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Evaluation of plasma enzyme activities using gas chromatography–mass spectrometry based steroid signatures

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ABSTRACT

The simultaneous quantification of 65 plasma steroids, including 22 androgens, 15 estrogens, 15 corticoids and 13 progestins, was developed using gas chromatography-mass spectrometry (GC–MS). The extraction efficiency of the catechol estrogens was improved by the addition of L-ascorbic acid in several steps. All steroids, as their trimethylsilyl derivatives, were well separated with good peak shapes within a 50 min run. The devised method provided good linearity (correlation coefficient, $r^2 > 0.993$), while the limit of quantification ranged from 0.2 to 2.0 ng mL^{-1}. The precision (% CV) and accuracy (% bias) were 2.0–12.4% and 93.5–109.2%, respectively. The metabolic changes were evaluated by applying this method to plasma samples obtained from 26 healthy male subjects grouped according to the pre- and post-administration of dutasteride, which inhibits 5α-reductase isoenzyme types 1 and 2. The levels of three plasma steroids, such as dihydrotestosterone, 5α-androstanedione and allotetrahydrocortisol, were decreased significantly after drug administration, while the levels of testosterone and 5β-androstane-3β,17α-diol were increased. In addition, the ratios of the steroid precursors and their metabolites, which represent the activities of the related enzymes, were *z*-score transformed for visualization in heat maps generated using supervised hierarchical clustering analysis. These results validated the data transformation because 5α-reductase is an indicator for the biological actions of dutasteride. GC–MS base quantitative visualization might be found in the integration with the mining biomarkers in drug evaluations and hormone-dependent diseases.

1. Introduction

Endogenous steroids are synthesized from cholesterol mainly by endocrine glands, such as the adrenal cortex, testes and ovaries, and are then released into the blood circulation [1,2]. These steroids play a variety of important physiological roles on the peripheral target tissues or central nervous system [3]. The steroid metabolism is important for the production of these hormones and the regulation of their cellular physiological actions. The steroid metabolic pathways are involved in two major types of enzymes, cytochrome P450 and other steroid oxidoreductases. Many endocrine disorders can be attributed to defects in these enzymes that lead to a hormonal imbalance and serious consequence [4–10]. As an example, the enhanced androgenic activity by the conversion of testosterone to an active form, dihydrotestosterone (DHT), by 5α-reductase is closely related to prostate diseases and male-pattern baldness [11–13]. Therefore, enzymes involved in the androgen metabolism can be therapeutic targets, and the activities of enzymes, including 5α-reductase, can be monitored by evaluating the drug efficacy in androgen-dependent diseases [14,15].

The enzyme activity profiles can be used to explain the functional diversity of biological systems according to their genetic diversity. Many methods have been used to measure the enzyme activity, including radioimmunoassay (RIA) or enzyme immunoassays (EIA). However, these methods have limited applicability due to cross-reacting antibodies and the fact that only single enzymes can be measured at one time [16–18]. In contrast to conventional enzyme assays, mass spectrometry based techniques offer better quantitative reproducibility and generate multi-targeted profiling analysis [16–22]. Gas chromatography–mass spectrometry (GC–MS) profiling analysis is a powerful technique that is widely used for steroid analysis [20–22]. It can be applied to large-scale clinical studies to present the concentrations of individual steroids as well as the ratio of steroid metabolites to the precursor, thus providing a measure of enzyme activities [23].

Plasma is more useful for revealing biological alterations than other biological fluids because steroids are released into the blood circulation immediately in their active form after biosynthesis. Biological alterations are generally expressed in tables or bar graphs that show the changes in few analytes across the groups of interest

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[6,12,18,22]. However, a large number of quantitative data sets are difficult to present visually. Statistical clustering offers one such approach, and has been introduced in genomic and proteomic studies to reveal the variables that are responsible for group discrimination and to effectively visualize such differentiations [24–26]. In a similar fashion, quantitative results obtained by metabolite profiling can be directly compared between samples and utilized as metabolic biomarkers. In recent reports, MS based quantitative data generated by HCA is subjected to pattern analysis for metabolite profiling [27,28]. The heat map as a concept of "metabolite signature" is a result of this process, and such signatures are useful for measuring and visualizing the relative analyte concentrations. However to date, there are no reports of the plasma steroids signature generated by heat map using HCA.

In this study, a GC–MS approach was used for the quantitative profiling of plasma steroids, including 22 androgens, 15 estrogens, 15 corticoids and 13 progestins to evaluate HCA as a form of multiple-substrate enzyme assay. This study focused on the development of quantitative GC–MS profiling in plasma and evaluating the usefulness of the steroid signature in explaining both the concentrations of individual steroids and the activities of the enzymes responsible for steroidogenesis. In addition, the prevention of oxidative decomposition of catechol estrogens during sample preparation was examined. This method could be used to evaluate the biological actions of dutasteride on healthy male subjects, and may be a useful tool for monitoring the efficacy of drugs used to treat abnormalities of the steroid metabolizing enzymes.

2. Experiment

2.1. Chemicals

Reference standards of the 65 steroids examined in this study (Table 1) were obtained from Sigma (St. Louis, CA, USA) for An, Etio, ααβ-diol, Epi-An, A-diol, 5α-dione, Epi-T, αββ-diol, A-dione, 11β-OH-An, 11β-OH-Etio, 17α-E2, E1, 17β-E2, 4-MeO-E1, 2-OH-E2, E3, 2-OH-E3, E, 5β-DHP, P-one, Preg, 5α-DHP and Prog; Steraloids (Newport, RI, USA) for other steroids. The internal standards used were 16,16,17-d_3-testosterone from NARL (Sydney, Australia) and methyltestosterone from Steraloids for the 22 androgens, 2,4,16,16-d_4-estradiol from C/D/N isotopes (Pointe-Claire, Quebec, Canada) for the 15 estrogens, 9,11,12,12-d_4-cortisol from C/D/N isotopes for the 15 corticoids, 2,2,4,6,6,17α,21,21,21-d_9-progesterone from C/D/N isotopes and 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone from C/D/N isotopes for the 13 progestins. For solid-phase extraction (SPE), an Oasis HLB cartridge (3 mL, 60 mg; Waters, Milford, MA, USA) was preconditioned with 2 mL of methanol followed by 2 mL of deionized water. Sodium acetate (reagent grade), acetic acid (glacial, 99.99+%) and L-ascorbic acid (reagent grade) were obtained from Sigma (St. Louis, MO, USA). The trimethylsilylating (TMS) agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH_4I), and dithioerythritol (DTE) were purchased from Sigma. All organic solvents were of analytical or HPLC grade, and purchased from Burdick & Jackson (Muskegan, MI, USA). Deionized water was prepared using a Milli-Q purification system (Millipore, Billerica, MA, USA).

2.2. Standard solutions and quality-control samples

Stock solutions of all the reference standards were prepared at a concentration of 1000 mg L^{-1} in methanol, and working solutions were made up with methanol to concentrations ranging from 1 to 10 mg L^{-1}. L-Ascorbic acid (1 g L^{-1}) was added to prevent the oxidation of catechol estrogens. All standard solutions were stored at −20 °C until needed and were stable for a minimum of 3 months. A commercially available steroid-free serum (Scipac; Sittingbourne, UK) was used for calibration and quality-control (QC) purposes instead of the plasma samples.

2.3. Subjects and sample-collection

Human plasma samples were obtained from 26 healthy male subjects (age: 37.7 ± 5.7 years), which were divided into pre- and post-administration groups, at the Department of Dermatology at Kyung Hee University Medical Center (Seoul, Korea). Plasma samples were collected immediately before as a control and 6 months after the daily oral administration of Avodart (soft gelatin capsule, 0.5 mg dutasteride; GlaxoSmithKline, Research Triangle Park, NC, USA). Samples were stored at −20 °C until required for analysis.

2.4. Sample preparation

Quantitative metabolite profiling of the plasma steroids was based on previous reports [6,29,30]. Briefly, plasma samples (0.4 mL) were diluted with 2.6 mL acetate buffer (0.2 M, pH 5.2) and 100 μL of aqueous 0.2% ascorbic acid, and spiked with 20 μL of the 6 internal standards (d_3-T and d_4-17β-E2, 0.2 mg L^{-1}; d_4-F and d_8-17α-OH-Prog, 1 mg L^{-1}; and methyltestosterone and d_9-Prog, 2 mg L^{-1}). The samples were extracted using Oasis HLB™ SPE cartridges coupled to a small peristaltic pump, which was operated at a low flow rate (<1 mL min^{-1}) to improve the extraction efficiency during the SPE process. After loading the sample onto the cartridge, it was washed with 2 mL water and eluted twice with 2 mL of methanol. The combined methanol eluates were evaporated under a nitrogen stream and the dried eluates were then dissolved in 1 mL of a 0.2 M acetate buffer (pH 5.2) and 100 μL of aqueous 0.2% ascorbic acid. The solution was then extracted with 2.5 mL of ethyl acetate: *n*-hexane (2:3, v/v) twice. The combined organic solvents were evaporated using an N_2 evaporator at 40 °C and further dried in a vacuum desiccator over P_2O_5–KOH for at least 30 min. Finally, the dried residue was derivatized with MSTFA/NH_4I/DTE (30 μL; 500:4:2, v/w/w) at 60 °C for 20 min, and 2 μL of the resulting mixture was subjected to GC–MS in selected-ion monitoring (SIM) mode. To maximize the extraction efficiency, the anti-oxidation effect with L-ascorbic acid was tested by addition immediately before SPE and/or liquid–liquid extraction (LLE) in the three different concentrations (2, 10 and 50 μg L^{-1}).

2.5. Instrumental conditions

GC–MS was performed using an Agilent 6890 Plus gas chromatograph interfaced with a single-quadrupole Agilent 5975 MSD at an electron energy of 70 eV and an ion source temperature of 230 °C. Each sample (2 μL) was injected in split mode (8:1) at 280 °C and separated using an Ultra-1 capillary column (25 m × 0.2 mm i.d., 0.33 μm film thickness; Agilent Technologies; Palo Alto, CA, USA). The GC oven temperature was set initially to 215 °C, ramped to 260 °C at 1 °C/min, increased to 320 °C at 15 °C/min and then held at that temperature for 1 min. The carrier gas was helium at a constant head pressure of 255.1 kPa. For quantitative analysis, the characteristic ions of each steroid were determined as their TMS derivatives. The peak identifications were achieved by comparing the retention times and matching the height ratios of the characteristic ions (Table 1).

2.6. Method validation

The QC samples containing all 65 analytes were quantified using the MS peak height ratios versus the IS. Calibration samples were prepared at 12 different concentrations depending on the sensitivity and reference values of the plasma steroids. Least-squares

Table 1
GC–MS information for quantitative analysis of the plasma steroids studied.

Steroids	Abbreviation	Molecular ion (m/z)[a]	Ion selected[b]	Retention time (min)	Compounds (trivial name)	Abbreviation	Molecular ion (m/z)	Ion selected	Retention time (min)
Androgens					*Estrogens*				
5β-Androstan-3α,17α-diol	βαα-diol	436	256	11.46	17α-Estradiol	17α-E2	416	416	17.85
5β-Androstan-3β,17α-diol	ββα-diol	436	256	12.18	Estrone	E1	414	414	18.42
Androsterone	An	434	434	14.57	17β-Estradiol	17β-E2	416	416	19.26
Etiocholanolone	Etio	434	434	14.75	4-Methoxyestrone	4-MeO-E1	444	444	22.06
5α-Androstan-3α,17β-diol	ααβ-diol	436	241	15.24	4-Methoxy-17β-estradiol	4-MeO-E2	446	446	22.98
5β-Androstan-3α,17β-diol	βαβ-diol	436	256	15.36	2-Methoxyestrone	2-MeO-E1	444	444	23.85
5α-Androstan-3β,17α-diol	αβα-diol	436	241	16.28	2-Methoxy-17β-estradiol	2-MeO-E2	446	446	24.84
Epidihydrotestosterone	Epi-DHT	434	434	16.70	2-Hydroxyestrone	2-OH-E1	502	502	25.16
11-Keto-androsterone	11-keto-An	520	520	16.75	2-Hydroxy-17β-estradiol	2-OH-E2	504	504	26.02
11-Keto-etiocholanolone	11-keto-Etio	520	520	16.82	4-Hydroxyestrone	4-OH-E1	502	502	26.62
Dehydroepiandrosterone	DHEA	432	432	17.10	4-Hydroxy-17β-estradiol	4-OH-E2	504	504	27.70
Epiandrosterone	Epi-An	434	419	17.33	Estriol	E3	504	504	29.15
Androstenediol	A-diol	434	434	17.82	16-Keto-17β-estradiol	16-keto-E2	502	487	29.43
5α-Androstanedione	5α-dione	432	432	17.86	16α-Hydroxyestrone	16α-OH-E1	502	487	29.43
Epitestosterone	Epi-T	432	432	17.98	2-Hydroxyestriol	2-OH-E3	592	592	36.72
5α-Androstan-3β,17β-diol	αββ-diol	436	241	18.09					
Dihydrotestosterone	DHT	434	434	18.57					
Androstenedione	A-dione	430	430	19.05					
Testosterone	T	432	432	19.72					
11β-Hydroxyandrosterone	11β-OH-An	522	522	19.97					
11β-Hydroxyetiocholanolone	11β-OH-Etio	522	522	20.32					
16α-Hydroxy-DHEA	16α-OH-DHEA	520	520	27.74					
Corticoids					*Progestins*				
Tetrahydrodeoxycortisol	THS	638	548	34.28	5β-Dihydroprogesterone	5β-DHP	460	445	19.17
Tetrahydrodeoxycorticosterone	THDOC	550	550	35.55	Epipregnanolone	Epi-P-one	462	447	22.46
Tetrahydrocortisone	THE	724	634	38.01	Pregnanolone	P-one	462	462	22.78
Tetrahydrocortisol	THF	726	636	40.75	Allopregnanolone	Allo-P-one	462	447	23.13
Dihydrodeoxycorticosterone	DHDOC	548	548	41.48	Pregnanediol	P-diol	464	269	24.18
Allotetrahydrocortisol	Allo-THF	726	636	41.84	Pregnanetriol	P-triol	552	435	25.48
21-Deoxycortisol	21-deoxyF	634	634	41.96	Pregnenolone	Preg	460	445	26.62
11-Deoxycortisol	11-deoxyF	634	544	42.24	5α-Dihydroprogesterone	5α-DHP	460	445	27.76
11-Deoxycorticosterone	11-deoxyB	546	546	42.98	Progesterone	Prog	458	458	29.11
Cortisone	E	720	615	45.71	20α-Hydroprogesterone	20α-DHP	460	460	29.48
11-Dehydrocorticosterone	11-dehydroB	632	617	46.63	17α-Hydroxypregnenolone	17α-OH-Preg	548	548	31.90
Allodihydrocorticosterone	Allo-DHB	636	636	46.73	17α-Hydroxyprogesterone	17α-OH-Prog	546	546	35.01
Allodihydrocortisol	Allo-DHF	724	634	46.90	11β-Hydroxyprogesterone	11β-OH-Prog	546	531	40.73
Corticosterone	B	634	634	47.72					
Cortisol	F	722	632	47.85					

[a] Molecular weight as the TMS derivatives of the steroids.
[b] Quantitative ions as the TMS derivatives of the steroids.

regression analysis was performed on the peak height ratios at increasing analyte levels to obtain calibration linearity. The 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. The precision and accuracy are expressed as the coefficients of variation (% CV) and percentage relative errors (% bias), respectively, and were determined using the QC samples at three different concentrations (low, medium, and high): 1, 10 and 100 μg L^{-1} for An, Epi-An, 5α-dione, Epi-T, A-dione, T, 16α-OH-DHEA, E1, 17β-E2, 2-MeO-E1, 2-MeO-E2, 2-OH-E1, 2-OH-E2, 4-OH-E1, 4-OH-E2, 16-keto-E2 and 16α-OH-E1, THS, THDOC, THF, Allo-THF, Allo-DHF, 5β-DHP, Epi-P-one, P-triol, Preg and 20α-DHP; 2, 20 and 100 μg L^{-1} for Etio, αβα-diol, Epi-DHT, 11-keto-An and Etio, DHEA, A-diol, DHT, 11β-OH-An, 11β-OH-Etio, 17α-E2, 4-MeO-E1, 4-MEO-E2, E3, 2-OH-E3, THE, DHDOC, 21-deoxyF, 11-deoxyF, 11-deoxyB, Cortisone (E), 11-dehydroB, Allo-DHB, Corticosterone (B), Cortisol (F), P-one, Allo-P-one, 5α-DHP, Prog and 17α-OH-Preg; 5, 20 and 100 μg L^{-1} for βαα-diol, ββα-diol, ααβ-diol, βαβ-diol, αββ-diol, P-diol and 17α-OH-Prog; and 10, 50 and 200 μg L^{-1} for 11β-OH-Prog. The within-day repeatability was determined by analyzing four replicates, while the day-to-day reproducibility was measured by running the samples on four different days.

The extraction recoveries were determined using the QC samples at three different concentrations in triplicate for each steroid by adding known amounts of mixed working solutions to the steroid-free serum samples. The absolute recovery was calculated by comparing the peak height ratios of the extracted samples with their non-extracted counterparts of same concentration of reference standards.

2.7. Statistical analysis and steroid signatures

Data manipulation was performed using Excel 2007 spreadsheets (Microsoft Corp., Seattle, WA, USA), Sigmaplot (SYSTAT Software Inc., San Jose, CA, USA) and SIMCA software (Umetrics Inc., Umeå, Sweden). The quantitative results are expressed as the mean ± SD, and group comparisons were made using paired two-tailed Student's *t*-test. *P*-values <0.01 were considered significant.

A supervised hierarchical clustering algorithm based on Pearson's correlation coefficient was used with a TIBCO Spotfire DecisionSite™ Browser (TIBCO Spotfire, Inc., Somerville, MA, USA) to visualize the altered steroid concentrations. The concentrations of individual steroids and the metabolite to precursor ratios (as an indicator of the enzyme activity) in the plasma samples obtained from volunteers, pre- and post-administration of dutasteride, were compared using the statistically significant variables. For clustering analysis, all quantitative results of the steroids measured and their ratios were log transformed and normalized using the *z*-scores by subtracting the population means from the individual raw scores and dividing the result by the population standard deviation. A *z*-score transformation [*z* = (observed value–baseline median)/baseline standard variation] ensures that each analyte in the subject population has a median value of 0 and a standard deviation of 1. In the heat map generated by HCA, the color coding in maps indicating the auto-range by the difference of concentration in each compound was gradated at three points; red, blue and white indicate *z*-scores >0, *z*-scores <0, and *z*-scores ≈0, respectively, of a row (subjects) across all columns (plasma steroids).

3. Results and discussion

3.1. Optimization of sample preparation

Many enzymes responsible for the steroid metabolism play a key role in hormone-dependent conditions, and their activities can be evaluated using the individual concentrations and metabolite to precursor ratios. To assess the multiple-substrate enzyme activities by GC–MS, a comprehensive steroid profiling that deals with diverse steroid metabolites including androgens, estrogens, corticoids and progestins is required. Although many techniques showed good validation results in steroid analysis extracted from biological fluids [6,29–31], the extraction of catechol estrogens, such as 2-OH-E1, 2-OH-E2, 4-OH-E1, 4-OH-E2, and 2-OH-E3, was relatively lower than the other steroids. Catechol estrogens are quite susceptible to oxidation because of the unique catechol structure. In addition, they have lower recoveries [32] and are degraded when exposed to light or a pH > 9.5 [33]. A solution of L-ascorbic acid, as an antioxidant, was used to prevent oxidative decomposition during sample preparation procedures [34,35]. Therefore, L-ascorbic acid was added under the following different conditions: (1) before SPE extraction, (2) before LLE extraction and (3) both steps. When L-ascorbic acid was added in any of these steps, there was an improvement in the extraction recovery of the catechol estrogens. The best recovery (74.9–100.5%) was observed by addition in both steps except for 2-OH-E3 (44.1%; Fig. 1). The recovery of the other steroids was not affected by the addition of L-ascorbic acid. Under optimized conditions, all plasma steroids studied were extracted in good yield (73.5–105.7%), except for 2-OH-E3, which was still detectable when 0.4 mL of plasma was prepared (Fig. 2).

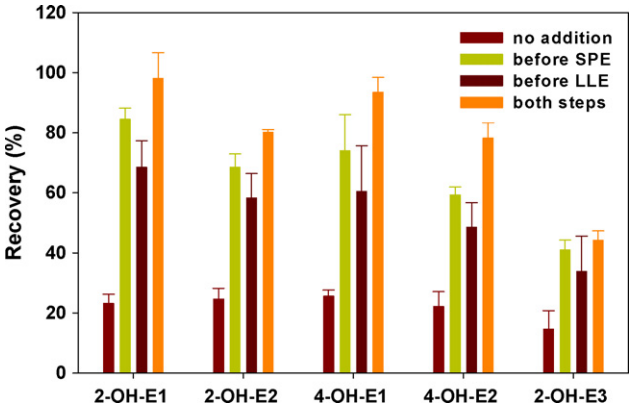

Fig. 1. The effects of L-ascorbic acid on the extraction recovery of catechol estrogens ($n = 4$).

3.2. Gas chromatography–mass spectrometry

GC-SIM/MS was used to analyze 65 steroid hormones with a 50 min chromatographic-run. Under these conditions, each steroid hormone was clearly identified from their TMS derivatives and the peak identification was straightforward using the retention times, characteristic ions, chromatograms and mass spectra: the latter of which was compared with the reference standards. The GC oven program resulted in well separated, good peak shapes for most steroids with retention times ranging from 11.57 min for 5β-androstan-3α,17α-diol to 47.85 min for cortisol. On the other hand, 11-keto-An and 11-keto-Etio, and 16-keto-E2 and 16α-OH-E1 co-eluted and were estimated as a total (see supporting information Fig. S-1). However, some partially overlapped compounds (e.g., 5α-dione and 17α-E2, 4-OH-E1 and Preg) were differentiated by SIM using their different characteristic ions (Table 1).

While most steroids were monitored using their molecular ions as base peaks, other ions were chosen as the abundant ions, such as βαα-diol, ββα-diol, βαβ-diol with $[M-180]^+$ ion at *m*/*z* 256, for the some steroids (Table 1). In the cases of pregnanediol, pregnanetriol and 11β-OH-Prog, less intense fragments at *m*/*z* 269, 435 and 531, respectively, were chosen instead of the most intense peaks (*m*/*z*

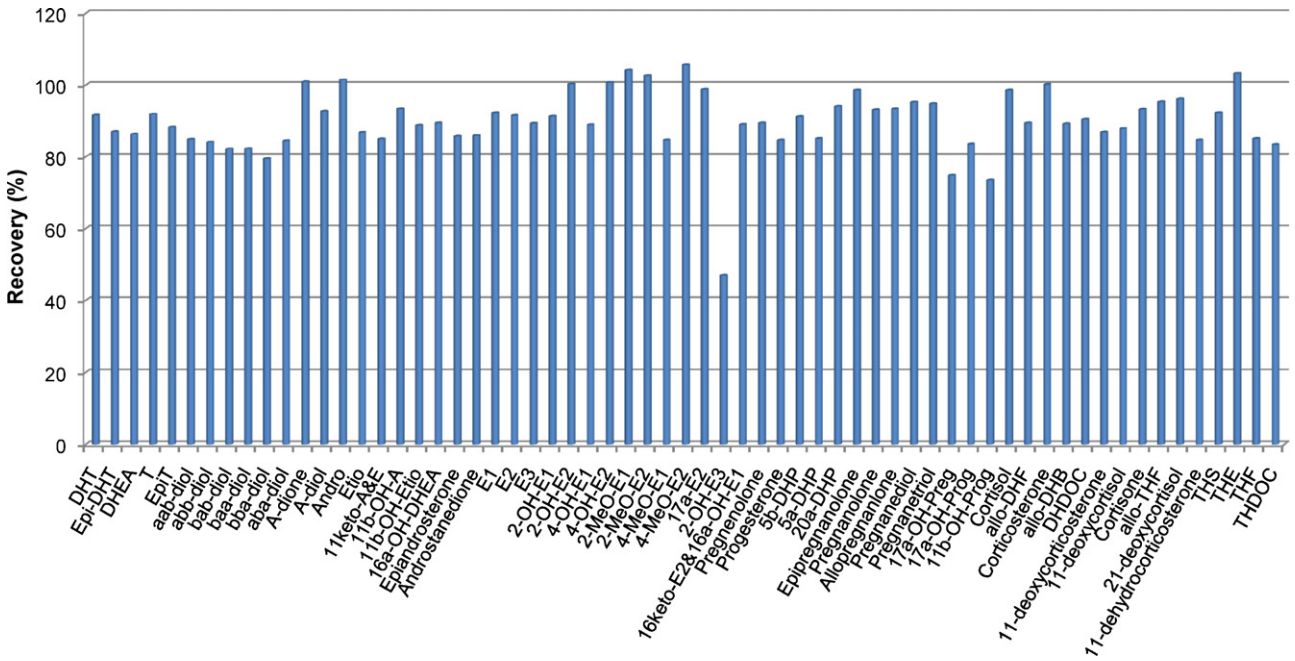

Fig. 2. Extraction recovery of 65 plasma steroids analyzed using the described method. The absolute recovery was calculated by comparing the peak height ratios of the extracted samples with their non-extracted counterparts.

117, 255 and 546) to improve the selectivity; this was attributed to matrix interference.

3.3. Method validation

Method validation requires an evaluation of the linearity, LOD, LOQ, precision, and accuracy using calibration samples prepared from a steroid-free serum. Calibration curves were generated for all analytes using the QC samples fortified with all reference standards at different concentrations. The devised method was linear (correlation coefficient, $r^2 > 0.993$; range 0.2–200 ng mL^{-1}) for all analytes. The LOD and LOQ were also evaluated (Table 2). Most compounds had an LOQ in the 0.2–2.0 μg L^{-1} range except for 11β-OH-Prog of 5 μg L^{-1}. When the chromatograms of blank plasma was compared with standard spiked plasma at a concentrations of related LOQ, only some compounds, βαα-diol, ββα-diol, ααβ-diol, βαβ-diol, αβα-diol, αββ-diol and 17α-OH-Prog were affected by plasma matrix. And these compounds have relatively high LOQ. But the 11β-OH-prog might have originated from the low sensitivity instead of plasma matrix interference (see supporting information Fig. S-1).

The assay precision and accuracy were determined by analyzing three QC samples at different concentrations of individual steroids (1, 2, 5, 10, 20, 50, 100 and 200 μg L^{-1}). The intra-day ($n = 4$) precision (expressed as % CV) ranged from 2.4% to 12.4%, whereas the accuracy (expressed as % bias) ranged from 93.5% to 109.2%. The inter-day ($n = 4$) precision (% CV) and accuracy (% bias) ranged from 2.0% to 9.9% and 94.2% to 108.5%, respectively (Table 2).

3.4. Quantitative results of dutasteride administration

Validated GC–MS based quantitative profiling was used to evaluate the drug efficacy of dutasteride on the plasma samples obtained from 26 healthy male subjects. Dutasteride is a 5α-reductase inhibitor that blocks the action of 5α-reductase enzymes that mainly convert testosterone to DHT [36]. It is used to treat diseases caused by enhanced androgenic activity, such as benign prostatic hyperplasia [37–39]. All quantitative results between the plasma steroid levels in pre- and post-administration were differentiated using Student's *t*-test (Table 3). The DHT concentration decreased significantly ($P = 5.27 \times 10^{-6}$), while the testosterone level was increased ($P = 3.35 \times 10^{-3}$), which concurs with previous findings [37,38]. In addition, there were significant changes in ββα-diol, 5α-dione and allo-THF after dutasteride administration (Table 3). This might be associated with the drug action because these metabolites are also affected by 5α-reductase in the steroid metabolic pathway. The other steroids were not changed significantly.

The quantitative results obtained in this profiling method can be expressed in the form of multiple-substrate enzyme activity. The plasma concentration ratio of the steroid metabolite and precursor as an indicator of the enzyme activity are also presented in Table 3. While most results were not changed significantly, only the DHT/T, 5α-dione/A-dione and allo-THF/F responsible for the activity of 5α-reductase were reduced after the dutasteride treatment ($P = 4.89 \times 10^{-5}$–8.39×10^{-9}). The change in enzyme activity according to the individual subjects was examined by analyzing the average ratios according to the post-/pre-value of metabolite/precursor in each subject. The indicators of 5α-reductase only had low values, which suggest a decrease in enzyme activity, and other indicators had a near value of 1, which indicates an unchanged character. The quantitative change in the steroid metabolites and multi-substrate enzyme activities agreed well with the specific action of dutasteride.

3.5. Steroid signatures by hierarchical clustering analysis

The steroid profiling method described in this study was successfully used to measure the concentrations of steroid metabolites simultaneously and multi-substrate enzyme activities in plasma. However, it is difficult to present individual differences and identify the critical meaning of the results because of the large volume of quantitative data. Hierarchical clustering involves calculating the distance matrices of the data objects and then merging the objects that are close to each other to form sub-clusters. In this study, after determining the relative levels of each steroid, all data was *z*-score transformed to produce a heat map using a supervised HCA

Table 2
The validation results of the overall method with the intra- and inter-day assays.

Compounds (trivial name)	LOD[a] (μg L^{-1})	LOQ[b] (μg L^{-1})	Calibration range (μg L^{-1})	Linearity (r^2)	Intra-day (n = 4)		Inter-day (n = 4)	
					CV[c] (%)	Accuracy[c] (%)	CV (%)	Accuracy (%)
Androgens								
βαα-diol	1.0	2.0	2–200	0.998	5.9	98.3	7.6	98.8
ββα-diol	1.0	2.0	2–200	0.993	9.2	102.6	8.2	103.7
An	0.1	0.2	0.2–200	0.993	6.6	94.2	7.1	94.6
Etio	0.2	0.5	0.5–200	0.996	6.8	101.0	5.1	100.2
ααβ-diol	2.0	2.0	2–200	0.993	7.6	101.6	8.1	104.3
βαβ-diol	1.0	2.0	2–200	0.993	11.9	107.3	9.1	106.4
αβα-diol	0.5	1.0	1–200	0.993	7.6	100.7	5.5	102.9
Epi-DHT	0.2	0.5	0.5–200	0.994	7.4	98.9	8.9	106.5
11-keto-An and Etio	0.2	0.5	0.5–200	0.995	4.6	103.0	5.3	107.7
DHEA	0.2	0.5	0.5–200	0.994	6.5	100.3	8.9	98.5
Epi-An	0.1	0.2	0.2–200	0.994	6.7	93.5	7.1	95.1
A-diol	0.5	1.0	1–200	0.993	6.4	109.2	6.5	108.5
5α-dione	0.1	0.2	0.2–200	0.995	9.0	99.2	9.0	99.8
Epi-T	0.1	0.2	0.2–200	0.995	6.1	97.9	7.0	101.6
αββ-diol	2.0	2.0	2–200	0.997	3.5	94.4	4.6	97.6
DHT	0.2	0.5	0.5–200	0.996	5.3	104.7	7.0	102.5
A-dione	0.1	0.2	0.2–200	0.995	9.4	101.1	4.5	101.5
T	0.1	0.2	0.2–200	0.995	7.3	95.7	5.0	104.3
11β-OH-An	0.2	1.0	1–200	0.993	7.7	106.6	5.8	104.0
11β-OH-Etio	0.2	1.0	1–200	0.993	8.4	104.6	9.9	102.5
16α-OH-DHEA	0.1	0.2	0.2–200	0.994	12.4	98.0	8.8	100.2
Estrogens								
17α-E2	0.2	0.5	0.5–200	0.996	6.5	98.3	6.0	97.8
E1	0.1	0.2	0.2–200	0.997	5.0	97.2	5.7	100.1
17β-E2	0.1	0.2	0.2–200	0.996	4.8	96.8	4.6	101.0
4-MeO-E1	0.5	1.0	1.0–200	0.996	5.8	98.0	4.1	98.6
4-MeO-E2	0.2	0.5	0.5–200	0.997	7.0	99.1	4.7	99.0
2-MeO-E1	0.1	0.2	0.2–200	0.996	3.3	95.1	4.5	100.2
2-MeO-E2	0.1	0.2	0.2–200	0.996	3.6	100.1	4.9	98.4
2-OH-E1	0.1	0.2	0.2–200	0.998	7.4	102.3	7.9	97.8
2-OH-E2	0.1	0.2	0.2–200	0.999	5.3	100.4	5.8	95.9
4-OH-E1	0.1	0.2	0.2–200	0.998	5.5	95.5	4.1	94.2
4-OH-E2	0.1	0.2	0.2–200	0.997	6.3	105.9	3.4	104.7
E3	0.2	0.5	0.5–200	0.995	7.5	101.7	4.8	103.8
16-keto-E2 and 16α-OH-E1	0.1	0.2	0.2–200	0.998	5.9	103.9	5.4	100.0
2-OH-E3	0.2	0.5	0.5–200	0.999	2.4	99.7	6.1	97.8
Corticoids								
THS	0.1	0.2	0.2–200	0.998	5.7	100.5	2.0	101.5
THDOC	0.1	0.2	0.2–100	0.999	2.5	99.3	4.1	95.2
THE	0.5	1.0	1–200	0.997	5.4	99.0	6.6	100.0
THF	0.1	0.2	0.2–100	0.999	5.3	102.1	7.2	103.1
DHDOC	0.2	0.5	0.5–100	0.998	6.5	95.0	8.9	102.6
Allo-THF	0.1	0.2	0.2–200	0.995	6.9	99.6	6.0	106.4
21-deoxyF	0.2	0.5	0.5–200	0.995	11.3	100.2	4.2	96.4
11-deoxyF	0.2	0.5	0.5–200	0.997	7.5	105.5	4.2	106.3
11-deoxyB	0.2	0.5	0.5–100	0.999	6.3	107.5	8.4	97.4
Cortisone (E)	0.5	1.0	1–500	0.995	5.2	102.0	7.3	108.0
11-dehydroB	0.2	0.5	0.5–100	0.999	10.1	107.7	6.0	101.4
Allo-DHB	0.2	0.5	0.5–100	0.999	4.8	99.0	4.3	98.2
Allo-DHF	0.1	0.2	0.2–200	0.996	6.4	101.5	9.0	105.4
Corticosterone (B)	0.2	0.5	0.5–100	0.999	6.4	104.4	4.9	104.9
Cortisol (F)	0.2	0.5	0.5–200	0.995	9.2	103.2	4.3	100.7
Progestins								
5β-DHP	0.1	0.2	0.2–200	0.999	7.4	98.8	5.4	102.5
Epi-P-one	0.1	0.2	0.2–200	0.999	11.6	96.0	2.5	98.6
P-one	0.5	1.0	1–200	0.999	5.2	94.7	5.4	98.9
Allo-P-one	0.5	1.0	1–200	0.999	7.0	102.8	6.7	98.6
P-diol	1.0	2.0	2–200	0.999	6.0	103.1	4.4	103.6
P-triol	0.1	0.2	0.2–200	0.999	4.0	103.9	6.9	96.6
Preg	0.1	0.2	0.2–200	0.999	9.9	101.2	4.0	99.0
5α-DHP	0.2	0.5	0.5–200	0.999	5.1	96.6	4.8	96.0
Prog	0.5	1.0	1–200	0.999	8.2	101.7	5.2	104.2
20α-DHP	0.1	0.2	0.2–200	0.999	7.2	105.6	8.6	99.8
17α-OH-Preg	0.2	0.5	0.5–100	0.998	5.5	100.6	5.7	97.4
17α-OH-Prog	0.5	2.0	2–200	0.997	8.6	109.0	4.0	107.6
11β-OH-Prog	2.0	5.0	5–200	0.997	7.4	105.0	4.5	103.1

[a] The limit of detection was measured with a S/N ratio >3.
[b] The limit of quantification was measured with a S/N ratio >10.
[c] Precision and accuracy are expressed as the mean values of the data obtained from three different concentrations of each analyte.

Table 3
The plasma steroid concentrations and enzyme activities in the pre- and post-administration of dutasteride.

Steroids	Steroid concentrations ($\mu g\,L^{-1}$)			Enzymes		Enzyme activities (metabolite/precursor)			
	Pre-value	Post-value	*P*-value			Pre-value	Post-value	*P*-value	Ratio[a]
DHT	2.01 ± 0.63	1.11 ± 0.38	5.27E−06	5α-reductase	DHT/T	0.46 ± 0.14	0.18 ± 0.06	8.39E−09	0.45 ± 0.20
DHEA	5.61 ± 4.70	4.98 ± 4.19	0.60		5α-dione/A-dione	1.28 ± 0.64	0.50 ± 0.36	4.89E−05	0.53 ± 0.46
T	4.59 ± 1.88	6.47 ± 2.63	3.35E−03		Allo-THF/F[b]	0.07 ± 0.03	0.02 ± 0.02	1.50E−07	0.29 ± 0.36
Epi-T	0.50 ± 0.18	0.51 ± 0.19	0.86	5β-reductase (+ 3β-HSD)	THE/E	0.12 ± 0.07	0.11 ± 0.07	0.65	1.14 ± 1.13
αββ-diol	16.25 ± 10.25	15.08 ± 7.01	0.64		THF/F	0.01 ± 0.01	0.01 ± 0.01	0.65	1.69 ± 1.71
βαα-diol	1.31 ± 1.66	2.24 ± 1.59	0.08	3α-HSD	An/5α-dione	0.93 ± 1.52	1.46 ± 1.10	0.20	1.52 ± 1.36
ββα-diol	0.52 ± 1.04	2.67 ± 1.49	1.24E−06		5α-dione/An	4.53 ± 10.73	1.20 ± 0.88	0.12	0.90 ± 1.18
A-dione	1.23 ± 0.50	1.40 ± 0.58	0.18	3β-HSD	αββ-diol/DHT	8.69 ± 5.19	14.52 ± 7.77	9.26E−03	0.99 ± 0.61
A-diol	1.82 ± 1.12	1.58 ± 0.94	0.40		T/A-diol	3.69 ± 2.67	7.08 ± 10.31	0.13	1.10 ± 1.57
An	0.83 ± 0.51	0.78 ± 0.55	0.73		A-dione/DHEA	0.26 ± 0.10	0.32 ± 0.12	0.08	1.32 ± 0.41
16α-OH-DHEA	0.46 ± 0.33	0.50 ± 0.55	0.75		17 α-OH-Prog/17 α-OH-Preg	4.09 ± 8.29	10.69 ± 38.59	0.41	1.06 ± 2.39
Epi-An	0.47 ± 0.25	0.46 ± 0.25	0.82	17α-HSD	Epi-T/A-dione	0.43 ± 0.13	0.38 ± 0.12	0.31	0.92 ± 0.20
5α-dione	1.47 ± 0.66	0.63 ± 0.44	4.52E−05	17β-HSD	T/A-dione	4.61 ± 1.76	4.20 ± 1.24	0.39	0.92 ± 0.21
E1	0.13 ± 0.14	0.16 ± 0.19	0.50		A-dione/T	0.24 ± 0.08	0.26 ± 0.08	0.56	1.12 ± 0.24
Preg	1.32 ± 1.01	0.98 ± 0.89	0.19		A-diol/DHEA	0.38 ± 0.24	0.37 ± 0.24	0.83	1.08 ± 0.60
P-triol	1.80 ± 0.96	1.77 ± 0.75	0.91		DHEA/A-diol	4.41 ± 4.08	5.04 ± 6.91	0.67	1.24 ± 0.74
17 α-OH-Preg	2.52 ± 4.03	1.99 ± 3.16	0.62		DHT/5α-dione	2.15 ± 1.63	1.98 ± 1.00	0.65	1.07 ± 0.52
17 α-OH-Prog	1.74 ± 1.72	1.38 ± 1.02	0.40		5α-dione/DHT	0.58 ± 0.21	0.63 ± 0.30	0.50	1.50 ± 2.21
F	103.05 ± 58.84	122.59 ± 53.96	0.24	11β-HSD	11-dehydro B/B	0.72 ± 0.67	0.56 ± 0.41	0.30	1.52 ± 2.81
B	3.98 ± 3.65	4.84 ± 5.09	0.47		E/F	0.35 ± 0.13	0.31 ± 0.08	0.28	0.96 ± 0.37
E	33.95 ± 17.37	35.36 ± 14.94	0.73	aromatase	E1/A-dione	0.20 ± 0.15	0.19 ± 0.13	0.64	1.12 ± 1.46
Allo-THF	7.70 ± 4.59	2.11 ± 3.23	1.03E−07	17 α-hydroxylase	17 α-OH-Preg/Preg	2.54 ± 3.36	3.78 ± 5.36	0.30	1.51 ± 2.46
11-dehydro B	1.82 ± 1.18	2.05 ± 1.45	0.57	16 α-hydroxylase	16α-OH-DHEA/DHEA	0.10 ± 0.07	0.11 ± 0.07	0.81	1.13 ± 0.27
THE	4.40 ± 3.58	4.35 ± 4.22	0.96	Epimerase	An/Epi-An	2.10 ± 1.10	2.80 ± 3.40	0.32	2.59 ± 9.01
THF	1.76 ± 1.68	2.55 ± 2.60	0.27		Epi-An/An	0.79 ± 0.98	1.45 ± 3.71	0.39	1.54 ± 1.99

[a] Average ratios are presented as the pre-/post-value of metabolite/precursor in each subject.
[b] Allo-THF was converted from F by 5α-reductase and 3β-HSD.

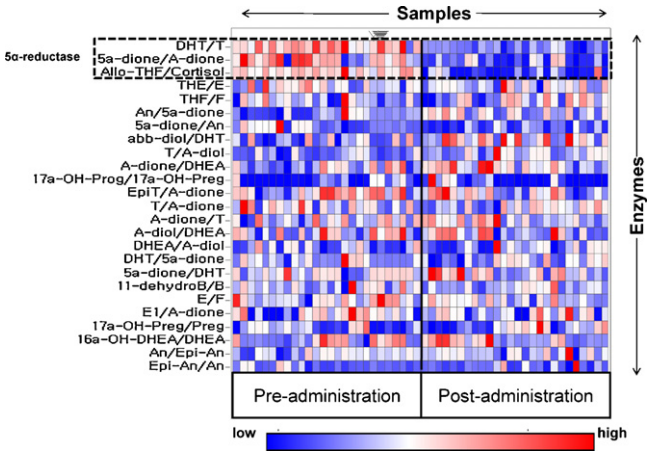

Fig. 3. Hierarchically clustered heat map of the detectable multiple-substrate enzyme activities of volunteers, pre- and post-administration of dutasteride on male subjects. A heat map for profiling the enzyme activities related to the steroid metabolism without clustering after dutasteride administration. The rows represent the subjects and column the metabolic ratio. (For interpretation of the references to color in the text, the reader is referred to the web version of this article.)

based on Pearson's correlation coefficient. The clustering method and similarity measure were set to the unweighted average and Euclidean distance, respectively. The heat maps were compared with respect to the individual quantitative results, which indicated the effectiveness of the steroid signatures.

In order to highlight the usefulness of a steroid signature, the steroid metabolite to precursor ratios, which reflects the enzyme activity (Fig. 3), were represented by colors in the heat map. Each ratio was represented by a single row of colored boxes, whereas columns represented different subjects. The cluster and heat maps of all samples were not clustered between the groups in terms of these ratios (data not shown). This is because most indicators showed no significance except for those of 5α-reductase. The heat map without clustering showed altered ratios of the enzyme indicators with respect to the individual quantitative results after dutasteride administration as well as changes in all enzyme activities and a decrease in 5α-reductase in most subjects individually (Fig. 3). Therefore, this method can be used to visualize a multi-substrate enzyme activity profile in a single graphic using a hierarchically clustered heat map.

4. Conclusions

Improved extraction recovery of catechol estrogens from sample preparations was obtained by the addition of L-ascorbic acid before the SPE and LLE steps, which is very simple and useful for profiling 65 plasma steroids. The devised method was validated for linearity, precision, accuracy, LOQ and LOD. Profiling analysis involves the collation of quantitative results for a broad series of metabolites to gain an overall understanding of the metabolism. This is in contrast to conventional approaches, which focus mainly on single enzymes, single metabolic reactions and kinetic properties. The multiple-substrate enzyme activity profiles based on the steroid signatures, as represented by hierarchically clustered heat maps, use colors instead of numbers to communicate the underlying quantitative results. This profiling method was employed to evaluate the drug efficacy of dutasteride, a 5α-reductase inhibitor. The quantitative results of the steroid metabolites and enzyme activities were accordance with the specific action of dutasteride. The heat map showed not only the changes in all enzyme activities measured, but also the individual changes due to the drug.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jchromb.2009.11.010.

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