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Comprehensive and Highly Sensitive Urinary Steroid Hormone Profiling Method Based on Stable Isotope-Labeling Liquid Chromatography–Mass Spectrometry

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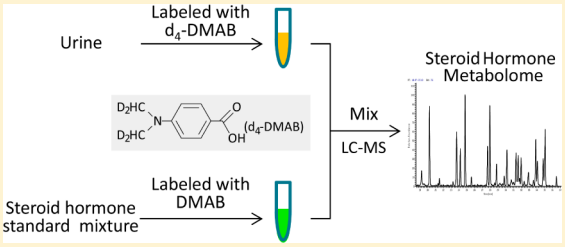
Supporting Information

ABSTRACT: Steroid hormones are crucial substances that mediate a wide range of vital physiological functions of the body. Determination of the levels of steroid hormones plays an important role in understanding the mechanism of the steroid hormone-related diseases. In this study, we present a novel targeted metabolic profiling method based on the introduction of an easily protonated stable isotope tag to a hydroxyl-containing steroid hormone with a synthesized derivatization reagent, deuterium 4-(dimethylamino)-benzoic acid (d_4-DMBA), and liquid chromatography–mass spectrometry (LC-MS). Different from other reported derivatization reagents that have been used to enhance the sensitivities for estrogens or androgens, our method is comprehensive with the capability of covering hydroxyl-containing androgens, estrogens, corticoids, and progestogens. Furthermore, the nonderivatized steroid hormones (e.g., 17α-hydroxyprogesterone, progesterone, and androstenedione) were not destroyed during the derivatization process, and their levels could still be obtained in one LC-MS run. We were able to detect 24 steroid hormones at subng/mL levels (the lower limit of detection could reach 5 pg/mL for estrone and 16α-hydroxy estrone, which is equivalent to 0.1 pg on column) with maximum sensitivity enhancement factors of more than 10^3- to 10^4-fold after derivatization. The method was successfully applied to the measurement of free (unconjugated) steroid hormones in urine samples of males, females, and pregnant women. Because the significant role the steroid hormone pathway plays in humans, a comprehensive, sensitive, specific, and accurate method for profiling the steroid hormone metabolome shall offer new insights into hormone-related diseases.



Steroid hormones are among the most important species of endogenous metabolites, regulating the life action for human beings by participating in the maintenance of secondary sexual characteristics, modulation of gene transcription, and regulation of endocrine and immune functions, etc. Disorders of the steroid hormones homeostasis are related to various diseases. Recent studies showed that the steroid hormones were implicated in the process of cancer diseases. It was reported that breast cancer is often correlated with high circulating and urinary endogenous estrogens levels.[1−3] Sex hormones, especially androgens, are involved in the etiology of prostate cancer.[4−6] Steroid hormones are also involved in the homeostasis of immune/inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus.[7,8] Quantitatively measuring a series of steroid hormones (ideally the steroid hormone metabolome) will play a vital role in understanding disease progression and individual biochemical responses to the disease, as well as in achieving better personalized medicine.

In previous studies, analytical methods to quantify one or several steroid hormones have been widely employed in pathological studies and clinic diagnoses.[9,10] However, steroid hormone levels can vary a great deal from one individual to another and can also be affected by the menstrual cycle. Therefore, attention needs to be paid to the risk of a pathological result obtained from the measurement of single hormone metabolites. In contrast, detecting the steroid hormone metabolome is helpful for holistically understanding the alteration of the steroid hormone pathway, and leads to a reduction in the risk of false positive/negative diagnosis error. However, due to their extremely low abundance in body fluids, the detection of steroid hormones is still a very challenging task.[11]

The current methods for measuring endogenous steroid hormones have involved immunoassay, gas chromatography–mass spectrometry (GC-MS), and liquid chromatography–mass spectrometry (LC-MS).[12] Although immunoassay can analyze one hormone sensitively, it suffers from poor specificity, accuracy, and reproducibility because of matrix interferences and the high lot-to-lot variability of antibodies.[13,14] The specificity of the measurements of steroid hormones significantly improves when using GC-MS and LC-MS. However,

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because of limited sensitivity and labor-intensive and time-consuming sample preparation, the GC-MS approach has not become a routine method for measuring steroid hormones in clinical laboratories.[12,14] LC-MS provides good specificity, high sensitivity, and the ability to measure multiple analytes. Therefore, LC-MS is a preferable choice for profiling the steroid hormone metabolome.[15] A number of LC-MS-based metabolomic studies have been carried out to analyze as many steroid hormones as possible.[13,16−20] However, it is unable to profile the steroid hormone metabolome with only one LC-MS platform. It is necessary to use various types of ionization sources and polarities to achieve satisfactory sensitivity.[15,21]

Derivatization is a widely used approach to improve ultraviolet (UV), fluorescence (FL), and mass spectrometry (MS) signals. In LC-MS-based studies, the derivatization reagent reacts with a functional group of the targeted compounds, such as an amino, carboxyl, hydroxyl, or carbonyl group, to improve column retention and ionization efficiency and to overcome the switch of the ionization mode.[22] Dansyl chloride is the most popular derivatization reagent used for esterifying the hydroxyl group to enhance the sensitivity for the measurement of estrogens[17−19] and unactivated alcohols including testosterone, 6β-OH testosterone, and hydrocortisone.[23] Some novel derivatization reagents, such as picolinic acid,[9] *N*,*N*-dimethylglycine,[24] 1,2-dimethylimidazole-4-sulfonyl chloride,[25] and Sanger's reagent and analogue,[26] have also been used to react with steroid hormones to introduce a high ionization tendency moiety for sensitivity enhancement.

Serious matrix effects caused by endogenous metabolites occur during the measurement of low abundance metabolites (e.g., steroid hormones) in LC-MS-based complex sample studies. Stable isotope internal standards are widely used to overcome the matrix effects for the accurate absolute/relative quantification. However, it is impossible to obtain all the stable isotope internal standards of a metabolome. The stable isotope-tagged derivatization is an alternative way to introduce a stable isotope-tagged moiety to the endogenous compounds and therefore perform as stable isotope internal standards. This isotope labeling method has been applied in metabolomics[27−32] and proteomics[33−35] studies.

Urine is a sample that is very easy and noninvasive to obtain. Detecting urinary steroid hormones has shown potential in disease biomarker discovery.[36] In this study, we present a method that integrates LC-MS with solid phase extraction (SPE) and derivatization to profile free (unconjugated) endogenous steroid hormones in urine. Deuterium 4-(dimethylamino)-benzoic acid was synthesized as a novel derivatization reagent to label steroid hormones for sensitivity enhancement and stable isotope-based quantification.

EXPERIMENTAL SECTION

Chemicals. Estrone (E_1), β-estradiol (E_2), 2-hydroxy estrone (2-OH E_1), 16α-hydroxy estrone (16α-OH E_1), estriol (E_3), testosterone, dehydroepiandrosterone (DHEA), 5a-androstan-17b-ol-3-one (5α-DHT), 5b-androstan-17b-ol-3-one (5β-DHT), mesterolone, pregnenolone, deoxycorticosterone (DOC), 17α-hydroxypregnenolone (17OH-Preg), corticosterone, cortexolone, tetrahydrodeoxycortisol (THS), hydrocortisone, androsterone, progesterone, 17α-hydroxyprogesterone (17-OHP), D-androsten-3b,17b-diol (5-androstenediol), 5a-tetrahydrocorticosterone (5a-THB), and aldosterone were purchased from the International Laboratory (South San Francisco, CA, USA). Androstenedione was purchased from Dr. Ehrenstorfer (Augsburg, Germeny). Dichloromethane of HPLC grade was purchased from Tedia (Fairfield, OH, USA). Acetonitrile and methanol of HPLC grade were purchased from Merck (Darmstadt, Germany). 4-Dimethylaminopyridine (DMAP) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were purchased from Sigma Chemical Co. (St. Louis). 4-(Dimethylamino)-benzoic acid (DMAB) was purchased from Alfa Aesar (Ward Hill, MA, USA).

Synthesis of d_4-4-(Dimethylamino)-benzoic Acid. Figure 1A shows the synthetic scheme for d_4-4-(dimethylamino)-benzoic acid (d_4-DMAB). The detailed procedures of the synthesis and purification are described in the Supporting Information. The confirmation and purity was tested against commercial 4-(dimethylamino)-benzoic acid by thin layer chromatography (TLC), UV, LC-UV-MS, and nuclear magnetic resonance (NMR).

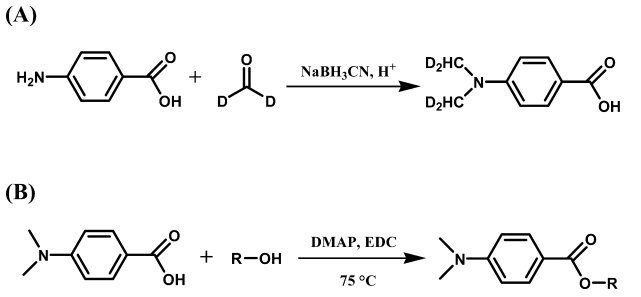

Figure 1. Reaction schemes for (A) synthesis of the stable isotope-labeling reagent, d_4-DMAB, and (B) steroid hormones derivatized by DMAB.

Urine Sample Collection. The urine samples were collected from volunteers after a morning of fasting. The volunteers included five males, five nonpregnant females, and two pregnant women (3 and 4 months pregnant). The ages of the 12 volunteers ranged from 23 to 28 years. The urine samples were centrifuged at 10 000 rpm for 10 min. The supernatants were stored at −20 °C.

Three pooled urine samples were prepared (before the steroid hormone extraction and derivatization) by pooling the five samples from the male volunteers into the first sample, the five samples from the female volunteers into the second sample, and the two samples from the pregnant volunteers into the third sample.

Extraction of Urinary Steroid Hormones. An optimized SPE procedure was applied for the removal of the bulk of the polar solutes in urine. The SPE extraction cartridge (Waters Oasis HLB, 30 mg) was activated with 2 mL of methanol and was then washed with 2 mL of water. The mixed solution of 0.5 mL of urine and 0.4 mL of methanol was loaded onto the SPE extraction cartridge. Washing with 3 mL of 45% methanol prior to the elution with 1.5 mL of 90% methanol caused the eluted solution to be lyophilized pending derivatization.

Derivatization. The derivatization scheme for the steroid hormones using DMAB in the presence of DMAP and EDC is displayed in Figure 1B. The procedure was optimized as follows: 80 μL of 2 mg/mL DMAB solution, 80 μL of 2 mg/mL DMAP solution, and 80 μL of 5 mg/mL EDC solution were added into the lyophilized sample. Dichloromethane was used as the solvent. After spirited vortex, the solution was transferred into a glass vial and sealed. The vial was kept at 75 °C for 5 h. The dichloromethane was then evaporated at 45 °C.

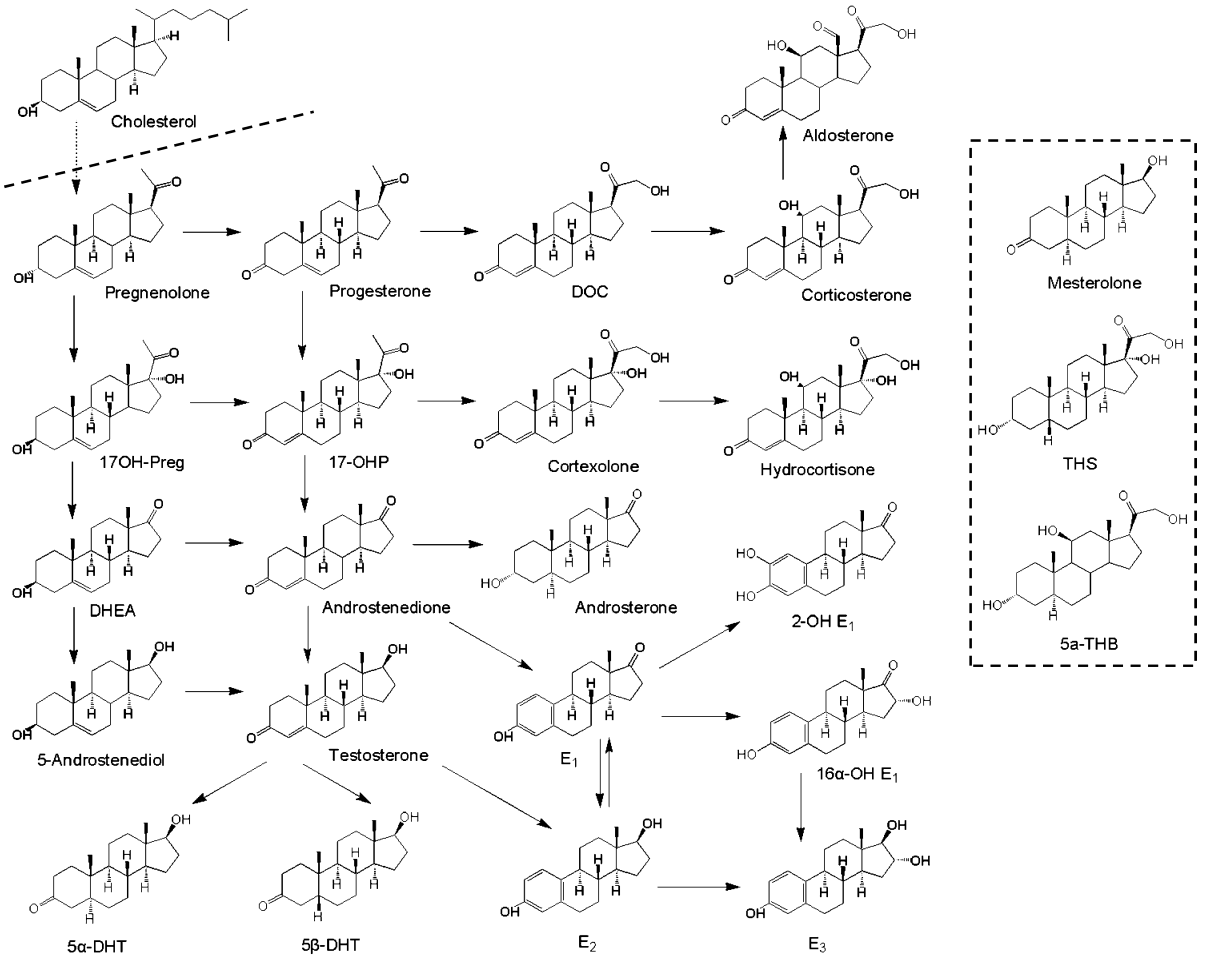

Figure 2. Structures and the related steroid hormones biosynthesis pathway of the 24 selected standards. THS, 5a-THB, and mesterolone were listed with a dashed line frame because they were not found in the given pathway from the KEGG database.

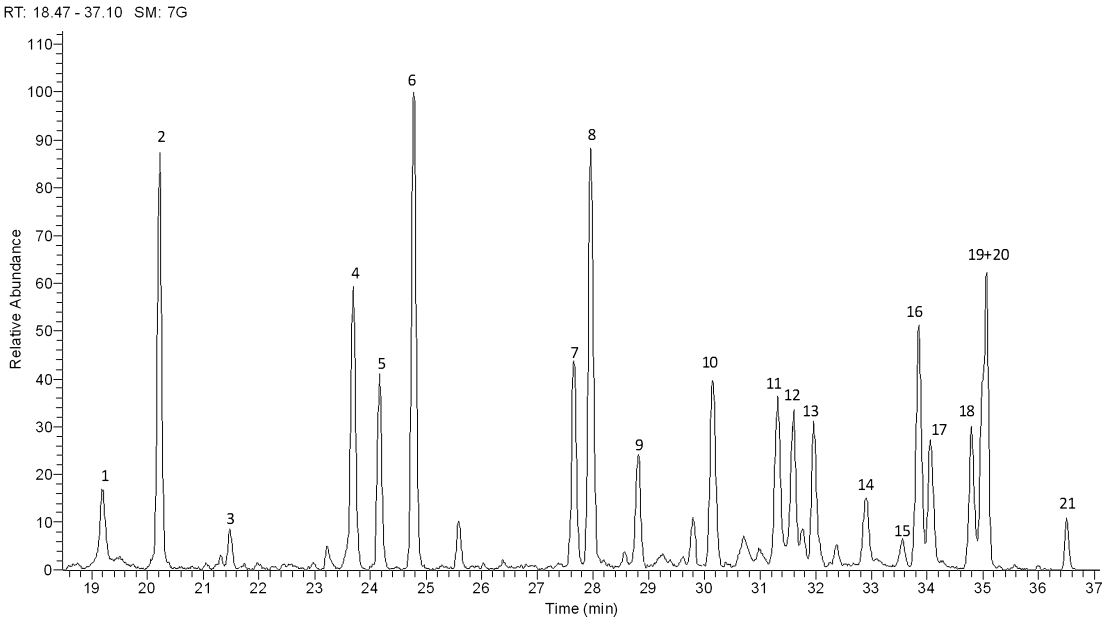

Figure 3. Extracted ion chromatogram (EIC) of the 21 DMAB derivatized steroid hormones in urine spiked with 20 ng/mL standards mixture by LC-MS. Identification: 1, Aldosterone; 2, hydrocortisone; 3, E_3; 4, corticosterone; 5, 5a-THS; 6, cortexolone; 7, THS; 8, DOC; 9, E_2; 10, E_1; 11, testosterone; 12, 5-androstenediol; 13, 2-OH E_1; 14, androsterone; 15, 17OH-Preg; 16, DHEA; 17, 5α-DHT; 18, 5β-DHT; 19, 16α-OH E_1; 20, mesterolone; 21, pregnenolone.

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Table 1. LLOD, Intrabatch Precision, Interbatch Precision, Linear Range, Recovery, and Stability within 48 h of Urinary Steroid Hormones Measurement, Including SPE Extraction, Derivatization, and LC-MS Analysis Steps

							recovery (%)			
no.	steroid hormones	LLOD (pg/mL)	intrabatch precision (%, n = 6)	interbatch precision (%, n = 6)	linear range (pg/mL)	regression coefficient (r)	4 ng/mL	8 ng/mL	16 ng/mL	stability within 48 h (%)
1	aldosterone	200	10.2	11.8	200−20000	0.967	134.9	109.5	101.4	4.6
2	hydrocortisone	20	2.3	5.2	10−20000	0.995	137.4	94.9	102.2	3.1
3	E_3	10	13.3	21.6	10−5000	0.999	106.1	88.8	86.0	5.9
4	corticosterone	10	3.8	4.3	10−20000	0.996	102.7	91.1	91.8	3.6
5	5a-THB	100	7.0	9.1	100−5000	0.999	119.4	106.0	107.3	6.6
6	cortexolone	10	3.4	4.9	10−20000	0.996	100.1	90.0	92.2	6.5
7	THS	50	9.1	14.8	100−20000	0.996	106.6	90.9	89.5	5.5
8	DOC	10	1.3	3.6	5−20000	0.998	103.5	91.8	90.9	5.5
9	E_2	10	5.1	9.3	10−20000	0.993	108.5	95.4	94.5	6.3
10	E_1	5	4.3	5.1	5−20000	0.997	106.8	93.7	94.0	7.7
11	testosterone	20	13.6	19.3	20−10000	0.999	88.5	95.5	100.6	6.9
12	5-androstenediol	10	4.9	4.1	20−20000	0.998	97.7	92.3	98.2	10.8
13	2-OH E_1[a]	50	7.4	6.9	500−20000	0.970	8.7	7.9	11.8	5.1
14	androsterone	500	16.4	19.9	500−10000	0.995	96.6	96.5	109.9	4.2
15	17OH-Preg	500	13.7	16.6	500−10000	0.998	99.5	91.6	102.5	9.7
16	DHEA	100	16.1	14.5	100−10000	0.999	121.8	101.2	112.2	7.3
17	5α-DHT	100	12.5	20.3	50−10000	0.995	95.2	90.4	105.3	6.7
18	5β-DHT	100	14.6	18.2	500−10000	0.993	93.2	96.3	101.4	6.4
19	mesterolone	50	13.8	20.0	100−10000	0.994	97.0	96.1	107.4	5.5
20	16α-OH E_1	5	7.0	7.7	5−20000	0.999	112.3	99.5	96.8	12.4
21	pregnenolone	500	11.8	16.9	500−10000	0.986	104.8	99.0	105.5	14.8

[a]2-OH E_1 with very low recoveries could only be used for semi-quantitative analysis.

The residue was dissolved with 200 μL of 50% acetonitrile pending LC-MS analysis.

LC-MS Analysis. The MS instrument LTQ-Orbitrap XL (Thermo Fisher Scientific, Rockford, IL, USA) settings were as follows: capillary temperature 325 °C, source voltage 4.5 kV, and capillary voltage 49 V for ESI+ analysis. The mass range was set at 260−700 m/z. The resolution of the Orbitrap was set at 15 000. Chromatographic separation was performed on a BEH C18 column (10 cm × 2.1 mm, 1.7 μm; Waters, Milford, MA, USA) using a Thermo Fisher Accela LC 1000 system with an injection volume of 20 μL. The linear gradient program was set as follows with mobile phase (A) 0.1% formic acid solution and phase (B) acetonitrile: 0 min, 20% B; 8 min, 30% B; 33 min, 75% B; 35 min, 98% B; 41 min, 98% B; 41.01 min, 20% B; 48 min, 20% B. The flow rate was 0.25 mL/min.

RESULTS AND DISCUSSION

Coverage of DMAB-Derivatized Steroid Hormones. The metabolic pathway shown in Figure 2 was modified according to the pathway of steroid hormone biosynthesis in the Kyoto Encyclopedia of Genes and Genomes database (KEGG, http://www.genome.jp/kegg-bin/show_pathway?ko00140+C00280). It represents the core metabolic pathway of steroid hormones biosynthesis containing most of the well-known steroid hormones. Mesterolone, 5a-THB, and THS are listed in Figure 2 with a dashed line frame because they were not found in the given pathway from the KEGG database. To establish an analytical method to cover as many steroid hormones as possible, 24 steroid hormone standards (listed in Figure 2) covering the species of estrogen, androgen, glucocorticoid, mineralocorticoid, and progestogen were used to evaluate the performance of the derivatization and the LC-MS analysis. All the selected hormone standards, with the exception of the progesterone and androstenedione, contained at least one hydroxyl moiety in their chemical structure that was expected to be esterified by DMAB (Figure 1B).

The results show that all of the hydroxyl-containing steroid hormones could be derivatized by DMAB in the presence of DMAP and EDC in dichloromethane, with the exception of 17-OHP. The failure of 17-OHP to react with DMAB was attributed to the steric hindrance of the hydroxyl moiety. The extracted ion chromatogram (EIC) of the 21 DMAB derivatized steroid hormones by LC-MS is displayed in Figure 3. All of the derivatized steroid hormones were separated well in the retention time range from 19 to 37 min. Isomers, for example, 5α-DHT and 5β-DHT with a difference only in the orientation of one hydrogen atom, could be separated on the baseline. Because of the feasibility of the derivatization of hydroxyl-containing steroid hormones by DMAB, we can expect that obtaining a detailed pathway including more steroid hormones can be accomplished by enlarging the steroid hormone standards library.

Determination of Nonderivatized Steroid Hormones. A solution containing a mixture of progesterone, 17-OHP, and androstenedione (200 ng/mL) was tested to investigate the percentage of nonderivatized steroid hormones that are destroyed when subjected to the derivatization procedure. Compared with intact standards solution (200 ng/mL), the recoveries (n = 3) of progesterone, 17-OHP, and androstenedione were 95.6%, 102.1%, and 102.0%, respectively. This result indicated that these nonderivatized steroid hormones were not destroyed during the process of derivatization. In other words, the initial levels of progesterone, 17-OHP, and androstenedione could still be obtained in the derivatized urine in one LC-MS run. Therefore, the method presented herein has the capability of profiling all the steroid hormones listed in Figure 2. Fortunately, the nonderivatized steroid hormones progesterone, 17-OHP, and androstenedione had the lowest lower limit

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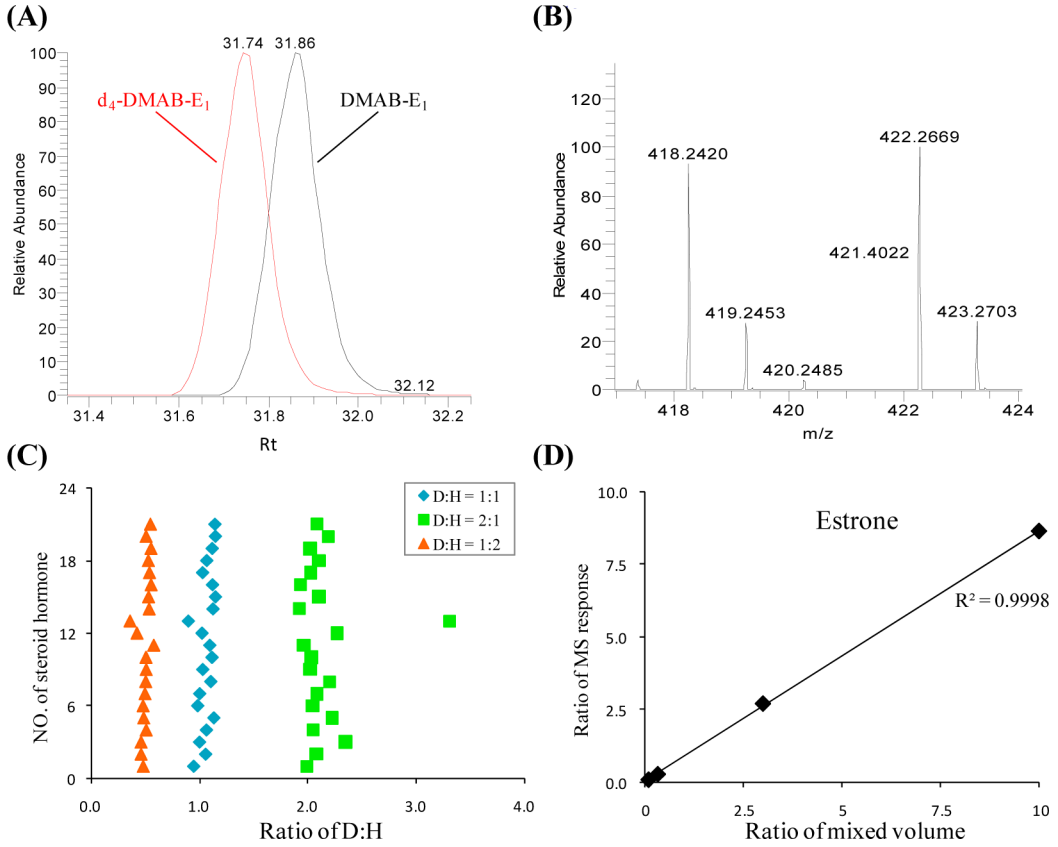

Figure 4. Results of relative quantification based on D/H stable isotope DMAB labeling: (A) Extracted ion chromatogram of heavy and light labeled estrone; (B) mass spectrum of the ion pair of heavy and light labeled estrone; (C) intensity ratios of 21 heavy/light labeled steroid hormones with the amount of 1:2, 1:1, and 2:1 (the number of the *Y*-axis represents the steroid hormone in Table 1); (D) ion pairs of light/heavy labeled estrone in 1:10, 1:3, 1:1, 3:1, and 10:1 mixed solution showed a good linear regression.

of detections (LLODs, 20 pg/mL) when compared with the other intact steroid hormones.

Enhancement of Sensitivity. The detection sensitivities for intact steroid hormones and DMAB-derivatized steroid hormones by LC-MS are compared in Supplemental Table S1. Because the baseline of the EIC was horizontal for the high-resolution mass spectrometer, the signal-to-noise ratio (S/N) cannot be calculated. Herein, the lowest concentration corresponding to the signal that can be detected by the EIC was defined as the LLOD. The LLODs of the 21 steroid hormones without SPE extraction are summarized (Supplemental Table S1). The LLODs of the DMAB-derivatized steroid hormones ranged from 5 to 500 pg/mL (equivalent to 0.1−10 pg on column), while the LLODs of their corresponding intact steroid hormones ranged from 50 pg/mL to >200 ng/mL by LC-MS. The sensitivity, therefore, was improved by 1- to 10 000-fold using the derivatization method. For example, for the improvement in LLOD for 5-androstenediol, 5a-THB, and 16α-OH E_1, the improvement folds of LLOD was observed to be more than 3−4 orders of magnitude. There were two major aspects that contributed to the improved sensitivity after the DMAB derivatization: (1) the introduction of an easily protonated tertiary amine moiety by derivatization with DMAB greatly increased the ionization efficiency for steroid hormones and (2) the decrease in the polarity and the elution with a higher percentage of organic phase led to an increase in the nebulization efficiency.

Analytical Characteristics of the Method. Steroid hormone mixture solutions of 5, 10, 20, 50, 100, 200, 500, 1000, 2000, 5000, 10 000, and 20 000 pg/mL were subjected to SPE extraction, derivatization, and LC-MS analysis steps to investigate the LLODs and linear ranges for the 21 derivatized steroid hormones. The results (Table 1) show that the 21 derivatized steroid hormones were all detected at subng/mL levels. The LLODs ranged from 5 to 500 pg/mL, equivalent to 0.1 to 10 pg being injected onto the column.

The derivatized steroid hormones show good linear behavior with concentration within a range of 2−4 orders of magnitude. The regression coefficients ranged from 0.967 to 0.999 (Table 1).

A 20 μL portion of a steroid hormone mixture solution (200 ng/mL) was spiked into 500 μL of urine and was then subjected to the SPE extraction-derivatization-LC-MS analysis procedure to evaluate the intrabatch and interbatch precision (n = 6). The relative standard deviation (RSD) for the intrabatch precision ranged from 1.3% to 16.4%, with an average RSD of 9.1% for the 21 steroid hormones. The RSD for the interbatch precision ranged from 3.6% to 21.6%, with an average RSD of 12.1% for the 21 steroid hormones.

Steroid hormone mixture solutions of different volumes (10, 20, and 40 μL at 200 ng/mL) were spiked into 500 μL urine samples and were then subjected to the SPE extraction-derivatization-LC-MS analysis procedure to evaluate the recovery. The recoveries of the 21 steroid hormones with spiked standard levels of 4, 8, and 16 ng/mL in urine ranged from 88.5% to 137.4%, from 88.8% to 109.5%, and from 86.0% to 112.2%, respectively. The steroid hormone 2-OH E_1 was the

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only exception, with very low recoveries that could only be used for semiquantitative analysis.

A 20 μL portion of the steroid hormone mixture solution (200 ng/mL) was spiked into 500 μL of urine. After being derivatized, the sample was analyzed at 0, 24, and 48 h to test the stability at 4 °C within 2 days (n = 3). The RSD of the 21 derivatized steroid hormones were all <15%, with an average RSD of 6.9%. This result indicated that the derivatized urine sample was stable while waiting for the LC-MS run in the autosampler within 48 h.

Stable Isotope-Based Quantification. After the SPE extraction and lyophilization, 8, 16, and 32 ng/mL steroid hormone mixtures were derivatized with d_4-DMAB. The derivatized mixtures were then mixed with an equal volume of a DMAB-derivatized 16 ng/mL steroid hormone mixture. The mixed solution contained 1:2, 1:1, and 2:1 d_4-DMAB/DMAB derivatized steroid hormones. These mixed solutions were then analyzed to investigate the MS intensity ratios of ion pairs of d_4-DMAB derivatized (heavy labeled) and DMAB derivatized (light labeled) steroid hormones.

Here, we can look at estrone as an example. In Figure 4A, the ion pair of the d_4-DMAB and the DMAB derivatized steroid hormone was found in one LC-MS run. DMAB-estrone was determined to have an m/z = 418, while d_4-DMAB-estrone showed an m/z = 422. The difference in m/z values indicates the replacement of four hydrogen atoms by four deuterium atoms (Figure 4B). The feasibility of stable isotope labeling-based relative quantification was evaluated in two ways: (1) 1:2, 1:1, and 2:1 steroid hormones were derivatized with d_4-DMAB and DMAB (the results are shown in Figure 4C) and (2) the d_4-DMAB and DMAB derivatized solutions were mixed with volume ratios of 1:10, 1:3, 1:1, 3:1, and 10:1 (the results are shown in Figure 4D). After the SPE extraction, lyophilization, derivatization, and mixing, the intensity ratios of ion pairs were approximately 1 (ranging from 0.90 to 1.14) with an average ratio of 1.06 for the 21 steroid hormones when the D/H = 1:1 (Figure 4C). Additionally, the quantitative ratios were approximately 2 (except for the 3.31 observed for 2-OH E_1, which could only be used for semiquantitative analysis) and 0.5 when the D/H = 2:1 and 1:2, respectively. In Figure 4D, the intensity ratios of the ion pairs in the 1:10, 1:3, 1:1, 3:1, and 10:1 mixed d_4-DMAB/DMAB derivatized solutions showed a good linear regression. With use of estrone as an example, the R^2 value was 0.9998 (the linear regression of the other 20 steroid hormones is shown in Figure S1 of the Supporting Information). The linear regressions indicated that good relative quantification results can be obtained based on the stable isotope-labeling strategy.

These results show that the heavy labeled urine sample mixed with the light labeled steroid hormone standards of known concentrations as stable isotope internal standards can be used to provide accurate absolute measurements of steroid hormones.

Quantifying the Steroid Hormones in Male, Female, and Pregnant Volunteer Urine Samples. To test the new method's utility for the quantification of steroid hormones in actual clinical samples, urine samples from five males, five nonpregnant females, and two pregnant females were analyzed. As shown in Figure 5, the pooled urine from male, female, and pregnant women was heavy labeled (n = 3), while 4 ng/mL steroid hormone standards were light labeled. The heavy labeled urine and light labeled standards were mixed with a volume ratio of 3:1 and then analyzed by LC-MS.

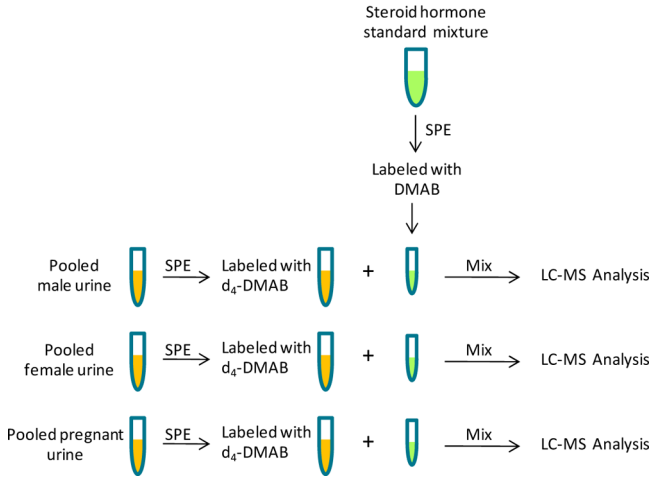

Figure 5. Scheme of the procedure for the absolute quantification of free (unconjugated) urinary steroid hormones of male, female, and pregnant female volunteers.

The analysis by LC-MS detected 17 derivatized free steroid hormones and 3 nonderivatized steroid hormones (17-OHP, androstenedione, and progesterone) in the nonhydrolyzed urine samples (the EICs of the detected steroid hormones are shown in Supplemental Figure S2). The levels of these steroids were absolutely quantified (the results of the concentrations are shown in Supplemental Table S2). As expected, the urine samples from the males had higher levels of androgens, while females excreted more estrogens than males. The pregnant women had many significant changes in urinary steroid hormones, offering a snapshot of the alteration in the steroid hormone pathway during pregnancy (Supplemental Figure S3). Pregnant women excreted much greater amounts of estrone, estradiol, estriol, 16α-OH E_1, and progesterone. These steroid hormones were increased more than 30-fold when compared to nonpregnant women. In particular, the level of free urinary estriol reached 187 ng/mL, increasing 510-fold compared with nonpregnant women. This result is consistent with the description that "during pregnancy, estriol constitutes 60−70% of the total estrogens, increasing to 300−500-fold in relation to non-pregnant women" in HMDB.

CONCLUSIONS

There is mounting evidence showing that steroid hormones play important roles in the development and progression of various diseases. Unfortunately, few approaches offer a comprehensive and highly sensitive means of detecting steroid hormones. In this work, we developed a stable isotope labeling-based quantitative profiling technique for targeted analysis of steroid hormones by LC-MS. The hormones studied include hydroxyl-containing androgens, estrogens, corticoids, and progestogens. Deuterium-4-(dimethylamino)-benzoic acid was synthetized as a novel derivatization reagent for labeling steroid hormones to improve the sensitivity 1- to >10 000-fold with LLODs ranging from 5 to 500 pg/mL. The method showed good intrabatch precision (average RSD = 9.1%), interbatch precision (average RSD = 12.1%), and stability within 48 h (average RSD = 6.9%), and 2−4 orders of magnitude of linear range. Furthermore, the accurate relative and absolute quantification can be achieved using a stable isotope-labeling strategy. We also noted that a small number of nonderivatized steroid hormones were not destroyed during the derivatization

process, and that their quantitative information can be obtained simultaneously in one LC-MS run. Therefore, our approach using a stable isotope-labeling method combined with SPE and LC-MS was able to obtain quantitative profiling of the steroid hormone metabolome with subng/mL LLODs. Furthermore, the method was successfully applied to analyze the free urinary steroid hormones of males, females, and pregnant women. Our future work will focus on building a larger library of derivatized steroid hormones and applying this method to take a snapshot of the steroid hormone pathway in hormone-related disease studies, such as ovarian cancer, prostate cancer, hepatocellular carcinoma, and many others.

■ ASSOCIATED CONTENT

Supporting Information

This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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