × 0.2 mm inner diameter, 0.33-μm film thickness; Agilent Technologies). The oven temperature was initially 215°C, which was ramped to 245°C at 1°C/min and then finally increased to 315°C (held for 2 minutes) using a 10°C/min ramping program. Ultra-high purity helium was used as the carrier gas with a column head pressure of 210.3 kPa (column flow: 1.0 mL/min at an oven temperature of 215°C). For quantitative analysis, the characteristic ions of the monitored steroids were determined as their TMS derivatives. Peak identification was achieved by comparing the retention times and matching the peak height ratios of the characteristic ions (Table 1).

Assay Validation

The urine samples for the calibration and the quality control (QC) were prepared with *in-house* steroid-free

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TABLE 1. The GC-MS Information for Quantitative Analysis of the Steroids Studied

Compounds (Trivial Name)	Abbreviation	Molecular Ion*	Characteristic Ion†	Quantitative Ion‡	Retention Time (min)
Androgens					
Androsterone	An	434	419, 434, 329	434	13.12
Etiocholanolone	Etio	434	419, 434, 329	434	13.28
Dehydroepiandrosterone	DHEA	432	432, 417, 327	432	15.46
Epitestostserone	Epi-T	432	432, 417, 327	432	16.32
7α-OH-dehydroepiandrosterone	7α-OH-DHEA	520	415, 430, 325, 520, 505	415	16.39
7α-OH-androstenedione	7α-OH-A-dione	518	428, 431	428	16.79
Androstenedione	A-dione	430	430, 415	430	17.27
7α-OH-testosterone	7α-OH-T	520	430, 415	430	17.52
7β-OH-dehydroepiandrosterone	7β-OH-DHEA	520	415, 430, 505, 520	415	17.78
Testosterone	T	432	432, 417	432	17.94
11β-OH-androsterone	11β-OH-An	522	522, 417, 507, 327, 432	522	18.20
11β-OH-etiocholanolone	11β-OH-Etio	522	522, 327, 507, 417, 432	522	18.52
19-OH-dehydroepiandrosterone	19-OH-DHEA	520	327, 340, 430	327	19.33
11α-OH-androstenedione	11α-OH-A-dione	518	518, 503	518	21.38
19-OH-androstenedione	19-OH-A-dione	518	428, 518, 503	518	21.63
15β-OH-testosterone	15β-OH-T	520	520, 430, 505, 415	520	22.08
19-OH-testosterone	19-OH-T	520	430, 520, 417, 505, 402, 327	520	22.40
11α-OH-testosterone	11α-OH-T	520	520, 505	520	22.69
6β-OH-androstenedione	6β-OH-A-dione	518	518, 503	518	23.57
11β-OH-androstenedione	11β-OH-A-dione	518	518, 503	518	23.98
6β-OH-testosterone	6β-OH-T	520	520, 505	520	24.38
4-OH-androstenedione	4-OH-A-dione	518	518, 503	518	25.06
11β-OH-testosterone	11β-OH-T	520	520, 415	520	25.09
16α-OH-dehydroepiandrosterone	16α-OH-DHEA	520	505, 520	505	25.52
16α-OH-testosterone	16α-OH-T	520	520, 505	520	27.59
16α-OH-androstendione	16α-OH-A-dione	518	503, 518	518	27.80
Estrogens					
Estrone	E1	414	414, 399, 309	414	16.66
17β-estradiol	E2	416	416, 285, 401	416	17.45
2-hydroxyestrone	2-OH-E1	502	502, 487, 397	502	23.08
2-hydroxy-17β-estradiol	2-OH-E2	504	504, 373, 489	504	23.89
4-hydroxyestrone	4-OH-E1	502	502, 487, 397	502	24.43
4-hydroxy-17β-estradiol	4-OH-E2	504	504, 373, 489	504	25.46
Estriol	E3	504	504, 345, 386, 414, 489	504	26.84
16α-hydroxyestrone	16α-OH-E1	502	487, 502	487	27.01
16-keto-17β-estradiol	16-keto-E2	502	487, 502	487	27.01
Progestins					
Pregnenolone	Preg	460	445, 460, 355	445	24.43
Progesterone	Prog	458	458, 443, 353	458	26.87
17α-hydroxypregnenolone	17α-OH-Preg	548	548, 443, 458	548	29.62
17α-hydroxyprogesterone	17α-OH-Prog	546	546, 441, 456	546	31.96
21-hydroxypregnenolone	21-OH-Preg	548	548, 533, 458	548	34.11
Corticoids					
21-deoxycortisol	21-deoxyF	634	634, 404	634	34.68
11-deoxycortisol	11-deoxyF	634	544, 529	544	34.98
Cortisone	E	720	615, 630	615	35.67
18-hydroxycortisol	18-OH-F	810	455, 648	455	36.27
Cortisol	F	722	632, 617	632	36.70
6β-hydroxycortisone	6β-OH-E	808	703, 718, 615	703	36.92
6β-hydroxycortisol	6β-OH-F	810	720, 632	720	37.89

*All steroids were derivitized with the trimethylsilylation agents, MSTFA/NH_4I/DTE (500:4:2, v/w/w) for both the hydroxy and the keto groups of the steroids.
†Characteristic ions are given as within 20% of the base peak.
‡Quantitative ions as the TMS derivatives of steroids.

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urine.[17,18] QC samples containing all 47 steroids were quantified using MS peak height ratios versus the IS. Calibration samples were prepared at 15 different concentrations depending on the sensitivity and reference values of the urine samples. Least square regression analysis was performed on the peak height ratios at increasing analyte levels to obtain calibration linearity. Limits of detection (LOD) and quantification (LOQ) were defined as the lowest concentration with a signal-to-noise (S/N) ratio >3 for LOD and >10 for LOQ. Imprecision is expressed as the coefficient of variation (% CV) and inaccuracies as percentage relative error (% bias), and each were determined using QC samples at 3 different concentrations (low, medium, and high) based on individual analyte calibration ranges. For the determination of within-day repeatability, 5 replicates were analyzed. Day-to-day reproducibility was measured by running samples on 5 different days. Extraction recoveries were determined using QC samples at 3 concentration levels in triplicate for each steroid by the addition of known amounts of mixed working solutions to steroid-free urine samples. Absolute recoveries were calculated by comparison of the peak height ratios of the extracted samples versus those of their nonextracted counterparts.

The stability of the analyte during sample collection and handling, which is a prerequisite of reliable quantification, was also evaluated. Stability was measured by comparison of the results of the samples analyzed before and after exposure to the stability assessment conditions at 3 different concentrations in triplicate. First, the stability of the solutions was tested by standing at room temperature for 6 hours over the time required for sample preparation. Second, the freeze-thaw stability was determined after 3 freeze-thaw cycles. Three aliquots of QC samples were stored at $-20°C$ for 24 hours and then thawed at room temperature. Once completely thawed, the samples were refrozen for 12 hours under the same conditions. These processes were repeated 3 times. Third, the short-term temperature stability of the QC samples was evaluated by thawing at ambient temperature and then leaving them to stand at this temperature for 6 hours. Fourth, the postpreparative stability was evaluated by re-injecting the prepared samples after 20 hours (after single-batch analysis of validation samples) and 40 hours (1 day after being placed in the sample tray of the auto-injector).

Specimens

Twelve healthy adult Korean males participated in this study and were managed by the Department of Pharmacology and Clinical Pharmacology at the Seoul National University College of Medicine (Seoul, Korea). All subjects gave informed consent for the study protocol, which conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and was approved by the local ethics committee. The clinical findings and laboratory tests of all subjects were normal. All volunteers received 600 mg of rifampicin orally every evening for 7 days and the control urine sample was obtained 12 hours before administration. The treated urine samples were collected over 12 hours after administration. All samples were stored at $-20°C$ until use. To compensate for variation in urine concentration, urinary steroid levels were corrected by the creatinine values determined according to the Jaffé method.[33]

Statistical Analysis and Steroid Signatures

Data manipulation was performed using Excel 2007 spreadsheets (Microsoft Corporation, Seattle, WA), Sigmaplot (SYSTAT Software Inc, San Jose, CA), and a TIBCO Spotfire DecisionSite Browser (TIBCO Spotfire Inc, Somerville, MA). The concentrations of the individual steroids and the metabolic ratios were calculated by dividing the concentration of substrate by that of its hydroxymetabolite (as an indicator of enzyme activity) in the urine samples obtained from volunteers before and after rifampicin administration, and these values were compared using a paired Student t test. Statistical significance was set at $P < 0.02$ to better reflect metabolic significance in this study.

To visualize steroid signatures between before and after administration, a supervised hierarchical clustering algorithm based on the Pearson correlation coefficient was used with a TIBCO Spotfire DecisionSite Browser. The metabolic ratios selected as statistically significant variables were log transformed and normalized using z scores by subtracting population means from individual raw scores and dividing the result by the population standard deviation. The z-score transformation [z = (observed value − baseline median)/baseline standard variation] ensures that each CYP metabolic ratio in the subject population has a median value of 0 and a SD of 1. In the heat map, color coding was used to indicate automatic ranges according to differences in metabolic ratios, which were graded at 3 points: red indicated z scores >0, blue indicated z scores <0, and white z scores ≈ 0 for a row (ie, subjects) across all columns (metabolic ratio, representing CYP enzymes).

RESULTS

GC-MS Characteristics of Steroids

Comprehensive steroid profiling that deals with diverse substrates and their hydroxymetabolites, including androgens, estrogens, corticoids, and progestins, is required to identify CYP-mediated steroid signatures. In this study, 47 steroid hormones associated with human CYP activities were analyzed as their TMS derivatives (**Table, Supplemental Digital Content 1**, http://links.lww.com/TDM/A48). As summarized in Figure 1, the major CYP-mediated hydroxymetabolites of the steroid substrates were as follows: testosterone (6β-, 7α-, 11β-, 15β-, and 16α-OH-T), androstenedione (4-, 6β-, 7α-, 11β-, and 16α-OH-A-dione), dehydroepiandrosterone (7α-, 7β-, and 16α-OH-DHEA), estrone (2-, 4-, and 16α-OH-E1), 17β-estradiol (2-, 4-, and 16α-OH-E2 [E3]), pregnenolone (17α- and 21-OH-Preg), progesterone (17α-OH-Prog), cortisol (6β- and 18-OH-F), and cortisone (6β-OH-E). Peak identification was straightforward using retention times, characteristic ions and mass spectra, and the latter were compared with those of reference calibrators (Table 1). The GC oven program resulted in good chromatographic separation and peak shapes for all steroids with retention times from 13.12 minutes for androsterone to 37.89 minutes for 6β-hydroxycortisol except for 16-keto-E2 and 16α-OH-E1, which were co-eluted and estimated for quantification (**Figure, Supplemental Digital Content 2**, http://links.lww.com/TDM/A48).

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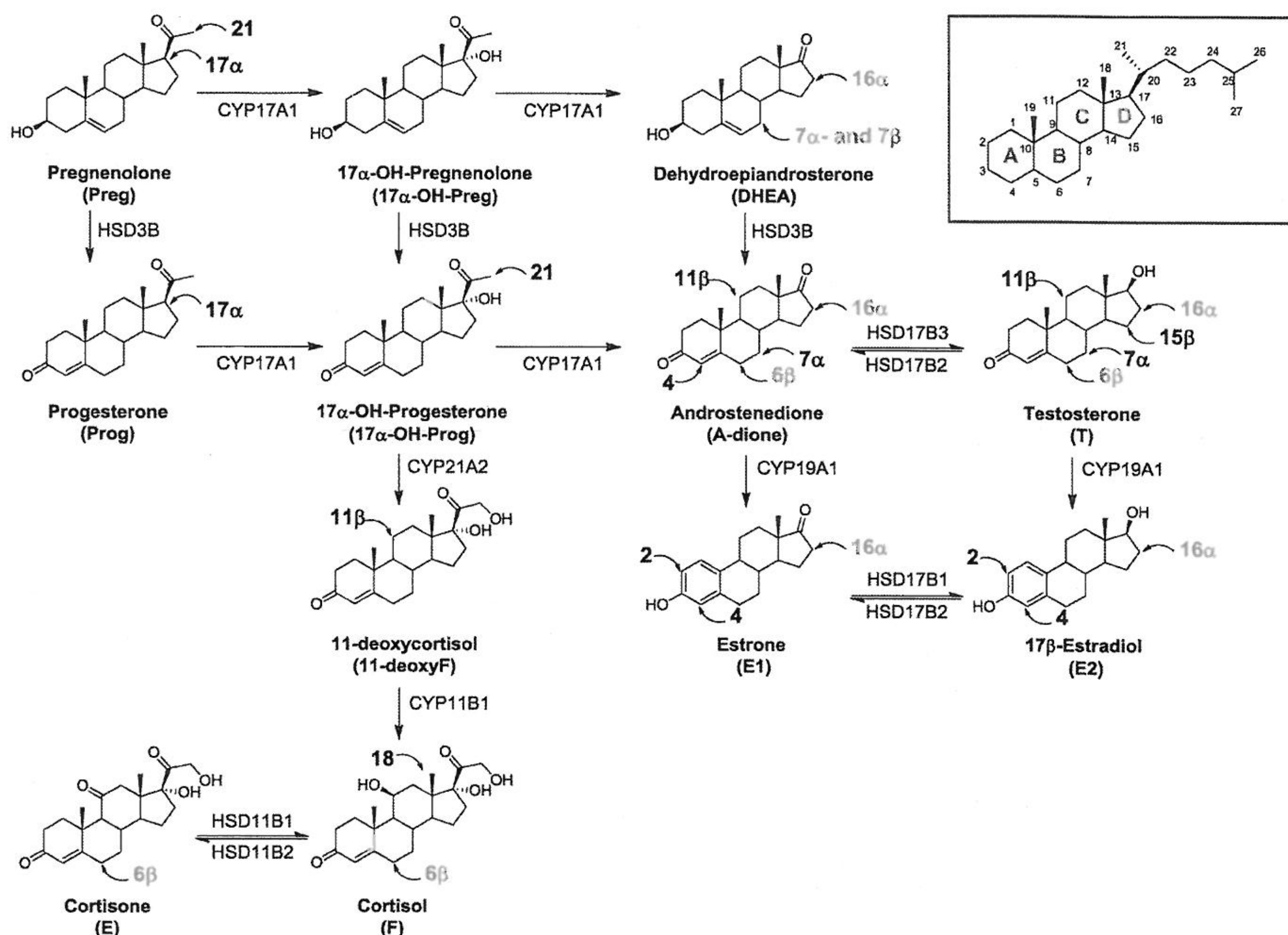

FIGURE 1. The regioselective hydroxylation in steroid metabolism mediated by human CYP enzymes. Note that a bold letter is used to denote the CYP isoform that has either high activity or a distinct regioselectivity for catalyzing a given metabolic reaction. In particular, a red bold letter indicates the main hydroxylation position affected by rifampicin.

Most steroids were monitored with their molecular ions as the base peaks except for the following: 7α-OH-A-dione and 7α-OH-T, which had an abundant (M-90; M-OTMS)$^+$ ion at *m/z* 428 and 430, respectively; 7α-OH-DHEA and 7β-OH-DHEA with an (M-90-15; M-OTMS-CH_3)$^+$ ion at *m/z* 415; 16α-OH-DHEA with an (M-15; M-CH_3)$^+$ ion at *m/z* 505; 16-keto-E2 and 16α-OH-E1 with an (M-15)$^+$ ion at *m/z* 487; Preg with an (M-15)$^+$ ion at *m/z* 445; 11-deoxyF, cortisol (F), and 6β-OH-F with an (M-90)$^+$ ion at *m/z* 544, 632 and 720, respectively; and cortisone (E) and 6β-OH-E with an (M-90-15)$^+$ ion at *m/z* 615 and 703, respectively. 19-OH-DHEA has an *m/z* 327 (M-90-103)$^+$ ion as major fragments formed by a typical loss of 90 Da (-OTMS) from its molecular ion (*m/z* 520) and the elimination of 103 Da (TMS-O-$CH_2^{\bullet}$) from the C-19 primary alcohol. Also, 18-OH-F was monitored at an *m/z* 455 [M-90-103-TMS-O-Si$(CH_3)_2$-CH_3]$^+$ ion (Table 1).

Optimization of Steroid Profiling

The profiling method described above was optimized for simultaneous quantification of the investigated steroid hormones. After solid-phase extraction and enzymatic hydrolysis, 5 different organic extraction solvents—*n*-hexane, MTBE, and 3 mixtures of ethyl acetate, and *n*-hexane (2:3, 3:2, and 4:1 v/v)—were tested to compare both extraction efficiency and matrix interference. The extraction with *n*-hexane offered better selectivity for some androgens for removal of the interference background signals but showed a poor recovery of <24.5% for most of the hydroxylated metabolites, while the extraction efficiencies increased with increasing solvent polarity. Thus, MTBE was chosen considering the overall recoveries and chromatographic properties (data not shown). Using optimized conditions, all the studied urinary steroids were extracted with good yields >70.4% except for 6β-OH-F at 53.7% (Table 2).

Assay Validation

Assay validation requires evaluation of imprecision, inaccuracy, selectivity, sensitivity, reproducibility, and stability using spiked samples, prepared (in this case) with steroid-free urine. The LOD and LOQ were also evaluated (Table 2) using sample concentrations that provided an S/N ratio of >3 and >10, respectively, and the resulting LOQs of their TMS derivatives were 0.2–2.0 ng/mL. The calibration curve consisted of

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TABLE 2. The Validation Results of the Overall Method in the Intra- and Interday Assays

Compounds (Abbreviation)	LOD* (ng/mL)	LOQ† (ng/mL)	Recovery‡ (%)	Linearity§ (r^2)	Intraday (n = 5)		Interday (n = 5)	
					CV¶ (%)	Bias¶ (%)	CV (%)	Bias (%)
Androgens								
An	0.2	0.5	103.7	0.9999	3.3	99.8	3.8	100.2
Etio	0.2	0.5	109.8	0.9929	2.7	100.1	3.3	98.6
DHEA	0.2	0.5	104.7	0.9994	5.8	104.1	4.3	103.6
Epi-T	0.1	0.2	126.9	0.9996	11.0	99.9	11.5	98.7
7α-OH-DHEA	1.0	2.0	70.4	0.9986	10.6	88.2	6.9	100.4
7α-OH-A-dione	0.1	0.2	125.1	0.9998	16.6	124.9	15.2	107.3
A-dione	0.1	0.2	107.2	0.9999	4.4	102.4	4.8	104.8
7α-OH-T	0.2	0.5	118.1	0.9992	16.9	118.4	15.6	103.9
7β-OH-DHEA	0.5	1.0	107.9	0.9987	4.8	101.0	4.8	104.7
T	0.1	0.2	103.7	0.9989	4.4	106.2	3.9	104.6
11β-OH-An	0.2	0.5	135.7	0.9968	3.9	96.8	3.1	98.4
11β-OH-Etio	0.2	0.5	127.3	0.9967	2.8	95.7	3.0	101.6
19-OH-DHEA	0.2	0.5	106.8	0.9997	5.2	99.6	4.3	101.1
11α-OH-A-dione	0.1	0.2	105.6	0.9999	4.8	105.7	4.2	100.3
19-OH-A-dione	0.2	0.5	106.5	0.9972	2.4	107.5	5.6	101.8
15β-OH-T	0.5	1.0	103.5	0.9989	3.2	105.3	4.6	101.3
19-OH-T	0.2	0.5	100.7	0.9975	4.0	108.7	4.4	103.1
11α-OH-T	0.1	0.2	105.5	0.9995	5.7	114.3	4.4	103.2
6β-OH-A-dione	0.1	0.2	115.0	0.9996	5.7	107.2	5.5	102.5
11β-OH-A-dione	0.1	0.2	112.0	0.9999	5.8	112.0	4.8	103.6
6β-OH-T	0.1	0.2	114.3	0.9994	4.0	105.0	5.4	101.4
4-OH-A-dione	0.1	0.2	117.7	0.9997	5.2	109.4	4.5	103.6
11β-OH-T	0.1	0.2	125.1	0.9997	5.5	110.4	5.0	103.4
16α-OH-DHEA	0.1	0.2	109.9	0.9940	5.5	104.3	4.9	100.3
16α-OH-T	0.1	0.2	107.1	0.9995	4.8	109.4	4.3	106.1
16α-OH-A-dione	0.2	0.5	109.9	0.9979	3.9	106.9	4.4	102.6
Estrogens								
E1	0.1	0.2	106.1	0.9999	4.1	108.3	5.3	104.2
E2	0.1	0.2	105.6	0.9993	2.9	104.8	5.9	100.1
2-OH-E1	0.1	0.2	105.5	0.9999	7.2	108.2	6.7	99.6
2-OH-E2	0.1	0.2	104.1	1.0000	7.1	109.5	7.2	100.3
4-OH-E1	0.1	0.2	103.8	0.9999	7.9	107.3	5.7	102.8
4-OH-E2	0.1	0.2	106.4	0.9994	6.1	108.3	6.0	102.0
E3	0.5	1.0	108.5	0.9995	5.3	108.7	5.7	101.7
16α-OH-E1 and 16-keto-E2	0.1	0.2	107.1	0.9992	3.7	106.8	3.6	101.0
Progestins								
Preg	0.2	0.5	93.0	0.9990	6.1	104.2	4.3	103.0
Prog	0.2	0.5	99.0	0.9995	6.6	107.4	4.6	106.8
17α-OH-Preg	0.2	0.5	102.6	0.9960	9.1	106.4	7.5	98.0
17α-OH-Prog	0.2	0.5	112.1	0.9970	7.0	113.2	6.9	102.5
21-OH-Preg	0.1	0.2	110.3	0.9990	9.8	105.2	9.8	104.0
Corticoids								
21-deoxyF	0.2	0.5	126.9	0.9992	7.8	109.0	7.6	100.7
11-deoxyF	0.1	0.2	108.0	0.9947	8.1	100.9	7.7	99.3
E	0.2	1.0	124.3	0.9945	6.6	92.2	6.6	103.2
18-OH-F	0.2	0.5	63.7	0.9991	7.4	91.4	9.1	109.2
F	0.1	0.2	105.0	0.9998	5.5	102.8	5.3	100.3
6β-OH-E	0.2	1.0	79.1	0.9958	5.7	109.9	6.2	103.9
6β-OH-F	0.2	0.5	53.7	0.9998	7.7	108.1	7.6	99.1

*The LOD was measured at S/N ratio >3.

†LOQ was measured at S/N ratio >10.

‡Absolute recoveries were calculated by comparing the peak height ratios of the samples using the described method with those of their nonextracted counterparts from 3 different QC concentrations in 5 replicates.

§Calibration ranges were from the LOQ level to expected concentrations in healthy human urine for each analyte and the linearity was measured as the mean levels of data through 5 different days.

¶Imprecision and inaccuracy were expressed as the mean values of data obtained from 3 QC samples through intra- and interday assays.

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a blank sample (a matrix sample processed without an IS), a zero sample (a matrix sample processed with an IS), and 14 samples from the LOQ to the range expected in the sample. The devised method was found to give a linear relationship, with a correlation coefficient (r^2) >0.994 for all of the analyzed steroids. Assay imprecision and inaccuracy were determined by analysis of the QC samples acquired for the intra- and interday assays at 3 different levels of the individual CYP-mediated steroids (0.5, 1, 2, 5, 15, 40, 80, 150, 400, and 2000 ng/mL). Intraday (n = 5) imprecision (expressed as % CV) was 2.4%–16.9% and inaccuracy (expressed as % bias) was 88.2%–124.9%, whereas interday (n = 5) imprecision (% CV) and inaccuracy (% bias) were 3.0%–15.6% and 98.0%–109.2%, respectively (Table 2).

The stability test was conducted for the reliable quantification of CYP-mediated steroids, including calibrator solution storage, short-term storage (bench-top at room temperature), freeze and thaw cycles, and the analytical process as described in the experimental section. Stock solutions and QC samples were freshly prepared and L-ascorbic acid (1 mg/mL) was added to prevent oxidative degradation of the catechol estrogens. The calibrator solutions were stable at −20°C for 3 months and at room temperature for 6 hours. The short-term stability, which was tested by thawing the QC samples at 25°C and leaving them to stand for 6 hours, showed no significant changes within about 20% deviation in concentration under the tested conditions. The freeze/thaw stability was evaluated from the aliquot concentration that had not been subjected to freeze/thaw cycles as a reference. The stability of these compounds was also demonstrated in urine samples subjected to 3 freeze/thaw cycles (82.1%–104.7% yields), and the overall differences were within the criteria of acceptance of at least 20% deviation in the nominal concentrations of the QC samples. Three repeated freeze/thaw cycles did not seem to affect the concentration of substrates and their hydroxysteroids. Because instability can occur in both the sample matrix and the prepared samples, it is important to test postpreparative stability under the analysis conditions, including the autosampler for the expected maximum time of an analytical run, to determine whether the analytical run can be performed again in the case of instrument failure. The results showed that the TMS derivatives of hydroxysteroids were quite stable when the prepared samples were injected 40 hours after being placed in the sample trays (<±13.6% relative standard deviation; **Table, Supplemental Digital Content 3**, http://links.lww.com/TDM/A48).

Assay Application

Validated CYP-mediated steroid signatures were used to evaluate whether rifampicin (a known CYP3A4 inducer) alters steroid metabolism in urine samples obtained from human subjects before and after administration. All quantitative results were normalized to urinary creatinine levels, summarized as mean ± SD (ng/mg creatinine), and excretion pattern differences between before and after treatment conditions were evaluated using the Student t test (Table 3). The induction ratio, indicating the fold change in the mean concentration value, was calculated by dividing the level after treatment by the level before treatment. Among the steroid substrates of CYP, 7 compounds—An (P = 0.0006), Etio (P = 0.0004), DHEA (P = 0.00009), A-dione (P = 0.0006), T (P = 0.01), E1 (P = 0.000003), and E2 (P = 0.002)—were significantly decreased, whereas F was increased (P = 0.007). In the case of CYP-mediated hydroxysteroids, the urinary levels of 4-, 16α-, and 6β-hydroxymetabolites of some substrates were increased [eg, 4-OH-A-dione (P = 0.01, 1.8-fold), 16α-OH-A-dione (P = 0.0003, 2.0-fold), 16α-OH-DHEA (P = 0.0001, 3.7-fold), 6β-OH-E (P = 0.00004, 3.8-fold), and 6β-OH-F (P = 0.00001, 5.4-fold)], whereas 11β-OH-Etio (P = 0.007) and 2-OH-E1 (P = 0.00002) were significantly decreased. The other substrates and their detected hydroxymetabolites did not change significantly.

On the basis of the quantitative results of the enzyme activity assay, the hydroxymetabolite to substrate ratio was examined to determine the effect of CYP enzymes on steroid metabolism (**Table, Supplemental Digital Content 4**, http://links.lww.com/TDM/A48). Statistically significant changes are shown in Figure 2, which displays mean values marked with a line in the individual subject. The 11β-hydroxylation of A-dione and T, representing 11β-hydroxylase, displayed a statistically significant increase after rifampicin treatment (Fig. 2A, P = 0.0006, 3.0-fold; Fig. 2B, P = 0.0003, 1.3-fold). The 7α- and 7β-hydroxylation of DHEA was significantly different before versus after treatment (Fig. 2C, P = 0.0005, 1.6-fold; Fig. 2D, P = 0.0001, 1.5-fold), whereas no significant change was observed in the 7α-hydroxylation of A-dione or T. For 16α-hydroxylase, all groups showed significantly increased 16α-OH-DHEA to DHEA (Fig. 2E, P = 0.0001, 6.8-fold), 16α-OH-A-dione to A-dione (Fig. 2F, P = 0.00003, 3.1-fold), 16α-OH-T to T (Fig. 2G, P = 0.003, 1.4-fold), and 16α-OH-E1 plus 16-keto-E2 to E2 (Fig. 2H, P = 0.001, 2.5-fold) ratios. In addition, the 6β-hydroxylation of A-dione, cortisone (E), and cortisol (F) was remarkably induced after rifampicin administration, as were the ratios of 6β-OH-A-dione to A-dione (Fig. 2I, P = 0.001, 1.9-fold), 6β-OH-E to E (Fig. 2J, P = 0.000005, 3.2-fold), and 6β-OH-F to F (Fig. 2K, P = 0.000001, 3.4-fold). The 4-hydroxylation of A-dione showed a statistically significant increase as well (Fig. 2L, P = 0.000007, 2.6-fold). In contrast, aromatase (an indicator of CYP19A activity) was significantly reduced after rifampicin administration, as were the ratios of E1 to A-dione (P = 0.001) and E2 to T (P = 0.02). Estrogen 2-hydroxylase, progestin 17α-hydroxylase, and cortisol 18-hydroxylase were not changed between the 2 groups (**Table, Supplemental Digital Content 4**, http://links.lww.com/TDM/A48). Other metabolic ratios related to the reductive 17β-HSD type (HSD17B3 and HSD17B1; T/A-dione and E2/E1, P < 0.005 for both comparisons), the reductive 11β-HSD type (HSD11B1; F/E and 6β-OH-F/6β-OH-E, P < 0.02 for both comparisons), and 3β-HSD (HSD3B2; A-dione/DHEA, P = 0.04) were slightly decreased by rifampicin.

To illustrate the CYP signatures, the metabolic ratios, which reflect CYP activities altered by rifampicin, were grouped according to statistical significance (P < 0.02) and represented by colors in a heat map (Fig. 3). In the same manner as in Figure 2, the 12 metabolic ratios from 11β-OH-A-dione/A-dione to 4-OH-A-dione/A-dione showed distinctly increased patterns after rifampicin treatment in this study. These results support the validity of the data transformation. Furthermore, other altered metabolic ratios in the CYP signature study

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TABLE 3. Urinary Steroid Concentrations in 12 Healthy Volunteers Before and After Administration of Rifampicin

Steroids	Before Rifampicin (Mean ± SD)*	After Rifampicin (Mean ± SD)	Induction Ratio† (After/Before)	P
An	1827.9 ± 365.9	1206.1 ± 308.3	0.7 ± 0.2	0.0006
Etio	1351.3 ± 397.9	861.5 ± 217.9	0.7 ± 0.2	0.0004
DHEA	31.4 ± 10.0	16.8 ± 3.5	0.6 ± 0.2	0.00009
Epi-T	27.5 ± 18.0	29.9 ± 14.2	1.3 ± 0.4	0.11
7α-OH-DHEA	3.0 ± 1.0	2.6 ± 0.5	0.9 ± 0.3	0.10
7α-OH-A-dione	9.3 ± 6.1	8.6 ± 4.9	1.2 ± 0.8	0.46
A-dione	1.4 ± 0.5	0.9 ± 0.2	0.7 ± 0.2	0.0006
7α-OH-T	1.6 ± 0.7	1.7 ± 1.2	1.1 ± 0.6	0.66
7β-OH-DHEA	94.9 ± 47.5	136.4 ± 57.7	1.7 ± 0.8	0.04
T	9.3 ± 6.3	8.1 ± 5.9	0.8 ± 0.2	0.01
11β-OH-An	488.0 ± 190.8	361.4 ± 141.8	0.9 ± 0.5	0.11
11β-OH-Etio	212.8 ± 157.4	52.1 ± 19.2	0.5 ± 0.4	0.007
6β-OH-A-dione	2.1 ± 0.7	2.3 ± 0.7	1.2 ± 0.5	0.46
11β-OH-A-dione	5.5 ± 2.6	9.3 ± 3.8	2.0 ± 1.0	0.04
4-OH-A-dione	7.2 ± 4.1	10.7 ± 3.0	1.8 ± 0.9	0.01
11β-OH-T	116.7 ± 21.5	124.9 ± 29.8	1.1 ± 0.4	0.46
16α-OH-DHEA	5.8 ± 2.1	21.8 ± 14.7	3.7 ± 1.8	0.0001
16α-OH-T	20.8 ± 5.6	24.0 ± 10.1	1.2 ± 0.5	0.32
16α-OH-A-dione	5.2 ± 1.8	10.0 ± 4.2	2.0 ± 0.8	0.0003
E1	5.4 ± 1.6	2.2 ± 0.5	0.4 ± 0.2	0.000003
E2	1.2 ± 0.3	0.9 ± 0.2	0.7 ± 0.2	0.002
2-OH-E1	2.3 ± 1.4	0.7 ± 0.3	0.3 ± 0.2	0.00002
2-OH-E2	0.5 ± 0.3	0.4 ± 0.1	0.9 ± 0.4	0.29
E3	4.5 ± 2.9	4.2 ± 3.0	1.1 ± 0.6	0.69
16α-OH-E1 and 16-keto-E2	0.6 ± 0.3	0.7 ± 0.6	1.1 ± 0.6	0.55
Preg	0.9 ± 0.3	1.0 ± 0.3	1.2 ± 0.8	0.68
17α-OH-Preg	1.6 ± 0.8	1.7 ± 1.2	1.1 ± 0.7	0.92
E	58.8 ± 32.0	65.6 ± 27.9	1.2 ± 0.4	0.27
18-OH-F	84.7 ± 39.7	152.1 ± 214.0	1.7 ± 1.5	0.24
F	17.9 ± 12.2	26.1 ± 12.6	1.7 ± 0.6	0.007
6β-OH-E	22.5 ± 5.6	83.1 ± 37.6	3.8 ± 1.7	0.00004
6β-OH-F	202.2 ± 104.6	1060.6 ± 655.9	5.4 ± 2.3	0.00001

*Concentrations are expressed as ng/mg creatinine (mean ± SD) and analyzed by the Student *t* test.
†Induction ratio, indicating the fold change in the mean value of the concentration, was calculated by dividing the level of after treatment by that of before rifampicin using each subject.

(eg, CYP19A1, HSD11B1, and HSD17B1/3) showed unexpected effects of rifampicin on steroid metabolism. The heat map showed not only changes in multiple CYP activities but also in the individual changes induced by drug treatment. Therefore, GC-MS–based CYP signatures can be used to efficiently and rapidly visualize the complex steroid hydroxylation altered by drug treatment.

DISCUSSION

A novel approach to identifying CYP-mediated steroid signatures was achieved using a GC-MS–based steroid profiling method. Steroid hormones are divided into androgens, estrogens, corticoids, and progestins and have a variety of physiologic and pharmacologic functions.[21,22] Human CYP enzymes present in the endoplasmic reticulum are responsible for drug oxidation.[2] Due to the chemical and physical diversity of steroids, an efficient extraction method was optimized for steroid substrates and their hydroxymetabolites, which can reveal the activity of members of the CYP family, including CYP1A, CYP1B, CYP2B, CYP2C, CYP3A, CYP11B, CYP17A, CYP19A, and CYP21A isoforms. In addition, a β-glucuronidase solution for enzymatic hydrolysis was used to prevent the systematic error that can arise from the unexpected transformation of 3β-hydroxysteroids to 3-oxo-steroids with β-glucuronidase/arylsulfatase extracted from *Helix pomatia*, thus leading to a misinterpretation of clinical implications.[32] Because of its very polar structure, the recovery of 6β-OH-F with MTBE extraction was relatively low compared with that of other hydroxysteroids, but its urinary concentration remained quantifiable.

Steroid hydroxylation affected by human CYPs has been widely evaluated.[23–29] However, these CYP assays have been performed with either assays of a single CYP enzyme or single metabolic reactions, and no studies to date have reported the assessment of multiple CYP enzymes on steroid metabolism. In general, urinary steroids are converted to more biologically

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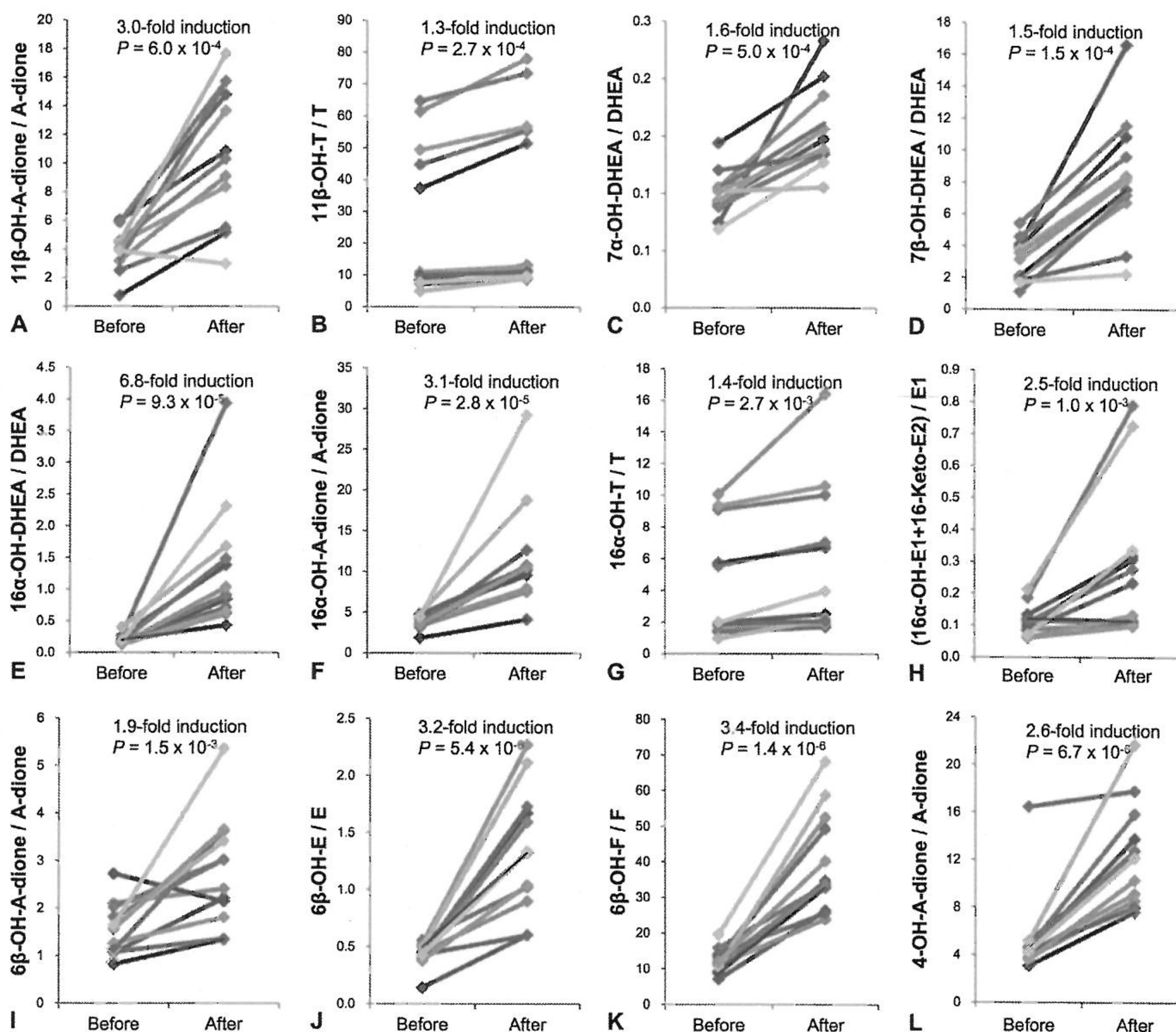

FIGURE 2. Urinary metabolic ratios before and after treatment with daily doses of 600 mg of rifampicin. Mean values are marked with a line. Statistically significant changes calculated using a paired Student *t* test are shown. In addition, statistically significant induction ratios in individual subjects are shown and were calculated by dividing the level after rifampicin treatment by that measured before treatment.

active metabolites by the CYP enzymes (**Table, Supplemental Digital Content 1**, http://links.lww.com/TDM/A48). First, testosterone (T) is primarily metabolized into 6β-, 11β-, and 15β-OH-T by CYP3A4,[24,28,34] and 16α-OH-T by CYP2B6/2C8/2C19.[34] The major hydroxymetabolites of DHEA are 7α-OH-DHEA produced by CYP3A4 and CYP7B1, and 7β- and 16α-OH-DHEA by CYP3A4 in adult liver microsomes, whereas 16α- and 7β-OH-DHEA, which is produced by the fetal/neonatal form CYP3A7.[26,27] With androstenedione (A-dione), 6β-hydroxylation also occurs by CYP3A4.[25] Estrone (E1) and 17β-estradiol (E2) are hydroxylated on the C-2, C-4, and C-16α positions by CYP1A1, CYP1B1, and CYP3A4, respectively.[29] Both cortisol (F) and cortisone (E) are mainly metabolized by CYP3A4, resulting in 6β-OH-F and 6β-OH-E.[11] There are the 11β-hydroxylation of 11-deoxyF and A-dione by CYP11B1, 17α-hydroxylation of Preg and Prog by CYP17A1, and 18-hydroxylation of F by CYP11B2. In addition, A-dione and T transformation into estrogens by aromatase, E1 and E2 metabolism by CYP19A1, and 21-hydroxylation of 17α-OH-Prog by CYP21A2 were also found.[21,22,35] 7α-OH-A-dione and 7α-OH-T, which were detected in this study, but have not been previously identified in humans, have been reported to be produced by CYP2A1 in rat testes.[36] Furthermore, whether 4- and 16α-hydroxylation of A-dione, which was present in considerable amounts in the urine, are catalyzed by CYP3A4 or another enzyme in

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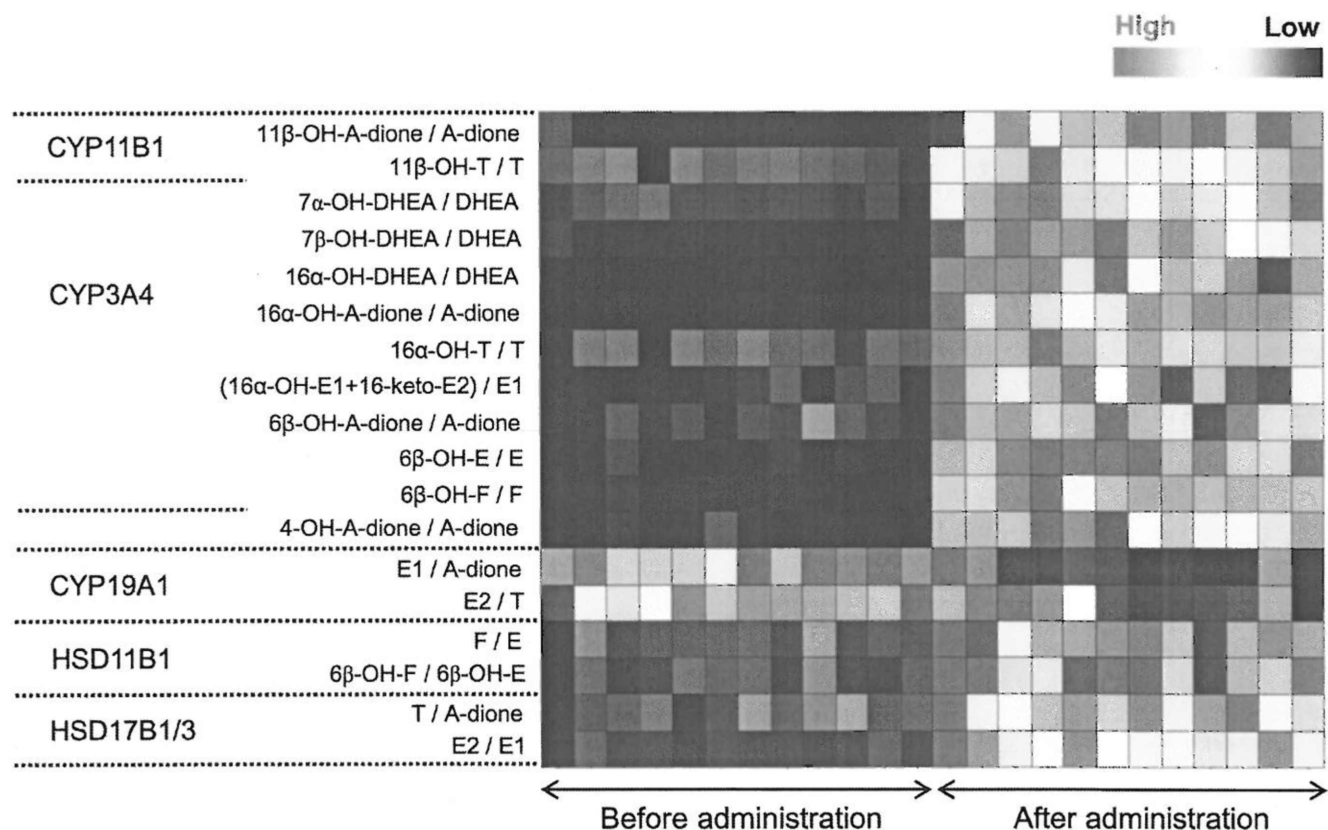

FIGURE 3. A heat map for the multiplexed CYP enzyme activities on steroid metabolism in urine samples obtained from 12 male volunteers before and after rifampicin administration. The ratio of hydroxymetabolite to substrate was calculated, and only metabolic ratios with significance levels at $P < 0.02$ were selected. The rows represent subjects and the columns represent the metabolic ratio. Red represents a high ratio, whereas blue represents a low ratio.

humans is also unclear. Therefore, further studies must be carried out to determine the exact human CYP isoforms involved in A-dione hydroxylation reactions. In the case of 19-hydroxylation, 19-OH-androgens (19-OH-DHEA, 19-OH-A-dione, and 19-OH-T) are known to be intermediary metabolites in the aromatizing reaction by the human ovary;[37] however, these compounds were not detected in this study. Moreover, 6β- and 16α-OH-hydroxylation of Prog by CYP3A4[24] were excluded because the 2 peaks of 6β- and 16α-OH-Prog were not separated in the analytical conditions as described above.

On the basis of the role of these CYP activities on steroid metabolism, the validated CYP signatures were applied into 12-pair urine samples obtained 12 hours before and after oral administration of rifampicin (600 mg every evening for 7 day). Rifampicin, an antibiotic used in treating diseases such as tuberculosis, is a known inducer of CYP3A4, CYP2B6, and CYP2C9.[38] Although elucidating clinical implications was not the purpose of this study, some features were notable and significant differences were found (Table 3). Significant metabolic changes among steroid substrates were seen in 16α-OH-DHEA, 6β-OH-E, and 6β-OH-F with a >3.7-fold induction because these hydroxysteroids are highly affected by CYP3A4, which concurs with previous findings.[11,26] The levels of DHEA, A-dione, and T after rifampicin treatment were reduced by multihydroxylation on 1 substrate by one or more CYP enzymes; for example, DHEA (7α-, 7β-, and 16α-hydroxylation), A-dione (4-, 6β-, 7α-, 11β-, and 16α-hydroxylation), and T (7α-, 11β-, and 16α-hydroxylation). Urinary E1 and E2 also showed significant decreases that might be associated with lower aromatase activity and/or higher estrogen 16α-hydroxylation by rifampicin. In addition, increased F levels could be supported by the activation of pregnane X receptor (PXR) by rifampicin, which markedly increases glucocorticoid concentration through increased expression of adrenal steroidogenic enzymes, including CYP11B1 and HSD3B.[39] Other steroid changes observed in this study (decreased levels of An, Etio, 11β-OH-Etio, and 2-OH-E1 and increased levels of 4-, 11β-, and 16α-OH-A-dione) have not been reported to date.

The present quantitative profiling revealed the phenotypes of multiplex CYPs using the metabolic ratio of its CYP-mediated hydroxymetabolite and steroid substrate. As expected, both 6β-OH-E/E (Fig. 2J) and 6β-OH-F/F (Fig. 2K) produced by 6β-hydroxylase showed remarkable induction in all subjects and also 6β-OH-A-dione/A-dione displayed a similar pattern, except for 1 subject (Fig. 2I). The urinary excretion ratio of 6β-OH-F/F and 6β-OH-E/E is an indicator of CYP3A4 activity in the evaluation of drug–drug interactions associated with inhibition

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and induction.[7,8,11] Thus, our CYP-mediated steroid signatures (Fig. 3) concurred with previous findings that 3-keto-4-ene steroids (eg, F, E, A-dione, and T) are preferentially hydroxylated at the 6β-position.[40] Due to the low urinary concentration of 6β-OH-T, 6β-OH-T/T was not evaluated. Similar to 6β-hydroxylase as biomarker of CYP3A4 activity, the other 9 metabolic ratios showed significant differences before and after dosing (Fig. 3). CYP3A4 induction by rifampicin can be evaluated using additional metabolic ratios consisting of testosterone 11β-hydroxylation, DHEA 7α-/β- and 16α-hydroxylation, and estrone 16α-hydroxylation.[26,28,29] Although testosterone 16α-hydroxylation (an indicator of CYP2B6 activity) showed a small induction, a significant increase in 16α-OH-T/T may be responsible for the effects that rifampicin can induce with regard to the expression of CYP2B6 and CYP2C8/9, in addition to CYP3A4.[38] Furthermore, a significant increase in androstenedione 11β-hydroxylation may be attributable to PXR activation through rifampicin, which is associated with an upregulation of CYP11B1.[35,39] Differences between the 2 groups with regard to 4- and 16α-hydroxylation of A-dione were also seen, but these effects of rifampicin have not been previously reported. However, measurement of these factors could be used to further implicate altered steroid metabolism by the CYP3A4 inducer rifampicin.

On the basis of the substrate induction ratios observed in this study, the reaction preference for major androgen hydroxylation by the CYP3A4 inducer was as follows: DHEA ($16\alpha >> 7\beta > 7\alpha$), A-dione ($16\alpha \geq 11\beta > 6\beta$), T ($16\alpha \geq 11\beta$), and E1 ($16\alpha >> 2$). These findings may suggest that steroid 16α-hydroxylation represents a useful biomarker of CYP3A4 activity in urine samples similar to 6β-hydroxylation. Because CYP3A4, the most abundant CYP enzyme, metabolizes nearly 50% of administered drugs, differences in CYP3A4 expression can affect drug efficacy, drug toxicity, and therapeutic outcomes and thus can have serious clinical consequences.[1] Therefore, these CYP3A4-related metabolic ratios can be efficiently used to study drug metabolism and metabolic interactions in the early stages of drug discovery and development. The lack of significant changes in the 7α-hydroxylation of A-dione or T could be explained by the fact that the specific CYP enzyme catalyzing the 7α-hydroxylation of both steroids is different from the enzyme associated with 6β-hydroxylation induced by rifampicin, but further investigation is needed to identify the exact CYP types involved in these hydroxylation reactions.[23]

The metabolic ratios associated with the role of aromatase (CYP19A1) as a key enzyme in estrogen biosynthesis (ie, the conversion of A-dione and T to E1 and E2, respectively) were significantly decreased by rifampicin. T and A-dione have shown specific stimulatory effects on CYP19 mRNA concentrations,[41] whereas 4-OH-A-dione is a potent aromatase inhibitor used in the treatment of breast cancer and has been shown to significantly decrease estrogen receptor concentrations in vivo.[42,43] Consequently, our findings explain the lower A-dione and T levels and/or increased inhibitory effect of aromatase by higher 4-OH-A-dione levels after drug administration. Moreover, HSD11B1, which plays a role in converting inactive forms (E and 6β-OH-E) into active forms (F and 6β-OH-F), was slightly increased. Furthermore, reductive HSD17B3/1 (T/A-dione and E2/E1), which catalyzes the final step in the biosynthesis of the active gonadal steroid hormones E2 and testosterone T, was significantly induced (Fig. 3), a finding that has not been previously reported. Thus, this quantitative steroid profiling method reveals the novel metabolic pathways altered by PXR activation—CYP19A1, HSD11B1, HSD17B1. Further mechanistic studies are required to confirm these findings because, at present, these results remain descriptive.

CONCLUSIONS

The CYP-mediated steroid signatures reflect a broad spectrum of CYP expression on steroid metabolism affected by rifampicin administration. To our knowledge, this is the first study showing simultaneous drug effects of rifampicin that is not the result of a single effect on 1 enzyme system but, rather, indicates the possibility of multiple effects. It seems that rifampicin treatment is not specifically perturbing CYP3A4 activity but causes significant effects on other drug-metabolizing CYPs (CYP2C9 and CYP2B6) and UGTs through the PXR pathway. The steroid signatures observed probably reflect the broad and complex effects of PXR activation rather than a validation test for CYP3A4 induction.

Our data suggest that CYP signatures may be useful in drug evaluation as a screening assay. Compared with conventional CYP assays, the GC-MS based profiling method is capable of simultaneous assessment of a diversity of CYP enzymes, leading to an overall understanding of drug/steroid metabolism within a single experiment. The devised method could serve as a noninvasive monitoring tool to predict the activities of drugs on steroid-metabolizing CYP enzymes and may also help to identify new CYP enzyme markers in drug response studies and cross-validation of pharmacogenomics-based CYP biomarkers.

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