
Heat-Map Visualization of Gas Chromatography-Mass Spectrometry Based Quantitative Signatures on Steroid Metabolism

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Abnormalities in steroid hormones are responsible for the development and prevention of endocrine diseases. Due to their biochemical roles in endocrine system, the quantitative evaluation of steroid hormones is needed to elucidate altered expression of steroids. Gas chromatographic-mass spectrometric (GC-MS) profiling of 70 urinary steroids, containing 22 androgens, 18 estrogens, 15 corticoids, 13 progestins, and 2 sterols, were validated and its quantitative data were visualized using hierarchically clustered heat maps to allow "steroid signatures". The devised method provided a good linearity ($r^2 > 0.994$) with the exception of cholesterol ($r^2 = 0.983$). Precisions (% CV) and accuracies (% bias) ranged from 0.9% to 11.2% and from 92% to 119%, respectively, for most steroids tested. To evaluate metabolic changes, this method was applied to urine samples obtained from 59 patients with benign prostatic hyperplasia (BPH) versus 41 healthy male subjects. Altered concentrations of urinary steroids found and heat maps produced during this 70-compound study showed also differences between the ratios of steroid precursors and their metabolites (representing enzyme activity). Heat maps showed that oxidoreductases clustered (5α -reductase, 3α -HSD, 3β -HSD, and 17β -HSD, except for 20α -HSD). These results support that data transformation is valid, since 5α -reductase is a marker of BPH and 17β -HSD is positively expressed in prostate cells. Multitargeted profiling analysis of steroids generated quantitative results that help to explain correlations between enzyme activities. The data transformation and visualization described may to be found in the integration with the mining biomarkers of hormone-dependent diseases. (J Am Soc Mass Spectrom 2009, 20, 1626–1637) © 2009 American Society for Mass Spectrometry

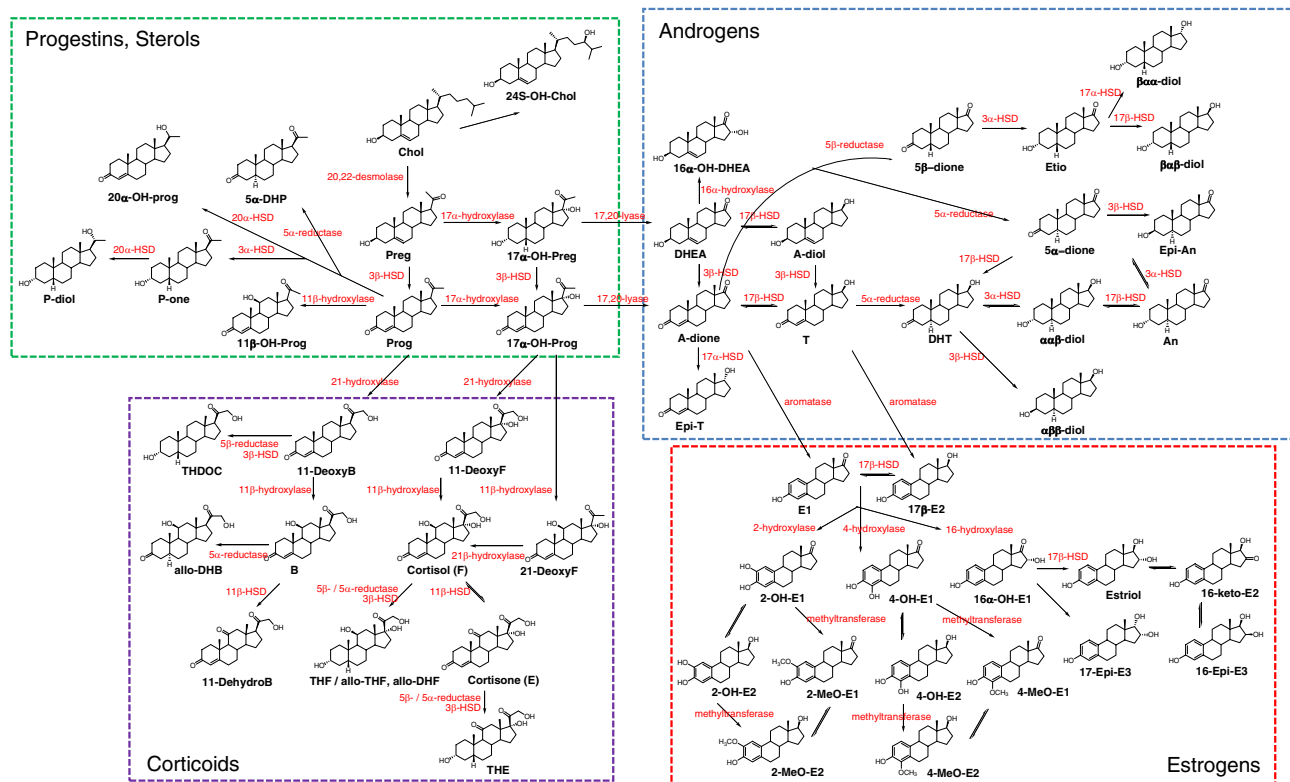
Mass spectrometry based metabolite profiling reveals the metabolic states of biological systems and provides comprehensive insights by allowing comparisons between many metabolites simultaneously present in cells, tissues, or organisms [1, 2]. This technique promotes the establishment of relationships between phenotypes and metabolisms by providing descriptions of the distributions of metabolites and their biological functions. In fact, quantitative analyses of sets of metabolites in biochemical pathways have been used for physiological monitoring, toxicological evaluations, and clinical diagnosis [3–6].

Many naturally occurring steroids with similar chemical structures could yield biological information [7]. Endogenous steroids are divided into five groups, namely, androgens, estrogens, corticoids, progestins, and sterols, which are generally synthesized from cholesterol in the adrenal cortex, ovaries, and testes (Scheme 1). In biosynthetic pathways of steroid hor-

none, two major types of enzymes are involved, cytochrome P450 and steroid oxidoreductase. Abnormalities of these enzymes often lead to hormonal imbalances that have serious consequences, and which are responsible for the development of hormone-dependent diseases (see Supplementary Table 1, which can be found in the electronic version of this article). For example, concentrations of corticoids and their metabolic ratios provide diagnostic evidence of apparent mineralocorticoid excesses caused by 11β -HSD deficiency [8] and congenital adrenal hyperplasia, which are caused by deficiencies of enzymes like hydroxylase (at C-11, 17, and 21) or 3β -HSD [9]. In addition, enhanced androgen activity generated by the conversion of testosterone to dihydrotestosterone (DHT) by 5α -reductase was utilized to allow early therapeutic intervention in young men [10].

Enzyme activity profiles can be used to describe the functional diversities of biological systems, which are driven by genetic diversity. Although enzyme activities have been monitored by following reactions between targeted enzymes and substrate molecules, overestimations by radioimmunoassay (RIA) and enzyme immunoassay (EIA), because of cross reacting antibod-

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Scheme 1. General scheme for steroid metabolism in man. See Supplementary Table 1 for the full names of steroid hormones.

ies, limit the applicability of these assays, and furthermore, only single enzymes can be estimated at a time [11–14]. In contrast to conventional enzyme assays, gas chromatography-mass spectrometry (GC-MS)-based techniques have better quantitative reproducibility [15, 16]. For these reasons, GC-MS profiling has been widely used for steroid analysis [17–20], and offers the basis for techniques that can be applied to large-scale clinical studies [21].

Clinical significances are generally expressed in tables or bar graphs that show changes in analytes across groups of interest. For studies involving few compounds, these visualizations are enough to differentiate classes showing metabolic differences. However, quantitative datasets of multiple compounds are much more difficult to represent visually. Statistical clustering offers one such approach, and has been utilized to support genomic and proteomic studies [22–24]. In a similar fashion, quantitative results obtained by metabolite profiling can be directly compared between samples and utilized as metabolic biomarkers. In recent, MS-based quantitative data generated by hierarchical clustering analysis (HCA) has been subjected to pattern analysis for metabolite profiling [2, 25]. The concept of “metabolite signature” is a result of this process, and such signatures are useful for measuring and visualizing the relative analyte concentrations. Although many

groups have used GC-MS based steroid analysis for mining biomarkers, technical improvements that more effectively allow the visualization of steroid metabolites are required [26]. However, no studies have reported MS-based steroid signatures generated by HCA to date.

Here, we introduce the GC-MS quantitative profiling of 70 urinary steroids, including 22 androgens, 18 estrogens, 15 corticoids, 13 progestins, and 2 sterols, generated by HCA to evaluate metabolic changes and enzyme reactions in steroid analysis. The aim of this study was to validate a GC-MS profiling method and a method that allows the quantitative visualization of multiple urinary steroids. To visualize quantitative results, a microarray map (a type of heat map) was designed to present the urine sample results of 59 patients with benign prostatic hyperplasia (BPH) and 41 healthy male subjects; BPH was chosen because steroid metabolism is known to play a role in the progress of prostate diseases [27–29]. This study focused on illustrating the usefulness of steroid signatures for explaining both the concentrations of individual steroids and the activities of enzymes correlated global steroidogenesis in BPH, and suggest that enzyme activity profiling may be a useful diagnostic tool and provide a means of identifying mining biomarkers in hormone-dependent diseases.

Experimental

Chemicals and Materials

Reference standards of the 70 steroids examined in this study (Table 1) were obtained from Sigma (St. Louis, MO, USA), Steraloids (Newport, RI, USA), or NARL (Pumble, Australia). The internal standards used were, 16,16,17-*d*₃-testosterone and methyltestosterone for 22 androgens, 2,4,16,16-*d*₄-estradiol for 18 estrogens, 9,11,12,12-*d*₄-cortisol for 15 corticoids, 2,2,4,6,6,17 α ,21,21,21-*d*₉-progesterone and 2,2,4,6,6,21,21,21-*d*₈-17 α -hydroxyprogesterone for 13 progestins, and 2,2,3,4,4,6-*d*₆-cholesterol for 2 sterols were purchased from NARL and C/D/N isotopes (Pointe-Claire, Quebec, Canada). For solid-phase extraction (SPE), Oasis HLB cartridge (3 mL, 60 mg; Waters, Milford, MA, USA) 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 (St. Louis, MO, USA). A solution of β -glucuronidase/arylsulfatase from *Helix pomatia* was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The trimethylsilylating (TMS) agents, *N*-methyl-*N*-trifluorotrimethylsilyl acetamide (MSTFA), ammonium iodide (NH₄I), and dithioerythritol (DTE) were purchased from Sigma. All organic solvents used were of analytical or HPLC grade and were purchased from Burdick and Jackson (Muskegan, MI, USA). Deionized water was prepared using the Milli-Q purification system (Millipore, Billerica, MA, USA).

Standard Solutions and Quality-Control Samples

Stock solutions of all reference standards were prepared at a concentration of 1000 μ g/mL in methanol and working solutions were made up with methanol at concentrations in the range 0.1 to 10 μ g/mL. L-Ascorbic acid (1 mg/mL) was added to prevent the oxidations of labile steroid metabolites. All standard solutions were stored at -20°C until required and all were stable for a minimum of 3 mo. The urine samples used for calibration and quality-control (QC) purposes were prepared in house from steroid-free urine [30]. Steroids-free urine samples were prepared by percolating urine samples through Serdolit PAD-1 (0.1–0.2 mm analytical grade; Serva, Heidelberg, Germany). In this way, urinary steroids and other potential interfering compounds are retained in the cartridges and the eluates were collected. After checking for endogenous steroids with negative result following the solvent extraction procedure, they were used as matrix for steroid profiling.

Subjects and Sample Collection

First-morning urine samples were collected from 59 BPH patients (age: 65.3 ± 8.2 y male) and 41 healthy male subjects (age: 56.7 ± 7.1 y) in the Department of Urology at Severance Hospital (Seoul, Korea). All patients underwent a transrectal ultrasound-guided oc-

tant biopsy and had either a suspicious finding by digitorectal examination or an elevated level of serum prostate-specific antigen (PSA). The age-matched normal controls showed no evidence of serum PSA level elevation or of BPH. Steroid levels in urine were normalized versus urinary creatinine values using Jaffe method [31].

Sample Preparation

Quantitative metabolite profiling of urinary steroids was based on previous reports [4, 8, 32]. Briefly, urine samples (2 mL) spiked with 20 μ L of the 7 internal standards (*d*₃-testosterone and *d*₄-estradiol; 1 μ g/mL, *d*₄-cortisol and *d*₈-17 α -hydroxyprogesterone; 5 μ g/mL, methyltestosterone, *d*₉-progesterone, and *d*₆-cholesterol to 10 μ g/mL) were extracted with Oasis HLB SPE cartridges coupled to a peristaltic pump. After loading a sample onto a cartridge, the cartridge was washed with 2 mL water and eluted twice with 2 mL of methanol. Combined methanol eluates were evaporated under a stream of nitrogen and then 1 mL of 0.2 M acetate buffer (pH 5.2), 100 μ L of aqueous 0.2% ascorbic acid, and 50 μ L of β -glucuronidase/arylsulfatase were added. After incubation at 55°C for 3 h, the solution was extracted twice with 2.5 mL of ethyl acetate: *n*-hexane (2:3, vol/vol). Combined organic solvents were evaporated using a N₂ evaporator at 40°C and further dried in a vacuum desiccator over P₂O₅-KOH for at least 30 min. Finally, the dried residue was derivatized with MSTFA/NH₄I/DTE (40 μ L; 500:4:2, vol/wt/wt) at 60°C for 20 min, and 2 μ L of the resulting mixture was subjected to GC-MS in selected-ion monitoring (SIM) mode. To maximize extraction efficiencies, two organic solvents, tetrahydrofuran (THF) and ethyl acetate, were evaluated in the pH range 5 to 9. In addition, the potential uses of diethyl ether, methyl *tert*-butyl ether, and *n*-hexane were investigated for promoting the partition of water-miscible THF and ethyl acetate.

Instrumental Conditions

GC-MS was performed with 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 μ L) was injected in split mode (10:1) at 280°C and separated through an Ultra-1 capillary column (25 m \times 0.2 mm i.d., 0.33 μ m film thickness; Agilent Technologies; Palo Alto, CA, USA). The GC oven temperature was initially set at 215°C , then ramped to 260°C at $1^{\circ}\text{C}/\text{min}$, and finally increased to 320°C at $15^{\circ}\text{C}/\text{min}$ and held for 1 min. The carrier gas was helium at a column head pressure of 210.3 kPa (column flow: 1.0 mL/min at oven temperature of 215°C). For quantitative analysis, characteristic ions of each steroid were determined as their TMS derivatives. Peak identifications were achieved by

Table 1. The GC-MS information for quantitative analysis of steroids studied

Compounds (trivial name)	Abbreviation	Ion selected ^a	Retention time (min)	Compounds (trivial name)	Abbreviation	Ion selected	Retention time (min)
Androgens				Estrogens			
5 β -androstan-3 α ,17 α -diol	$\beta\alpha\alpha$ -diol	256	12.12	17 α -estradiol	17 α -E2	416	18.60
5 β -androstan-3 β ,17 α -diol	$\beta\beta\alpha$ -diol	256	12.87	Estrone	E1	414	19.12
Androsterone	An	434	15.27	17 β -estradiol	17 β -E2	416	19.98
Etiocholanolone	Etio	434	15.44	2-methoxy-17 β -estradiol-3-methylether	2-MeO-E2-3-methylether	388	23.40
5 α -androstan-3 α ,17 β -diol	$\alpha\alpha\beta$ -diol	241	16.00	4-methoxy-17 β -estradiol	4-MeO-E2	446	23.73
5 β -androstan-3 α ,17 β -diol	$\beta\alpha\beta$ -diol	256	16.07	2-methoxyestrone	2-MeO-E1	444	24.57
5 α -androstan-3 β ,17 α -diol	$\alpha\beta\alpha$ -diol	241	17.04	2-hydroxy-17 β -estradiol-3-methylether	2-OH-E2-3-methylether	446	25.06
Epidihydrotestosterone	Epi-DHT	434	17.46	2-methoxy-17 β -estradiol	2-MeO-E2	446	25.53
11-keto-androsterone	11-keto-An	520	17.55	2-hydroxyestrone	2-OH-E1	502	25.92
11-keto-etiocholanolone	11-keto-Etio	520	17.60	2-hydroxy-17 β -estradiol	2-OH-E2	504	26.75
Dehydroepiandrosterone	DHEA	432	17.85	4-hydroxyestrone	4-OH-E1	502	27.34
Epiandrosterone	Epi-An	419	18.10	4-hydroxy-17 β -estradiol	4-OH-E2	504	28.42
Androstenediol	A-diol	434	18.58	17-epiestriol	17-epi-E3	504	29.19
5 α -Androstanedione	5 α -dione	432	18.60	Estriol	E3	504	29.82
Epitestosterone	Epi-T	432	18.77	16-keto-17 β -estradiol	16-keto-E2	487	30.06
5 α -androstan-3 β ,17 β -diol	$\alpha\beta\beta$ -diol	241	18.83	16 α -hydroxyestrone	16 α -OH-E1	487	30.06
Dihydrotestosterone	DHT	434	19.34	16-epiestriol	16-epi-E3	504	31.16
Androstenedione	A-dione	430	19.80	2-hydroxyestriol	2-OH-E3	592	37.30
Testosterone	T	432	20.52	d4-17 β -estradiol ^b	d4-E2	420	19.88
11 β -hydroxyandrosterone	11 β -OH-An	522	20.73				
11 β -hydroxyetiocholanolone	11 β -OH-Etio	522	21.05				
16 α -hydroxy-DHEA	16 α -OH-DHEA	505	28.49				
d3-testosterone ^b	d3-T	435	20.44				
Methyltestosterone ^b	Methyl-T	446	24.21				
Corticoids				Progestins			
Tetrahydrodeoxycortisol	THS	548	34.98	5 β -dihydroprogesterone	5 β -DHP	445	20.01
Tetrahydrodeoxycorticosterone	THDOC	550	36.26	Epipregnanolone	Epi-P-one	447	23.33
Tetrahydrocortisone	THE	634	38.66	Pregnanolone	P-one	447	23.62
Tetrahydrocortisol	THF	636	41.30	Allopregnanolone	Allo-P-one	447	23.97
Dihydrodeoxycorticosterone	DHDOC	548	42.03	Pregnanediol	P-diol	269	25.03
Allotetrahydrocortisol	Allo-THF	636	42.39	Pregnanetriol	P-triol	435	26.34
21-deoxycortisol	21-deoxyF	634	42.55	Pregnenolone	Preg	445	27.36
11-deoxycortisol	11-deoxyF	544	42.76	5 α -dihydroprogesterone	5 α -DHP	445	28.57
11-deoxycorticosterone	11-deoxyB	546	43.51	Progesterone	Prog	458	29.90
Cortisone	E	615	46.03	20 α -hydroprogesterone	20 α -DHP	445	30.20
11-dehydrocorticosterone	11-dehydroB	617	46.80	17 α -hydroxypregnenolone	17 α -OH-Preg	548	32.68
Allodihydrocorticosterone	Allo-DHB	636	46.87	17 α -hydroxyprogesterone	17 α -OH-Prog	546	35.70
Allodihydrocortisol	Allo-DHF	634	47.01	11 β -hydroxyprogesterone	11 β -OH-Prog	531	41.32

Table 1. Continued

Compounds (trivial name)	Abbreviation	Ion selected ^a	Retention time (min)	Compounds (trivial name)	Abbreviation	Ion selected	Retention time (min)
Corticosterone	B	634	47.79	d9-progesterone ^b	d9-Prog	465	29.70
Cortisol	F	632	47.90	d8-17 α -hydroxyprogesterone ^b	d8-17 α -OH-Prog	552	35.53
d4-cortisol ^b	d4-F	636	47.86	Sterols			
				Cholesterol	Chol	458	40.80
				24S-hydroxycholesterol	24S-OH-Chol	413	48.99
				d6-cholesterol ^b	d6-Chol	464	40.55

^aQuantitative ions as the TMS derivatives of steroids.

^bAn internal standard used in steroid profiling.

comparing retention times and matching the height ratios of characteristic ions (Table 1).

Method Validation

QC samples containing all 70 analytes were quantified using MS peak height ratios versus the IS. Calibration samples were made up at 15 different concentrations, which depended on the sensitivity and reference values of the urinary steroids. Least-squares regression analysis was performed on 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 of S/N ratio > 10 for LOQ. Precisions are expressed as coefficients of variation (% CV) and accuracies as percent relative errors (% bias), and were determined using QC samples at three different concentrations (low; 2 ~ 50 ng/mL, medium; 20 ~ 200 ng/mL, and high; 100 ~ 2000 ng/mL) depending on the sensitivity and reference values of the urinary steroids. To determine within-day repeatabilities, four replicates were analyzed, whereas day-to-day reproducibility was measured by running samples on 5 different days.

Extraction recoveries were determined using QC samples at three concentration levels in triplicate for each steroid by adding known amounts of mixed working solutions to steroid-free urine samples. Absolute recoveries were calculated by comparing peak height ratios of extracted samples versus those of their nonextracted counterparts, using the IS added just before the derivatization step.

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). Quantitative results are expressed as means \pm SD, and group comparisons are made using the unpaired two-tailed Student's *t*-test. Statistical significance was considered at $P < 0.01$.

To visualize differences between the steroid signatures of BPH patients and healthy subjects, 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). Concentrations of steroids and ratios of metabolites to precursors (an indicator of enzyme activity) in BPH patients and healthy subjects were compared using the Student's *t*-test, and statistically significant variables were selected and processed. For clustering analysis, all prefiltered quantitative results of steroids measured and their ratios 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.

Z-score transformation [$z = (\text{observed value} - \text{baseline median}) / \text{baseline standard variation}$] 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 using HCA, color coding in maps indicating auto range by difference of concentration in each compound was gradated at three point; red indicates z -scores > 0 , blue z -scores < 0 , and white z -scores ≈ 0 of a row (subjects) across all columns (urinary steroids).

Results and Discussion

Urinary Steroid Profiling

Steroid hormones of significance under different biological conditions fall into several distinct groups, and are frequently used to evaluate endocrine functions. Comprehensive extraction methods for GC-MS based steroid analysis mainly focus on specific steroid metabolites which have the same biological function and chemical structures [15–17, 19]. Due to the chemical and physical diversities within androgens, estrogens, corticoids, progestins, and sterols, the described profiling technique was devised to simultaneously quantify steroid analytes as diverse as lipophilic cholesterol and hydrophilic corticoids in a single extract.

In SPE experiment, urinary steroids are purified using a unique copolymer sorbent, Oasis HLB, which allows high and reproducible results to be obtained [8, 30, 32, 33]. In general, SPE absorbent retains steroids based on hydrophobic interactions with free and conjugated steroids. The following two extractions methods were therefore investigated: (1) 2 mL of urine was processed with a SPE cartridge and the sample obtained was hydrolyzed, and (2) urine was first hydrolyzed and then processed in SPE. However, difficulties in loading samples into SPE cartridges after hydrolyzing them with β -glucuronidase/arylsulfatases, due to an increase in the viscosity of urinary solution hydrolyzed, were observed. This result was not observed if β -glucuronidase only was present in the enzyme solution [6]. Therefore, SPE procedure was processed first in sample preparation steps.

Although catechol estrogens, such as 2-OH-E1, 2-OH-E2, 4-OH-E1, 4-OH-E2, and 2-OH-E3, are degraded when exposed to light or to a pH higher than 9.5 [34], 0.2% aqueous L-ascorbic acid was added to prevent oxidative decomposition before enzymatic hydrolysis. After enzymatic hydrolysis, deconjugated steroids were extracted with an ethyl acetate: *n*-hexane (2:3, vol/vol) mix. In extraction, THF and ethyl acetate were primarily investigated, and diethyl ether, *t*-butyl methyl ether, and *n*-hexane were added to expedite THF and ethyl acetate partitioning. When relatively polar organic solvents, such as diethyl ether and *t*-butyl methyl ether, were used, chromatographic interference was encountered. Although both THF and ethyl acetate had high extraction efficiencies, ethyl acetate was finally selected because of the objectionable smell and high polarity of THF.

Overall extraction efficiencies were not significantly different for all steroids studied at pH values of 5 to 7. The recoveries of catechol estrogens (10.4% ~ 48.9%) were poor at pH 9 because they are oxidized to quinones at this pH [35]. In addition, different ratios of ethyl acetate to *n*-hexane were examined and a 2:3 (vol/vol) mix was chosen having considered extraction yield and chromatographic properties. Using optimized conditions, all urinary steroids studied were extracted in good yields, except 2-OH-E3 and 24S-OH-Chol, which are still detectable in urine after extraction (Figure 1).

Gas Chromatography-Mass Spectrometry

Using the described GC-SIM/MS conditions, 70 steroid hormones were analyzed as their TMS derivatives and peak identification was straightforward using retention times, characteristic ions, chromatograms, and mass spectra, the latter were compared with those of reference standards. The GC oven program used resulted in well separated, good peak shapes for most steroids with retention times from 12.12 min for $\beta\alpha\alpha$ -diol to 48.99 min for 24S-OH-Chol. On the other hand, 11-keto-An and 11-keto-Etio, and 16-keto-E2 and 16 α -OH-E1 were co-eluted and estimated totally in quantification (Figure 2). However, some partial-overlapped compounds (e.g., 5 α -dione and 17 α -E2, 2-OH-E2-3-methylether and P-diol, 4-OH-E1 and Preg) were differentiated by SIM using their different characteristic ions (Table 1).

Most steroids were monitored using their molecular ions as base peaks, except: $\beta\alpha\alpha$ -diol, $\beta\beta\alpha$ -diol, $\beta\alpha\beta$ -diol, which had an abundant $[\text{M}-180; \text{M}-2\text{OTMS}]^+$ ion at m/z 256; $\alpha\alpha\beta$ -diol, $\alpha\beta\alpha$ -diol, $\alpha\beta\beta$ -diol with an $[\text{M}-195; \text{M}-2\text{OTMS}-\text{CH}_3]^+$ ion at m/z 241; 5 β -DHP, 5 α -DHP, Preg with an $[\text{M}-15; \text{M}-\text{CH}_3]^+$ ion at m/z 445; Epi-P-one, P-one, and Allo-P-one with an $[\text{M}-15]^+$ at m/z 447; 16-keto-E2, 16 α -OH-E1 with an $[\text{M}-15]^+$ ion at m/z 487; THS with an $[\text{M}-90; \text{M}-\text{OTMS}]^+$ ion at m/z 548; THE and Allo-DHF with an $[\text{M}-90]^+$ ion at m/z 634; THF and Allo-THF with an $[\text{M}-90]^+$ ion at m/z 636; 11-deoxyF with an $[\text{M}-90]^+$ ion at m/z 544; cortisone (E) with an $[\text{M}-90-15; \text{M}-\text{OTMS}-\text{CH}_3]^+$ ion at m/z 615; and 11-dehydroB with an $[\text{M}-15]^+$ at m/z 617. In the cases of P-diol, P-triol, and 11 β -OH-Prog, less intense fragments at m/z 269, 435 and 531, respectively, were chosen to improve selectivity rather than most intense peaks (m/z 117, 255, and 546); this was attributed to urinary interference. Regarding the TMS ethers of P-diol and P-triol, the characteristic ions at m/z 269 and 435 were monitored, which are attributed to $[\text{M}-195; \text{M}-2\text{OTMS}-\text{CH}_3]^+$ and $[\text{M}-117; \text{M}-\text{CH}[\text{OSi}(\text{CH}_3)_3]\text{CH}_3 \text{ side-chain}]^+$ ions. The base peak of P-diol is m/z 117 and other important fragments are m/z 269 $[\text{M}-90-90-15]^+$, m/z 284 $[\text{M}-90-90]^+$, m/z 347 $[\text{M}-117]^+$, and m/z 449 $[\text{M}-15]^+$. Among these fragments, m/z 269 instead of m/z 117 was chosen to improve selectivity from urinary backgrounds. Also the P-triol has m/z 255 $[\text{M}-117-90-90]^+$, m/z 345 $[\text{M}-117-90]^+$, and m/z 435 $[\text{M}-117]^+$ as major fragments formed

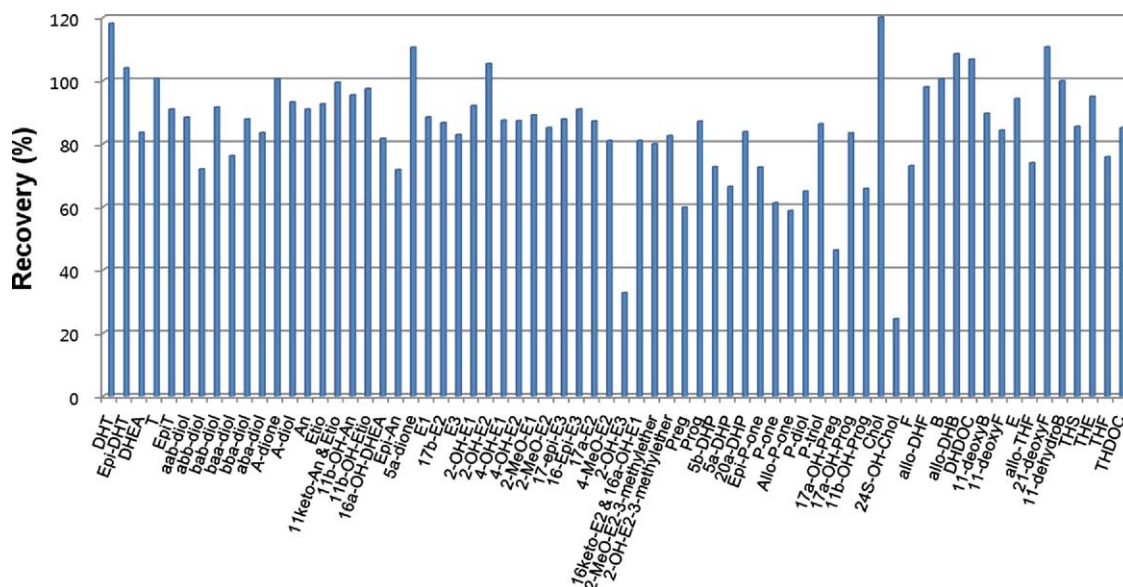


Figure 1. Extraction recoveries of 70 urinary steroids analyzed using the described method. Absolute recoveries were calculated by comparing peak height ratios of extracted samples versus their non-extracted counterparts.

by the loss of 117 mass units, but m/z 435 was monitored to improve selectivity. In addition, 11β -OH-Prog elutes THF at the similar retention time (41.30 min), but a typical loss of 90 Da (-OTMS) from the THF (m/z 636) would interfere the detection of 11β -OH-Prog in case of using m/z 546, the molecular ion. Therefore, m/z 531 $[M-90-15]^+$, which rarely did not exist in THF, was chosen instead of m/z 546 (see Supplementary Figure 1).

Method Validation

Method validation requires the evaluations of linearity, LOD, LOQ, precision, and accuracy using calibration samples prepared from in-house steroid-free urine [30]. Calibration curves were generated for all analytes using QC samples fortified with all reference standards at

different concentrations. The devised method was found to be linear (correlation coefficient, $r^2 > 0.994$) for all analytes, except for cholesterol ($r^2 = 0.983$). Most compounds had an LOQ in the 0.05 ~ 2.0 ng/mL range, while those of DHEA, Epi-An, 5α -dione, $\alpha\beta$ -diol, 11-dehydroB, 17α -OH-Preg, and 11β -OH-Prog ranged between 5.0 and 50 ng/mL (Table 2).

Assay precisions and accuracies were determined by analyzing three QC samples at different concentrations of the individual steroids (5, 20, 100, 200, 500, and 2000 ng/mL). Intra-day ($n = 4$) precisions (expressed as % CV) ranged from 0.9% to 11.2%, whereas accuracies (expressed as % bias) ranged from 95.8 to 119.1%, and inter-day ($n = 5$) precisions (% CV) and accuracies (% bias) ranged from 1.6% to 10.5% and from 91.6% to 115.5%, respectively (Table 2). Especially, the accuracy

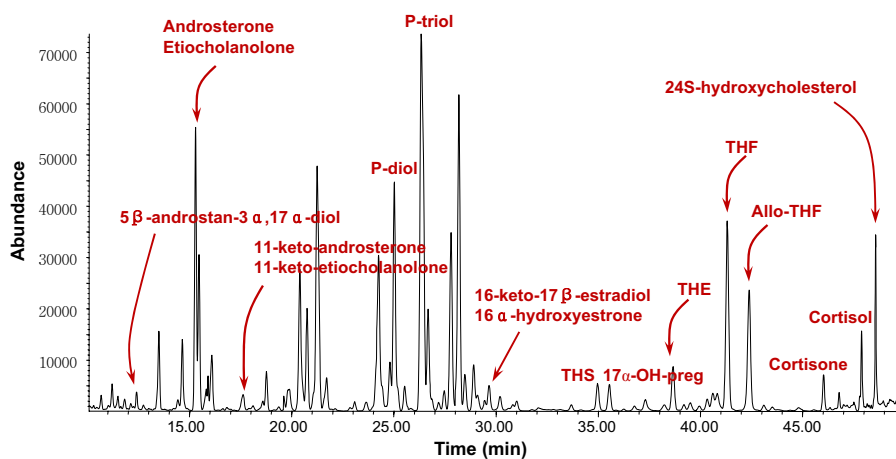


Figure 2. A representative total ion chromatogram obtained from a healthy male subject for detection of 70 urinary steroids.

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

Compounds (trivial name)	LOD ^a (ng/mL)	LOQ ^b (ng/mL)	Calibration range (ng/mL)	Linearity (r^2)	intra-day ($n = 4$)		inter-day ($n = 5$)	
					CV ^c (%)	Accuracy ^c (%)	CV (%)	Accuracy (%)
Androgens								
$\beta\alpha$ -diol	0.5	1.0	1–200	0.998	5.9	104.0	3.8	97.9
$\beta\beta$ -diol	0.2	0.5	0.5–200	0.998	5.7	107.8	3.1	105.0
An	0.1	0.5	0.5–3000	0.996	3.2	119.1	3.5	108.8
Etio	0.1	0.5	0.5–3000	0.998	2.5	106.1	2.2	109.7
$\alpha\alpha\beta$ -diol	0.5	1.0	1–200	0.998	6.8	111.4	3.3	109.6
$\beta\alpha\beta$ -diol	0.2	0.5	0.5–200	0.998	4.4	106.2	3.0	109.1
$\alpha\beta\alpha$ -diol	1.0	2.0	2–200	0.995	5.2	107.5	3.4	103.4
Epi-DHT	0.1	0.2	0.2–200	0.994	8.0	106.0	3.4	108.9
11-keto-An & Etio	0.1	0.5	0.5–1000	0.996	3.7	108.4	3.4	112.3
DHEA	5.0	50.0	50–3000	0.997	3.5	106.6	3.3	108.2
Epi-An	2.0	5.0	5–1000	0.994	2.5	98.0	2.1	109.5
A-diol	0.5	2.0	2–200	0.996	7.4	116.3	10.5	97.2
5 α -dione	0.5	5.0	10–200	0.995	4.8	106.2	2.2	111.8
Epi-T	0.05	0.1	0.1–200	0.997	3.8	100.7	1.6	100.8
$\alpha\beta\beta$ -diol	10.0	20.0	20–500	0.998	6.9	104.0	3.0	104.0
DHT	0.1	0.2	0.2–200	0.998	4.7	109.6	2.4	102.7
A-dione	0.05	0.1	0.1–200	0.999	5.7	103.4	2.4	101.7
T	0.05	0.1	0.1–200	0.998	4.7	106.1	1.9	102.5
11 β -OH-An	0.2	0.5	0.5–3000	0.997	3.4	104.6	5.8	112.9
11 β -OH-Etio	0.2	0.5	0.5–3000	0.998	2.7	101.3	4.1	113.4
16 α -OH-DHEA	0.1	0.2	0.2–1000	0.996	4.0	104.3	1.7	105.8
Estrogens								
17 α -E2	0.05	0.1	0.1–200	0.998	3.9	98.6	2.6	96.3
E1	0.1	0.2	0.2–200	0.998	2.9	99.0	2.0	97.3
17 β -E2	0.05	0.1	0.1–200	0.997	3.5	99.8	2.3	97.2
2-MeO-E2-3-methylether	0.1	0.2	0.2–200	0.998	2.9	97.3	2.1	96.4
4-MeO-E2	0.2	0.5	0.5–200	0.998	2.1	98.7	1.8	95.6
2-MeO-E1	0.05	0.1	0.1–200	0.998	1.9	99.0	1.8	95.8
2-OH-E2-3-methylether	0.1	0.2	0.2–200	0.998	4.2	101.3	2.4	97.6
2-MeO-E2	0.05	0.1	0.1–200	0.998	4.3	100.2	2.2	97.1
2-OH-E1	0.1	0.2	0.2–200	0.998	4.9	104.7	3.7	99.5
2-OH-E2	0.1	0.2	0.2–200	0.998	6.1	106.4	3.4	99.3
4-OH-E1	0.1	0.2	0.2–200	0.998	2.9	103.2	2.5	98.8
4-OH-E2	0.1	0.2	0.2–200	0.998	4.6	100.1	4.2	97.3
17-epi-E3	0.5	1.0	1–200	0.997	2.4	106.7	2.4	100.3
E3	0.2	0.5	0.5–200	0.997	2.7	101.6	2.8	99.7
16-keto-E2 and 16 α -OH-E1	0.1	0.2	0.2–500	0.998	2.7	98.7	8.8	92.5
16-epi-E3	0.5	1.0	1–200	0.997	3.5	103.2	2.3	100.5
2-OH-E3	0.5	1.0	1–100	0.997	5.0	107.4	5.3	101.0
Corticoids								
THS	0.1	0.2	0.2–200	0.997	0.9	99.2	3.0	98.4
THDOC	0.2	0.5	0.5–200	0.996	7.1	100.4	4.1	100.0
THE	1.0	2.0	2–500	0.997	7.6	151.4	9.9	151.3
THF	0.2	0.5	0.5–200	0.998	6.6	106.1	4.9	104.0
DHDOC	0.5	1.0	1–200	0.997	4.2	101.2	4.1	104.9
Allo-THF	0.2	0.5	0.5–500	0.996	4.8	103.9	2.6	102.6
21-deoxyF	0.5	1.0	1–200	0.997	7.3	108.5	4.8	109.3
11-deoxyF	0.2	0.5	0.5–200	0.998	2.9	98.5	3.5	91.6
11-deoxyB	0.5	1.0	1–200	0.997	6.9	100.3	2.9	105.1
Cortisone (E)	0.2	0.5	0.5–500	0.998	3.2	101.2	2.7	99.8
11-dehydroB	0.2	5.0	5–200	0.994	2.1	101.8	2.7	100.4
Allo-DHB	0.5	1.0	1–200	0.997	6.9	113.1	5.3	108.0
Allo-DHF	1.0	2.0	2–200	0.997	2.3	97.5	2.9	107.3
Corticosterone (B)	0.2	0.5	0.5–200	0.997	3.3	99.0	3.3	100.3
Cortisol (F)	0.1	0.2	0.2–200	0.998	3.0	100.5	3.5	98.0
Progestins								
5 β -DHP	0.1	0.2	0.2–100	0.997	9.9	109.2	3.5	110.5
Epi-P-one	1.0	2.0	2–200	0.995	4.1	106.0	3.0	105.8
P-one	1.0	2.0	2–200	0.997	7.4	110.1	3.3	115.5
Allo-P-one	0.2	0.5	0.5–200	0.994	2.9	109.9	2.2	103.7

Table 2. Continued

Compounds (trivial name)	LOD ^a (ng/mL)	LOQ ^b (ng/mL)	Calibration range (ng/mL)	Linearity (<i>r</i> ²)	intra-day (<i>n</i> = 4)		inter-day (<i>n</i> = 5)	
					CV ^c (%)	Accuracy ^c (%)	CV (%)	Accuracy (%)
P-diol	0.5	1.0	1–500	0.997	6.2	105.6	3.6	107.5
P-triol	0.2	0.5	0.5–500	0.999	6.0	102.8	2.3	105.5
Preg	1.0	2.0	20–200	0.995	8.8	104.1	3.5	98.5
5 α -DHP	0.2	0.5	0.5–200	0.995	7.0	99.2	3.8	104.0
Prog	0.5	1.0	1–100	0.998	4.8	96.0	2.5	98.6
20 α -DHP	0.2	0.5	0.5–100	0.997	9.1	101.7	4.9	103.8
17 α -OH-Preg	5.0	10.0	20–500	0.998	3.6	98.7	3.0	102.9
17 α -OH-Prog	0.2	0.5	0.5–200	0.996	3.1	95.8	2.3	96.1
11 β -OH-Prog	2.0	5.0	5–500	0.996	11.2	103.9	7.3	94.3
Sterols								
Chol	1.0	2.0	50–1000	0.983	7.3	113.0	7.8	115.0
24S-OH-Chol	0.5	2.0	2–200	0.994	6.5	96.3	4.1	100.9

^aThe limit of detection was measured S/N ratio > 3.

^bThe limit of quantification was measured S/N ratio > 10.

^cPrecision and accuracy were expressed as the mean values of data obtained from three different concentrations for each analyte.

of THE resulted in abnormal values, and it might be produced by low calibration slope because of poor GC-MS properties.

Quantitative Results of Benign Prostatic Hyperplasia

The validated quantitative profiling method was applied to measure 70 endogenous steroids in urine samples obtained from 59 BPH patients and 41 age-matched healthy male subjects. All quantitative results, corrected by urinary creatinine levels, were compared, and differences between patients and healthy subjects were evaluated using the Student's *t*-test. Although it was not the purpose of this study to elucidate clinical implications, some features were notable, and significant differences were found between patients and healthy subjects (see Supplementary Figure 2).

In patients, urinary levels of T, Epi-T, $\alpha\alpha\beta$ -diol, $\beta\alpha\beta$ -diol, $\beta\beta\alpha$ -diol, 17 β -E2, 11 β -OH-Prog, and 16 α -OH-DHEA were significantly decreased ($P = 4.52 \times 10^{-4} \sim 2.77 \times 10^{-13}$), and those of 11 β -OH-Etio, $\alpha\beta\beta$ -diol, 2-OH-E3, 17 α -OH-Prog, and 11-deoxyF were significantly increased ($P = 4.56 \times 10^{-7} \sim 9.42 \times 10^{-18}$). Some other urinary steroids quantified were also significantly different in patients and controls ($P < 0.01$). Changes in the urinary levels of $\alpha\alpha\beta$ -diol and $\alpha\beta\beta$ -diol were in accord with previous findings [36, 37], but other steroid changes observed in this study have not been reported to date. In particular, urinary 17 β -E2 and 2-OH-E3 showed remarkable changes with *P* values of 2.77×10^{-13} and 4.25×10^{-9} , which might be associated with the different biochemical roles of estrogen receptor α (ER α) and β (ER β) in various types of prostate cells [28, 38, 39]. The involvements of other urinary steroids needs to future study.

Steroid Signatures by Hierarchical Clustering Analysis

The present MS-based steroid profiling has become an essential clinical tool and has produced many results in BPH, but it is difficult to identify critical differences between patients and controls because of the large number of results. Hierarchical clustering, which involves calculating the distance matrices of data objects and then merging objects that are close to each other to form sub-clusters, has been utilized in biomedical studies to interpret the expressions of biomolecules [2, 22–25]. In the present study, after determining the relative levels of each steroid, all data were z-score transformed to produce a heat map using a supervised HCA based on Pearson's correlation coefficient. The raw data were the relative concentrations of the 70 urinary steroids, and these were filtered by statistical significance ($P < 0.01$) using the Student's *t*-test. The clustering method and similarity measure were set unweighted average and Euclidean distance, respectively. Urinary concentrations were represented by colors in the heat map, and each steroid was represented by a single row of colored boxes, whereas columns represented different subjects. Heat maps were compared with respect to individual quantitative results, and indicated the effectiveness of steroid signatures.

Figure 3 illustrates in a heat map showing altered concentrations of urinary steroids in BPH patients and healthy subjects. The heat map readily shows changes in the concentrations of urinary steroids. Increased urinary excretion patterns of several steroids, from Epi-DHT down to 11-deoxyF, were detected in patients, while other steroid levels were significantly reduced. In addition, visual inspections of steroid signatures complemented quantitative statistics. Although 2-OH-E3 was present at a significantly higher level in patients ($P = 4.25 \times 10^{-9}$; see Supplementary Figure 2), these increases were only ob-

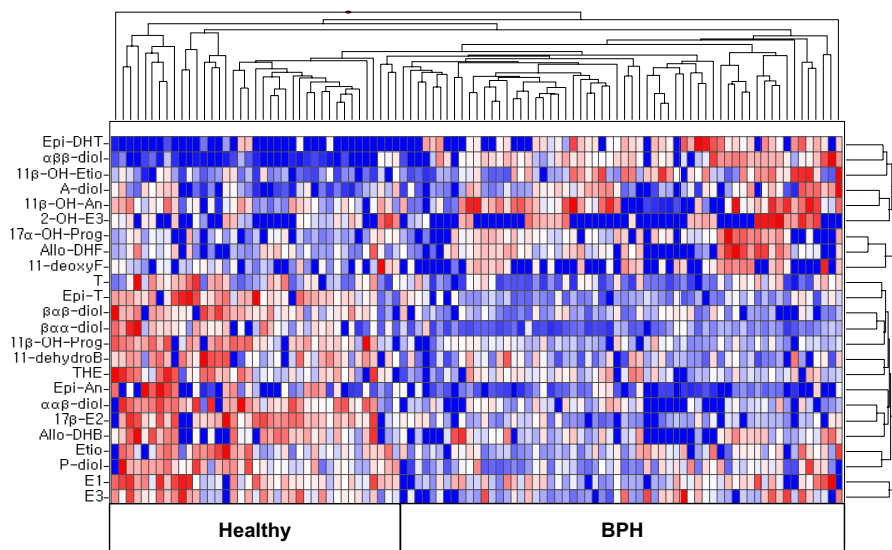


Figure 3. Hierarchically clustered heat map of the urinary steroids levels of 59 BPH patients and 41 healthy male subjects. Differences between steroid levels in controls and patients were examined using Student's *t*-test, and only those that were significantly different at the $P < 0.01$ level were selected. Row represents subjects and column urinary concentration of individual steroids. Cells are colored based on concentrations in urinary samples. Red represents highly excreted into urine and blue showed a concentration in urine.

served in some patients in the heat map. The levels of other steroids, 11β -OH-Etio and 11-deoxyF, were also inconsistency different in patients (Figure 3).

To illustrate further the usefulness of a steroid signature, the ratio of steroid metabolite to precursor, which reflects enzyme activity (Scheme 1), was examined. The cluster and heat maps of all analyzed samples in this 70 compound study showed differences between groups in these ratios (Figure 4). A dendrogram of ratios revealed that the oxidoreductases formed a cluster (5α -reductase, 3α -HSD, 3β -HSD, and 17β -HSD, except for 20α -HSD; see Supplementary Table 1 for full enzyme names), which are differentiated with a cytochrome P450 enzyme, 11β -hydroxylase. These results support the validity of the data transformation, since 5α -reductase is a marker of

BPH [27–29], and 17β -HSD is positively expressed in prostate cells [40, 41]. Other up-regulated enzymes in the map might be biomarkers of BPH. Furthermore, the method can be used to visualize quantitative steroid data in a single graphic using a hierarchically clustered heat map. The need for this type of visualization arises from the complexity of large experimental datasets. One important advantage of using a heat map is that it can be used to visualize clinical data across multiple subjects for many analytes, which allows patterns to be easily identified.

Conclusions

Due to the clinical requirement to quantify trace levels of urinary endogenous steroids, we devised an analyt-

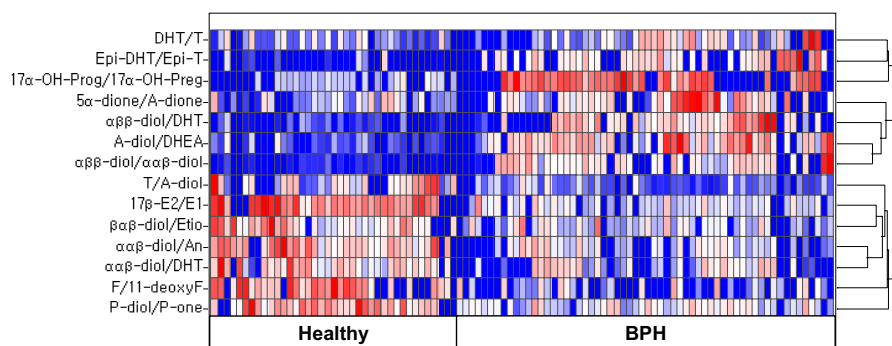


Figure 4. A heat map for measuring enzyme activities related to steroid metabolism. The ratio of precursor to metabolite for each steroid was calculated, and only steroids with level differences significant at the $P < 0.01$ level were selected. Rows represent subjects and column metabolic ratio. Red represents a high ratio and blue a low ratio.

ical method to measure the individual concentrations of 70 urinary steroids and several enzyme activities based on steroid signatures using a single assay, which substantially reduces analytical time and sample size. In contrast to classical biochemical approaches using RIA and EIA that mainly focus on single enzymes, single metabolic reactions, and kinetic properties, profiling analysis involves the collation of quantitative results for a broad series of metabolites to gain an overall understanding of metabolism.

Steroid signatures as represented by hierarchically clustered heat maps use color to communicate underlying quantitative results, which are much harder to appreciate when presented numerically. This method is simple to implement and maintains the biological integrity of data. Using this technique, inter-relationships between absolute concentrations and enzyme activities in samples from different sources can be recognized. The combined GC-MS profiling technique described has adequate sensitivity that could be used for high throughput analysis, and could be adopted to enable the quantifications of urinary steroids and indicate metabolic alterations at biologically relevant levels.

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Appendix A Supplementary Material

Supplementary material associated with this article may be found in the online version at [doi:10.1016/j.jasms.2009.04.020](https://doi.org/10.1016/j.jasms.2009.04.020).

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