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Simultaneous measurement of 18 steroids in human and mouse serum by liquid chromatography–mass spectrometry without derivatization to profile the classical and alternate pathways of androgen synthesis and metabolism

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ABSTRACT

Background: The recently identified alternate, or backdoor, pathway of DHT synthesis provides important novel information on androgen biosynthesis beyond the classical pathway. We report a rapid and versatile liquid chromatography–tandem mass spectrometry (LC–MS/MS) method to simultaneously and accurately quantify key steroids in human or mouse serum involved in either the classical or backdoor androgen synthesis pathways.
Methods: Serum (200 µL) fortified with isotopically labelled internal standards underwent liquid–liquid extraction (LLE) with MTBE and extracts were analysed on a LC–MS/MS. The targeted steroids for quantification were testosterone (T), dihydrotestosterone (DHT), 5α-androstane-3α,17β-diol (3α diol), 5α-androstane-3β,17β-diol (3β diol), dehydroepiandrosterone (DHEA), androstenedione (A4), androsterone (AD), estradiol (E2), estrone (E1), progesterone (P4), pregnenolone (P5), androstenediol (Adiol), 17-hydroxyprogesterone (17-OHP4) and 17-hydroxypregnenolone (17-OHP5), corticosterone (B), cortisol (F), allopregnanolone (Allo-P5) and dihydroprogesterone (DHP).
Results: The limits of quantification (LOQ) were 5 pg/mL for E2 and E1, 25 pg/mL for T, 50 pg/mL for A4 and 0.10 ng/mL for DHT, 17OHP5, P4, P5, AD, Adiol, DHEA, AlloP5 and 0.20 ng/mL for 17OHP4, 3α diol, 3β diol, DHP, 0.25 ng/mL for B and 1 ng/mL for F. Accuracy, precision, reproducibility and recovery were within acceptable limits for bioanalytical method validation. The method is illustrated in human and mouse, male and female serum.
Conclusions: The presented method is sufficiently sensitive, specific and reproducible to meet the quality criteria for routine laboratory application for accurate quantitation of 18 steroid concentrations in male and female serum from humans or mice for the purpose of profiling androgen synthesis and metabolism pathways.

1. Introduction

Since the invention of the steroid immunoassay, around 1970 [1,2], antibody-affinity-based methods have been widely used for measurement of bioactive steroids in biological fluids due to their high sensitivity. However, steroid immunoassays are subject to antibody-dependent epitope specificity allowing cross-reaction with structurally related steroids (such as precursors or metabolites) that results in loss of specificity and, often, overestimated levels [3–5]. Additionally, steroid immunoassays require an individually optimized assay for each steroid and, for many precursors and metabolites of bioactive steroids, specific immunoassays have never been widely available. These limitations have been increasingly recognized in scientific position statements [6,7] and editorials in leading endocrinology [8] and reproductive medicine [9] journals.

Steroid mass spectrometry has also been available since the 1970s [10], but the advent of bench-top equipment in recent decades has led to a much wider availability of liquid chromatography – tandem mass

Abbreviations: 17OHP4, 17-hydroxyprogesterone; 17OHP5, 17hydroxypregnenolone; 3α diol, 5α-androstane-3α17β-diol; 3β diol, 5α-androstane-3β17β-diol; A4, androstenedione; AD, androsterone; Adiol, androstenediol; AlloP5, allopregnanolone; B, corticosterone; DHEA, dehydroepiandrosterone; DHP, dihydroprogesterone; F, cortisol; T, testosterone; DHT, dihydrotestosterone; E1, estrone; E2, estradiol; P4, progesterone; P5, pregnenolone; IS, internal standard; LOD, lower limit of detection; LOQ, lower limit of quantification; ME, matrix effect; MTBE, methyl *tert*-butyl ether; CSP, Charcoal Stripped Plasma; APPI, atmospheric pressure photoionization; NMI, National Measurement Institute; S/N, signal-to-noise ratio

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spectrometry (LC–MS/MS), which has become the method of choice for steroid measurement in clinical research and practice [11,12]. LC–MS/MS combines reference level specificity with high sensitivity, matching or exceeding the best steroid immunoassays. Furthermore, steroid mass spectrometry has long been available [10] to provide multi-analyte capabilities that provide steroid profiling from a single sample, thereby facilitating greater insight into complex steroid pathway fluxes than available through the measurement of individual steroids by multiple immunoassays, where they exist. For example, the spectrum of diagnostically important metabolites in a serum steroid profile is particularly helpful for correct differential diagnosis of non-classical forms of complex steroid disorders, such as congenital adrenal hyperplasia [13] or other disorders of sexual differentiation [14,15]. However, steroid analysis by LC–MS/MS requires efficient sample preparation [16,17] and chromatographic separation in order to reduce matrix effects [18–20] and to resolve structurally similar (isobaric) molecules [21,22].

Characterizing androgen action in humans and other mammals requires accurate measurement of the two potent natural endogenous androgens, T and DHT. The major route of androgen synthesis in males is the classic, or canonical, pathway that involves the Δ4 steroids P4, 17OHP4 and A4, but recent discoveries have highlighted alternate "backdoor" routes to synthesize DHT that bypass the usual Δ 4 intermediates, including T (Fig. 1) [23,24]. Even 5 decades after the discovery of DHT [25] as the most potent natural androgen [26], the lack of specific DHT immunoassays with only very few laboratories offering DHT measurement by LCMS has left a dearth of information on its biology [27], illustrated by the recent discovery of multiple pathways of DHT synthesis [23,28,29] and novel findings on the human health impacts of DHT measurement [30–32]. Hence, to characterize the flux and the variations of steroid synthesis involving both classical and alternative backdoor pathways, it is necessary to measure the key intermediate steroids simultaneously. On the occasion of the US Endocrine Society's Fred Conrad Koch Lifetime Achievement Award to Walter L Miller, he highlighted, as a high priority that much needs to be learned about, the backdoor pathway of androgen synthesis and how it integrates into a more complete picture of male and female development, as well as pathophysiology of reproductive biology and medicine [33]. To help meet this need we extended our LC–MS method [34] to measure additional key steroids to characterize the classical and alternate backdoor pathways for T and DHT synthesis. This stable-isotope dilution LC–MS method uses atmospheric pressure photoionization (APPI) to quantify circulating levels of T and DHT, as well as E2, E1, 3α diol, 3β diol, DHEA, F, B, 17OHP4, 17OHP5, A4, P4, P5, AD, Adiol, AlloP5 and DHP from liquid:liquid extracts of 200 μL of human or mouse serum. Sample preparation is rapid and efficient while the LC–MS method is sensitive, specific, and robust, meeting all relevant laboratory quality criteria for routine use.

2. Materials and methods

Steroid reference material was obtained from different sources. T, DHT, 3αDiol, 3βDiol, DHEA, A4 and AD were obtained from the National Measurement Institute (NMI; Sydney, Australia). E2, E1 and P4 were obtained from Cerillant (Round Rock, Texas, USA). P5, Adiol, 17-OHP4 and 17-OHP5, F, Allo-P5 and DHP were obtained from

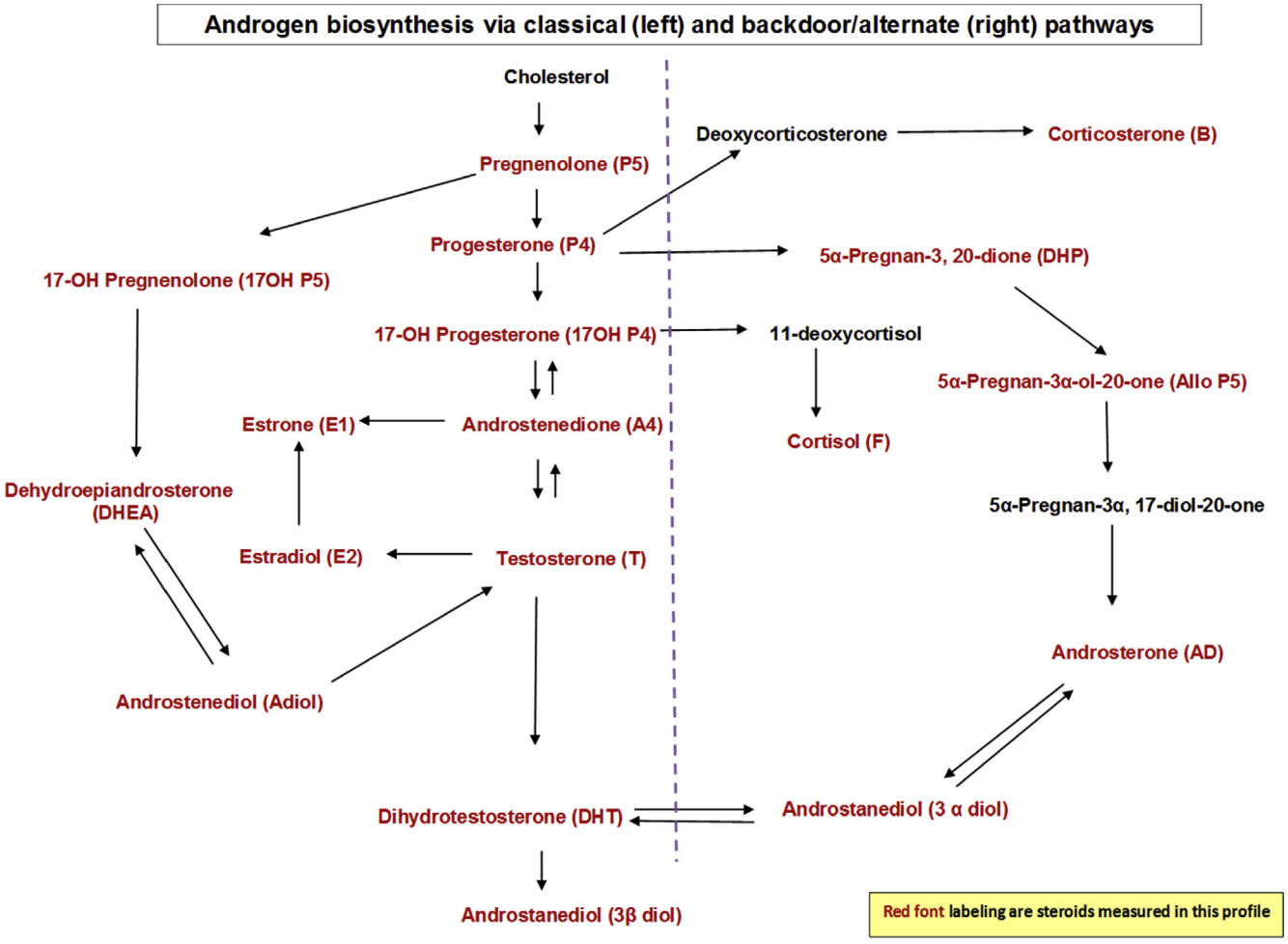

Fig. 1. DHT biosynthesis via the "backdoor" and "classical" pathway.

Table 1
Optimized settings for MRM transitions of steroids quantified. The declustering potential (DP), entrance potential (EP) and exit potential (CXP) were 80, 10 and 12 in positive mode and −80, −10 and −12 in negative mode, respectively, for all analytes.

	Steroid	Ionization mode	RT (min)	Precursor Ion	Quantifier MRM transition (Q1 → Q3)	CE (Quantifier ion)	Qualifier MRM transition (Q1 → Q3)	CE (Qualifier ion)
1	F	+APPI	5.04	[M + H]+	363.2 → 121.3	35	363.2 → 327.4	30
	d4-F	+APPI	4.99	[M + H]+	367.3 → 121.3	45	367.3 → 331.4	30
2	E2	−APPI	6.87	[M − H]−	271.0 → 145.0	−57	271.0 → 183.0	−57
	d4-E2	−APPI	6.87	[M − H]−	275.0 → 147.0	−57	275.0 → 187.0	−58
3	3α Diol	+APPI	9.12	$[M - 2H_2O + H]^+$	257.0 → 161.0	28	257.0 → 175.1	24
	d3-3α Diol	+APPI	9.06	$[M - 2H_2O + H]^+$	260.0 → 164.0	28	260.0 → 178.1	24
4	3β Diol	+APPI	8.83	$[M - 2H_2O + H]^+$	257.0 → 161.0	28	257.0 → 175.1	24
	d3-3β Diol	+APPI	8.8	$[M - 2H_2O + H]^+$	260.0 → 164.0	28	260.0 → 178.1	24
5	17-OHP5	+APPI	8.35	$[M - 2H_2O + H]^+$	297.2 → 104.9	55	297.2 → 159.3	35
	d3-17-OHP5	+APPI	8.3	$[M - 2H_2O + H]^+$	300.3 → 105.2	55	300.3 → 159.2	35
6	B	+APPI	9.45	[M + H]+	347.2 → 121.2	30	347.2 → 311.3	22
	d8-B	+APPI	9.38	[M + H]+	355.2 → 125.1	35	355.2 → 319.2	45
7	Adiol	+APPI	7.88	$[M - 2H_2O + H]^+$	255.3 → 91.1	68	255.3→159.3	28
	d3-Adiol	+APPI	7.82	$[M - 2H_2O + H]^+$	258.3 → 91.1	61	258.3→159.2	30
8	E1	−APPI	10.09	[M − H]−	269.1 → 144.9	−53	269.1 → 143.0	−75
	d4-E1	−APPI	10.02	[M − H]−	273.2 → 147.1	−53	273.2 → 145.0	−80
9	T	+APPI	11.66	[M + H]+	289.0 → 109.0	35	289.0 → 96.1	36
	d3-T	+APPI	11.56	[M + H]+	292.0 → 109.0	35	292.0 → 96.2	36
10	DHT	+APPI	12.61	$[M - 2H_2O + H]^+$	273.0 → 123.0	31	273.0 → 255.3	29
	d3-DHT	+APPI	12.51	$[M - 2H_2O + H]^+$	276.0 → 123.0	31	276.0 → 258.4	22
11	DHEA	+APPI	11.08	$[M - 2H_2O + H]^+$	253.1 → 197.1	30	253.1 → 167.2	59
	d2-DHEA	+APPI	11.03	$[M - 2H_2O + H]^+$	255.2 → 197.1	30	255.2 → 167.2	60
12	17-OHP4	+APPI	11.69	[M + H]+	331.3 → 97.1	37	331.3 → 109.1	39
	d8-17-OHP4	+APPI	11.62	[M + H]+	339.4 → 100.2	37	339.4 → 113.2	40
13	AD	+APPI	11.93	$[M - 2H_2O + H]^+$	255.3 → 199.1	29	255.3 → 105.1	48
	d4-AD	+APPI	11.85	$[M - 2H_2O + H]^+$	259.3 → 203.4	28	259.3 → 173.2	30
14	A4	+APPI	13.27	[M + H]+	287.1 → 97.1	34	287.1 → 109.1	36
	d3-A4	+APPI	13.15	[M + H]+	290.1 → 100.1	34	290.1 → 109.1	36
15	P5	+APPI	14.26	$[M - 2H_2O + H]^+$	281.1 → 171.1	35	281.1 → 156.3	55
	d4-P5	+APPI	14.16	$[M - 2H_2O + H]^+$	285.1 → 175.1	35	285.1 → 160.2	50
16	**AlloP5	+APPI	14.55	$[M - 2H_2O + H]^+$	305.5 → 135.2	28	305.5 → 283.2	22
17	P4	+APPI	17.34	[M + H]+	315.3 → 97.1	34	315.3 → 109.1	37
	d9-P4	+APPI	17.14	[M + H]+	324.3 → 100.1	34	324.3 → 113.1	37
18	**DHP	+APPI	18.27	[M + H]+	317.3 → 123.2	32	299.4 → 189.1	29

** For DHP the internal standard d9-P4 and for AlloP5 the internal standard was d4-P5 used for quantitation purposes as the deuterated internal standards for these 2 compounds were not available.

Steraloids (Newport, Rhode Island, USA) and B from Sigma-Aldrich (St Louis, MO, USA)/Merck (Kenilworth, New Jersey, USA). The deuterium-labeled internal standards, d3-testosterone (d3-T), d3-dihydrotestosterone (d3-DHT), d3-5α-Androstane-3α,17β-diol (d3-3αDiol), d3-5α-androstane-3β,17β-diol (d3-3βDiol), d2-dehydroepiandrosterone (d2-DHEA) and d3-androstenedione (d3-A4) were from NMI; d4-estradiol (d4-E2) was from Cambridge Isotope Laboratory (Andover, MA, USA); d9-androsterone (AD), d3-androstenediol (d3-Adiol) and d4-estrone (d4-E1) were from Steraloids, d9-progesterone (d9-P4), d4-pregenenolone (d4-P5), d8-17 hydroxyprogesterone (d8-17OHP4), d4-Cortisol (d4-F) and d3-17 hydroxypregnenolone (d3-17OHP5) were from CDN isotopes (Pointe-Claire Quebec, Canada), and d8-corticosterone (d8-B) was from Sigma-Aldrich. For DHP and Allo-P5, deuterated isotopes were not available, so we used d9-P4 and d4-P5, respectively, for calibration and quantitation purposes.

HPLC grade methanol and toluene were purchased from Lab-Scan Analytical Sciences (Dublin, Ireland). Autosampler microtitreplates were from Perkin Elmer (Waltham, Massachusetts, USA). Water of 18-MΩ quality was prepared by a Millipore Milli-Q system (Bedford, MA, USA). Lyophilized BSA (Cat #A7906) was purchased from Sigma-Aldrich/Merck, HPLC-grade toluene and premium-grade methyl *tert*-butyl ether (MTBE) and methanol were from RCI LabScan (Bangkok, Thailand). Serum and charcoal-stripped plasma used for the QC and validation samples were produced from pooled human serum or plasma (Concord Hospital blood bank) with charcoal-stripped plasma produced in-house by mixing human plasma with activated charcoal (Norit A, BDH Chemicals, London, England, UK) 50 mg/1 mL), stirring overnight at room temperature, centrifuging at 2700 × *g* for 10 min and successive filtering with 0.45 μm and 0.22 μm polyethersulfone filters until the filtrate was clear.

3. Preparation of calibration standards

Stock solutions of each unlabelled steroid were prepared gravimetrically by weighing out powder on a four-point balance, followed by dissolution in methanol. A working profile calibrator solution containing all of the monitored steroids was prepared by mixing appropriate volumes of individual steroid stock with methanol to give a final concentration; 1600 ng/mL T and DHT, 80 ng/mL E2 and E1, 800 ng/mL Adiol, 3αDiol, 3βDiol and 17-OH P4, 3200 ng/mL P4 and DHEA, 400 ng/mL P5, 17-OHP5, A4, AD, AlloP5 and DHP, 16,000 ng/mL B and 32,000 ng/mL F. Eight-point calibration curves (excluding zero and blank) were prepared by diluting the working profile calibrator solution with 4% bovine serum albumin (w/v) prepared in phosphate buffered saline. Aliquots (200 μL) of calibrators and quality control (QC) samples were stored in plastic tubes at −80 °C and thawed immediately before use. QC samples were prepared in charcoal-stripped plasma (CSP) at low, medium and high levels. Internal standard (IS) stock solutions were prepared in methanol, and to obtain a working solution were diluted with 20% (v/v) methanol: water (5 ng/mL d3-T, d9-P4, d8-17-OHP4, d4-P5, d3-A4, d8-B and 8 ng/mL d3-Adiol, d3-DHT, d9-AD d2-DHEA, d4-F; 10 ng/mL d3-17-OHP5, d3-3αdiol, d3-3βdiol, d4-E2 and d4-E1.

Table 2
Limit of detection (LOD), limit of quantification (LOQ) and detectability (> LOD) for steroids in human and mouse serum from males and females.

Analytes	LOD ng/ml	LOQ ng/ml	% detectable* Human (M/F)	% detectable# Mouse (M/F)
T	0.01 (0.035 nM)	0.025 (0.087 nM)	100/100	100/88
DHT	0.05 (0.172 nM)	0.1 (0.344 nM)	75/94	47/33
DHEA	0.05 (0.173 nM)	0.1 (0.347 nM)	100/100	27/9
A4	0.025 (0.087 nM)	0.05 (0.175 nM)	100/100	40/48
3α diol	0.05 (0.171 nM)	0.2 (0.684 nM)	77/100	42/20
3β diol	0.05 (0.171 nM)	0.2 (0.684 nM)	77/100	16/13
17OH-P4	0.05 (0.151 nM)	0.2 (0.605 nM)	100/100	36/43
P4	0.05 (0.159 nM)	0.1 (0.318 nM)	58/0	79/100
F	0.25 (0.690 nM)	1.0 (2.759 nM)	100/100	~
B	0.1 (0.289 nM)	0.25 (0.722 nM)	~	100/100
17OH-P5	0.05 (0.150 nM)	0.1 (0.301 nM)	54/100	59/60
Adiol	0.05 (0.171 nM)	0.1 (0.342 nM)	100/100	52/24
AD	0.05 (0.172 nM)	0.1 (0.344 nM)	54/100	11/4
P5	0.05 (0.158 nM)	0.1 (0.316 nM)	100/79	32/37
AlloP5	0.05 (0.157 nM)	0.1 (0.314 nM)	100/74	60/74
DHP	0.1 (0.316 nM)	0.2 (0.632 nM)	84/23	97/78
E2	0.0025 (0.009 nM)	0.005 (0.018 nM)	100/100	Below LOQ
E1	0.0025 (0.009 nM)	0.005 (0.018 nM)	100/100	Below LOQ

~ Cortisol (F) was measured in human sera while corticosterone was measured in mouse samples.
Conversion factor to multiply ng/ml (all non-estrogen steroids) or pg/ml (estradiol, estrone) to get SI units were T (3.47); DHT (3.44); DHEA (3.47); A4 (3.49); 3α diol (3.42); 3β diol (3.42); E2 (3.67); E1 (3.70); 17OHP4 (3.03); P4 (3.18); F (2.76); B (2.89); 17OHP5 (3.01); Adiol (3.44); AD (3.44); P5 (3.16); AlloP5 (3.14) and DHP (3.16).
* Based on 13 healthy young men (M) and 19 healthy young women (F).
# Based on 73 male (M) and 46 female (F) mice.

4. Serum samples and preparation

Aliquots (200 µL) of thawed serum, standards or QC samples were transferred into 5 mL glass tubes. To this was added 50 µL of the deuterated steroid IS solution prepared in 50% (v/v) methanol: water (d3-T, d3-DHT, d9-P4, d8-17OHP4, d4-P5, d3-A4, d4-AD, d3-Adiol, d3-17OHP5 at 0.25 ng/mL; d4-E2, d4-E1, d3-3α Diol, d3-3β Diol at 0.15 ng/mL; d2-DHEA at 0.4 ng/mL; d8-B and d4-F at 0.5 ng/mL). The samples were vortex mixed and left at 4 °C for 15 min before addition of 1 mL MTBE followed by vigorous mixing for 1 min to extract steroids into the organic solvent (upper layer). Tubes were then covered with parafilm and allowed to stand at 4 °C for 1 h to allow phase separation. Tubes were then placed in a −80 °C freezer for 30 min to freeze the lower aqueous layer, with the upper organic layer then decanted into clean glass tubes. The solvent extracts, maintained at 40 °C, were allowed to evaporate in a fume hood overnight. For analysis, the dried samples were resuspended in 75 µL of 20% (v/v) methanol:water. Tubes were mixed for 1 min and the entire volume was then transferred into the well of a 96-well microtitre plate. A 50 µL aliquot was injected for LC–MS/MS analysis.

5. LC–MS/MS conditions

The LC–MS analysis was based on reversed-phase chromatographic separation of the injected sample using ultra-pressure liquid chromatography (UPLC) followed by gradient elution and detection of the 18 targeted steroids using triple quadrupole mass spectrometric analysis. The liquid chromatography system was a Shimadzu Nexera UHPLC (Shimadzu Scientific Instruments, Columbia, MD, USA) with a Restek Raptor biphenyl column (100 cm × 2.1 mm, 2.7 µm; cat#9309A12) with Raptor biphenyl guard cartridge (cat# 9309A0252). The solvents used were; A: Milli-Q water, B: methanol, and C: toluene (dopant). The chromatographic conditions used comprised of initially as 10% B then 0.11–2.0 min 25–50% B, 2.01–10.0 min 50–60% B, 10.01–13.5 min 65–69% B, 13.51–18.0 min 69–75% B, 18.01–20.0 min 85–95% B, then 100% B until 20.5 min and then back to 10% B for 1 min. A flow rate of 0.7 mL/min was used and the total run time was 21.5 min. The column temperature was maintained at 40 °C and the auto-sampler was held at 4 °C.

An API-5000 triple-quadruple mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario, Canada) equipped with an atmospheric pressure photoionization (APPI) source was operated in both positive and negative ion modes for the analysis. The APPI system consisted of a 10 eV krypton discharge lamp with dopant (toluene) delivery set to 75 µL/min. Nitrogen and zero grade air were supplied using a PEAK A320DR/NM20ZL unit (Peak Scientific Instruments, Renfrewshire, Scotland). Nitrogen was used for curtain gas (12), ion source gas 1 (55), ion source gas 2 (50) and collision gas (6). The APPI probe temperature was 500 °C and the ion spray voltage was set to 750 V in positive and −750 V in negative mode. Steroids were quantified by multiple reaction monitoring (MRM) using settings for the various transitions optimized by infusing pure steroid into the mass spectrometer. Unit mass resolution was used in both mass-resolving quadruples Q1 and Q3. One qualifier and quantifier ion was optimized for each analyte. All MRM transitions and other instrument parameters are in Table 1. Data acquisition and processing was performed with Analyst 1.6.2 (AB SCIEX). Peak area ratios of analyte and IS quantifier transitions were calculated as a function of analyte concentration. Calibration curves and limits of quantification (LOQ) were defined according to the FDA guidance [35].

6. Method validation

Reproducibility was investigated for each steroid quantified at three QC levels in CSP with each analysed in replicate to determine within-day (n = 6) and between-day (n = 3 on 5 different days) precision. Limits of detection (LOD) and lower limits of quantification (LOQ) were determined by adding the analytes at progressively lower levels to steroid-free serum and analysing samples in replicate (n = 6). The limit of detection (LOD) was defined as the lowest analyte concentration giving a minimum signal-to-noise ratio (S/N) > 3:1 in replicate, and the LOQ as the lowest concentration with a CV < 20% and accuracy within ± 20% of the target concentration. The standard curves were generated from five independent runs (n = 5) and the mean correlation coefficients (R) for each analyte were evaluated from multiple curves.

Accuracy was assessed by spiked absolute recovery from pooled serum (containing endogenous steroids) spiked with known amounts of steroid, as mentioned in Table 3. Absolute recovery was estimated by [(final observed concentration − initial concentration)/spiked concentration] (spiked level) at three QC levels and expressed as a percentage. The recoveries for all 18 target steroids were investigated separately for mouse and human serum.

The extraction recovery was studied by comparing pooled serum samples that were spiked pre-and post-extraction, with spiking post extraction corresponding to 100% extraction recovery.

Matrix effect was also studied in the same experiment, but by comparing post-spiked extracted serum with neat standard solution

Table 3
Accuracy, Precision and Recovery.

Steroid	Nominal concentration (ng/mL)	Within Day (n = 5)		Between day (n = 15)		Recoveries		Equation fit
		Accuracy %	Precision %CV	Accuracy %	Precision %CV	Human serum %	Mouse serum %	
E2	0.02	95	10	92	13	86	84	Linear
	0.08	109	8	103	10	88	87	
	0.4	104	5	98	7	89	89	
E1	0.02	99	6	93	8	85	85	Linear
	0.08	108	8	105	11	92	87	
	0.4	109	4	106	7	93	89	
T	0.4	110	8	104	6	97	96	Quadratic
	1.6	106	6	101	3	95	93	
	8	108	2	94	5	93	92	
DHT	0.4	92	13	89	11	86	90	Linear
	1.6	108	9	101	10	92	94	
	8	109	7	103	9	97	88	
3α diol	0.2	108	11	103	8	94	94	Linear
	0.8	111	7	105	5	99	92	
	4	108	5	102	9	92	92	
3β diol	0.2	95	12	98	9	93	94	Linear
	0.8	109	8	105	5	102	89	
	4	111	6	103	2	92	91	
DHEA	0.8	101	7	114	6	90	98	Quadratic
	3.2	108	6	114	3	89	98	
	16	103	4	111	3	91	97	
F	8	111	12	108	13	89	86	Quadratic
	32	110	9	105	12	99	89	
	160	109	13	109	11	94	90	
B	4	89	11	92	12	94	91	Quadratic
	16	88	11	85	9	98	90	
	80	97	8	85	7	92	87	
17OH P5	0.1	109	9	112	7	102	90	Linear
	0.4	92	6	89	11	98	88	
	2	91	3	94	2	91	90	
17OH P4	0.2	94	13	97	9	103	88	Linear
	0.8	96	11	92	11	104	89	
	4	89	7	85	9	89	88	
A4	0.1	107	8	102	11	99	90	Linear
	0.4	107	6	92	9	97	106	
	2	108	4	98	2	96	89	
AD	0.1	111	4	107	8	89	94	Linear
	0.4	106	8	102	11	90	97	
	2	101	3	97	4	88	98	
P4	0.8	101	11	114	7	100	102	Quadratic
	3.2	108	2	105	9	99	100	
	16	105	6	98	7	92	108	
P5	0.1	92	8	85	9	103	107	Linear
	0.4	103	10	112	11	92	98	
	2	109	7	97	6	91	88	
Adiol	0.4	90	12	94	9	92	90	Linear
	1.6	95	7	98	6	96	93	
	8	105	3	103	5	94	97	
AlloP5	0.1	89	7	86	9	85	83	Linear
	0.4	101	7	97	8	88	86	
	2	101	2	93	5	94	92	
DHP	0.2	95	4	98	7	86	85	Linear
	0.4	93	9	89	11	88	87	
	2	90	3	94	6	86	102	

without matrix.

Selectivity for structurally similar isobaric pairs of steroids (e.g., T and DHEA; AD and DHT, P5 and DHP) was ensured by chromatographic resolution and was verified by analysis of neat solutions of these pairs of steroids. In addition, 21 hydroxy progesterone did not interfere with 17OHP4. Interference was suspected if the peak area ratio of quantifier to qualifier transitions deviated more than ± 20% from the mean ion ratio calculated from the standards.

Stability was investigated by the mean of triplicate analysis of QCs at three concentration levels after storage under different conditions; ambient temperature for 48 h and frozen at −20 °C for 6 months and reconstituted samples for at least 24 h at 4 °C.

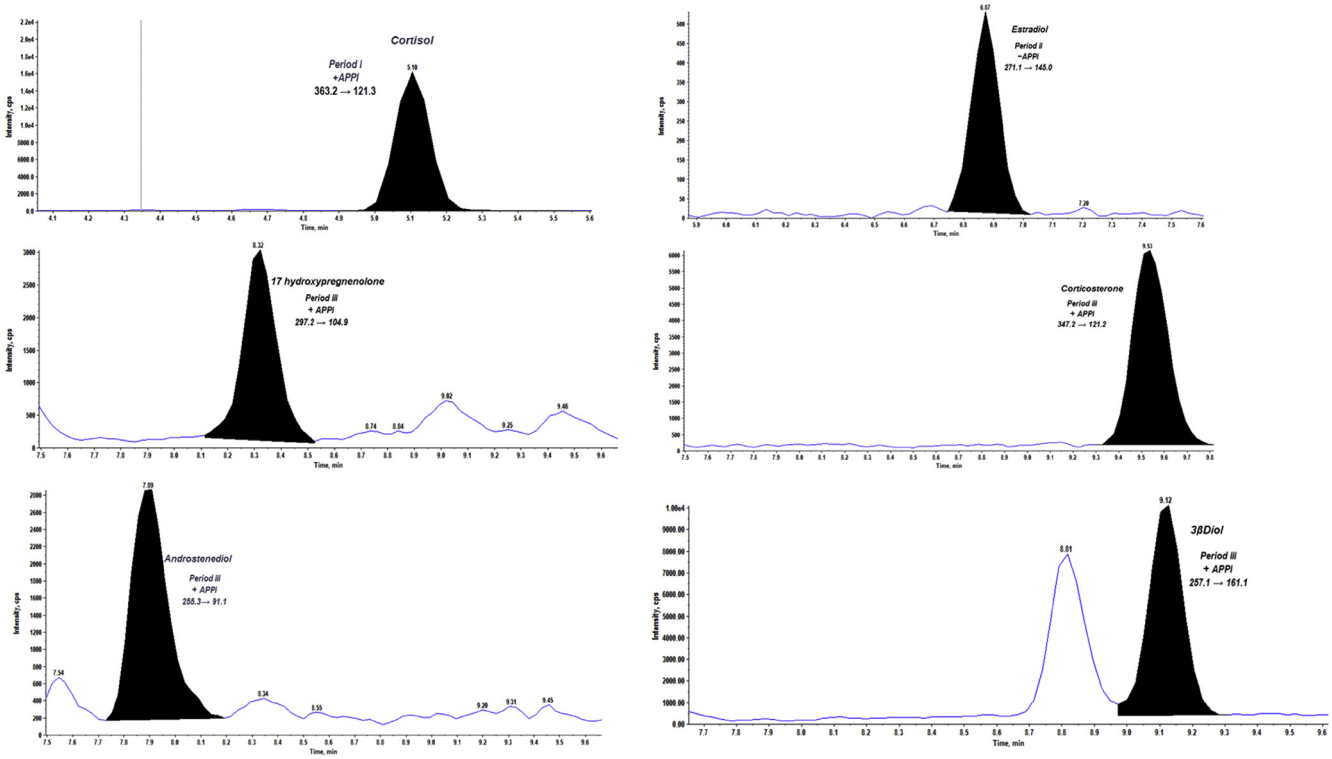

Fig. 2. Extracted ion chromatograms of target steroids from a liquid–liquid extracted (LLE) at LOQ for all compounds. The LC–MS/MS analysis was divided into seven periods to allow analysis of estrogens by −APPI (Period II and IV) and androgens by +APPI (Period I, III, V, VI and VII).

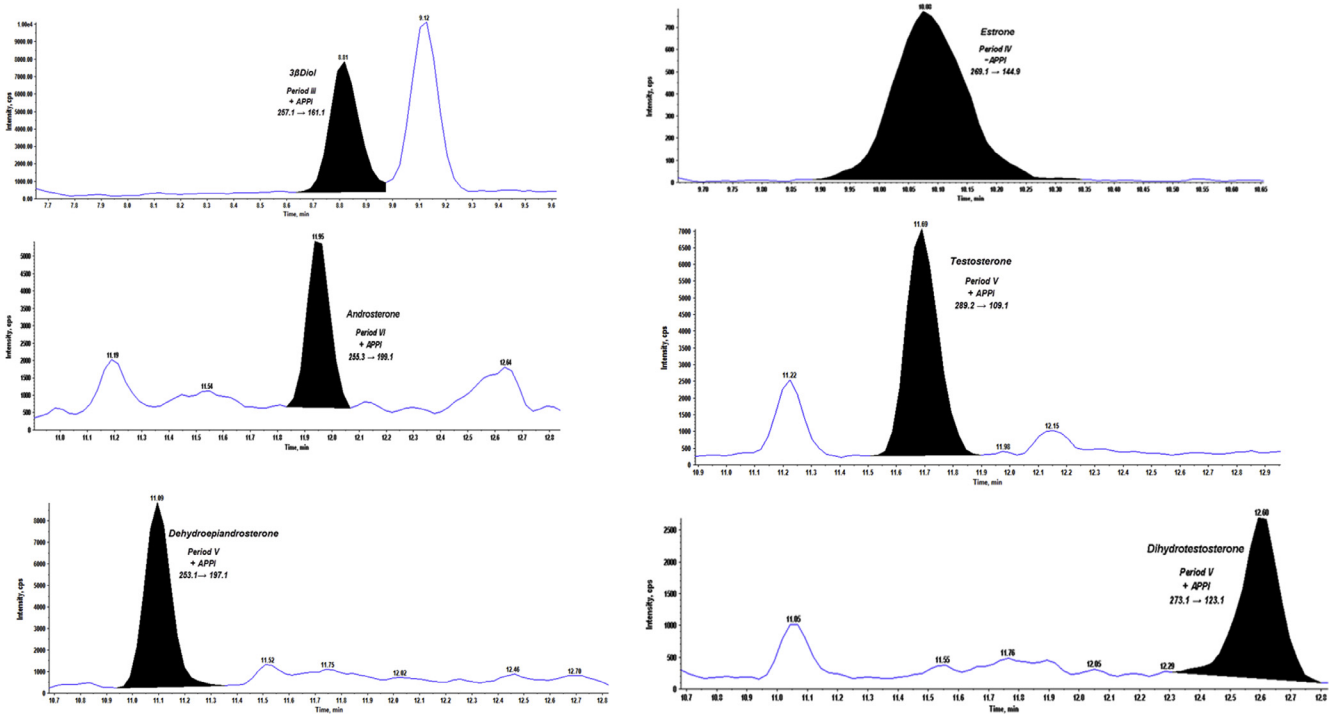

Fig. 2. (*continued*)

Freeze–thaw stability was determined after two cycles of freezing to −20 °C. Carry-over was examined by injecting a solvent blank after the highest calibrant in three runs and comparing the analyte peak areas in the blank with that of the calibrant.

Dilution effect was investigated to ensure that samples could be diluted with blank matrix without affecting the final concentration. Steroid-spiked human and mouse serum samples prepared at two concentrations (2× and 4× ULOQ) for respective steroids were diluted with steroid free serum for human and pooled mouse serum at dilution factors of 5 and 10 in six replicates and analyzed. As part of the validation, the replicates had to have both a precision and accuracy of ± 15%.

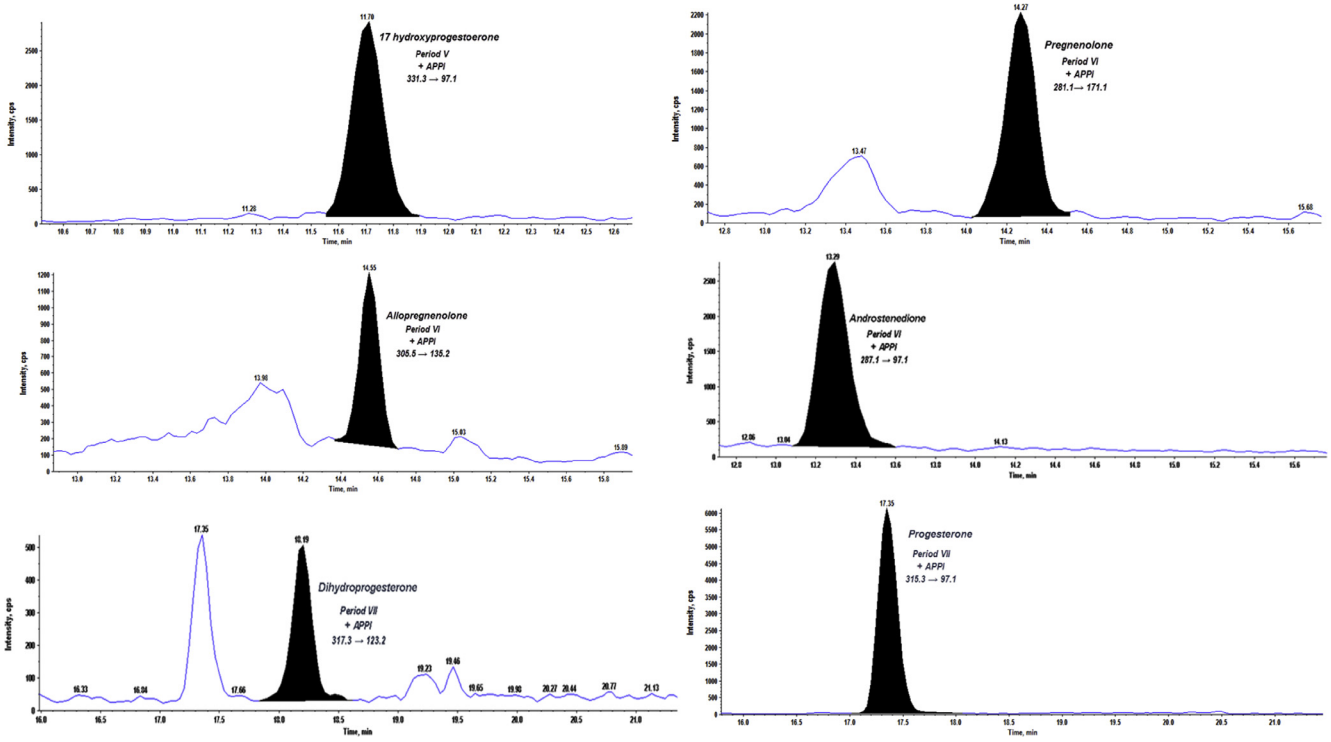

Fig. 2. (*continued*)

7. Serum samples

Stored serum samples were obtained from previous human and mouse studies. The clinical studies provided 19 samples from individual healthy young female controls without polycystic ovary syndrome (PCOS) from an observational study of PCOS [36] and 13 baseline (pre-treatment) samples from healthy eugonadal young men [37]. Mouse serum samples (n = 119; 41 female, 73 male) were obtained from littermates in the ANZAC Research Institute's animal facility [38].

8. Results

8.1. Calibration curve and quantification range

Linear calibration curves were fitted for target compounds across the concentration range tested using a weighted (1/×) regression for E2 (2.5–800 pg/mL), E1 (2.5–800 pg/mL), DHT (0.05–16 ng/mL), 3β diol (0.05–8 ng/mL), 3α diol (0.05–8 ng/mL), 17OH P5 (0.05–16 ng/mL), A4 (0.025–16 ng/mL), AD (0.05–16 ng/mL), P5 (0.05–16 ng/mL), AlloP5 (0.05–16 ng/mL) and DHP (0.1–16 ng/mL). A weighted (1/×) quadratic fit, which produced a superior goodness of fit, was used for T (0.01–64 ng/mL), DHEA (0.05–64 ng/mL), F (0.25–160 ng/mL), B (0.1–160 ng/mL), 17OHP4 (0.05–32 ng/mL), P4 (0.05–64 ng/mL) and Adiol (0.05–32 ng/mL). The deviation of the calibration standards from their nominal concentrations at the LOQ was always less than 20% and less than 15% at all other concentration levels. The standard curves generated from five independent runs had mean correlation coefficients (R) between 0.9991 and 0.9999 for measured analytes.

8.2. Sensitivity

The LOD and LOQ for each analyte (Table 2) were determined by adding the analytes at progressively lower levels to steroid-free serum and analysing samples in replicate (n = 6). The LOQ had precision of less than 20% and accuracy of 80–120%. The sensitivity for E2 and E1 was not sufficient to allow detection in mouse serum, but was suitable for human serum. F was not detected in mouse samples and B in human samples. For all other steroids we have clinically relevant sensitivity for both mice and human serum.

8.3. Extraction recovery for accuracy and matrix effect

The amount of analyte recovered after extraction and processing of the samples, represents the extraction recovery of an analytical procedure. The recovery of all steroids in mouse and human serum was determined to evaluate the measurement accuracy of the analytes spiked in serum.

Six replicates of pooled serum samples at three QC levels were evaluated for each steroid separately for mouse and human serum. Good absolute recoveries were observed for all 18 steroids at the concentrations tested, with accuracy ranging between 85 and 104% for human serum and 83–108% for mouse serum samples (Table 3).

The extraction recoveries ranging between 80 and 98% for human serum and 77–94% for mouse serum. No clear ion suppression or enhancement was observed for any of the targeted analytes during matrix effect analysis. The matrix effect % ranged from 86 to 97% for human serum and 81 to 92% for mouse serum.

8.4. Precision

For all steroids, the within-run accuracy was 88–111% with CVs between 2 and 13%. Between-run accuracy was 85–114% with CVs between 2 and 13% (Table 3).

8.5. Stability and carry-over

Stock solutions of all steroids were stable for at least twelve months at −20 °C and working standard solutions were stable for at least six months at −20 °C when compared with freshly prepared solutions. In addition, the QCs analysed for all monitored steroids were robust (± 15% of nominal levels) to at least two freeze-thaw cycles, after 48 h of storage at ambient temperature and reconstituted samples were stable for at least 24 h at 4 °C when compared with freshly prepared calibrators and QCs.

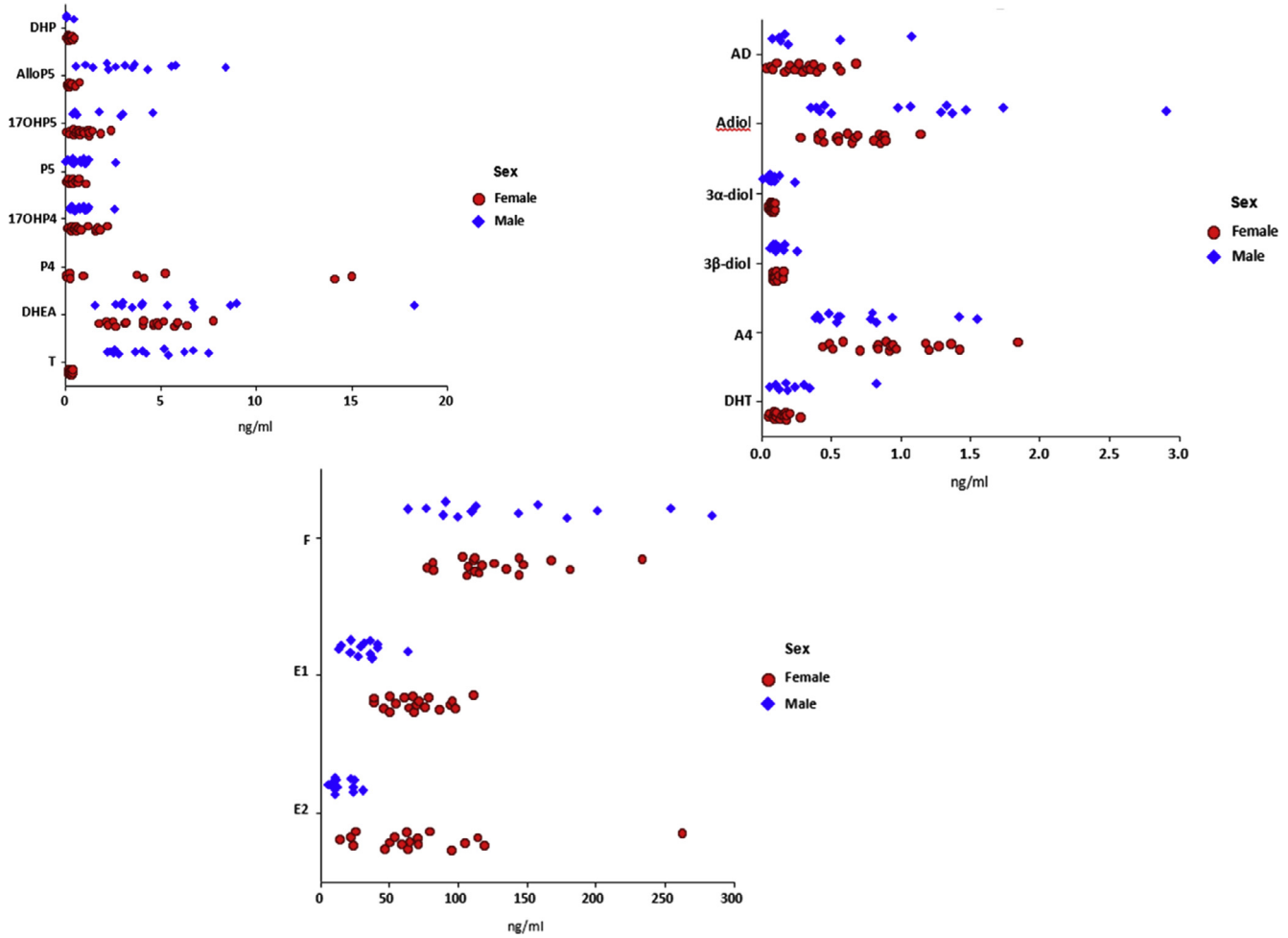

Fig. 3. Dot plots of human serum in males (blue diamonds, n = 13) and female (pink circles, n = 19) for the following steroids (lower panel) cortisol (F), estrone (E1) and estradiol (E2), (upper left panel) dihydroprogesterone (DHP), allopregnanolone (Allo-P5), 17-hydroxypregnenolone (Hydroxy P5), pregnenolone (P5), 17-hydroxyprogesterone (Hydroxy P4), progesterone (P4), dehydroepiandrosterone (DHEA), testosterone (T), (upper right panel) androsterone (AD), androstenediol (Adiol), 5α-androstane-3β,17β-diol (A 3β diol), 5α-androstane-3α,17β-diol (A 3α diol), androstenedione (A4), dihydrotestosterone (DHT).

No carry-over was detected when a blank solvent was injected after the highest calibrator.

8.6. Selectivity

For each pair of isobaric steroids (i.e., T and DHEA, AD and DHT, P5 and DHP), baseline chromatographic separation was observed indicating that each steroid would not interfere with the quantification of its isobaric pair (Fig. 2). The quantifier: qualifier peak area ratio was within ± 20% of the expected value for all the analytes.

8.7. Dilution integrity

Dilution integrity tested on six replicates of two- (2× ULOQ) and four- (4× ULOQ) dilutions. The accuracy and precision of all diluted levels was 90–104% with CVs between 6 and 9% for human serum, and 87–107% with CVs between 4 and 12% for mouse serum. The results show that the method is accurate, precise, and reproducible in assays of diluted samples.

8.8. Application

We analyzed serum samples from humans (n = 33, Fig. 3, Supplementary Table-1) and mice (n = 119, Fig. 4, Supplementary Table-2) to show feasibility and sensitivity (Table 2) of our method for serum from both males and females. All steroids were measured for both sexes other than the species-specific predominant glucocorticoid B for mouse and F for humans. For each steroid, the conversion factor to multiply ng/ml (all non-estrogen steroids) or pg/ml (E2, E1) to get SI units was T (3.47); DHT (3.44); DHEA (3.47); A4 (3.49); 3α diol (3.42); 3β diol (3.42); e2 (3.67); e1 (3.70); 17OHP4 (3.03); P4 (3.18); F (2.76); B (2.89); 17OHP5 (3.01); Adiol (3.44); AD (3.44); P5 (3.16); AlloP5 (3.14) and DHP (3.16).

9. Discussion

We report here a general profiling method for the analysis of 18 steroids by LC–MS/MS in MRM mode that can be used for both human and mouse serum. The species-specific B (Corticosterone) was not measured in human samples and F (Cortisol) was not measured for mouse samples.

Steroid measurement by LC–MS/MS is now widely accepted as the method of choice for quantifying endogenous steroids including bioactive androgens (e.g., T, DHT), as well as their precursors (e.g., including pro-androgens A4, DHEA) and metabolites [11]. It features high levels of specificity, sensitivity and accuracy that is well-served for the role for diagnosis of a wide range of endocrine and reproductive disorders through multi-analyte profiling [13–15]. While many influential papers reporting foundational LC–MS/MS methods have focused on a limited number of steroids [21,39–42], we have developed an

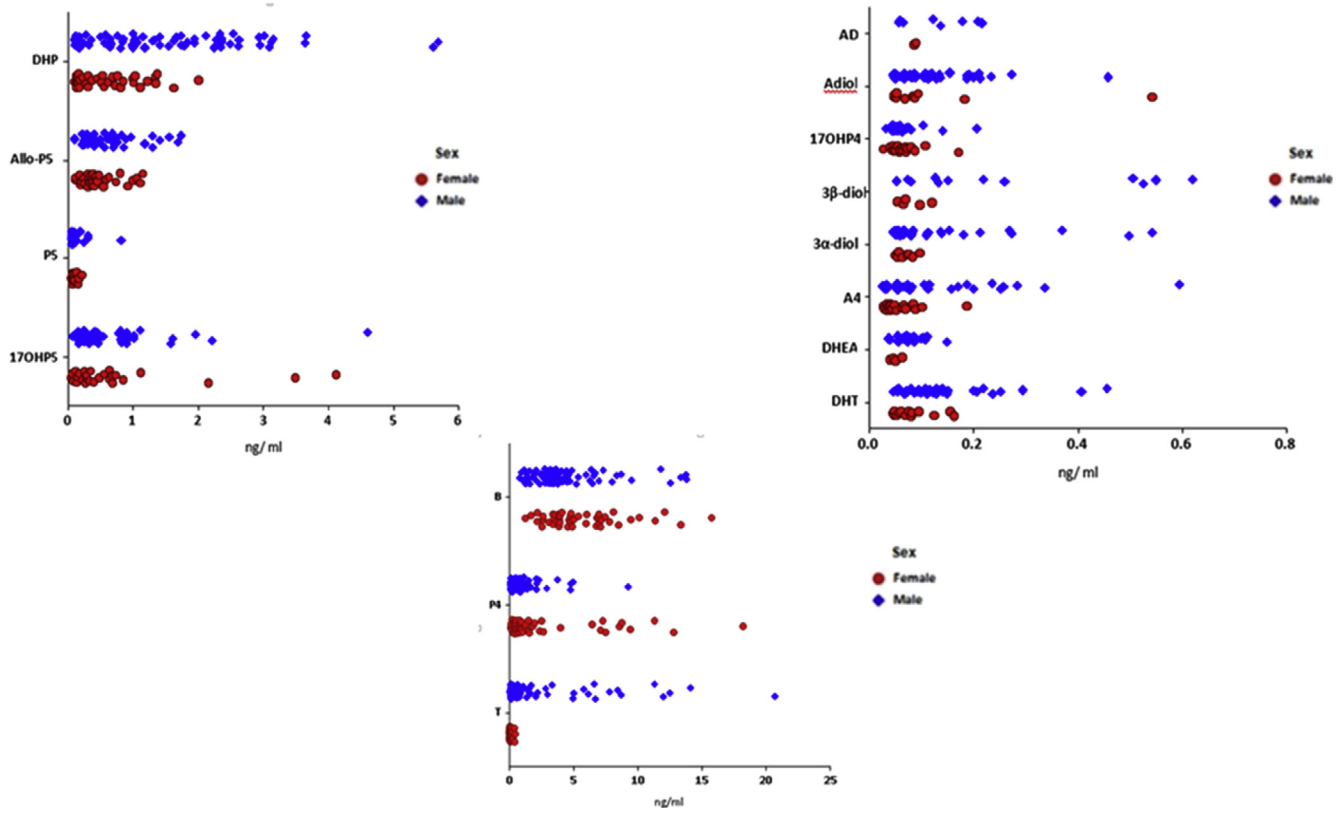

Fig. 4. Dot plots of mouse serum in males (blue diamonds, n = 73) and female (pink circles, n = 41) for the following steroids (lower panel) corticosterone (B), testosterone (T), progesterone (P4), (upper left panel) dihydroprogesterone (DHP), allopregnanolone (Allo-P5), pregnenolone (P5), 17-hydroxypregnenolone (Hydroxy P5), (upper right panel) androsterone (AD), androstenediol (Adiol), 17-hydroxyprogesterone (Hydroxy P4), 5α-androstane-3β,17β-diol (A 3β diol), 5α-androstane-3α,17β-diol (A 3α diol), androstenedione (A4), dehydroepiandrosterone (DHEA), dihydrotestosterone (DHT). Note that for B the values are divided by 20 to fit the scaling on the graph.

extended simple, sensitive, specific and robust method for quantifying a wide range of steroids with a focus on bioactive androgens together with pro-androgens, precursors and metabolites, so as to provide a snapshot of the multiple synthesis and metabolism pathways from a single, small serum sample. This method can measure concentrations of 18 steroids in a single volume of 0.2 mL serum without derivatization using a liquid:liquid extraction followed by chromatographic separation and quantitation using LC–MS/MS (Fig. 2).

This method avoids column-switching techniques [22,43] and derivatization [44–50], and achieves chromatographic separation of all steroids under analysis using a robust single column and HPLC pump setup. Ionization using APPI reduces matrix interferences, while improving ionization and, thereby, sensitivity [40,49,50]. Although, APPI ionisation is not the most popular technique, it has several advantages over conventional ESI and APCI techniques that have been previously described [11,12,50–52], highlighting its propensity to increase sensitivity for non-polar and poorly ionizable molecules, such as steroids, through a more selective ionization process. Our extensive steroid profile includes virtually all intermediates involved in the "classical" and alternate "backdoor" pathway of androgen synthesis and metabolism (Fig. 1). In humans, DHT, generated by the classical pathway, plays an indispensable role in male fetal sexual development, but the emerging role of the backdoor pathway in developing and mature females remains to be fully clarified [23,33,53]. The present study did not evaluate any potential impact on steroid measurement of drug interference, chronic organ failure, pregnancy or altered circulating protein concentrations.

To our knowledge, this is the first method reported to include all androgens and estrogens involved in both the "classical" and "backdoor" androgen synthesis pathway, as well as metabolites of the "backdoor" pathway for the biosynthesis of DHT. Previously, steroids along the alternative backdoor pathway have not been routinely measured in serum samples. The ability to easily collect this information may help explain divergences between virilization and androgen levels seen in clinical practice [53,54].

We conclude that this method is accurate, sensitive, specific and robust enough for simultaneous measurement of 18 steroids without derivatization in human and mouse serum samples. The method exhibited excellent performance in terms of selectivity, linearity, accuracy, precision, recovery, stability, detection limit and quantitation limit, thereby giving confidence in the analytical results generated. The inclusion of AlloP5, DHP, Diols and AD in the method allows an assessment of abnormalities, or variations, in steroid hormone production that result in potentially profound and complex effects.

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Conflict of interest

None of the authors has any conflicts of interest to disclose.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clinms.2018.12.003.

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