Journal of the Endocrine Society, 2024, **8**, 1–9
https://doi.org/10.1210/jendso/bvad155
Advance access publication 21 December 2023
Clinical Research Article

Multiplexed Serum Steroid Profiling Reveals Metabolic Signatures of Subtypes in Congenital Adrenal Hyperplasia

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Abstract

Context: Altered metabolic signatures on steroidogenesis may characterize individual subtypes of congenital adrenal hyperplasia (CAH), but conventional diagnostic approaches are limited to differentiate subtypes.

Objective: We explored metabolic characterizations and identified multiple diagnostic biomarkers specific to individual subtypes of CAH.

Methods: Liquid chromatography-mass spectrometry-based profiling of 33 adrenal steroids was developed and applied to serum samples obtained from 67 CAH patients and 38 healthy volunteers.

Results: Within- and between-run precisions were 95.4% to 108.3% and 94.1% to 110.0%, respectively, while all accuracies were <12% and the correlation coefficients (r^2) were > 0.910. Metabolic ratios corresponding to 21-hydroxylase characterized 21-hydroxylase deficiency (21-OHD; n = 63) from healthy controls (area under the curve = 1.0, $P < 1 \times 10^{-18}$ for all) and other patients with CAH in addition to significantly increased serum 17α-hydroxyprogesterone ($P < 1 \times 10^{-16}$) and 21-deoxycortisol ($P < 1 \times 10^{-15}$) levels. Higher levels of mineralocorticoids, such as corticosterone (B) and 18-hydroxyB, were observed in 17α-hydroxylase deficiency (17α-OHD; N = 3), while metabolic ratio of dehydroepiandrosterone sulfate to pregnenolone sulfate was remarkably decreased against all subjects. A patient with 11β-hydroxylase deficiency (11β-OHD) demonstrated significantly elevated 11-deoxycortisol and its metabolite tetrahydroxy-11-deoxyF, with reduced metabolic ratios of 11β-hydroxytestosterone/testosterone and 11β-hydroxyandrostenedione/androstenedione. The steroid profiles resulted in significantly decreased cortisol metabolism in both 21-OHD and 17α-OHD but not in 11β-OHD.

Conclusion: The metabolic signatures with specific steroids and their corresponding metabolic ratios may reveal individual CAH subtypes. Further investigations with more substantial sample sizes should be explored to enhance the clinical validity.

Key Words: congenital adrenal hyperplasia, adrenal steroids, metabolic signatures, hydroxylase deficiency, cortisol

Abbreviations: 11β-OHD, 11β-hydroxylase deficiency; 11-deoxyF, 11-deoxycortisol; 17α-OHD, 17α-hydroxylase deficiency; 17α-OHP4, 17α-hydroxyprogesterone; 20α-DHE, 20α-dihydrocortisone; 20α-DHF, 20α-dihydrocortisol; 21-deoxyF, 21-deoxycortisol; 21-OHD, 21-hydroxylase deficiency; allo-THF, allo-tetrahydrocortisol; B, corticosterone; CAH, congenital adrenal hyperplasia; CV, coefficient of variation; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; DOC, deoxycorticosterone; E, cortisone; F, cortisol; HPLC, high-performance liquid chromatography; LC-MS, liquid chromatography-mass spectrometry; LOQ, limit of quantification; QC, quality control; THF, tetrahydrocortisol.

Congenital adrenal hyperplasia (CAH) is an autosomal recessive disorder distinguished by enzymatic deficiencies, and each subtype exhibits abnormal steroid metabolism depending on the location of the enzymatic impediment. Diminished cortisol production across all CAH subtypes, including glucocorticoid deficiency, leads to an excessive release of ACTH from the pituitary gland, resulting in compromised hormonal secretions [1, 2]. Metabolic productions of mineralocorticoids and androgens can be also altered based on the specific subtype involved (Fig. 1).

The 21-hydroxylase deficiency (21-OHD) is the most common cause of CAH, and it shows decreased glucocorticoid and mineralocorticoid levels with a significant 17α-hydroxyprogesterone elevation, which is further metabolized to adrenal androgens, leading to androgen excess and virilization in female patients [3, 4]. Among the rare CAH subtypes, 17α-hydroxylase deficiency (17α-OHD) is characterized by increased serum mineralocorticoid levels, including 11-deoxycorticosterone and corticosterone, while cortisol and dehydroepiandrosterone sulfate (DHEA-S) are decreased [1, 5]. The significantly increased 11-deoxycortisol (11-deoxyF) levels are representatively observed in 11β-hydroxylase deficiency (11β-OHD [1, 6]).

Immunoassays have been practically used to measure individual steroids in clinical laboratories, but overestimation

Received: 11 August 2023. **Editorial Decision:** 3 December 2023. **Corrected and Typeset:** 21 December 2023

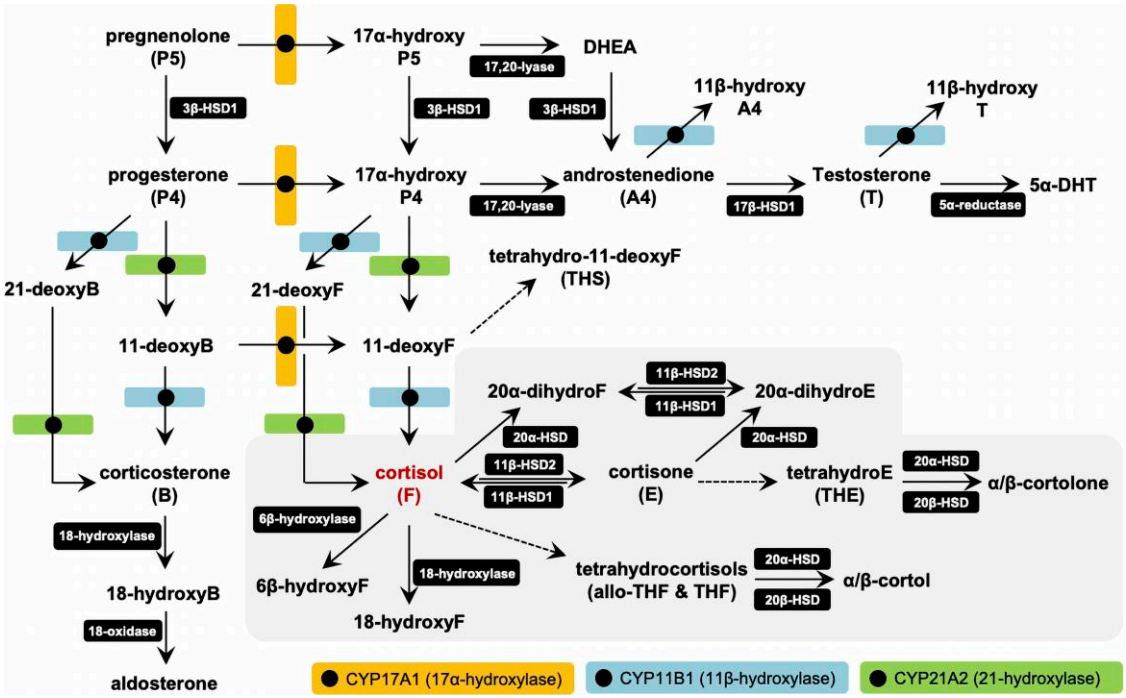

Figure 1. Metabolic pathways of adrenal steroids and steroidogenic enzymes associated with individual CAH subtypes. A qualitative enzyme 17α-hydroxylase (CYP17A1) mainly inhibits androgen biosynthesis as well as cortisol production, in addition to desmolase, which is a quantitative enzyme on steroidogenesis. The inhibited 11β-hydroxylase activity reduces 21-deoxycorticosterone and 21-deoxycortisol as well as corticosterone and cortisol, while 11-hydroxylated androgens are also decreased. As the main cause of CAH, 21-hydroxylase also reduced the production of corticosterone and cortisol through their 11-deoxy precursors.
Abbreviations: CAH, congenital adrenal hyperplasia.

derived from cross-reactivity in a high degree of structural similarity to target steroids pose an analytical accuracy [7, 8]. Endogenous precursors and metabolites of cortisol, such as 21-deoxycortisol (21-deoxyF) and dihydro-/tetrahydro-cortisols, could be estimated 2 times higher quantity in cortisol immunoassay [9, 10]. The capability of multiplexed profiling of steroids has been applied to characterize metabolic signatures of individual CAH subtypes as the main advantages of mass spectrometry over the immunoassay [3-6, 11], and a logistic regression model combined with steroid profiles has been recently suggested [12].

The present study primarily focused on the metabolic profiles of cortisol, given that all CAH subtypes exert an impact on cortisol production. Furthermore, enhancing the chromatographic separation of adrenal steroids was imperative, necessitating a refinement over our previous methodology [5]. Therefore, the partial method validation for 33 serum steroids, including 6 newly added cortisol metabolites and 2 11-oxygenated androgens, was processed. Then, the devised method was applied to characterize the differential metabolic signatures along with CAH subtypes. This study aimed to devise a methodology aimed at offering significant insights into the characterization and subtyping of CAH.

Methods

Study Participants

This study retrospectively enrolled 67 patients diagnosed with classic CAH (28 males and 39 females, aged 21-57 years) at the Department of Internal Medicine, Seoul National University Hospital (Seoul, Korea) between 2018 and 2020. The diagnosis of CAH was established through a combination of clinical and biochemical tests during the neonatal period. Furthermore, confirmation of the diagnosis was achieved by whole exome sequencing, which revealed mutations in CYP21A2, CYP11B1, and CYP17A1 genes. All participants were aged over 20 years and were on glucocorticoid (hydrocortisone/prednisolone) and/or mineralocorticoid (fludrocortisone) therapy, while no patients were subjected to sex hormone therapy. Patients were grouped with 63 patients with 21-OHD (27 males and 36 females, aged 21-53 years), 3 patients with 17α-OHD (3 females, aged 24-57 years), and a 39-year-old male patient with 11β-OHD (Table 1). The clinical subtype of 21-OHD, including salt wasting and simple virilizing forms, was identified at the time of diagnosis. Patients who had undergone adrenalectomy were excluded from this study (Fig. 2). The control group consisted of 18 males and 20 females (aged 22-74 years) with normal blood pressure and without any symptoms of adrenal hyperplasia. None of the healthy volunteers had taken any steroid therapies before, including oral contraceptives.

Blood sampling was conducted between 8 AM and 9 AM following an overnight fast, prior to initiating steroid replacement because spironolactone affects the levels of mineralocorticoids and sex steroids, and the serum was separated immediately and stored at −80 °C until being used. The study was approved by the Institutional Review Board of Seoul National University Hospital (IRB No. 2004-147-1118) and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study subjects.

Chemicals

The reference standards of 33 steroids (Supplementary Table S1 [13]) were purchased from Sigma (St. Louis, MO,

Table 1. Baseline characteristics of subjects enrolled

	Healthy	Patients with CAH				*P*-value[a]	*P*-value[b]
		Total (n = 67)	21-OHD (n = 63)	17α-OHD (n = 3)	11β-OHD (n = 1)		
Age (year)	36.8 ± 12.5	30.9 ± 8.1	30.4 ± 7.4	40.0 ± 16.5	39	.018	.220
Male (%)	18 (47.3)	28 (41.8)	27 (42.9%)	0 (0%)	1 (100%)	.580	.152
BMI (kg/m^2)	26.1 ± 5.4	25.2 ± 5.2	25.3 ± 5.3	23.9 ± 3.8	24.7	.466	.885
Hydrocortisone (%)	—	6 (9.0)	4 (6.3)	1 (33.3)	1 (100)	—	.037
Prednisolone (%)	—	62 (92.5)	60 (95.2)	2 (66.7)	0 (0)	—	.025
Fludrocortisone (%)	—	50 (74.6)	50 (79.4)	0 (0)	0 (0)	—	.003
Spironolactone (%)	—	4 (6.0)	0 (0.0)	3 (100)	1 (100)	—	<.001
CAH phenotype							
Simple virilizing (%)	—		17 (27.0)				
Salt wasting (%)	—		46 (73.0)				
ACTH (ng/mL)	—	349.5 ± 697.3	332.0 ± 749.4	367.0 ± 119.5	87.1		.941
17α-OHP4 (ng/mL)	—		68.6 ± 42.2				
17α-OHP4 < 10 ng/mL (%)	—		5 (7.9)				
DHEA-S (ng/mL)	—	1190.1 ± 1596.9	1246.1 ± 1632.5	147.5 ± 40.3	1043.0		.639
Plasma renin activity (ng/mL)	—	10.8 ± 11.6	11.6 ± 11.7	1.2 ± .2	0.1		.162

Abbreviations: 17α-OHP4, 17α-hydroxyprogesterone; BMI, body mass index; CAH, congenital adrenal hyperplasia; DHEA-S, dehydroepiandrosterone sulfate.
[a]The value for comparison between healthy and all patients with CAH.
[b]The value for comparison among 11β-hydroxylase deficiency, 17α-hydroxylase deficiency, and 21-hydroxylase deficiency.

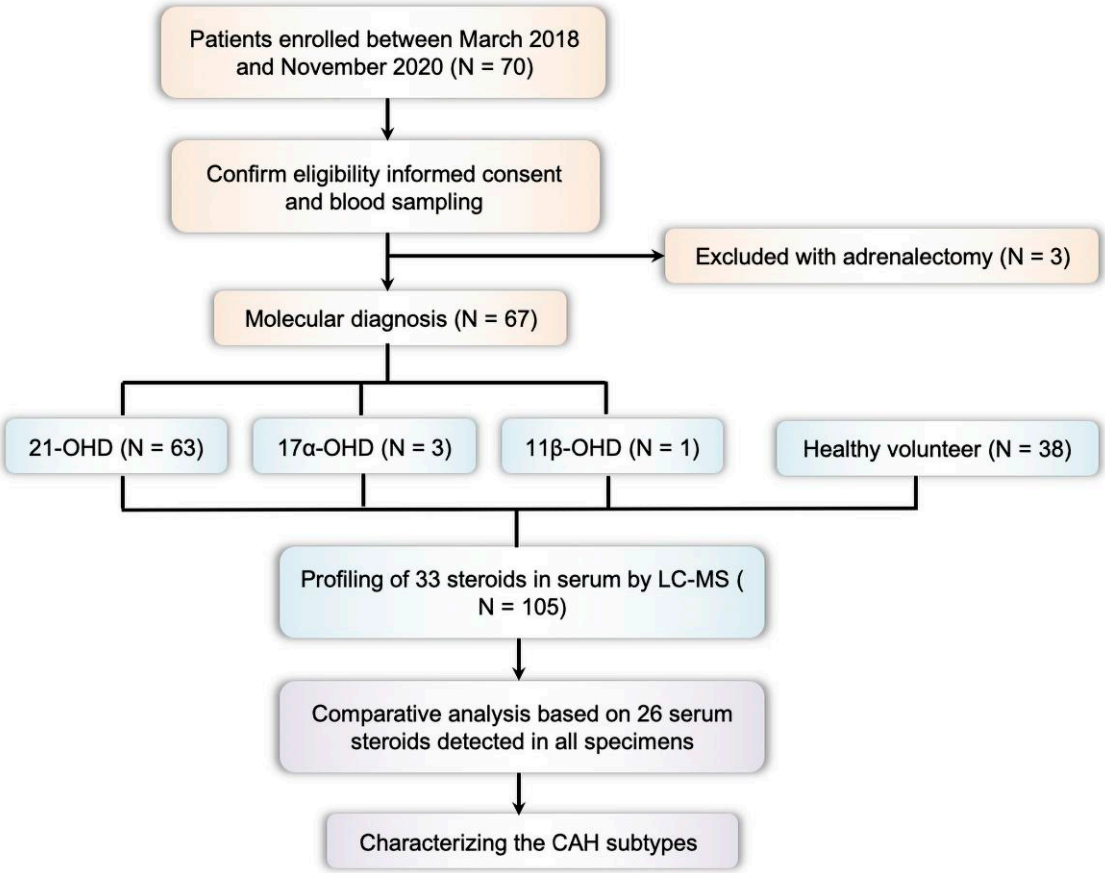

Figure 2. An overview of the study design.
Abbreviations: 11β-OHD, 11β-hydroxylase deficiency; 17α-OHD, 17α-hydroxylase deficiency; 21-OHD, 21-hydroxylase deficiency.

USA) and Steraloids (Newport, RI, USA). Eight internal standards of 9,11,12,12-d_4-cortisol; 2,2,4,6,6,17α,21,21,21-d_9-progesterone; 2,2,4,6,6,21,21,21-d_8-17α-hydroxyprogesterone; 16,16,17-d_3-testosterone; 2,2,3,4,4,6-d_6-dehydroepiandrosterone (DHEA); 16,16,17-d_3-testosterone sulfate; 17α,21,21,21-d_4-pregnenolone; and 21,21,21-d_3-17α-hydroxypregnenolone were obtained from C/D/N isotopes (Pointe-Claire, Quebec, Canada).

All organic solvents were of analytical and high-performance liquid chromatography (HPLC) grades (Burdick & Jackson; Muskegon, MI, USA). Sodium phosphate monobasic (reagent grade), sodium phosphate dibasic (reagent grade), and dimethyl sulfoxide (for HPLC) were purchased from Sigma. Formic acid (HPLC grade, 99%) was obtained from Wako (Osaka, Japan). A 50% glycerol solution of β-glucuronidase from *Escherichia coli* (140 U/mL) was purchased from Roche Diagnostics GmbH (Mannheim, Germany). The Oasis HLB™ solid-phase extraction cartridge (3 mL, 60 mg) was purchased from Waters (Milford, MA, USA). The ultrafree-MC centrifugal filters (PVDF, 0.1 μm) were applied from Millipore (Billerica, MA, USA).

Calibration and Quality Control Samples

Stock solutions for all reference standards were prepared at a concentration of 1000 μg/mL in a methanol and chloroform mixture (9:1, *v/v*), except α-/β-cortol in a mixture of methanol and chloroform (1:1, *v/v*) and α-/β-cortolone in chloroform. Working solutions were prepared at concentrations ranging from 1 to 1000 ng/mL in a methanol and chloroform mixture (9:1, *v/v*). All standard solutions were stored at −20 °C until required. Steroid-free serum was prepared from commercially available steroid-depleted serum (BBI solutions, Cardiff, UK) based on a previous method for calibration and quality control (QC) purposes [14].

Sample Pretreatment

Sample preparation was based on a previous assay [5]. Briefly, 200 μL of serum samples spiked with 20 μL of the internal standard mixtures (d_4-cortisol, d_8-17α-OH-progesterone, and d_4-pregnenolone of 0.2 μg/mL; d_6-DHEA of 0.5 μg/mL; d_9-progesterone and d_3-17α-OH-pregnenolone of 0.1 μg/mL; d_3-testosterone sulfate of 1 μg/mL; d_3-testosterone of 0.02 μg/mL) were added to 1.8 mL of 0.2 *M* phosphate buffer (pH 7.2). Then, the sample was incubated for 1 hour at 55 °C with 50 μL of β-glucuronidase. The hydrolyzed samples were further processed with an Oasis HLB™ cartridge preconditioned with 4 mL of methanol followed by 4 mL of water. The cartridge was washed twice with 0.7 mL of 10% methanol after sample loading, and then all analytes were eluted with 1 mL of methanol in duplicate. The eluate was evaporated under a nitrogen stream at 40 °C, and the dried extracts were reconstituted with 50 μL of methanol and transferred to the ultrafree-MC centrifugal filter. After centrifugation at 14 000 rpm for 5 min, 50 μL of 10% dimethylsulfoxide in water (*v/v*) was added into the filter and centrifuged again at 14 000 rpm for 5 min. Aliquots of 5 μL were injected into the liquid chromatography-mass spectrometry (LC-MS) system.

Analytical Parameters

The LC-MS system (LCMS-8050, Shimadzu Corp., Kyoto, Japan) consists of a Shimadzu Nexera ultra-high-performance liquid chromatograph with an 8050 triple quadrupole mass spectrometer combined with electrospray ionization. Hypersil Gold-C18 (1.9 μm particle size, 100 × 2.1 mm i.d.; Thermo Fisher Scientific, Waltham, MA, USA) was used at a flow rate of 0.25 mL/min with eluent A (0.1% formic acid in 5% acetonitrile) and eluent corticosterone (B) (0.1% formic acid in 95% acetonitrile) as the mobile phases at an oven temperature of 25 °C. The following gradient was used (percentages represent eluent B): 15% at 0 min; 15% to 25% at 0 to 5 min; 25% to 30% at 5 to 8 min; 30% to 40% at 8 to 12 min; 40% to 70% at 12 to 16 min; 70% to 100% at 16 to 18 min. The gradient was returned to the initial conditions (B 100–15% at 22-26 min) after holding for 2 min and then held again for 2 min.

All free steroids were analyzed and quantified in the multiple-ion monitoring mode, and 2 sulfated steroids were monitored in the selected-ion monitoring mode. All peaks were identified by comparison of the retention times and matching the height ratios of characteristic ions. The Lab Solutions LC version 5.85 software (Shimadzu Corp.) was used for data acquisition.

Method Validation

The limit of quantification (LOQ), calibration linearity (r^2), precision [% coefficient of variation (CV)], and accuracy (% bias) were assessed for intra- and interday assays over 4 replicates on 4 different days to validate the devised method. Calibration samples were prepared at 9 different concentrations, and QC samples were prepared at 4 different concentrations, including LOQ, low (3- or 5- fold excess of LOQ), medium, and high QC. Extraction recoveries were determined by adding known amounts of mixed working solutions using QC samples for 3 different concentrations (low, medium, and high QC) in 3 replicates.

Statistical Analysis

Data were analyzed using SPSS (v 22.0; SPSS Inc., Chicago, IL, USA) and GraphPad Prism (v. 8.2; GraphPad Software Inc.; San Diego, CA, USA). The ratios of metabolite to the corresponding precursor, which indicate enzyme activities, were examined based on the quantitative results of individual steroids. Nonparametric Mann–Whitney *U* test was used to evaluate group differences between healthy controls and 21-OHD patients as well as between well-treated 21-OHD patients and patients who were poorly controlled. All quantitative results are expressed as means ± SD, and statistical significance was considered at $P < .01$.

Results

Chromatographic Properties

The chromatographic parameters on column length, mobile phase gradients, and column temperatures were optimized for better separation from isobaric species and endogenous interference against a previous method [5]. In particular, 2 newly added cortisol metabolites of α- and β-cortolone, which are further metabolized from tetrahydrocortisol (THF) catalyzed by 20α- and 20β-reductases, respectively, have the same molecular weight with allo-tetrahydrocortisol (allo-THF) and THF. These 4 steroids were eluted at 6.4 to 6.9 min through a previously used 5 cm long column (2.1 mm i.d. × 1.9 μm particle size) with a coelution of β-cortolone and allo-THF (Fig. 3A), while both were not separated under modified mobile phase gradients (data not shown). The 10 cm long columns with the same dimension were tested under the same gradient elution and chosen to provide excellent chromatographic resolution for these steroids at 8.5 to 9.3 min (Fig. 3B). The established analytical parameters

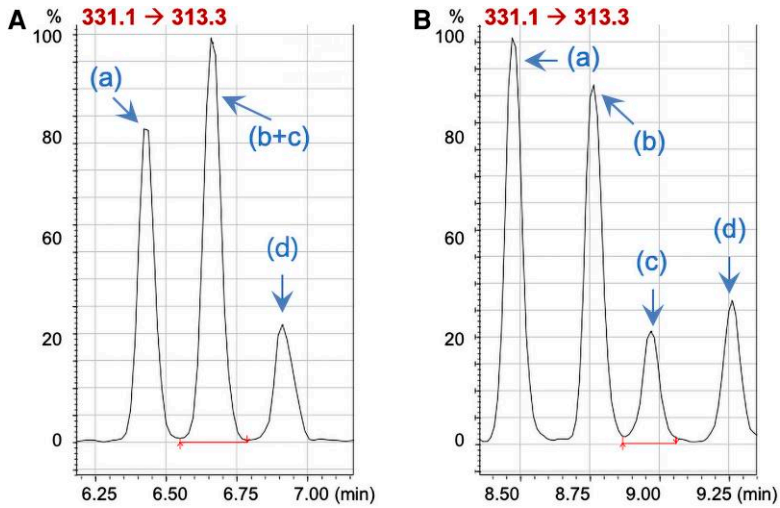

Figure 3. The comparative LC-MS chromatograms to minimize the effects of serum isobaric compounds. Among steroids, which have the same molecular weight (366 Da) and precursor/product ions (*m/z* 331.1 → *m/z* 313.3) in MS/MS analysis, of (A) α-cortolone, (B) β-cortolone, (C) allo-THF, and (D) THF, both β-cortolone and allo-THF were coeluted in (A) a previous method [5], whereas (B) newly optimized chromatographic parameters revealed excellent baseline separation of them all.
Abbreviations: allo-THF, allo-tetrahydrocortisol; LC-MS, liquid chromatography-mass spectrometry; MS/MS, tandem mass spectrometry; THF, tetrahydrocortisol.

provided excellent chromatographic separation and mass spectrometric detection properties for all steroids (Supplementary Table S1 [13]).

Method Validation

The validation process was conducted by evaluating LOQ, linearity, accuracy, and precision using spiked samples prepared from QC samples (Supplementary Table S2 [13]). The LOQ was defined as a signal-to-noise ratio of > 10 (range: 0.1-2.0 ng/mL) except for THS of 5.0 ng/mL from the 200 μL serum sample. The devised methods were linear ($r^2 > 0.910$) over the calibration ranges using a weighted regression model of $1/x^2$. Precisions and accuracies were determined by analyzing QC samples at 4 different concentrations of LOQ (0.1, 0.2, 0.5, 1, 2, and 5 ng/mL), low (0.5, 1, 2, 5, 10, and 20 ng/mL), medium (5, 10, 20, 25, 50, and 100 ng/mL), and high (20, 50, 200, 250, and 500 ng/mL), according to individual analytical sensitivity and calibration range. The intraday (n = 4) precisions (expressed as % CV) ranged from 2.1% to 10.2%, while accuracies (expressed as % bias) ranged from 95.4% to 108.3%, and interday (n = 4) precisions (% CV) and accuracies (% bias) ranged from 2.3% to 11.0% and 94.1% to 110.0%, respectively. The overall QC concentrations corresponded to the calculated amounts with an acceptable CV and bias of 12% for all steroids.

Quantification of Serum Steroids and Clinical Characteristics of the Study Subjects

Among the 33 steroids monitored, 26 serum steroids were quantitatively detected in all subjects (Table 2), while other steroids, including aldosterone, were only detected in certain subjects. Interestingly, 4 metabolites of cortisol, including 20α-dihydrocortisol (20α-DHF), 20α-dihydrocortisone (20α-DHE), α-cortolone, and β-cortolone, which are known as major urinary metabolites of cortisol [15, 16], were also comprehensively detected and compared. The validated LC-MS-based profiling assay was then applied to 67 patients with CAH and 38 healthy controls to characterize the individual CAH subtypes.

The general characteristics of the enrolled subjects are listed in Table 1. The mean age of patients was 30.9 ± 8.1 years, which was lower than that of the healthy controls (36.8 ± 12.5 years, $P = .018$). Body mass index and percentage of male patients were similar between patients with CAH and healthy controls. Most patients (92.5%) took prednisolone for glucocorticoid replacement therapy. The simple virilizing subtype was identified in 27.0% (n = 17) of the 21-OHD patients, whereas the salt-wasting subtype was found in 73.0% (n = 46) of the 21-OHD patients. Among the patients with 21-OHD, 5 patients exhibited well-controlled conditions.

The serum levels of all serum steroids were lower in patients with 21-OHD, which in turn exhibited good control because their 17α-hydroxyprogesterone (17α-OHP4) levels were below 10 ng/mL [17], as opposed to patients with 17α-OHP4 levels of 10 ng/mL or higher. Significantly decreased levels of androgens and their 11β-hydroxylated metabolites were observed in well-controlled patients ($P < .002$ for all; Supplementary Table S3 [13]). Some mineralocorticoids and steroid precursors were also decreased, while cortisol precursors and metabolites, including 21-deoxyF, cortisol (F), cortisone (E), tetrahydrocortisone, 20α-DHE, and β-cortolone, were lower in patients with well-controlled 21-OHD patients compared to those in poorly controlled ones.

Serum Metabolic Signatures of 21-OHD

The significantly increased 17α-OHP4 (66.69 ± 69.77 ng/mL, $P < 1 \times 10^{-16}$) and 21-deoxyF (12.85 ± 15.46 ng/mL, $P < 1 \times 10^{-15}$) levels were observed in a group of 21-OHD among healthy controls and all patients with CAH, while serum cortisol and its metabolites, including E, THF, allo-THF, tetrahydrocortisone, 20α-DHF, 20α-DHE, α-cortolone, and β-cortolone, were remarkably decreased against healthy controls ($P < 1 \times 10^{-15}$ for all; Table 2). Both DHEA and DHEA-S were decreased in patients with 21-OHD ($P < 1 \times 10^{-9}$ for both) against healthy controls. Additionally, higher serum P4 levels were found, and the statistical difference was obtained from only female participants ($P < .0003$) because

Table 2. Comparative serum concentrations of adrenal steroids from all subjects

Compounds		Serum Concentration (ng/mL, mean ± SD)				
		Healthy (n = 38)	21-OHD (n = 63)	17α-OHD (n = 3)	11β-OHD (nn = 1)	*P*-value[a]
Steroid precursors						
Pregnenolone	P5	3.11 ± 1.67	3.52 ± 2.87	5.34 ± 2.61	N.D.	.713
Pregnenolone sulfate	P5-S	50.83 ± 25.32	234.82 ± 305.19	794.88 ± 588.37	420.12	.013
Progesterone	P4[b]	2.77 ± 6.62	4.01 ± 5.21	1.35 ± 1.39	0.40	<.0005
17α-Hydroxyprogesterone	17α-OHP4	0.47 ± 0.36	**66.69 ± 69.77**	0.09 ± 0.04	3.33	$<1\times10^{-16}$
Dehydroepiandrosterone	DHEA	6.18 ± 2.98	2.97 ± 1.02 (N = 21)	N.D.	N.D.	$<1\times10^{-9}$
Dehydroepiandrosterone sulfate	DHEA-S	2733.49 ± 1395.59	646.25 ± 768.74	**0.46 ± 0.17**	650.89	$<1\times10^{-12}$
Mineralocorticoids						
11-Deoxycorticosterone	DOC	0.07 ± 0.01	0.08 ± 0.01	**1.16 ± 1.44**	**1.60**	.892
Corticosterone	B	5.28 ± 3.62	2.16 ± 1.92	**208.89 ± 233.12**	N.D.	.127
18-Hyrdoxycorticosterone	18-OHB	0.97 ± 0.44	1.01 ± 0.44	**6.58 ± 5.39**	N.D.	.763
Glucocorticoids						
11-Deoxycortisol	11-deoxyF	0.41 ± 0.19	0.22 ± 0.13	0.08 ± 0.11	**17.93**	.061
21-Deoxycortisol	21-deoxyF	0.04 ± 0.07	**12.85 ± 15.46**	0.05 ± 0.01	0.06	$<1\times10^{-15}$
Tetrahydrodeoxycortisol	THS	1.98 ± 2.24 (n = 6)	2.01 ± 1.85 (n = 10)	N.D.	**377.88**	.893
Cortisol	F	102.11 ± 36.24	5.03 ± 6.17	5.58 ± 6.25	71.86	$<1\times10^{-17}$
Cortisone	E	21.13 ± 5.88	1.30 ± 1.34	0.34 ± 0.30	22.21	$<1\times10^{-17}$
Tetrahydrocortisol	THF	21.02 ± 7.54	1.78 ± 1.23	2.65 (N = 1)	24.35	$<1\times10^{-15}$
Allo-tetrahydrocortisol	allo-THF	20.33 ± 10.29	2.02 ± 1.82	1.93 ± 1.32	18.87	$<1\times10^{-15}$
Tetrahydrocortisone	THE	27.16 ± 11.79	2.52 ± 2.78	1.76 ± 1.26	38.68	$<1\times10^{-17}$
20α-Dihydrocortisol	20α-DHF	2.99 ± 1.20	0.20 ± 0.18	0.17 ± 0.14	3.52	$<1\times10^{-17}$
20α-Dihydrocortisone	20α-DHE	4.93 ± 2.00	0.30 ± 0.37	0.15 ± 0.12	6.19	$<1\times10^{-17}$
α-Cortolone	—	5.54 ± 2.85	0.81 ± 0.66	0.92 ± 0.60	9.05	$<1\times10^{-16}$
β-Cortolone	—	5.74 ± 2.46	0.68 ± 0.95	0.44 ± 0.30	7.41	$<1\times10^{-16}$
18-Hydroxycortisol	18-OHF	0.81 ± 0.43	0.44 ± 0.39	0.06 ± 0.02	N.D.	.011
Androgens						
Androstenedione	A4	0.62 ± 0.31	2.27 ± 2.40	0.10 ± 0.04	4.98	$<1\times10^{-5}$
Testosterone	T	2.70 ± 2.86	2.39 ± 2.41	1.51 ± 2.48	0.74	.939
11β-Hydroxyandrostenedione	11β-OHA4	2.86 ± 1.61	8.28 ± 11.58	0.01 ± 0.001	0.05	.105
11β-Hydroxytestosterone	11β-OHT	0.34 ± 0.19	1.23 ± 2.29	0.03 ± 0.003	0.03	.259

Entries in bold indicate significant changes.
Abbreviations: 11β-OHD, 11β-hydroxylase deficiency; 17α-OHD, 11-hydroxylase deficiency; 21-OHD, 21-hydroxylase deficiency; N.D., not detected.
[a]Statistical differences between the patients with 21-OHD and the healthy controls.
[b]The serum levels of progesterone were compared in females only.

Table 3. Activities of steroidogenic enzymes calculated by metabolic ratios between groups studied

Enzyme activity	Metabolic ratio (mean ± SD)				
	Healthy (n = 38)	21-OHD (n = 63)	17α-OHD (n = 3)	11β-OHD (n = 1)	*P*-value[a]
21-Hydroxylase					
11-deoxyF/17α-OHP4	1.10 ± 1.08	**0.0026 ± 0.0028**	1.11 ± 1.70	5.39	$<1\times10^{-17}$
F/21-deoxyF	4811.19 ± 2835.28	**1.51 ± 2.89**	125.79 ± 150.28	21.58	$<1\times10^{-18}$
21-Hydroxylase + 11β-Hydroxylase					
(17α-OHP4 + 21-deoxyF)/F	0.01 ± 0.01	**33.96 ± 88.62**	0.21 ± 0.34	0.05	$<1\times10^{-18}$
17α-Hydroxylase[b]					
17α-OHP4/P4	5.13 ± 10.01	18.41 ± 12.79	**0.15 ± 0.12**	8.38	$<1\times10^{-8}$
17,20-Lyase					
A4/17α-OHP4	2.88 ± 7.15	**0.08 ± 0.13**	12.13 ± 8.00	1.50	$<1\times10^{-16}$
17α-Hydroxylase + 17,20-Lyase					
DHEA-S/P5-S	58.88 ± 29.16	7.16 ± 9.13	**0.0009 ± 0.0006**	1.55	$<1\times10^{-15}$
11β-Hydroxylase					
11β-OHA4/A4	5.62 ± 3.78	3.36 ± 2.80	0.18 ± 0.08	**0.01**	<.001
11β-OHT/T	1.29 ± 4.40	0.81 ± 0.76	0.25 ± 0.23	**0.04**	.159
11β-Hydroxysteroid dehydrogenase					
F/E	4.84 ± 1.43	3.33 ± 1.17	13.41 ± 6.22	3.24	$<1\times10^{-5}$
20α-Hydroxysteroid dehydrogenase					
20α-DHF/F	0.03 ± 0.01	0.09 ± 0.13	0.13 ± 0.18	0.05	<.0001
20α-DHE/E	0.24 ± 0.11	0.23 ± 0.10	1.20 ± 1.27	0.28	.481
α-Cortolone/THE	0.21 ± 0.06	1.16 ± 2.26	0.55 ± 0.05	0.23	<.00001
20β-Hydroxysteroid dehydrogenase					
β-Cortolone/THE	0.22 ± 0.05	0.36 ± 0.37	0.30 ± 0.17	0.19	.025

Entries in bold indicate significant changes.
Abbreviations: 11β-OHD, 11β-hydroxylase deficiency; 11-deoxyF, 11-deoxycortisol; 17α-OHD, 11-hydroxylase deficiency; 17α-OHP4, 17α-hydroxyprogesterone; 21-deoxyF, 21-deoxycortisol; 20α-DHE, 20α-dihydrocortisone; 20α-DHF, 20α-dihydrocortisol; 21-OHD, 21-hydroxylase deficiency; DHEA-S, dehydroepiandrosterone sulfate; E, cortisone; F, cortisol; THF, tetrahydrocortisol.
[a]Statistical differences between the patients with 21-OHD and the healthy controls.
[b]The values were compared in females only.

its level in healthy males was not measurable. The group differences between healthy controls and patients of both sexes had similar patterns in statistical analysis (Supplementary Table S4 [13]). The significantly increased serum 17α-OHP4 and 21-deoxyF levels and the decreased cortisol metabolites were observed in both sexes. Therefore, the statistical differences were processed without sexual distinction.

Two metabolic ratios of 11-deoxyF/17α-OHP4 (0.0026 ± 0.0028) and F/21-deoxyF (1.51 ± 2.89), indicating 21-hydroxylase activity, in patients with 21-OHD were significantly decreased compared to those in both controls (1.10 ± 1.08 and 4811.19 ± 2835.28, respectively; $P<1\times10^{-17}$ for both) and other patient groups. Therefore, the receiver operating characteristic curve analysis was conducted with individual concentrations of 17α-OHP4, 21-deoxyF, and the sum of 17α-OHP4 and 21-deoxyF, and metabolic ratios of 11-deoxyF/17α-OHP4, F/21-deoxyF, and (17α-OHP4 + 21-deoxyF)/F. All metabolic ratios resulted in a good diagnostic performance with an area under the curve of 1.0, while individual concentrations were obtained at 0.997 ($P<1\times10^{-16}$) for 17α-OHP4, 0.993 ($P<1\times10^{-15}$) for 21-deoxyF, and 0.997 ($P<1\times10^{-16}$) for 17α-OHP4 + 21-deoxyF against healthy controls. Another metabolic ratio of F to the sum of 17α-OHP4 and 21-deoxyF, which can be responsible for a combination of 21-hydroxylase with 11β-hydroxylase, was significantly increased in 21-OHD (33.96 ± 88.62) against all participants (Table 3). Both metabolic ratios, indicating 17α-hydroxylase and 17,20-lyase, which are associated with CYP17A1 regulation, demonstrated remarkable changes, while other metabolic ratios corresponding for enzymes, such as 11β-hydroxylase, 11β-HSD, 20α-HSD, and 20β-HSD, were slightly differentiated.

Metabolic Characteristics of Other CAH Subtypes

Two subtypes of 17α-OHD (n = 3, all female) and 11β-OHD (n = 1, male) were compared with both groups of the control and the 21-OHD but revealed no statistical differences because of the small number of patients (Tables 2 and 3). In patients with 17α-OHD, the ACTH levels were unexpectedly elevated, while renin activity was found to be suppressed. However, these patients demonstrated a significant increase in serum levels of B (208.89 ± 233.12 ng/mL) and 18-OHB (6.58 ± 5.39 ng/mL), which could be differentiated from healthy controls (5.28 ± 3.62 ng/mL and 0.97 ± 0.44 ng/mL, respectively) and both subtypes of the 21-OHD (2.16 ± 1.92 ng/mL and 1.01 ± 0.44 ng/mL, respectively) and 11β-OHD (undetectable due to the lower LOQ) groups. In particular, the extremely lower (~1000 folds in mean) DHEA-S levels (0.46 ± 0.17 ng/mL) were found in patients with 17α-OHD, while decreased androgens levels were also detected (Table 2). Two metabolic ratios of 17α-OHP4 to P4 and DHEA-S to P5-S were significantly

decreased in the 17α-OHD (0.15 ± 0.12 and 0.0009 ± 0.0006, respectively) compared with all others (Table 3).

A patient with 11β-OHD resulted significantly increased serum 11-deoxyF (17.93 ng/mL) and THS (378.61 ng/mL) levels against the controls (0.41 ± 0.19 ng/mL and 1.98 ± 2.24 ng/mL, respectively) and other patient groups (Table 2). In addition, a precursor of B, deoxycorticosterone (DOC) (1.60 ng/mL), was higher than both the control (0.07 ± 0.01 ng/mL) and 21-OHD (0.08 ± 0.01 ng/mL) groups. Serum B and 18-OHB levels, as sequential metabolites of DOC catalyzed by 11β-hydroxylase, were not detectable in a patient with 11β-OHD. The decreased 11β-OHA4 and 11β-OHT levels were demonstrated compared with controls and patients with 21-OHD but not with patients with 17α-OHD. However, metabolic ratios of 11β-OHA4/A4 (0.01) and 11β-OHT/T (0.04), indicating 11β-hydroxylase, were remarkably lower in a patient with 11β-OHD than all other participants (Table 3).

Discussion

Recently, a combination of machine-learning algorithms with mass spectrometry-based steroid profiling methods have been successfully introduced for differentiating adrenal diseases, including CAH, Cushing's syndrome, and primary aldosteronism [12, 18, 19]. These platforms could be cutting-edge diagnostic tools when the data-learning algorithms are based on reliable data sets of metabolic signatures. Method development and validation in steroid profiling were therefore accomplished, enabling us to evaluate more adrenal steroids compared to previous assays [5, 12, 18, 19]. Due to much matrix interference derived from endogenous steroids in the blood, improved chromatographic separation was necessary. Especially the quantitative results of serum cortisol can be compromised with its precursors and metabolites in immunoassays [9, 10], while 20α-DHE and 20β-DHE may also interfere in LC-MS analysis [20]. However, all precursors and metabolites of cortisol were selectively isolated in our LC-MS instrumentation, and their serum levels were quantitatively measured.

Significantly increased 17α-OHP4 and 21-deoxyF levels were found in 21-OHD compared to healthy controls and other patients in addition to decreased serum progesterone and DHEA levels against only healthy controls. The elevated 17α-OHP4 level is monitored in the conventional diagnostic test of 21-OHD [1-3]; however, the increased 17α-OHP4 levels were observed in this study because 17α-OHP4 is metabolized to 21-deoxyF by 11β-hydroxylase [6]. Initial screening with 17α-OHP4 immunoassays may have false-positive results in the second test by either immunoassay or LC-MS in clinical practice [21]. Serum 21-deoxyF levels were elevated only in patients with 21-OHD, not healthy participants or participants with other CAH subtypes, and it has been suggested as a better biomarker for nonclassical 21-OHD with an ACTH stimulation test [21, 22]. The increased serum level of 21-deoxyB was also found in adults with mildly defective 21-OHD [23] and in newborn screening [24], but it was not detectable in most subjects of this study. The main goals of steroid therapy for patients with 21-OHD are to suppress excess adrenal production of androgens, while the therapeutic outcomes can be monitored through serum levels of T, DHEA-S, 21-deoxyF, and 11-oxygenated androgens [25, 26]. The well-controlled 21-OHD patients had the most significantly lower serum levels among all steroids monitored, including recommended steroids and other potent biomarkers, than those of the suboptimally controlled counterparts.

CYP17A1 is associated with the qualitative regulation of steroidogenesis, and it determines which classes of steroids could be processed in contrast to mitochondrial CYP11A1 in the StAR/P450scc system. It encodes both 17α-hydroxylase and 17,20-lyase, and its deficiency can be caused either partially or completely according to the mutation, which results in decreased glucocorticoid and adrenal androgen production [27]. Patients with 17α-OHD showed lower serum glucocorticoid levels than healthy controls and a patient with 11β-OHD, while metabolic signatures of decreased cortisol metabolites were similar to those in the 21-OHD group. Significantly decreased androgen levels were also found in patients with 17α-OHD compared to both the control and 21-OHD groups, which are similar to the levels from a patient with 11β-OHD. However, significantly decreased DHEA-S levels could be characterized in all subjects. The metabolic ratios of DHEA-S/P5-S and 17α-OHP4/P4 were remarkably reduced, which is accordance with our previous report [5]. As expected, mineralocorticoids of B and 18-OHB were significantly elevated in patients with 17α-OHD, while serum DOC levels were significantly elevated compared to both the control and 21-OHD groups.

A patient with 11β-OHD demonstrated increased serum 11-deoxyF levels as the substance of 11β-hydroxylase and decreased 11β-hydroxylase products, 11β-OHA4 and 11β-OHT. Both 11β-hydroxylase metabolites could be also decreased in 17α-OHD, whereas metabolic ratios of 11β-OHA4/A4 and 11β-OHT/T, indicating 11β-hydroxylase activity, were significantly differentiated a patient with 11β-OHD from patients with 17α-OHD. Additionally, decreased mineralocorticoids of B and 18-OHB were observed in a patient with 11β-OHD, but they could be reduced in 21-OHD. In particular, remarkably elevated THS, as a metabolite of 11-deoxyF, clearly characterize 11β-OHD, which is following the increased urinary excretion in patients with 11β-OHD [28] and pregnancies with fetuses affected by 11β-OHD [29]. The elevated 11-deoxyF level could be used for discriminating 11β-OHD from 21-OHD [6].

The present work was designed to provide metabolic signatures associated with individual CAH subtypes; however, this retrospective study has several limitations. First, the small number of patients with 17α-OHD and 11β-OHD failed to provide statistical significances, resulting in the lack of data-driven computational approaches. Second, the age difference in both sexes might affect the statistical significance due to different biosynthetic rates along with age and sex in functional limitations, especially for patients with 17α-OHD and 11β-OHD. Third, this cross-sectional population contained patients pretreated with steroid replacement, which may change serum steroid levels associated with enzymatic imbalance. Long-term steroid replacement therapy could affect the metabolic signatures of adrenal steroids, although the blood sampling was done before taking medications.

Despite the previously described limitations, the present study suggests feasible biomarkers for characterizing individual subtypes of CAH and monitoring the treatment outcomes in patients with 21-OHD. Comparing to a recent study on CAH subtyping that employed a multiclassifier system based on 13 steroids [12], the current study overviewed 26 adrenal steroids and focused more on cortisol metabolism because all CAH subtypes represent hypocortisolism. In addition to monitoring classical biomarkers for 3 different CAH subtypes [12], the remarkably increased biomarkers of 21-deoxyF, DHEA-S, and THS in patients with 21-OHD, 17α-OHD, and 11β-OHD, respectively, were investigated to improve the diagnostic feasibility in the present study. Moreover, metabolic changes in cortisol biosynthesis may

support characterizing different subtypes, and reduced levels of α-cortolone and β-cortolone were observed only in patients with both 21-OHD and 17α-OHD and not 11β-OHD, while the metabolic ratios corresponding to 20alpha-hydroxysteroid dehydrogenase were significantly decreased in healthy controls.

In summary, metabolic changes of adrenal steroids characterized the CAH subtypes of 21-OHD, 17α-OHD, and 11β-OHD in a single analytical run with an overview of the steroidogenesis using LC-MS-based steroid profiling. Further studies on establishing therapeutic monitoring processes based on steroid profiling will be conducted with different steroids, especially replacement therapies with the salt-wasting variety treatment with glucocorticoids and mineralocorticoids.

Acknowledgments

The authors thank the volunteers who participated in this study.

Funding

This study was supported by the Institutional Program of Korea Institute of Science and Technology (2E31093) and the Korea Health Industry Development Institute R&D Project (HI21C0032) through the Ministry of Health and Welfare of the Republic of Korea.

Disclosures

The authors declare that there are no conflicts of interest.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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