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High-Resolution, Accurate-Mass (HRAM) Mass Spectrometry Urine Steroid Profiling in the Diagnosis of Adrenal Disorders

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BACKGROUND: Steroid profiling is a promising diagnostic tool with adrenal tumors, Cushing syndrome (CS), and disorders of steroidogenesis. Our objective was to develop a multiple-steroid assay using liquid-chromatography, high-resolution, accurate-mass mass spectrometry (HRAM LC-MS) and to validate the assay in patients with various adrenal disorders.

METHODS: We collected 24-h urine samples from 114 controls and 71 patients with adrenal diseases. An HRAM LC-MS method was validated for quantitative analysis of 26 steroid metabolites in hydrolyzed urine samples. Differences in steroid excretion between patients were analyzed based on *Z*-score deviation from control reference intervals.

RESULTS: Limits of quantification were 20 ng/mL. Dilution linearity ranged from 80% to 120% with means of 93% to 110% for all but 2 analytes. Intraassay and interassay imprecision ranged from 3% to 18% for all but 1 analyte. Control women had lower excretion of androgen and glucocorticoid precursors/metabolites than men ($P < 0.001$), but no difference in mineralocorticoids was seen ($P = 0.06$). Androgens decreased with age in both sexes ($P < 0.001$). Compared with patients with adrenocortical adenoma (ACA), patients with adrenocortical carcinoma (ACC) had 11 steroids with increased *Z* scores, especially tetrahydro-11-deoxycortisol (14 vs 0.5, $P < 0.001$), pregnanetriol (7.5 vs −0.4, $P = 0.001$), and 5-pregnenetriol (5.4 vs −0.4, $P = 0.01$). Steroid profiling also demonstrated metabolite abnormalities consistent with enzymatic defects in congenital adrenal hyperplasia and differences in pituitary vs adrenal CS.

CONCLUSIONS: Our HRAM LC-MS assay successfully quantifies 26 steroids in urine. The statistically significant differences in steroid production of ACC vs ACA, adrenal vs pituitary CS, and in congenital adrenal hyperplasia should allow for improved diagnosis of patients with these diseases.

Adrenal steroid analysis plays an important role in the diagnosis of Cushing syndrome (CS)[5], disorders of steroidogenesis, and adrenal tumors *(1–4)*. Over time, steroid assays have improved in analytical sensitivity and specificity, with the current reference standard being LC-MS/MS *(5–8)*. However, it has been suggested that the clinical diagnostic performance of modern assays might have paradoxically worsened compared with older, less-specific immunoassays, in particular for CS *(9, 10)*, suggesting that it might be beneficial to measure multiple steroids and their metabolites simultaneously to achieve optimal diagnostic accuracy *(10)*. In addition, during the past 5 to 6 years, a GC-MS urinary 32-analyte steroid profile has shown promising results as a diagnostic tool for distinguishing adrenocortical carcinoma (ACC) from a benign adrenocortical adenoma (ACA) *(11–13)*, further emphasizing the potential importance of measuring multiple steroids simultaneously.

Based on these observations, steroid metabolomics might be poised to make a substantial impact on endocrine laboratory testing. Unfortunately, steroid profiles are difficult to implement in the clinical laboratory. Among the plethora of naturally occurring steroids and

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[5] Nonstandard abbreviations: CS, Cushing syndrome; HRAM, high-resolution, accurate-mass; ACC, adrenocortical carcinoma; ACA, adrenocortical adenoma; 11-OXO-ET, 11-oxoetiocholanolone; 11B-OH-AN, 11β-hydroxyandrosterone; 11B-OH-ET, 11β-hydroxyetiocholanolone; 16a-DHEA, 16α-hydroxydehydroepiandrosterone; 17HP, 17α-hydroxypregnanolone; 17OHPG, 17-hydroxyprogesterone; 5PT, 5-pregnenetriol; 5αTHA, 5α-tetra-11-dehydrocorticosterone; 5a-THF, 5α-tetrahydrocortisol; DHEA, dehydroepiandrosterone; Etio, etiocholanolone; PD, pregnanediol; PT, pregnanetriol; PTONE, pregnanetriolone; 5PD, 5-pregnenediol; THS, tetrahydrodeoxycortisol; THB, tetrahydrocorticosterone; THF, tetrahydrocortisol; THE, tetrahydrocortisone; THDOC, tetrahydrodeoxycorticosterone; An, androsterone; 6B-OH-cortisol, 6β-hydroxycortisol; IS, internal standard; MP, mobile phase; QC, quality control.

their metabolites, there are many compounds that are near isobaric, or are isomers. Many of these compounds cannot be distinguished from each other using the relative low-resolution mass-filtering mass spectrometers used in clinical laboratories, even when MS/MS is used, thus necessitating complete chromatographic separation before mass spectrometry detection. This often requires gas chromatography, a methodology technically far more demanding, labor-intensive, and time-consuming than most LC-MS/MS methods. Consequently, these promising assays are not yet extensively used, and have been scarcely evaluated for their clinical utility outside of the differential diagnosis of ACC vs ACA.

Liquid-chromatography, high-resolution, accurate-mass mass spectrometry (HRAM LC-MS) might be a tool that can overcome the hurdles for wider use of steroid profiles. This methodology is rapidly gaining in popularity for quantitative clinical analysis of small endogenous molecules, anabolic drug testing, and proteins *(14–25)*. HRAM can resolve all steroids and their metabolites except isomers, allowing the use of liquid chromatography, including multiplexed liquid chromatography setups, as a front-end instead of gas chromatography.

Therefore, we decided to use HRAM LC-MS to develop a novel 26-analyte, urine-based steroid panel, to determine sex- and age-based control reference intervals, and to perform a limited clinical evaluation of the assay in a cohort of patients with different adrenal diseases.

Materials and Methods

SUBJECTS

This study was approved by the Mayo Clinic Institutional Review Board.

We obtained 24-h urine samples from 114 volunteers (66 women, median age 47 years, range 25–83 years; 48 men, median age 42 years, range 24–83 years) to establish reference intervals. Exclusion criteria were the presence of any adrenal gland disorder, benign or malignant neoplasm of other endocrine glands and related structures, and secondary malignant neoplasm.

For clinical validation, we collected 24-h urine samples from 71 patients with adrenal diseases: 4 patients had adrenocorticotropic hormone-dependent pituitary hypercortisolism; 1 woman had newly diagnosed congenital adrenal hyperplasia; 5 patients were diagnosed with ACC and 61 with ACAs (4 with cortisol-producing ACAs and 57 with nonfunctioning ACAs). The final diagnosis in all cases was based on clinical, imaging, and pathology results.

We performed all steroid measurements blinded to the clinical information.

MATERIALS AND METHODS

11-Oxoetiocholanolone (11-OXO-ET), 11β-hydroxyandrosterone (11B-OH-AN), 11β-hydroxyetiocholanolone (11B-OH-ET), 16α-hydroxydehydroepiandrosterone (16a-DHEA), 17α-hydroxypregnanolone (17HP), 5-pregnenetriol (5PT), 5α-tetra-11-dehydrocorticosterone (5aTHA), 5α-tetrahydrocortisol (5a-THF), α-cortolone, β-cortol, β-cortolone, cortisol, cortisone, dehydroepiandrosterone (DHEA), etiocholanolone (Etio), pregnanediol (PD), pregnanetriol (PT), pregnanetriolone (PTONE), 5-pregnenediol (5PD), tetrahydrodeoxycortisol (THS), tetrahydrocorticosterone (THB), tetrahydrocortisol (THF), tetrahydrocortisone (THE), and tetrahydrodeoxycorticosterone (THDOC) were purchased from Steraloids. Androsterone (An), 6β-hydroxycortisol (6B-OH-cortisol), and dehydroepiandrosterone-2,2,3,4,4,6-d_6 (DHEA-d_6) were purchased from Sigma-Aldrich.

Pregnanetriol-d_5 (PT-d_5), tetrahydrocortisol-d5 (THF-d_5), tetrahydrocortisone-d_5 (THE-d_5), cortisone-$^{13}C_3$, cortisol-$^{13}C_3$, tetrahydrocorticosterone-d_5 (THB-d_5), and 11-deoxycortisol-$^{13}C_3$ were purchased from IsoSciences, while 5α-tetra-11-dehydrocorticosterone-d3 (5αTHA-d_3) was purchased from Medical Isotopes and etiocholanolone-d_5 (Etio-d_5) and tetrahydrodeoxycorticosterone-d_3 (THDOC-d_3) were purchased from C/D/N Isotopes.

Glusulase (glucuronidase and sulfatase activity) was from PerkinElmer. HPLC-grade methanol, acetonitrile, and ethyl acetate were purchased from Fisher Scientific.

We prepared calibration stocks from each of the purchased steroids in methanol (1 g/L) and combined aliquots from all stocks to create an intermediate concentration calibrator, containing 10 μg/mL of each steroid in methanol. Finally, we serially diluted the intermediate calibrator to generate working calibrators containing 5000, 2500, 1250, 625, 312.5, 156.25, 78.13, 39.06, and 19.53 ng/mL, respectively, of each steroid. This large number of calibrators was chosen to ensure linear detection of the large variation in concentrations of our different analytes.

Nonradioactive isotopic internal standard (IS) stock solutions were made to either 1 g/L or 5 g/L concentrations in 50% methanol. We combined aliquots of the stocks to create working ISs containing 400 ng/mL of each IS.

We created 3 distinct batches of quality control (QC) material to cover low, intermediate, and high analyte concentrations for each analyte by spiking calibration material for each analyte into charcoal-stripped urine, tailoring the concentrations of individual analytes to their expected concentration ranges; e.g., a low control might contain concentrations of approximately 20 ng/mL for 1 analyte and much higher concentrations for

another. Across all analytes and controls, a range of 20–3000 ng/mL was covered.

SAMPLE STORAGE AND PREPARATION

We aliquoted and froze (−70 °C or colder) all samples immediately after recording the total urine volume of each 24-h collection. Steroids and their metabolites exist in urine mainly as sulfate or glucuronide conjugates. Hydrolysis converts these conjugates back to the unconjugated metabolites, simplifying mass spectrometry analysis.

For hydrolysis and extraction, we combined 150 μL of thawed urine or QC material with 50 μL of working IS. Ideally, there should be an IS for each analyte. However, several ISs showed poor reproducibility although analyte response was linear. Therefore, we settled on a final method using 4 ISs: THB-d_4 (11.1 min), DHEA-d_6 (14.2 min), PT-d_5 (15.1 min), and Etio-d_5 (16.3 min) (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol63/issue12). We verified with dilution studies that these 4 ISs ensured linear responses for all analytes.

The samples were vortex-mixed and sat at ambient temperature for 10 min. We then added 50 μL of sodium acetate buffer (3 mol/L, pH 5.2) and 10 μL of Glusulase to the tubes, covered the tubes, and incubated them for 2 h at 50 °C (dry-heat block). We then removed the samples from the heat block and stopped each reaction with 50 μL of potassium carbonate.

Liquid-liquid extraction was then performed with 3 mL of ethyl acetate per sample, followed by 5 min of vortex-mixing on a multitube vortexer (speed approximately 1500 rpm) and 15 min of centrifugation at approximately 1500*g*. We then pipetted 2 mL of the organic layer of each sample into a clean glass tube and dried it at 40 °C under nitrogen for 30 min. These extracts were resuspended in 200 μL of 50% acetonitrile in water, vortex-mixed for 60 s, and transferred to a 96 deep-well plate (Chrom Tech) for analysis.

HRAM LC-MS METHOD

Steroid metabolites were measured using a Thermo UniCell Dionex UPLC system coupled to Thermo Q Exactive Plus HRAM hybrid quadrupole/orbital trap mass spectrometer with a heated electrospray ionization source (Thermo Scientific). Data were collected in full-scan mode with 70000 resolution (relative to 200 *m/z*). The temperature and gas settings are provided in Table 2 of the online Data Supplement.

We used reversed-phase chromatography with Zorbax Extend-C18, Rapid Resolution HT, 2.1 × 50 mm, 1.8-μm analytical columns (Agilent Technologies). Mobile phase (MP) A consisted of 10% acetonitrile with 0.1% formic acid. MP B consisted of 90% acetonitrile with 0.1% formic acid. The injection volume was 5 μL at a flow rate of 300 μL/min, using MP A for 1 min, followed by a 35-min gradient to 100% MP B and column reconditioning with MP A for 4 min. Retention times of each analyte were established and confirmed by single analyte injections (Fig. 1). An on-board diversion valve was used in line with the mass spectrometer between 2 and 4 min and then again for 7–18 min of the liquid chromatography method, keeping the instrument cleaner by diverting more polar compounds at the start of the chromatography and more nonpolar compounds as the gradient increased and the column was subsequently washed.

Mass spectra were processed with Xcalibur Qual Browser (Thermo) and quantified with TraceFinder Clinical 3.3 (Thermo). The theoretical mass of each analyte was calculated during method development using the chemical formula spectral simulation function in Xcalibur's Qual Browser and then confirmed by an injection of the respective calibration standard (Table 1). The in-use *m/z* for each analyte (Table 1) was found to be protonated, dehydrated, or double-dehydrated (the second-most intense ion was recorded for confirmation purposes described below). Quantification was performed against a calibration curve using analyte-to-IS peak area ratios with linear regression analysis and 1/× weighting. The mass tolerance was set to ±5 ppm, and the retention time window constraint was 10 s (5PD was 30 s to afford less user manipulation because peaks tended to be low). Confirmation-ion target ratios (ratio between the quantification ion and a secondary ion most often defined as the second-most intense experimental ion) varied between analytes (and were set according to observed intensities). Because some analytes produced little or no confirmation ion (e.g., cortisol, which produced predominantly monoisotopic ions), we also used isotopic pattern scores, which were flagged when they were <90; this score indicates the goodness of fit of the isotopic distribution of the observed data to the theoretically expected pattern, with scores ranging from 0 (no match) to 100 (complete match). Isotopic pattern scoring showed poor utility at concentrations below the lower limit of quantification.

ANALYTICAL VALIDATION

Our criteria for calibration acceptance of individual calibration curves was an R^2 >0.995. Calibration curves ranged from 19.53 to 5000 ng/mL (52–68 nmol/L to 13211–17349 nmol/L) using linear regression analysis with 1/× weighting.

For each analyte, the limit of quantification was arbitrarily defined as the concentration equivalent to that of the lowest calibrator [rounded to 20 ng/mL (53–69 nmol/L)]. The signal-to-noise ratio was >3 for all analytes at this concentration.

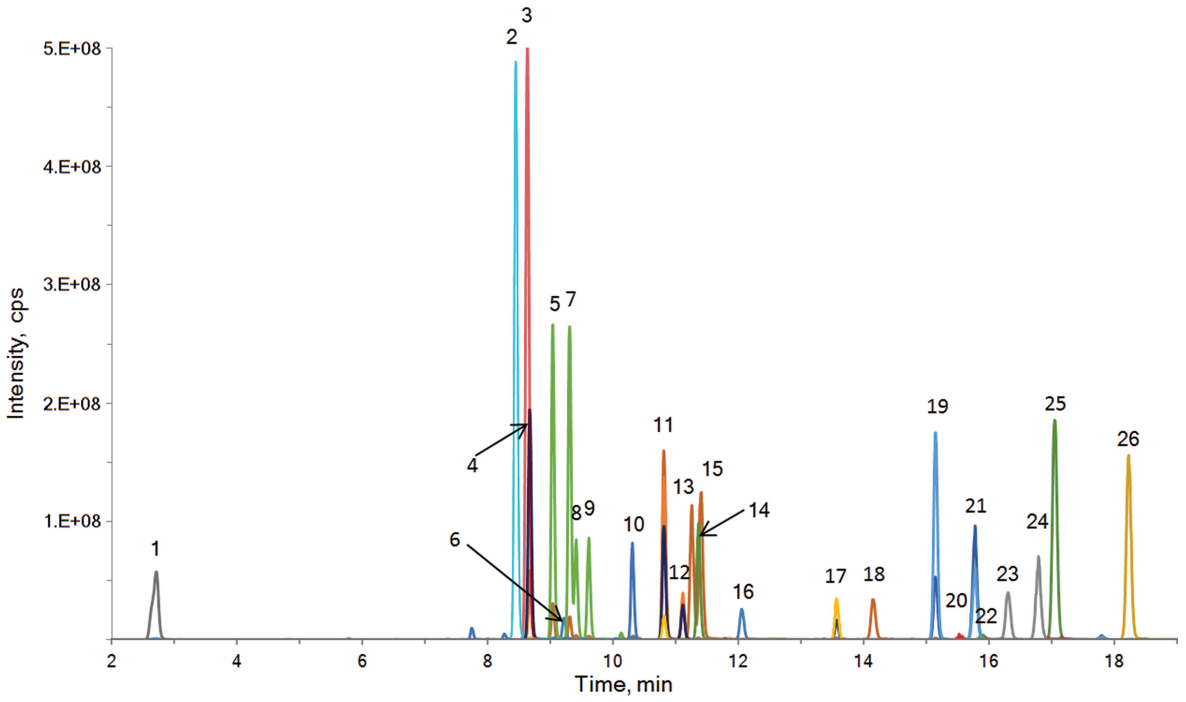

Fig. 1. Representative chromatogram indicating retention time and color-coded *m/z* used for analysis.

Peaks are numbered as listed in Table 1. The 26 compounds had 16 unique *m/z* among them. Isomeric compounds required chromatographic separation. The region without peaks between peak #1 (6B-OH-Cortisol) and peak #2 (cortisol) reflects use of the flow-divert valve mentioned in the main text, which was used to improve instrument robustness during gradient elution.

Linearity was determined by diluting urine samples with stripped urine at 2×, 4×, and 8× dilutions. Expected results were defined as the neat (undiluted) result divided by the dilution factor. Recovery was determined by spiking 250, 500, and 1000 ng/mL (661–867, 1321–1735, and 2642–3470 nmol/L) analytes into 16 different patient urine matrices. Expected values were determined by summing the spike with the neat value. Intraassay imprecision was determined by analyzing 20 QC samples within 1 assay, and interassay imprecision was calculated from 20 replicates assayed over a series of 20 batches. Any concentration with a mean that fell below the lower limit of quantification for a particular analyte was removed from the analysis, including the lower controls for 5PD, 5PT, DHEA, and PD. The imprecision was expressed as percentage CV.

CLINICAL VALIDATION

Any sample results that fell below the lower limit of quantification of the assay (19.53 ng/mL) were reported as <20 ng/mL (<53–69 nmol/L). Sample results that exceeded the upper limit of the calibration curve (5000 ng/mL, 13 211–17 349 nmol/L) were diluted in stripped urine before hydrolysis and extraction until they fell into the calibration range. Subsequent results were multiplied by the total volume recorded at the end of 24-h urine collection and divided by 1000 to give final results in micrograms per 24 h.

STATISTICS

Raw steroid results (ng/mL) were normalized to individual patients' total urine volume for final units of micrograms per 24 h. To account for differences in sex and age, individual steroid results for each patient were transformed into standard scores (Z scores), defined as: $Z = \frac{x - \mu}{\sigma}$, where x was the measured steroid value, and μ and σ were the mean steroid value and its SD, respectively, in controls of the same sex and age groups. Data were analyzed using JMP software, version 10 (SAS). Depending on the data distributions, descriptive statistics were used to determine mean and SD, or median and interquartile ranges (IQR 25%, 75%), respectively, and intergroup steroid differences were analyzed by Student t-tests or Wilcoxon/Kruskal–Wallis tests, respectively. P values <0.05 were considered significant.

Table 1. Analytes listed in order of retention time with peak number corresponding to Figure 1.

Peak number	Retention time, min	Analyte	Full name	Molecular formula	Ion formation	*m/z* used for quantification
1	2.70	6B-OH-Cortisol	6β-Hydroxycortisol	C21H30O6	$[M+H]^+$	379.212
2	8.44	Cortisol	Cortisol	C21H30O5	$[M+H]^+$	363.217
3	8.63	Cortisone	Cortisone	C21H28O5	$[M+H]^+$	361.201
4	8.67	B-Cortol	β-Cortol	C21H36O5	$[M+H-2H2O]^+$	333.242
5	9.02	a-Cortolone	α-Cortolone	C21H34O5	$[M+H-2H2O]^+$	331.227
6	9.21	16a-DHEA	16α-Hydroxydehydroepiandrosterone	C19H28O3	$[M+H-H2O]^+$	287.201
7	9.30	B-Cortolone	β-Cortolone	C21H34O5	$[M+H-2H2O]^+$	331.227
8	9.41	5a-THF	5α-Tetrahydrocortisol	C21H34O5	$[M+H-2H2O]^+$	331.227
9	9.60	THF	Tetrahydrocortisol	C21H34O5	$[M+H-2H2O]^+$	331.227
10	10.30	THE	Tetrahydrocortisone	C21H32O5	$[M+H]^+$	365.232
11	10.81	PTONE	Pregnanetriolone	C21H34O4	$[M+H-2H2O]^+$	315.232
12	11.12	THB	Tetrahydrocorticosterone	C21H34O4	$[M+H-2H2O]^+$	315.232
13	11.24	11B-OH-ET	11β-Hydroxyetiocholanolone	C19H30O3	$[M+H-2H2O]^+$	271.206
14	11.36	5PT	Pregnenetriol	C21H34O3	$[M+H-2H2O]^+$	299.237
15	11.40	11B-OH-AN	11β-Hydroxyandrosterone	C19H30O3	$[M+H-2H2O]^+$	271.206
16	12.04	11-OXO-ET	11-Oxoetiocholanolone	C19H28O3	$[M+H-H2O]^+$	287.201
17	13.56	THS	Tetrahydrodeoxycortisol	C21H34O4	$[M+H]^+$	351.253
18	14.16	DHEA	Dehydroepiandrosterone	C19H28O2	$[M+H-H2O]^+$	271.206
19	15.14	PT	Pregnanetriol	C21H36O3	$[M+H-2H2O]^+$	301.252
20	15.54	THDOC	Tetrahydrodeoxycorticosterone	C21H34O3	$[M+H-H2O]^+$	317.248
21	15.76	5PD	Pregnenediol	C21H34O2	$[M+H-2H2O]^+$	283.242
22	15.90	5a-THA	5α-Tetra-11-dehydrocorticosterone	C21H34O3	$[M+H-H2O]^+$	317.248
23	16.30	Etio	Etiocholanolone	C19H30O2	$[M+H-H2O]^+$	273.221
24	16.79	An	Androsterone	C19H30O2	$[M+H-H2O]^+$	273.221
25	17.03	17HP	17α-Hydroxypregnanolone	C21H34O3	$[M+H-2H2O]^+$	299.237
26	18.23	PD	Pregnanediol	C21H36O2	$[M+H-2H2O]^+$	285.258

Isomeric compounds are separated chromatographically.

Results

ANALYTICAL VALIDATION

All calibration curves met our acceptance criteria.

The %CV intraassay imprecision ranged from 3% to 10%, and interassay imprecision ranged from 6% to 18% for all analytes with the exception of 16a-DHEA, which had interassay CVs of 16% to 25% (Table 2).

Because not all steroid conjugates were available for purchase and synthesis was prohibitively costly, we opted to use unconjugated steroids as QC material. However, we did scan for glucuronidated and sulfated analytes in a selection of samples and found no residual conjugated analytes.

Dilution linearity met our acceptance criteria with results 80% to 120% of the predicted concentration for individual points, with means of 93% to 110% for all analytes except 5PD and 5PT, which met criteria for 2× dilutions but suffered from poor linearity with overall ranges of 63% to 93% (mean 79%) and 41% to 91% (mean 71%), respectively (Table 2).

Recovery experiments showed acceptable recoveries for all analytes except DHEA, 5PT, and 5PD (Table 2), which showed substantial loss during extraction. Loss of DHEA, 5PT, and 5PD was less pronounced when endogenous concentrations of DHEA, 5PT, and 5PD were high (see Table 6 in the online Data Supplement).

CLINICAL VALIDATION

The reference intervals showed differences between men and women, and among women based on pre/postmenopausal status. The most significant differences between sexes were seen in androgens (Table 3). Additionally, glucocorticoids and glucocorticoid precursors were generally higher in men. Analysis based on age was also performed, and subsequent *Z* scores were calculated based on sex, menopausal status, and age.

Table 2. Imprecision, dilution linearity and spiked recovery.

Analyte	Intraassay mean range, ng/mL (nmol/L)	Intraassay % CV range	Interassay mean range, ng/mL (nmol/L)	Interassay % CV range	Linearity range, ng/mL (nmol/L)	Linearity % of predicted value	Linearity mean	Recovery % of predicted value
An	340–1820 (1172–6271)	5–6%	348–1706 (1199–5878)	9–12%	30–2254 (103–7766)	85–110%	97%	81–111%
Etio	347–2962 (1196–10 206)	6–7%	368–2703 (1268–9313)	8–10%	82–6697 (283–23,075)	84–110%	99%	82–110%
DHEA	891 (3092)	6%	212–821 (436–2849)	9–17%	26–624 (90–2165)	100–120%	110%	19–91%
16a-DHEA	54–742 (178–2439)	7–10%	44–636 (145–2091)	16–25%	47–893 (155–2936)	93–118%	110%	80–119%
5PD	2307 (7249)	6%	2188 (6875)	16%	25–159 (79–500)	63–93%	79%	1–46%
5PT	1759 (5259)	6%	1610 (4813)	7%	32–574 (96–1716)	41–91%	71%	9–110%
5a-THA	39–72 (117–215)	8–9%	36–86 (108–257)	12–16%	36–128 (108–383)	113–119%	115%	80–120%
THB	27–74 (77–211)	5–10%	40–85 (114–243)	12–15%	29–368 (83–1050)	85–113%	102%	80–108%
THDOC	32–67 (96–200)	9%	35–67 (105–200)	12–15%	20–146 (60–436)	88–119%	108%	87–119%
PD	665 (2076)	5%	129–602 (403–1880)	11–15%	118–3929 (368–12,268)	88–114%	104%	80–107%
17HP	49–348 (147–1041)	8–10%	52–336 (156–1005)	8–13%	26–308 (78–921)	105–120%	109%	80–106%
PT	106–1710 (315–5082)	6–8%	109–1513 (324–4496)	6–10%	49–1809 (146–5376)	84–107%	95%	82–105%
PTONE	47–90 (134–257)	5–7%	40–84 (114–240)	7–12%	24–347 (68–990)	99–115%	108%	81–103%
THS	93–1694 (265–4833)	3–8%	29–1566 (83–4468)	11–15%	31–358 (88–1021)	84–107%	95%	82–118%
Cortisol	165–1972 (455–5441)	3–6%	150–1626 (414–4486)	10–11%	30–327 (83–902)	84–115%	104%	82–120%
6B-OH-cortisol	152–703 (402–1858)	6%	126–531 (333–1403)	16–17%	31–1021 (82–2698)	83–111%	96%	82–111%
Cortisone	175–1996 (486–5538)	3–6%	144–1502 (400–4167)	13–14%	24–499 (67–1384)	90–103%	95%	80–118%
THF	418–2030 (1141–5539)	3–7%	352–1580 (960–4311)	12–14%	112–3613 (306–9858)	81–110%	93%	86–109%
5a-THF	176–1905 (480–5198)	5–7%	149–1325 (407–3615)	9–14%	55–2249 (150–6137)	80–110%	94%	81–110%
THE	398–3036 (1092–8330)	5–8%	300–2141 (823–5874)	12–17%	295–9194 (809–25,226)	84–107%	93%	80–115%
B-Cortol	45–1077 (122–2923)	5–10%	37–804 (100–2182)	8–12%	20–608 (54–1650)	80–113%	102%	81–110%
a-Cortolone	494–2358 (1348–6434)	6–9%	406–1822 (1108–4971)	8–10%	109–1748 (297–4770)	99–118%	109%	81–112%
B-Cortolone	183–2447 (499–6677)	6–9%	160–1847 (437–5040)	6–9%	109–1748 (297–4770)	99–118%	109%	84–110%
11B-OH-AN	148–1902 (483–6211)	5–6%	167–1741 (545–5685)	8–9%	73–2385 (258–7789)	94–119%	108%	83–113%
11B-OH-ET	77–914 (251–2985)	5–8%	91–841 (297–2746)	8–14%	104–3521 (340–11,498)	88–117%	104%	86–115%
11-OXO-ET	68–1016 (244–3340)	7–10%	98–950 (322–3123)	10–18%	61–703 (201–2311)	85–112%	103%	88–117%

Linearity ranges include undiluted and subsequent diluted values. Recovery was performed on 16 unique urine samples spiked with 200, 500, and 1000 ng/mL of all analytes as a mixture.

Table 3. Control reference interval study.

	Analyte	Women premenopausal median, µg/24 h (IQR)	Women postmenopausal median, µg/24 h (IQR)	*P* value[a]	Men median, µg/24 h (IQR)	*P* value[b]
Androgens and precursors	An	370.3 (248.0, 805.0)	1288.7 (725.5, 1796.0)	<0.001	2150.7 (1650.3, 3417.8)	<0.001
	Etio	764.0 (528.7, 1403.0)	1737.3 (1118.8, 2242.6)	<0.001	2429.1 (1582.6, 3266.6)	<0.001
	DHEA	29.8 (21.4, 59.3)	79.1 (51.8, 115.5)	<0.001	149.4 (103.3, 235.8)	<0.001
	16a-DHEA	216.7 (101.7, 485.7)	552.0 (342.6, 778.4)	<0.001	562.1 (420.0, 918.1)	0.001
	5PD	23.4 (12.0, 36.8)	80.9 (37.5, 144.0)	<0.001	105.3 (72.7, 174.8)	<0.001
	5PT	26.1 (13.9, 67.9)	60.7 (32.5, 99.4)	0.01	84.4 (43.8, 163.5)	0.002
Mineralocorticoids and precursors	5a-THA	7.5 (3.8, 27.2)	22.4 (8.8, 49.7)	0.15	18.0 (5.7, 30.0)	0.27
	THB	101.0 (64.6, 185.1)	115.2 (76.1, 155.2)	0.79	128.5 (99.0, 196.4)	0.06
	THDOC	13.3 (7.6, 23.5)	25.0 (12.8, 48.6)	0.0071	17.2 (9.8, 36.2)	0.6
Glucocorticoid precursors	PD	114.5 (75.2, 196.6)	389.9 (152.4, 1326.1)	<0.001	222.9 (148.3, 326.4)	0.95
	17HP	38.4 (20.0, 69.1)	125.9 (52.6, 328.4)	<0.001	185.9 (157.0, 279.7)	<0.001
	PT	256.2 (170.7, 345.9)	544.4 (309.1, 868.8)	<0.001	715.3 (514.5, 938.3)	<0.001
	PTONE	31.6 (17.9, 49.7)	22.4 (15.3, 36.5)	0.13	25.7 (16.9, 39.4)	0.6
	THS	82.4 (55.3, 116.0)	70.9 (48.7, 91.9)	0.13	79.2 (61.3, 120.2)	0.17
Glucocorticoids	Cortisol	60.0 (45.5, 74.7)	70.3 (51.3, 99.1)	0.13	73.3 (59.1,95.2)	0.07
	6B-OH-cortisol	95.6 (70.2, 137.7)	107.7 (79.7, 131.1)	0.6	109.4 (80.8,159.3)	0.33
	Cortisone	101.5 (78.5, 125.0)	94.2 (79.1, 132.4)	0.93	122.5 (95.0, 162.2)	<0.001
	THF	1275.5 (1156.7, 1847.3)	1025.7 (839.2, 1379.7)	0.012	1566.0 (1310.7, 2154.0)	<0.001
	5a-THF	529.7 (324.5, 749.3)	379.9 (223.1, 794.4)	0.39	1127.4 (753.4, 1863.8)	<0.001
	THE	2674.2 (2015.5, 3476.9)	1851.8 (1166.6, 2985.9)	0.06	3078.8 (2359.8, 4328.9)	<0.001
	B-Cortol	242.7 (193.3, 388.2)	229.6 (168.8, 376.3)	0.68	435.5 (327.7, 598.9)	<0.001
	a-Cortolone	1319.1 (936.1, 1839.6)	1177.2 (853.4, 1839.2)	0.53	1487.9 (1065.8, 1805.9)	0.2
	B-Cortolone	541.3 (422.6, 756.0)	450.5 (307.4, 658.8)	0.12	852.5 (530.6, 1001.5	<0.001
	11B-OH-AN	557.9 (440.3, 844.4)	443.2 (330.3, 815.3)	0.17	984.1 (675.1, 1267.7)	<0.001
	11B-OH-ET	472.0 (246.2, 670.7)	382.7 (246.7, 559.7)	0.33	497.3 (308.7, 799.1)	0.18
	11-OXO-ET	560.4 (389.1, 712.2)	517.6 (307.7, 748.0)	0.66	771.8 (566.0, 1065.9)	<0.001

Age-, sex- and menopausal status-based *Z* scores for steroid metabolite analysis were calculated from urine testing of 114 controls (40 premenopausal women, 26 postmenopausal women, 48 men). IQR, interquartile range.

[a] *P* values between premenopausal and postmenopausal women.

[b] *P* values between men and women.

Steroid profiling demonstrated significant differences in patients with ACC when compared with patients with ACAs for 11 steroids, most notably in THS (median Z score of 14 for ACC vs 0.5 for ACA, $P < 0.001$), PT (median Z score of 7.5 vs −0.4, $P = 0.001$), 5PT (median Z score 5.4 vs −0.4, $P = 0.01$), and Etio (median Z score 5.4 vs −1.1, $P = 0.001$) (Fig. 2A; also see Table 3 in the online Data Supplement). PD, 5PD, DHEA, and 17HP also showed significant distinction from ACA with Z scores ranging from 1.1 to 3.3.

Patients with adrenocorticotropic hormone-dependent pituitary hypercortisolism (Cushing disease) had increased androgens (An, Etio, DHEA, 16a-DHEA, PT, and 5PD) and glucocorticoid metabolites (cortisol, 6B-OH-cortisol, THF, 5a-THF, B-cortol, 11B-OH-AN, 11B-OH-ET, cortisone, THE, a-cortolone, B-cortolone, and 11-OXO-ET), whereas patients with cortisol-producing adrenal adenomas had suppressed androgens and increased glucocorticoids, although to a smaller degree than Cushing disease patients (Fig. 2B; see also Table 4 in the online Data Supplement). The difference in 3 analytes met statistical significance: Etio, 11B-OH-AN, and a-cortolone.

The patient with confirmed 21-hydroxylase deficiency (see Table 5 in the online Data Supplement for clinical and genetic details) showed the expected *(26)* accumulation of metabolites upstream of the enzyme block, including 5PD, PD, 5PT, PT, 17HP, PTONE, DHEA, 16a-DHEA, An, Etio, 11B-OH-AN, 11B-OH-ET, and 11B-OXO (Fig. 3), with the most substantial increase being observed in PTONE (Z score 27.9). PT, DHEA, 17HP, and 11B-OH-AN were also substantially increased with Z scores of 16.8, 13.9, 12.9, and 7.1, respectively. Furthermore, metabolites found downstream of the defect were decreased, with Z scores ranging from −0.2 to −1.5. The 24-h urine sample for steroid analysis was collected before the patient received the first glucocorticoid dose.

Discussion

We have developed an HRAM LC-MS urinary 26-steroid quantitative assay and have illustrated its successful application in a selection of disorders affecting steroidogenesis.

Our HRAM LC-MS method demonstrated good linearity and intraassay- and interassay imprecision despite the quantification of multiple analytes in a single injection. However, some analytes did show inconsistent recoveries, namely, DHEA, 5PT, and 5PD. We attribute the poor recovery/linearity to loss during hydrolysis and extraction because matrix-free, deconjugated standards were prone to loss as well. We improved this to some extent by the addition of 0.1% estriol and 0.1% bovine serum albumin before analysis. Interestingly, recovery was better for DHEA, 5PT, and 5PD in a sample with exceedingly high endogenous steroids (see Table 6 in the online Data Supplement). To have precise and adequate recoveries, improvements to the procedure should be made before implementation in clinical practice and recoveries with conjugated steroids should be performed. Another caution is to confirm complete enzymatic hydrolysis because different conjugated steroid metabolites may be affected by the background matrix of the samples, including different preservatives. Understanding that there could be a range of responses to hydrolysis in the steroids, we optimized the hydrolysis conditions to a point by varying the volume of Glusulase (≥85 000 U/mL β-glucuronidase) added to each sample. A minimum of 10 μL was determined to be sufficient at achieving complete hydrolysis. To verify complete hydrolysis, we scanned for masses indicating glucuronidated or sulfated steroids in full-scan data (extended mass range) in a selection of hydrolyzed urine samples, but no ions indicating incomplete hydrolysis were found. An alternative would have been to forego hydrolysis and scan for, and quantify, both unconjugated and conjugated steroids by HRAM *(27, 28)*. We chose not to use this approach because it has its own limitations, namely, (a) variable glucuronide stability in biological matrices *(29)*, (b) the preference of some conjugates for negative ionization, which might compromise measurement of analytes preferring positive ionization, such as many unconjugated steroid metabolites, and (c) the increased complexity of the generated spectra, which might make data analysis more challenging. Additionally, retention time might be affected by conjugates, which can further complicate analysis *(28)*.

With regard to its clinical performance, establishing sex- and age-stratified control population reference intervals should help in the validation of our assay for the diagnosis of a variety of adrenal disorders.

Our assay confirmed the importance of steroid metabolite profiling in the ACA vs ACC noninvasive diagnosis, as previously shown using GC-MS urine steroid profiles *(11, 12, 26, 30)*. In our sampling of ACCs, we found Etio, DHEA, 5PT, 5PD, PD, 17HP, PT, and THS to be the strongest indicators of ACC, with THS being the most critical (Fig. 2A; also see Table 3 in the online Data Supplement). Because urine collection is far less invasive, costly, or traumatic to the patient than repeated imaging or adrenal biopsy, the urinary steroid metabolite panel has great diagnostic potential. Indeed, given that imaging and autopsy studies put the prevalence of incidental adrenal tumors at between 1% and 9% of the population *(31)*, with most of these tumors being benign ACA, the noninvasive differential diagnosis of ACA vs ACC is clearly an important future application of our HRAM LC-MS urine steroid panel.

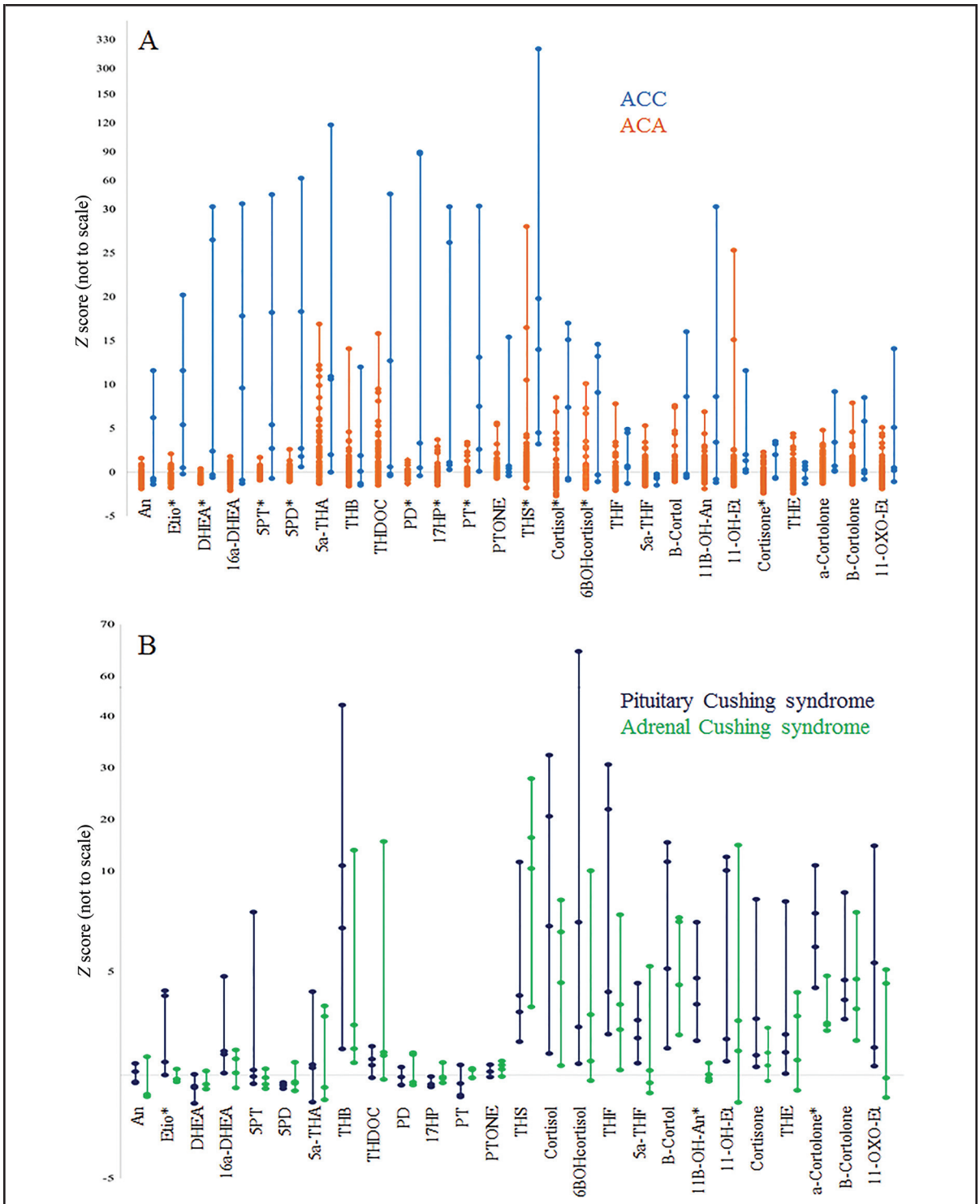

Fig. 2. Clinical significance of steroid metabolite profiling in adrenal disease states.

Comparison of *Z* score (*y* axis) between ACC and ACA (A) using HRAM LC-MS steroid profiling established 11 of 26 metabolites as statistically significant (*) in distinguishing ACC from ACA. Adrenal CS versus pituitary CS (B) showed 3 analytes with statistical significance (*). See Tables 3 and 4 in the online Data Supplement for details.

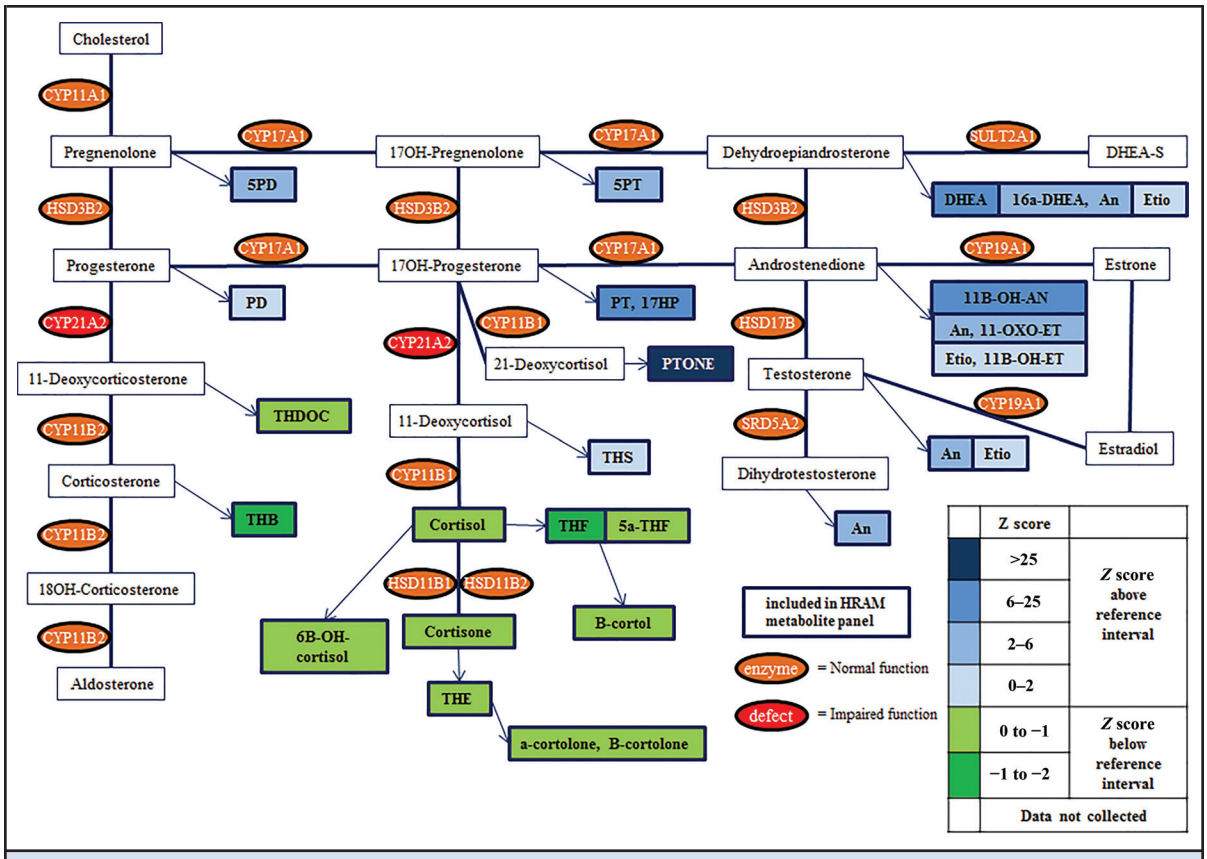

Fig. 3. Steroid pathway diagram indicating the observed changes in steroid metabolite concentrations in a patient with a CYP21A2 enzyme deficiency.

As expected, steroids and metabolites upstream from the CYP21A2 deficiency (confirmed through genetic testing) give increased *Z* scores (above reference interval), whereas those downstream of the enzyme deficiency showed a decrease compared with the reference interval.

Our steroid panel also allowed us to compare adrenocorticotropic hormone-dependent pituitary hypercortisolism and cortisol-producing adrenal adenomas. Distinguishing adrenocorticotropic hormone-dependent hypercortisolism from primary adrenal hypercortisolism remains one of the biggest challenges in the workup of CS. The results of the panel were promising in this regard; despite a sample size of only 4 patients in each group, we observed substantial differences in *Z* scores for multiple steroids, with 3 analytes showing statistically significant differences (Etio, 11B-OH-AN, and a-cortolone; Fig. 2B and also see Table 4 in the online Data Supplement).

The use of steroid profiling in congenital adrenal hyperplasia as a result of 21-hydroxylase (CYP21A2)[6] deficiency *(4, 32)* allowed for detailed characterization of adrenal steroidogenesis and showed the predicted response for all metabolites, both upstream and downstream from the enzyme blockade (Fig. 3). Moreover, in this single case, several metabolites, which are rarely, if ever, measured in CYP21A2 deficiency, displayed equal or greater changes from the normal state than the stalwart measurement targets 17-hydroxyprogesterone and androstenedione, suggesting that the profile might be useful for early diagnosis of subtle cases (e.g., nonclassical congenital hyperplasia).

There are some limitations to our study. Most importantly, the cohort of patients with various diseases, adrenal and other, will need to be extended.

With regard to the day-to-day operations, the assay run-time is relatively long. In part, this problem can be overcome by chromatography multiplexing, which was not possible with the instrumentation available to us at the time, but can theoretically be achieved with compatible multiplex liquid chromatography front-ends. Other operational issues include maintaining QC for 26 separate analytes.

[6] Human Gene: *CYP21A2*, cytochrome P450 family 21 subfamily A member 2, gene encoding 21-hydroxylase enzyme.

A limitation of steroid profiling is the large amount of data yielded. In a typical batch, including calibrators, controls, and approximately 50 patients, there are approximately 2000 chromatograms to quantify and confirm. We found the TraceFinder software to be superior at handling this amount of information compared with any of our triple quadrupole quantification programs. With our setup, retention times were reproducible, resulting in accurate computer-programmed peak selection and quantification with minimal user intervention. Furthermore, we assigned flagging rules for each analyte, including confirmatory ions (secondary ions), when available, and isotopic pattern scoring to further enhance analytical specificity.

Finally, with a panel of this size, creating an optimal reporting system may be challenging. Although itemized analyte results based on Z score could be a simple solution, a clear, comprehensive interpretation is probably required. Machine-learning *(11)* and heat-map analysis may offer some remedy to this. However, at this stage we remain unsure about how our final report to referring physicians will be structured.

In conclusion, we have developed a novel assay to quantify 26 steroid metabolites in urine using HRAM LC-MS. We have established control reference intervals and have demonstrated statistically significant differences in urinary steroid measurements in patients with ACC, adrenal and pituitary CS, and congenital adrenal hyperplasia. Although promising, we propose a larger validation in various adrenal diseases, which might ultimately allow this method to become a standard diagnostic tool for many adrenal diseases.

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