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Multi-steroid profiling by UHPLC-MS/MS with post-column infusion of ammonium fluoride

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ABSTRACT

Background: Multi-steroid profiling is a powerful analytical tool that simultaneously quantifies steroids from different biosynthetic pathways. Here we present an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) assay for the profiling of 23 steroids using post-column infusion of ammonium fluoride.

Methods: Following liquid–liquid extraction, steroids were chromatographically separated over 5 min using a Phenomenex Luna Omega C_{18} column and a water (0.1 % formic acid) methanol gradient. Quantification was performed on a Waters Acquity UHPLC and Xevo® TQ-XS mass spectrometer. Ammonium fluoride (6 mmol/L, post-column infusion) and formic acid (0.1 % (vol/vol), mobile phase additive) were compared as additives to aid ionisation.

Results: Post-column infusion of ammonium fluoride enhanced ionisation in a steroid structure-dependent fashion compared to formic acid (122–140 % for 3βOH-Δ5 steroids and 477–1274 % for 3-keto-Δ4 steroids). Therefore, we analytically validated post-column infusion of ammonium fluoride. Lower limits of quantification ranged from 0.3 to 3 nmol/L; All analytes were quantifiable with acceptable accuracy (bias range −14 % to 11.9 % for 21/23, −21 % to 11.9 % for all analytes). Average recovery ranged from 91.6 % to 113.6 % and average matrix effects from −29.9 % to 19.9 %. Imprecision ranged from 2.3 % to 23 % for all analytes and was < 15 % for 18/23 analytes. The serum multi-steroid profile of 10 healthy men and 10 healthy women was measured.

Conclusions: UHPLC-MS/MS with post-column infusion of ammonium fluoride enables comprehensive multi-steroid profiling through enhanced ionisation particularly benefiting the detection of 3-keto-Δ4 steroids.

1. Introduction

Steroid hormones are biosynthesised in the adrenal cortex and gonads via cascade-like, interlinked enzymatic pathways and undergo extensive metabolism with both activation and inactivation in peripheral tissues (Fig. 1) leading to a complex circulating steroid metabolome [1]. Steroid flux through the different pathways can be severely dysregulated in various conditions, requiring comprehensive steroid assessment to develop a mechanistic understanding of the condition and for diagnosis and treatment monitoring. Disorders of steroidogenesis with distinct steroid metabolome profiles include those with autonomous adrenal steroid production such as Cushing's syndrome or primary aldosteronism [2], adrenocortical carcinoma [3,4], polycystic ovary syndrome [5,6], idiopathic intracranial hypertension [7] and inborn enzymatic deficiencies such as congenital adrenal hyperplasia [2]. Steroid-dependent cancers, such as prostate cancer, can locally activate steroids and are treated with various pharmacological or surgical approaches to deplete the relevant steroids and their precursors in circulation [8].

Traditionally, individual steroids have been used for the assessment of a suspected underlying conditions (e.g. testosterone (T) for female androgen excess [9] and 17α-hydroxyprogesterone (17OHP) for congenital adrenal hyperplasia [10]). Mechanistic *in-vitro* studies often rely on the measurement of the end products of a biosynthesis pathway only. However, the determination of a multi-steroid profile has several advantages over the use of selected individual markers. First, steroid

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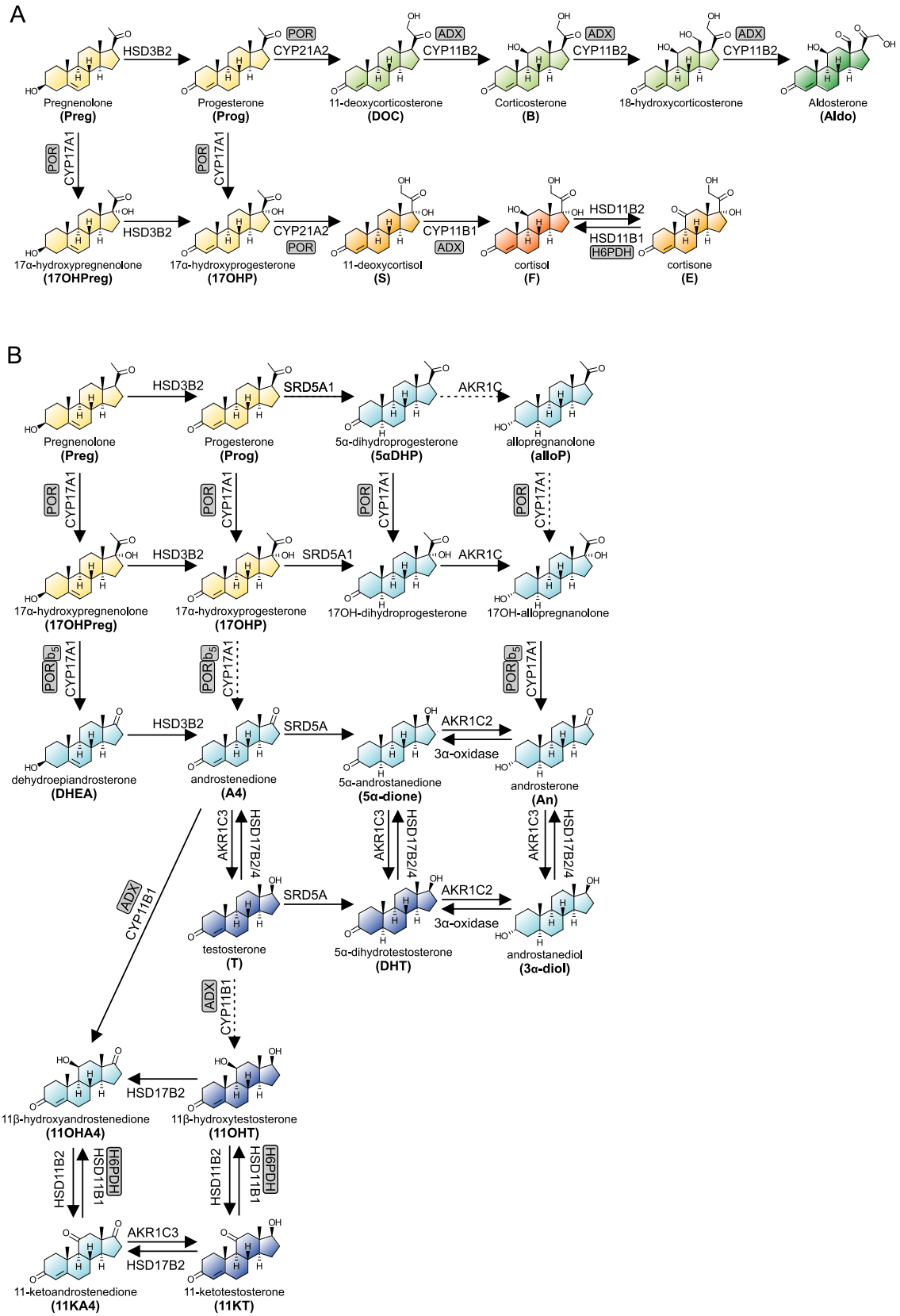

Fig. 1. Pathways of adrenal steroidogenesis (A) and androgen biosynthesis (B). General steroid precursors are shown in yellow, mineralocorticoids in green, glucocorticoids in orange, androgens in blue. Dark shades of each colour indicate active steroids, light shades inactive precursors. Abbreviations for steroids included in the UHPLC-MS/MS assay are shown in bold.

Table 1
Nomenclature of the 25 steroids analysed in this UHPLC-MS/MS assay.

Steroid abbreviation	Trivial name	Chemical name	Functional class
Preg	Pregnenolone	5-pregnene-3β-ol-20-one	General steroid precursor
Prog	Progesterone	4-pregnene-3,20-dione	Progestogen/ general steroid precursor
DOC	11-deoxycorticosterone	4-pregnene-21-ol-3,20-dione	Mineralocorticoid precursor
B	Corticosterone	4-pregnene-11β,21-diol-3,20-dione	Mineralocorticoid precursor
Aldo	Aldosterone	4-pregnene-11β,21-diol-3,20-dione-18-ol	Mineralocorticoid
17Preg	17α-hydroxypregnenolone	5-pregnene-3β,17α-diol-20-one	Glucocorticoid or androgen precursor
17OHP	17α-hydroxyprogesterone	4-pregnene-17β-ol-3,20-dione	Glucocorticoid precursor
S	11-deoxycortisol	4-pregnene-17α,21-diol-3,20-dione	Glucocorticoid precursor
F	Cortisol	4-pregnene-11β,17α,21-triol-3,20-dione	Glucocorticoid
E	Cortisone	4-pregnene-17α,21-diol-3,11,20-trione	Glucocorticoid metabolite
DHEA	Dehydroepiandrosterone	5-androstene-3β-ol-17-one	Classic androgen precursor
A4	Androstenedione	4-androstene-3,17-dione	Classic androgen precursor
T	Testosterone	4-androstene-17β-ol-3-one	Classic androgen
DHT	5α-dihydrotestosterone	5α-androstane-17β-ol-3-one	Classic androgen
5α-dione	5α-androstanedione	5α-androstane-3,17-dione	Classic androgen metabolite
3α-adiol	5α-androstanediol	5α-androstane-3α,17β-diol	Classic androgen metabolite/ alternative DHT pathway intermediate
An	5α-androsterone	5α-androstane-3α-ol-17-one	Classic androgen metabolite/ alternative DHT pathway intermediate
11KA4	11-ketoandrostenedione	4-androstene-3,11,17-trione	11-oxygenated androgen precursor
11KT	11-ketotestosterone	4-androstene-17β-ol-3,11-dione	11-oxygenated androgen
11OHA4	11β-hydroxyandrostenedione	4-androstene-11β-ol-3,17-dione	11-oxygenated androgen precursor
11OHT	11β-hydroxytestosterone	4-androstene-11β,17β-diol-3-one	11-oxygenated androgen
5αDHP	5α-dihydroprogesterone	5α-pregnane-3,20-dione	Progestogen metabolite /alternative DHT pathway intermediate
AlloP	Allopregnanolone	5α-pregnane-3α-ol-20-one	Progestogen metabolite/ alternative DHT pathway intermediate

precursor/product ratios can be calculated to identify distinct effects on individual enzymes [11] and to assess the degree of steroid precursor activation in conditions of steroid excess. For example, in serum the ratio of T to its less active precursor androstenedione (A4) and the ratio of T to the more potent androgen 5α-dihydrotestosterone (DHT) have been shown to be excellent markers for androgen excess and the associated adverse metabolic phenotype in polycystic ovary syndrome [6,12,13]. Secondly, multi-steroid profiling enables different pathways of steroidogenesis to be assessed simultaneously, which is essential due to the interlinked nature of steroidogenesis and the contribution of individual enzymes to several pathways (Fig. 1). Thirdly, multi-steroid profiling can be used to investigate off-target effects of inhibitors of steroidogenesis. Finally, multi-steroid profiling can be combined with machine learning approaches to generate powerful, unbiased and automated diagnostic steroid metabolomics tools [3,4].

In contrast to immunoassays, liquid chromatograhpy tandem-mass spectrometry allows for the high-throughput multiplexing of analytes that is required to effectively use multi-steroid profiling in clinical and research laboratories. Steroid analysis by mass spectrometry is often limited by poor sensitivity due to low analyte concentrations in biological samples and low ionisation efficiency of the analytes. Mobile phase additives can improve the chromatography (peak separation and shape), enhance the signal and, thereby, sensitivity. Formic (methanoic) acid, acetic acid and ammonium acetate or ammonium formate are common additives for corticosteroids and androgen analysis in the positive ionisation mode [14–17] as they can promote the formation of protonated molecular ions $[M + H]^+$. Ammonium fluoride (NH_4F) can aid the ionisation of steroids in electrospray ionisation (ESI) in negative mode [18] and hence improve the sensitivity of oestrogen measurements [19,20]. Additionally, NH_4F has been reported to augment the ionisation of steroids using ESI in positive ion mode in a structure-dependent manner, when coupled to supercritical fluid chromatography [21]. Here, we present an ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS) assay to measure 23 steroids (Table 1) from the mineralocorticoid, glucocorticoid and androgen biosynthetic pathways, including the 11-oxygenated androgen pathway and alternative pathway of DHT biosynthesis. The separation was achieved in 5 min using post-column infusion of NH_4F in positive ionisation mode to enhance ionisation.

2. Material and methods

2.1. Preparation of external standards and quality controls

Reference standards of all analytes were purchased as powders from Sigma-Aldrich (Gillingham, UK) and Steraloids (Newport, USA); for details see Supplemental Table 1. As certified reference material was not available for each steroid, we analysed all by gas chromatography mass spectrometry after derivatisation to form methyloxime-trimethylsilyl ethers [22]. Additional peaks indicating impurities were excluded by analysis of the chromatograms and mass spectra. Individual stock solutions at 1 mg/mL were prepared in UHPLC grade methanol (Biosolve,

Table 2
Retention times, quantifier and qualifier mass transitions, collision energies and cone voltages of target analytes and internal standards. Stable isotope labelled versions of each steroid were used as internal standards. Labelled versions were unavailable only for 5α-dione and 11OHT (*) therefore DHT-d3 and 11OHA4-d7 were used, respectively.

Analyte	Retention time (min)	Mass transition (*m/z*) Quantifier *Qualifier*	Cone voltage (V)	Collision energy (eV)
Preg	3.65	299.2 > 281.1	36	12
		317.1 > 281.1	16	14
Prog	3.37	315.1 > 97.1	20	20
		315.1 > 109.1	20	24
DOC	2.21	331.1 > 97.1	26	20
		331.1 > 109.1	26	24
B	1.52	347.2 > 121.1	20	24
		347.2 > 97.1	16	20
Aldo	0.86	361.2 > 343.2	40	16
		361.2 > 315.1	40	18
17Preg	2.42	315.2 > 297.2	8	12
		297.2 > 279.2	4	14
17OHP	2.45	331.1 > 97.1	16	22
		331.1 > 109.1	16	26
S	1.64	347.1 > 109.1	42	26
		347.1 > 97.1	42	22
F	1.06	363.3 > 121.1	42	22
		363.1 > 91.1	42	50
E	0.96	361.1 > 163.1	46	22
		361.1 > 121.01	46	38
DHEA	2.35	271.2 > 253.2	30	10
		289.2 > 253.2	12	8
A4	2.03	287.2 > 109.1	26	22
		287.2 > 97.1	26	22
T	2.26	289.2 > 97.1	40	20
		289.2 > 109.1	40	24
DHT	2.80	291.2 > 255.1	42	14
		291.2 > 159.1	42	22
5α-dione*	2.63	289.2 > 253.1	40	16
		289.2 > 271.1	40	10
3α-adiol	3.21	275.2 > 257	28	10
		275.2 > 81	28	28
An	3.34	291.2 > 273.2	24	8
		291.2 > 255.2	24	12
11KA4	1.06	301.1 > 121.1	44	22
		301.1 > 257.1	44	22
11KT	1.21	303.1 > 121	20	22
		303.1 > 259.1	20	22
11OHA4	1.34	303.1 > 285.1	30	14
		303.1 > 267.1	30	16
11OHT*	1.48	305.2 > 269.2	16	14
		305.2 > 121.1	16	20
5αDHP	4.01	317.1 > 281.1	38	12
		317.1 > 85.1	24	14
alloP	4.33	319.1 > 301.2	22	8
		319.1 > 283.2	22	14
Internal standard	**Retention time (min)**	**Mass transition (*m/z*) Quantifier *Qualifier***	**Cone voltage (V)**	**Collision energy (eV)**
Preg-d2-^{13}C2	3.64	321.1 > 303.1	28	8
Prog-d9	3.34	324.2 > 100.1	28	24
DOC-d8	2.18	339.1 > 100.1	26	24
B-d8	1.49	355.1 > 337.2	46	14
Aldo-d8	0.85	369.2 > 351.1	34	16
17Preg-d3	2.4	336.2 > 300.2	12	10
17OHP-d8	2.42	339.1 > 100.1	26	24
S-d2	1.62	349.1 > 97.1	42	22
F-d4	1.06	367.2 > 121.1	28	26
E-d7	0.95	369.1 > 169.1	40	24
DHEA-d6	2.33	277.2 > 219.1	22	14
A4-d7	2.01	294.2 > 109.1	26	22
T-d3	2.25	292.2 > 97.1	46	22
DHT-d3	2.79	294.1 > 258.1	22	16
3α-adiol-d3	3.20	278.2 > 150.2	20	20
An-d4	3.33	295.1 > 277.2	24	12
11K4-d10	1.04	311 > 265	46	16
11KT-d3	1.20	306.2 > 262.1	20	22
11OHA4-d7	1.30	310.1 > 292.1	30	14
5αDHP-d4	3.96	323.1 > 305.1	22	8
alloP-d4	4.31	323.1 > 305.1	22	8

Dieuze, France) and stored at −80 °C. Using the stock solutions, combined calibrators for all analytes were prepared by spiking phosphate buffered saline (PBS) pH 7.4 supplemented with 0.1 % (wt/vol) bovine serum albumin (BSA) (Sigma-Aldrich, Gillingham, UK) yielding 12 final concentrations from 0 to 250 ng/mL (Supplemental Table 2). PBS supplemented with BSA is a typical matrix used to mimic a serum environment in clinical biochemistry [14,23]. A combined internal standard stock solution containing 0.5 μg/mL of each internal standard was prepared in deuterated methanol (Sigma-Aldrich) to avoid hydrogen–deuterium exchange.

Quality control (QC) samples were prepared by spiking PBS 0.1 % BSA at four different concentrations covering the expected concentration range in human serum using independently prepared 1 mg/mL stock solutions (Supplemental Table 2). Additionally, pooled adult male human serum (Sigma-Aldrich) was aliquoted, stored at −80 °C and used as a biological QC in each analytical run. All calibrators, internal standards and QCs were stored at −20 °C.

2.2. Collection of serum samples

The collection of blood samples was approved by the authors' Institutional Review Board (Science, Technology, Engineering and Mathematics Ethical Review Committee of the University of Birmingham, ERN_17-0494). Informed, written consent was obtained from all individuals included in this study. Following venous puncture, blood was collected into gold top SST Vacutainers® (Becton, Dickinson, Wokingham, UK). Samples were spun and the serum removed and stored at −80 °C until analysis. The method was applied to 20 serum samples from healthy volunteers 10 female and 10 males (20–40 years).

2.3. Sample preparation

Steroids were extracted by liquid–liquid extraction. A set of calibrators, QCs and biological QCs were extracted with each batch of samples. Sample, calibrator, or QC (200 μL) were transferred into a hexamethyldisilazane-treated glass tube with 10 μL of the internal standard mixture containing all stable isotope labelled internal standards listed in Table S1. 50 μL of acetonitrile (Biosolve, Dieuze, FR) was added to precipitate the proteins and the samples were vortexed. 1 mL of *tert*-butyl methyl ether (Acros Organics, Fisher Scientific UK ltd, Loughborough, UK) was added to each sample and the samples were vortexed at 1000 rpm for 10 min on a Fisherbrand multi-tube vortex (Fisher, Loughborough, UK). The samples were then incubated at room temperature for at least 30 min to aid phase separation. The organic phase was subsequently transferred into the wells of a 2 mL square well 96-well plate (Porvair Sciences ltd, Wrexham, UK) containing 700 μL glass inserts (Randox, Crumlin, UK) and dried under a nitrogen stream at 45 °C. The dried extract was reconstituted in 100 μL of 50 % (vol/vol) UHPLC grade methanol (Biosolve) in UHPLC grade water (Biosolve) prior to analysis.

2.4. Ultra-high performance liquid chromatography

Chromatography was performed on a Classic Waters Acquity ultra performance liquid chromatography system with a 50 μL loop (UHPLC; Waters Ltd, Wilmslow, UK) using a Phenomenex Luna Omega column,

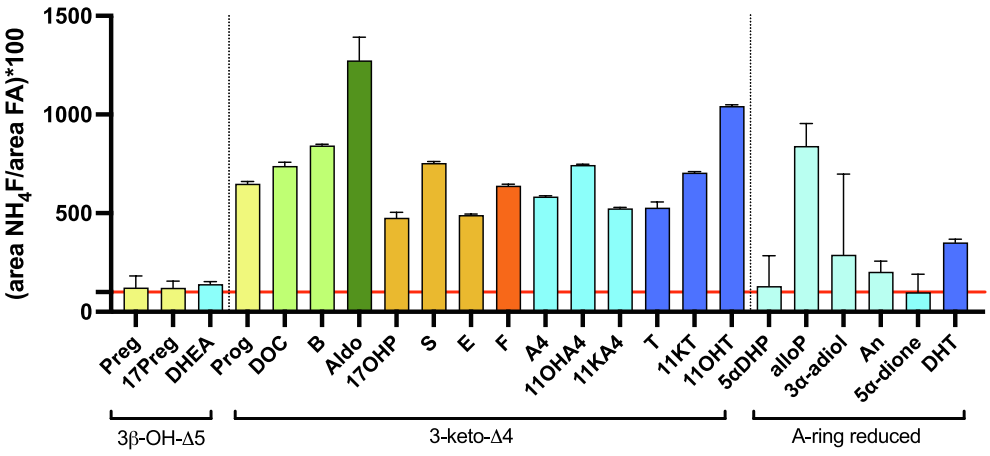

Fig. 2. Ammonium fluoride (NH_4F) post-column infusion enhances ionisation of steroids in a structure-dependent fashion as compared to mobile phase formic acid. The percentage increase in peak area of the quantifier transition when comparing post-column infusion of NH_4F (6 mmol/L, 5 μL/min) to the use of 0.1 % (vol/vol) formic acid in both the methanol and water mobile phase. Bars represent the mean percentage peak area and whiskers the relative standard deviation for serum samples from different individuals. Samples from all individuals with detectable peaks were included (Prog, DOC, B, 17Preg, 17OHP, S, F, E, DHEA, A4, T, DHT, An, 11KA4, 11KT, 11OHA4, 11OHT n = 74; Preg n = 73; Aldo n = 71; 3α-adiol n = 56; 5αDHP n = 48; alloP n = 15; 5α-dione n = 14). The red line indicates a relative peak area of 100 % (identical ionisation with both methods). The colour of the bars indicates the functional class of each analyte; yellow, general steroid precursor; light green, mineralocorticoid precursor; dark green, mineralocorticoid; orange, glucocorticoid precursor; red, glucocorticoid; turquoise, androgen precursor; dark blue, androgen; light blue, alternative DHT biosynthesis pathway intermediate.

1.6 μm, polar C18, 100 Å, 2.1 × 50 mm; (Phenomenex, Macclesfield, UK) at 60 °C. 10 μL of the reconstituted sample was injected. Mobile phase A consisted of UHPLC grade water (Biosolve) and mobile phase B of UHPLC grade methanol (Biosolve). An optimised method with a linear gradient from 45 % to 75 % of mobile phase B was applied over five minutes at a flow rate of 0.6 mL/min to separate the analytes followed by a wash step with 95 % B until 5.2 min and equilibration at starting conditions from 5.5 min until injection of the next sample. Total run time injection-to-injection was thus 6 min. The auto sampler was maintained at 10 °C. To investigate ionisation 0.1 % (vol/vol) formic acid (added to mobile phase A or both A and B) was compared to PCI of NH_4F.

2.5. Tandem mass spectrometry

The UHPLC eluate was injected into a XEVO® TQ-XS mass spectrometer (Waters Ltd) using ESI in positive ion mode. Post-column infusion 6 mmol/L NH_4F in 50 % (vol/vol) methanol in water was combined using the fluidics system on the mass spectrometer under full software control. The capillary voltage was maintained at 1.5 kV, the source temperature was 150 °C, desolvation temperature and gas flow were 600 °C and 1200 L/h, cone gas was 150 L/h. MassLynx 4.2 software (Waters ltd) was used for systems control and optimisation of mass transitions. Qualifier and quantifier mass transitions, cone voltages and collision energies (Table 2) were further manually optimised using direct infusion (20 μL/min) of a 500 nM solution of each steroid in 50 % (vol/vol) methanol in water containing 1 mM NH_4F and a UHPLC flow rate of 0.3 mL/min with a methanol/water mixture mimicking the elution conditions of the respective analyte. Dwell time was automatically calculated by MassLynx© for each transition with a minimum of twelve analytical points across each peak required for quantitative analysis.

TargetLynx software was used for data processing and quantification. Peak area ratios of analyte to internal standard were plotted against the nominal concentrations of the calibrators and 1/x weighting and linear least square regression were used to produce the standard curve. Ion-ratio error indicators are automatically calculated by the TargetLynx to allow for the detection of co-eluting steroid with cross-reactivity.

2.6. Validation

Validation was performed following protocols from published guidelines [24–26]. For each steroid, validation has been performed at different concentrations representing low, medium and high concentrations of that steroid in serum. While this method has utility for many biological matrices, we opted to validate for a serum application.

2.6.1. Recovery and matrix effects

Matrix effects and recovery were determined as previously described [27,28]. Six different human serum samples (four male and two female) were extracted (endogenous). A second aliquot from the same sample was then spiked at 5 ng/mL before extraction (pre extract) and after extraction and reconstitution (post extract). Additionally, the reconstitution solvent (50 % (vol/vol) UHPLC grade methanol in UHPLC grade water) was spiked with the same amounts of analytes (non extract). Percentage matrix effects and recoveries were calculated using the formulas below, comparing responses (matrix effects) or concentrations (recovery) from these samples. Quantification was completed after normalisation to the internal standard to realistically determine the impact of the matrix on the assay performance.

$$\%MatrixEffect = \left(\frac{(post\ extraction - endogenous) - non\ extraction}{non\ extraction}\right) \times 100$$

$$\%Recovery = \left(\frac{Concentration\ (pre\ extract)}{Concentration\ (post\ extract)}\right) \times 100$$

For matrix effects mean values between −15 and 15 % were considered ideal and −20 % and 20 % were acceptable. For recovery mean values between 80 % and 120 % were defined acceptable.

2.6.2. Limits of quantification

The lower limit of quantification (LLOQ) was defined as the lowest concentration for which 10 replicate samples of spiked surrogate matrix could be measured with an intra-assay (within-run) imprecision CV < 20 % and an accuracy (bias) between +20 % and −20 %.

The upper limit of quantification (ULOQ) as defined as the highest concentration for which 10 replicate samples of spiked surrogate matrix could be run with an intra-assay (within-run) imprecision CV < 15 % with an accuracy (bias) between +15 % and −15 %.

2.6.3. Linearity

Three calibration series were prepared by different scientists. For each analyte the ratio of analyte peak area to internal standard peak area was plotted against the nominal concentrations of the standard as described above. To be deemed an acceptable linear calibration series each data point above the LLOQ and below the ULOQ should have an

Table 3
Matrix effects, recovery and carry over for all steroids. Matrix effects and recovery were assessed at 5 ng/mL (approximately 17 nmol/L) in serum samples from six different donors.

Analyte	Matrix effects (%) mean (min–max)	Recovery (%) mean (min–max)	Carry over (%) (250 ng/mL)	Carry over (%) (1000 ng/mL)
Preg	19.9 (-0.4–43.8)	93.9 (75.3–110)	0.056	0.003
Prog	7.4 (-8.2–19.7)	98.9 (91.3–110)	0.201	0.012
DOC	14.4 (9.1–20.9)	113.6 (102.9–131.4)	0.124	0.017
B	13.5 (5.3–22.5)	91.3 (74.3–110.3)	0.091	0.021
Aldo	12.6 (8.7–17.5)	103.5 (92.4–111.8)	0.066	0.023
17Preg	−15.9 (-57.9–27.8)	105.5 (59.0–129.7)	0.064	0.024
17OHP	−11.4 (-31.3–24.2)	94.5 (78.0–108.6)	0.063	0.026
S	4.3 (-5.0–14.4)	99.9 (88.3–107.9)	0.069	0.028
F	−19.5 (–32.5–16.7	101.7 (7.5–108.4)	0.072	0.030
E	7.1 (-4.3–19.9)	100.7 (95.7–102.8)	0.064	0.031
DHEA	−1.4 (-20.1–12.9)	105.1 (95.0–118.2)	0.039	0.032
A4	12.7 (0.3–29.0)	95.2 (85.8–112.0)	0.129	0.035
T	0.5 (-8.3–4.9)	102.8 (97.9–106.7)	0.147	0.048
DHT	2.4 (-9.0–10.0)	102.7 (96.6–111.7)	0.058	0.049
5α-dione	19.2 (7.8–29.7)	104.6 (88.6–113.9)	0.024	0.056
3α-adiol	4.2 (-44.1–32.8)	109.1 (96.4–138.8)	0.684	0.057
An	13.0 (5.9–23.0)	98.1 (87.0–107)	0.008	0.073
11KA4	−2.6 (-25.4–33.6)	109.0 (77.3–133.9)	0.101	0.147
11KT	10.1 (4.7–16.6)	98.0 (89.3–102.1)	0.090	0.152
11OHA4	−3.6 (-17.4–17.2)	105.9 (94.1–117.8)	0.077	0.172
11OHT	1.7 (-6.7–6.1)	101.8 (96.5–110.2)	0.082	0.186
5αDHP	−29.9 (-49.9–−8.4)	113.3 (80.9–184.4)	0.015	0.201
alloP	16.0 (–22.3–40.6)	91.6 (74.0–124.1)	0.198	0.214

accuracy (bias) not exceeding ± 15 % compared to the nominal concentration. In addition, the correlation coefficient of the regression, R^2, generated from the calibration curve should be > 0.99.

2.6.4. Determination of imprecision

Intra-assay (within-run) imprecision.

Pooled surrogate matrix samples spiked with all analytes at four different concentrations (0.3, 1, 3 and 30 ng/mL) as well as pooled adult male serum samples (Sigma Aldrich) and pooled adult male serum (Sigma Aldrich) samples spiked with 5 ng/mL for all analytes were run ten times in the same batch to assess intra-assay (within-run) imprecision. The data for both biological and surrogate matrices is presented separately. A percentage deviation (CV) ≤ 15 % was considered acceptable.

Inter-assay (between-run) imprecision

Pooled adult male serum samples and pooled adult male serum samples spiked with 5 ng/mL of all analytes were extracted 10 times in two independent batches to assess inter-assay (between-run) imprecision. A percentage deviation (CV) ≤ 15 % was considered acceptable.

2.6.5. Accuracy

10 samples of surrogate matrix were spiked individually at four different concentrations (0.3, 1, 3 and 30 ng/mL). A percentage bias of the observed concentration to the nominal concentration between −15 % and + 15 % was considered optimal.

2.6.6. Carryover

Carryover was assessed by running a blank sample immediately after the injection of 250 and 1000 ng/mL steroid mixtures. Percentage carryover was calculated from the peak area of the analyte in the blank samples relative to the concentrated sample. Carryover of <2 % was deemed acceptable.

3. Results

3.1. Chromatographic separation

Analytes were separated using a linear gradient over five minutes followed by 0.5 min at starting conditions for equilibration resulting in a total run time injection-to-injection of 6 min. All analytes eluted as distinct, identifiable peaks (Supplemental Fig. 1). Two pairs of analytes co-eluted: (cortisol (*m/z* 363) and 11KA4 (*m/z* 301); 17OHP (*m/z* 330) and 17Preg (*m/z* 332)). No interference was observed due to the differences in *m/z* (Table 2). To confirm selectivity, the co-elution of endogenous steroids, that may be present at high concentrations, e.g. 21-deoxycortisol and 11-dehydrodeoxycorticosterone, were assessed. Due to the cross-reactivity of synthetic steroids, samples from patients on prednisone, prednisolone and dexamethasone cannot be analysed by the assay.

3.2. Comparison of additives to improve ionisation

The effect of NH_4F on signal intensity was assessed in comparison to our previously published method using 0.1 % (vol/vol) formic acid as mobile phase additive in both the methanol and water phases [6,7]. We chose to add NH_4F by post-column infusion as preliminary experiments with NH_4F as an additive led to increases in system pressure indicative of damage to the column material, with < 200 injections before the column pressure exceeded the limits of the system (>17000 psi) and chromatography was no longer reproducible. The intra-assay (within-run) imprecision using this modification was acceptable with a mean CV of the peak area of 7.0 % for all analytes (CV range 3.9 to 13.7 %; n = 60 injections of the same sample). Peak areas of all analytes in serum (n = 73) were compared between the assay with formic acid in both mobile phases and the assay using post-column infusion of NH_4F. NH_4F increased peak area in a structure-dependent fashion (Fig. 2, Supplemental Fig. 2). In comparison to formic acid, NH_4F induced significant increases in the peak area of steroids with 3-keto-Δ4 structure (Prog, DOC, B, Aldo, 17OHP, S, E, F, A4, 11OHA4, 11KA4, T, 11KT, 11OHT), with mean increases varying from 477 % (17OHP) to 1274 % (Aldo). NH_4F had a lower impact on the peak area of the majority of A-ring reduced steroids with changes varying between 100 % and 352 % (DHT, 5α-dione, 3α-diol, An, 5α-DHP), with the exceptions of alloP (841 %). NH_4F post-column infusion had only a very minor impact on the peak areas of 3β-OH-Δ5 steroids (Preg, 17Preg, DHEA). Our final optimised method employed 6 mmol/L NH_4F in 50 % (vol/vol) methanol in water introduced via post-column infusion at a flow rate of 5 μL/min, with 0.1 % (vol/vol) formic acid in the water mobile phase to limit the risk of microbial contamination.

3.3. Validation of the analytical performance

Matrix effects and recovery were assessed for six different serum samples (Table 3). Mean matrix effects ranged from −19.5 % to 19.9 % for all analytes, except for 5αDHP (-29.9 %), which was outside the desired range from −20 % to 20 %. Mean recovery was acceptable with a

Table 4

Accuracy (bias) and intra-assay (within-run) imprecision and limits of quantification. Accuracy and imprecision were determined at four concentrations spiked into surrogate matrix (n = 10). n/a, not applicable as concentration < LLOQ. The lower limit of quantification (LLOQ) was defined as the lowest concentration that can be assessed with appropriate accuracy (bias within ± 20 %) and imprecision (CV < 20 %). The upper limit of quantification (ULOQ) was defined as the highest concentration that can be assessed with appropriate accuracy (bias within ± 15 %) and imprecision (CV < 15 %).

	Bias (%)				**Imprecision, CV (%)**				**LLOQ**ng/mL (nM)	**ULOQ** ng/mL (nM)
Concentration (ng/mL)	**0.3**	**1**	**3**	**30**	**0.3**	**1**	**3**	**30**		
Preg	−9.6	−0.4	−1.4	−3.9	8.0	5.4	13.1	4.6	0.2 (0.6)	250 (791)
Prog	−6.2	1.6	1.3	−5.0	7.0	5.3	9.1	3.3	0.1 (0.3)	100 (318)
DOC	−0.7	−2.0	3.3	−0.2	7.4	7.6	11.6	6.0	0.2 (0.5)	100 (303)
B	−5.4	−6.5	−1.8	9.2	9.4	5.8	12.1	4.8	0.1 (0.3)	250 (722)
Aldo	−7.7	−2.1	0.1	0.8	7.0	5.1	9.6	5.0	0.2 (0.4)	250 (694)
17Preg	n/a	−17	3.8	−3.6	n/a	10.4	16.5	11.1	1.0 (3.0)	250 (753)
17OHP	4.9	−0.2	−5.6	0.2	5.7	10.0	9.4	6.2	0.2 (0.6)	100 (303)
S	8.4	3.9	6.8	1.8	5.5	4.8	8.3	3.8	0.2 (0.4)	250 (722)
F	2.6	7.7	10.3	2.7	5.5	4.9	4.1	2.3	0.1 (0.3)	250 (690)
E	0.7	4.7	7.2	5.0	6.7	4.5	8.2	4.3	0.1 (0.3)	250 (694)
DHEA	10.6	4.8	0.6	2.3	13.3	10.1	8.0	3.8	0.2 (0.7)	250 (868)
A4	6.1	−5.1	−0.4	−4.6	11.3	8.2	6.9	3.7	0.2 (0.7)	50 (175)
T	−1.8	5.3	7.5	0.6	7.6	5.6	8.2	3.8	0.1 (0.4)	50 (174)
DHT	1.3	6.4	10.2	11.9	6.7	5.1	8.4	3.2	0.1 (0.3)	250 (862)
5α-dione	−2.2	2.0	3.4	−8.0	7.8	9.5	11.2	8.0	0.2 (0.5)	250 (868)
3α-adiol	n/a	4.4	−6.1	2.5	n/a	17.8	11.5	6.5	1.0 (3.4)	250 (862)
An	2.5	1.8	−0.5	−2.9	15.2	12.2	9.1	3.5	0.2 (0.7)	250 (862)
11KA4	−9.8	−5.7	−0.5	−0.9	8.3	6.1	11.2	5.3	0.1 (0.3)	250 (833)
11KT	−8.5	0.5	2.3	−1.6	10.5	4.8	11.6	2.7	0.1 (0.3)	250 (828)
11OHA4	−4.1	1.6	2.8	−1.9	6.8	5.7	7.5	3.6	0.1 (0.3)	250 (828)
11OHT	0.2	5.6	10.9	4.0	11.6	6.8	8.0	5.0	0.1 (0.3)	100 (329)
5αDHP	−21.0	−8.2	−3.4	−4.6	14.3	12.5	11.6	14.4	0.2 (0.6)	250 (791)
alloP	−14.0	−10.7	−14.0	−11.5	20.5	16.1	15.7	15.1	0.2 (0.6)	250 (786)

range from 91.6 % to 113.6 %. Lower limits of quantification ranged between 0.1 ng/mL (~0.3 nmol/L) and 0.5 ng/mL (~1.5 nmol/L) for all analytes except for 17Preg and 3α-adiol, which had a limit of quantification of 1 ng/mL (corresponding to 3.01 and 3.42 nmol/L, respectively) (Table 4). ULOQ was 100 ng/mL for 11OHT, DOC, 17OHP and Prog; 50 ng/mL for T and A4 and 250 ng/mL for all other analytes (Table 4). Calibration curves were linear from the LLOQ to the ULOQ with an accuracy (bias) for each point not exceeding ± 15 % compared to the nominal concentration and an $R^2 \geq 0.99$ for all analytes. Carryover was ≤ 0.68 % for all analytes at both concentrations tested (Table 3).

The accuracy (bias) and imprecision of the assay were assessed with spiked PBS 0.1 % BSA samples at four different concentrations. The bias between the observed and nominal concentrations was calculated as a measure of accuracy and the coefficient of variation (CV) as a measure of imprecision (Table 4). Bias was within acceptable limits for the majority of analytes at all concentrations above the LLOQ ranging from −14.0 to 11.9 %, with the exception of 5αDHP at 0.3 ng/mL (-21 %) and 17OHPreg at 1 ng/mL (-17.0 %). Intra-assay imprecision determined for the spiked PBS 0.1 % BSA samples was within acceptable limits for the majority of analytes at concentrations above the LLOQ ranging from 2.3 to 15.2 % with the exception of 3α-adiol (17.8 % at 1 ng/mL), 17OHPreg (16.5 % at 3 ng/mL) and alloP (at all concentrations imprecision ranged from 15.1 to 20.5 %).

Additionally, imprecision was assessed using a pooled serum sample (Table 5). The levels in this pooled serum sample were below the limit of quantification for 9 analytes (Preg, Prog, Aldo, DOC, 5α-dione, 3α-adiol, An, 5αDHP and alloP). For the remaining analytes the intra-assay CV ranged from 2.4 to 16.3 % and the inter-assay CV from 3.5 to 16.1 %. For a pooled serum sample spiked with 5 ng/mL of all analytes the intra-assay CV was between 2.3 % and 12.5 % for all analytes. The inter-assay CV was < 15 % (3.2 to 14.7 %) for all steroids except 3α-adiol (23.0 %) and 5α-DHP (22 %).

3.4. Serum steroid profiling in healthy volunteers

Following validation, we applied this assay to the measurement of serum samples from 10 female (aged 23–39 years) and 10 male healthy volunteers (aged 28–37 years). The highest concentrations were

Table 5
Intra-assay (within-run) and inter-assay (between-run) imprecision of a pooled serum sample and a pooled serum sample spiked with 5 ng/mL of all analytes. (n = 10). n/a, not applicable as concentration below the lower limit of quantification (<LLOQ).

	Pooled serum			Pooled serum spiked at 5 ng/mL	
Analyte	Mean (ng/mL)	Inter-assay CV (%)	Intra-assay CV (%)	Inter-assay CV (%)	Intra-assay CV (%)
Preg	<LLOQ	n/a	n/a	8.2	4.9
Prog	<LLOQ	n/a	n/a	10.8	12.5
DOC	<LLOQ	n/a	n/a	5.9	2.7
B	2.3	11.2	7.3	12.5	3.7
Aldo	<LLOQ	n/a	n/a	5.6	3.7
17Preg	4.8	16.1	16.3	14.7	8.1
17OHP	1.2	5.5	4.2	12.1	4.5
S	0.2	8.1	6.9	5.8	3.5
F	75.2	3.5	2.4	3.7	2.5
E	9.7	4.2	3.4	4.5	3.1
DHEA	2.0	6.5	6.0	6.4	6.1
A4	0.6	15.3	8.0	9.8	9.0
T	4.0	4.7	3.5	3.2	2.3
DHT	0.4	7.5	5.4	5.4	4.1
5α-dione	<LLOQ	n/a	n/a	12.8	6.1
3α-adiol	<LLOQ	n/a	n/a	23.0	10.0
An	<LLOQ	n/a	n/a	4.0	2.8
11KA4	0.2	16.0	16.2	8.0	6.0
11KT	0.1	10.1	5.9	4.7	3.0
11OHA4	1.3	12.7	10.2	9.5	5.1
11OHT	0.1	14.9	8.2	5.3	3.2
5αDHP	<LLOQ	n/a	n/a	22	4.1
alloP	<LLOQ	n/a	n/a	8.1	6.1

observed for glucocorticoids (F and E), the adrenal androgen precursor DHEA and the mineralocorticoid precursor B. As expected, men had higher levels of T and DHT than women, the levels of all other adrenal derived steroids were similar in men and women (Fig. 3). Concentrations of Prog and its metabolites 5αDHP and alloP were below the LLOQ in men and in 8 of 10 women, which reflects that Prog is only produced at relevant levels in women during luteal phase of the menstrual cycle. In addition to these three analytes, serum levels of DOC, Aldo, DHT (in women), 5α-dione and 3α-diol were below the LLOQ for most samples.

4. Discussion

Multi-steroid profiling provides a wider insight than quantification of a single steroid marker. A multiplexed approach has extensive utility for the diagnosis, monitoring, and the development of a mechanistic understanding of steroid-associated conditions as it allows for the integration of steroid flux across all pathways of steroid biosynthesis and metabolism [2,29]. This has led several clinical laboratories to increase the number of analytes that are multiplexed in a single assay [14,30–32]. Here, we describe the development and validation of an UHPLC-MS/MS assay for the simultaneous measurement of 23 steroids from the mineralocorticoid, glucocorticoid and androgen biosynthetic pathways with a total run-time of 6 min.

Formic acid is a standard mobile phase additive for the analysis of steroids in positive ionisation mode as 3-keto-Δ4 steroids predominantly form protonated molecular ions $[M + H]^+$. However, the sensitivity achieved with formic acid as an additive can still be insufficient to accurately quantify the low levels of certain steroids present in serum. Hence, we tested the effect of post-column infusion of NH_4F on the signal of all 23 steroids in our assay and established large increases in peak area for all 3-keto-Δ4 analytes in the presence of NH_4F compared to formic acid, with lower impact observed on the ionisation of A-ring reduced and 3βOH-Δ5 steroids. These findings are consistent with published results on the effect of NH_4F on the ionisation of selected steroids in a supercritical fluid chromatography set up with ESI in positive mode [21]. 3-keto-Δ4 steroids predominantly form quasi molecular ions $[M + H]^+$ during ESI. Parr et al. [21] speculate that the signal enhancement observed for 3-keto-Δ4 is due to the aided formation of $[M + H]^+$ ions in the presence of NH_4F, as the proton affinity of 3-keto-Δ4 steroids is higher than that of ammonium. Ammonium, which is acidic in the gas phase, could function as a potential source of protons responsible to the increase in signal of $[M + H]^+$ ions. Another possible mechanism is that F^- ions capture potential Na^+ contaminations in the mobile phases, hence preventing the formation of $[M + Na]^+$ adducts.

While other assays for steroid analysis by ESI in positive mode use NH_4F as mobile phase additives [20,33], we chose to supply NH_4F by post-column infusion to allow for cost- and time efficient incorporation of the assay into lab workflows. As indicated by the system pressure increased in our own preliminary experiments with NH_4F in the mobile phase, NH_4F can have detrimental effects on column lifetime [34], which is circumvented by post-column infusion. If running different assays on the same LC-MS/MS system, post-column infusion of additives limits their variability in mobile phase compositions and could reduce the time needed for equilibration when switching between assays that require different additives. The use of the in-built fluidics system of this mass spectrometer for the post-column infusion is simple, robust, and fully automated.

Suitable analytical performance of the assay has been confirmed for all 23 analytes for concentrations between LLOQ and ULOQ through the validation described here. However, the application of the assay to serum samples from healthy male and female volunteers revealed that

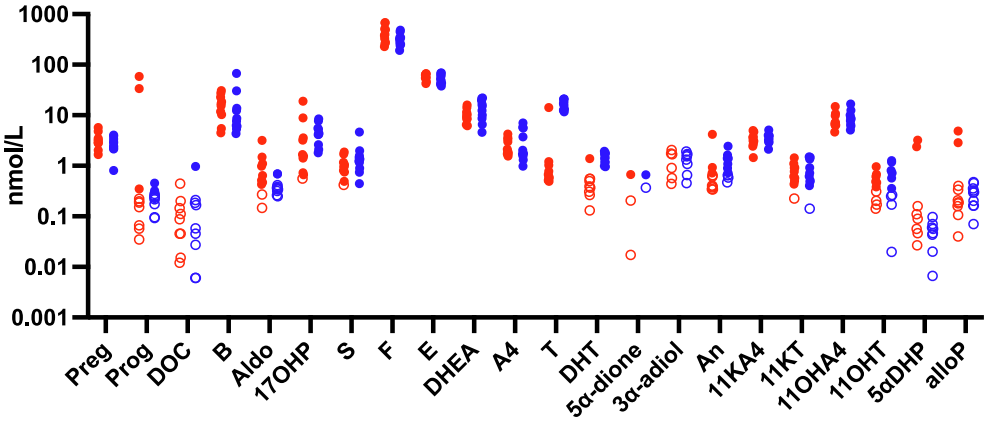

Fig. 3. Human Serum Steroid Profiles. Serum steroid profiles in healthy volunteers, female (red circles; n = 10, age range 20–40 years) and male (blue circles, n = 10, age range 30–40 years). Each symbol represents one participant; values < LLOQ are represented by open circles and undetectable levels were not included in the visualisation.

out of the 23 analytes only 14 for women and 15 for men can be quantified at the concentration ranges of the circulating steroid metabolome in healthy adults. Prog, DOC, Aldo, DHT (in women only), 5α-dione, 3α-diol, 5αDHP and alloP levels are below the LLOQ in a typical serum sample from the cohort studied here. Of note, these analytes might be quantifiable in steroid excess conditions potentially allowing for the assay to discriminate between samples from healthy individuals and those with excess activity of specific steroidogenic pathways.

When comparing the sensitivity of our assay with other assays in the literature it has to be considered that our assay represents a multi-analyte profiling method delivering information on the levels of analytes from all pathways of steroidogenesis in a single run. In order to achieve this, the assay has been optimised to provide sufficient analytical performance, including sensitivity, for all analytes as shown by the application to human adult serum samples resulting in a pareto-optimal solution. To accommodate the breadth of analytes it is not possible to have optimal extraction and mass spectrometry conditions for each individual analyte. However, when comparing LLOQs between our assay and more specialist assays in the literature, that target a lower number of analytes and/or structurally more similar analytes [35–38], our LLOQs are directly comparable or range within one order of magnitude demonstrating the competitive sensitivity of our assay despite the larger and structurally broader panel of analytes. Importantly, our sample preparation does not contain a derivatisation step as opposed to assays with similar LLOQs [35,36] thus reducing sources of variability and ensuring high selectivity of the MRMs.

It should be noted that the assay as presented here has been tailored to steroid analytes and concentrations relevant to adult circulation and has neither been designed nor validated to measure paediatric samples.

To the best of our knowledge, only two LC-MS/MS assays, which analyse 20 or more steroids in serum or plasma, have been published to date [39,40]. However, the run-times of these assays (16 min [39] and 8 min [40]) are significantly longer than that of our assay. Published assays with run-times $<$ 6 min cover a maximum of 16 analytes [37,41–43]. Additionally, while other assays focus mainly on adrenal steroids [16,32,41,44,45] or androgen panels [14,23], our assay comprehensively covers multiple steroid classes (Fig. 1). In addition to classic androgens, such as A4 and T, our assay covers the alternative pathway of DHT biosynthesis, which contributes to androgen excess in CAH [46,47], and the 11-oxygenated androgens, which have only recently been shown to have major relevance for the diagnosis and mechanistic understanding of androgen excess conditions [5,48–50].

Here we explored the application of this method to interrogate the complex matrix of serum, but with appropriate and newly validated adaptations of the sample preparation this UHPLC-MS/MS method can be applied to other matrices such as bio-fluids (saliva, follicular fluid, micro-dialysis fluid etc.), tissues (adipose, brain, prostate etc.) and *in-vitro* cell/organ culture experiments.

With possibly over 60 steroids that can be detected in biological samples [51] and the high frequency of patients taking synthetic steroids cross-reactivity of other steroids present in the sample needs to be excluded. As part of our validation, we established that the assay cannot be applied to serum from patients with intake of the synthetic steroids prednisone, prednisolone and dexamethasone and excluded cross reactivity of some endogenous steroids present in serum at high concentrations. In addition, in order to detect possible cross-reactivity in each sample run our data processing pipeline automatically generates ion-ratio error indicators for every analyte.

Our assay has limitations that might be considered to hamper its use in some laboratories: It uses liquid–liquid extraction, which is inexpensive, but time-consuming and labour-intensive, when performed manually, and can be challenging to automate. This assay can however be adjusted for an extraction technique with higher throughput and better potential for (semi)automation like supported liquid extraction or C_{18} solid phase extraction, used with satisfying results by other serum multi-steroid assays [14,16,32]. Moreover, post-column infusion as a mode of additive delivery is not available on all mass spectrometers, where this is the case external pumps can be purchased to enable post-column infusion.

Finally, not all analytes in our assay have their own stable isotope-labelled internal standard, which is usually considered a prerequisite to appropriately control for matrix effects and extraction losses and to allow for accurate and precise quantification [31,52]. At the time this assay was developed, no internal standards were commercially available for 5α-dione and 11OHT. Therefore, quantification was performed using the internal standard eluting closest to the analyte and our validation data proves this approach to be sufficient, however use of customised deuterium- or ^{13}C-labelled internal standards could improve accuracy and imprecision of these analytes.

5. Conclusions

UHPLC-MS/MS with post-column infusion of ammonium fluoride enables the high throughput profiling of 23 steroids. Use of NH_4F significantly increased sensitivity for most steroids. The method was analytically validated and applied to human serum.

CRediT authorship contribution statement

Lina Schiffer: Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Fozia Shaheen:** Methodology, Validation, Formal analysis, Investigation. **Lorna C. Gilligan:** Methodology, Validation, Formal analysis, Investigation, Writing – review & editing. **Karl-Heinz Storbeck:** Methodology, Writing – review & editing, Funding acquisition. **James M. Hawley:** Methodology, Validation, Writing – original draft, Writing – review & editing. **Brian G. Keevil:** Conceptualization, Validation, Writing – review & editing. **Wiebke Arlt:** Conceptualization, Methodology, Validation, Supervision, Project administration, Funding acquisition. **Angela E. Taylor:** Conceptualization, Methodology, Validation, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary material

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