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The development and validation of a turbulent flow chromatography–tandem mass spectrometry method for the endogenous steroid profiling of equine serum

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ABSTRACT

A method for the detection and quantitation of 35 endogenous steroids in equine serum was developed and validated. Androgens, estrogens, progestins and their metabolites potentially present in serum were simultaneously monitored in one method using on-line sample extraction by turbulent flow chromatography (TFC) on a 2-dimensional liquid chromatography system and detected on a triple-stage quadrupole mass spectrometer by electrospray ionization. Analytes were detected and quantitated by single-reaction monitoring or selected-ion monitoring. Limits of detection (range 0.025–10 ng mL^{-1}) and quantitation (range 0.125–25 ng mL^{-1}) along with recovery and matrix effects were determined for each analyte. Inter- and intra-day accuracy and precision was assessed for with the majority of analytes having %CV less than 20% and accuracy within 20% of the expected concentrations. Eight of the 35 analytes were unable to meet these guidelines across all of the quality control concentrations monitored for each analyte. This method was used to determine the endogenous steroid profiles of Thoroughbred horses and has been modified for use in non-human primates and cell culture.

1. Introduction

Detecting the abuse of endogenous anabolic androgenic steroids (AAS) such as synthetic testosterone and nandrolone is of great concern in all performance athletes, including horses. The endogenous steroids with a large possibility for abuse in horseracing include androstenedione, testosterone, boldenone, and nandrolone [1–6]. These steroids are naturally produced at varying levels in male and female horses. Detecting their abuse requires the monitoring of a large number of steroids involved in androgen biosynthesis in order to detect any changes in the steroid profile following administration of a synthetic AAS and/or precursors [2,7,8]. Previously, urinary profiling of endogenous steroids has been used to address the abuse of synthetic anabolic steroids and their precursors [7]. This methodology requires extensive sample cleanup steps that limit sample throughput and may cause degradation of unstable metabolites [9]. The use of serum/plasma based assays has recently gained acceptance, but has primarily focused on circulating levels of testosterone, boldenone, and nandrolone in intact male horses and not on determining steroid profiles [10]. To address these concerns, a highly sensitive method for profiling multiple endogenous steroids in equine serum was developed and validated. Endogenous steroids along with their conjugated polar metabolites were simultaneously monitored in one method using on-line sample extraction of diluted serum. This method uses turbulent flow chromatography (TFC) with detection by single-reaction monitoring (SRM) or selected-ion monitoring (SIM) on a triple quadrupole mass spectrometer.

Two-dimensional liquid chromatography (2D-LC) has found success in the separation of multiple classes of analytes and can provide additional selectivity and the ability for online sample extraction from complex biological matrices [11]. TFC has been

Abbreviations: ADD, 1,4-androstadien-3,17-one; 17P5, 17-hydroxypregenolone; 17P4, 17-hydroxyprogrestone; αE2, 17α-estradiol; βE2, 17β-estradiol; E2S, 17β-estradiol sulfate; 19dione, 19-norandrostendione; 19A, 19-norandrosterone; 19EA, 19-norepiandrosterone; Adiol, 5α-androstane-3α,17β-diol; 5αDHN, 5α-dihydronandrolone; 5αDHP, 5α-dihydroprogesterone; 5αDHT, 5α-dihydrotestosterone; 5αE2, 5α-estran-3β,17α-diol; 5βDHT, 5β-dihydrotestosterone; 6αA4, 6α-hydroxyandrostenedione; AP, allopregnanolone; A4, androstenedione; API, atmospheric pressure ionization; APPI, atmospheric pressure photoionziation; APCI, atmospheric pressure chemical ionization; Bold, boldenone; BS, boldenone sulfate; CID, collision induced dissociation; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; ESI, electrospray ionization; ENan, epi-nandrolone; ETest, epi-testosterone; E1, estrone; Ediol, etiocholan-3α-17β-diol; IS, internal standard; LC, liquid chromatography; MS, mass spectrometry; Nan, nandrolone; NG, nandrolone glucuronide; NS, nandrolone sulfate; P5, pregenolone; Pdiol, pregnanediol; P4, progesterone; QC, quality control; SIM, selected-ion monitoring; SRM, single-reaction monitoring; S/N, signal to noise; Test, testosterone; TG, testosterone glucuronide; TS, testosterone sulfate; TFC, turbulent flow chromatography; 2D-LC, two-dimensional liquid chromatography; LOD, limit of detection; LLOQ, lower limit of quantitation; ULOQ, upper limit of quantitation.

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used in the first dimension of 2D-LC systems allowing for on-line sample extraction by combing high flow rates (>2 mL min^{-1}) inside a small column filled with large stationary phase particles (>50 μm) creating a turbulent environment that selectively retains small molecules while allowing macromolecules to pass to waste [12]. Recently, the use of TFC allowing for on-line sample extraction of serum and plasma followed by detection using mass spectrometry of endogenous steroids and hormones has been explored [13–15]. However, none of these methods have focused on the collection of entire steroid profiles in the horse.

LC–MS has been the preferred alternative to both immunoassay (IA) and gas chromatography–mass spectrometry (GC–MS) in low level quantitation of steroids in serum/plasma in clinical and regulatory drug testing settings [16]. IA techniques suffer from problems with cross reactivity with other steroids, problems with low-level quantitation and the cost associated with running multiple IA tests on a single sample in order to analyze multiple steroids [16–18]. GC–MS cannot measure intact polar conjugated compounds, requires more extensive sample preparation steps, and complex derivatization prior to analysis making steroid analysis more difficult as compared to LC–MS [19,20].

The method developed and described in this paper, provides a high throughput, sensitive and selective analysis of 35 endogenous steroids in equine serum. This method demonstrates the feasibility of direct analysis of free and conjugated steroids simultaneously in one method with minimal sample preparation. The use of on-line sample extraction by a multiplexed TFC system and detection using a triple quadrupole mass spectrometer effectively analyzed over 117 samples a day by SRM and SIM.

2. Materials and methods

2.1. Standards and solutions

The following analytes used in this study were obtained from steraloids (Newport, RI): 17α-estradiol (αE2), 17β-estradiol (βE2), 17β-estradiol sulfate (E2S), 17-hydroxypregenolone (17P5), 17-hydroxyprogesterone (17P4), 19-norandrosterone (19A), 19-norepiandrosterone (19EA), 5α-androstane-3α,17β-diol (Adiol), 5α-dihydronandrolone (5αDHN), 5α-estran-3β,17α-diol (5αE2), 5α-dihydroprogesterone (5αDHP), 5β-dihydrotestosterone (5βDHT), 6α-hydroxyandrostenedione (6αA4), 6β-hydroxytestosterone, allopregnanolone (AP), androstenedione (A4), androstanedione, androsterone, boldenone sulfate (BS), cortisol, dehydroepiandrosterone (DHEA), dehydroepiandrosterone sulfate (DHEAS), d7-androstenedione (A4-d7), d3-testosterone sulfate (TS-d3), epi-androsterone, epinandrolone (ENan), epi-testosterone sulfate, estrone (E1), estrone sulfate, etiocholan-3α-17β-diol (Ediol), etiocholane-3,17-dione, etiocholanolone, nandrolone glucuronide (NG), nandrolone sulfate (NS), pregenolone (P5), pregnanediol (Pdiol), progesterone (P4), testosterone glucuronide (TG), and testosterone sulfate (TS). The following standards were obtained from Cerilliant (Round Rock, TX): 19-norandrostendione (19dione), 5α-dihydrotestosterone (5αDHT), boldenone (Bold), d3-boldenone (Bold-d3), d3-testosterone (Test-d3), nandrolone (Nan), testosterone (Test), and epi-testosterone (ETest). 1,4-androstadiene-3,17-dione (ADD) was obtained from Alltech (Deerfield, IL). For analytes purchased as powders, 1 mg mL^{-1} reference solutions were prepared by weighing 5 mg of reference standards and mixing with 5 mL either acetonitrile or methanol. Dilutions were made at 10^4, 10^3, 10^2 and 10 ng mL^{-1} in methanol.

Acetonitrile, methanol and water were of HPLC grade and obtained from Burdick and Jackson (Muskegon, MI). Acetone, isopropanol, and ammonium hydroxide were of Optima grade and obtained from Fisher Scientific (St. Louis, MO). Formic acid was of ACS grade and obtained from EMD (Gibbstown, NJ).

2.2. Collection of samples

Negative control serum was obtained by jugular venipuncture from two castrated male Thoroughbred horses at the University of California–Davis. Blood was allowed to coagulate in Corvac™ SST tubes (Tyco, Mansfield, MA) and centrifuged at approximately 2500 × *g* for 10 min and serum collected. Negative control serum was pooled and singly charcoal stripped to remove endogenous circulating steroids and frozen at −20 °C until analysis [21]. Castrated males were chosen due to their lack of gonadal production of steroids. Test samples were collected in a similar fashion as described above without the use of charcoal stripping and pooling of samples. Collection of control serum was approved by the University of California–Davis Institutional Animal Care and Use Committee.

2.3. Sample preparation

Three hundred μL of serum was added to autosampler vials followed by the addition of 150 μL of the IS solution in water. Samples were capped and vortexed for 30 s, followed by centrifugation at approximately 1000 × *g* for 3 min. Samples were stored at 7 °C in a temperature controlled sample compartment until a 75 μL injection introduced the diluted serum to the TFC-MS/MS system. Calibrators ($n = 9$) and QC samples ($n = 2$ per level) were prepared at the same time as the test samples by drying of standard solutions in autosampler vials using a Zymark Turbovap concentrator (Hopkinton, MA) at 40 °C with N_2. Calibrators and QC samples were re-dissolved with the addition of singly charcoal stripped serum and processed identically as the study samples. Calibration samples were run at the beginning and the end of each sample set while QC samples were interspersed throughout the run. Quantitation of analytes was determined by linear regression analysis of the ratio of analyte area to internal standard area using the equation $y = m(x) + b$. A minimum of a six-point calibration curve and maximum of nine points were used depending on the linear range of each analyte (Table 1).

2.4. Optimization of mass spectrometric parameters

The chromatographic conditions were optimized by a series of injections of standards comparing the peak areas and resolution. Mass spectrometric conditions were optimized by direct infusion (10^3 or 10^4 ng mL^{-1}) of monitored steroids at 5 μL min^{-1} to provide the settings with the lowest detection limits, highest sensitivity, and best selectivity for each analyte. The atmospheric pressure ionization (API) source was selected based on the best ion formation from both ESI and atmospheric pressure chemical ionization (APCI) using a Thermo Ion max source. Source temperature, spray voltage, ionization polarity, gas (aux, sweep, sheath) rates were investigated to provide the best precursor ion formation. Product ions and S-lens values were determined for each analyte following post-column infusion at 5 μL min^{-1} of either 10^3 or 10^4 ng mL^{-1} solution of each compound in methanol combined with 50/50 mobile phases A/B at 350 μL min^{-1} (Table 2). The most selective and sensitive product ions formed by collision-induced dissociation (CID) of the selected precursor ions were optimized for SRM analysis by increasing the collision energy.

2.5. TFC-MS/MS Analysis

Online sample extraction and separation by 2D-LC was accomplished using a multiplexed Thermo Aria TLX-2 TFC system

Table 1
Calibration and validation parameters. The limits of detection (LOD), lower limit of quantitation (LLOQ) and upper limit of quantitation (ULOQ) were experimentally determined for each analyte. One internal standard (ISTD) was used for each analyte. (1) d7-androstenedione, (2) d3-testosterone sulfate, (3) d3-testosterone, (4) d3-boldenone. The average and %CV for percent recovery (% recovery) along with the matrix factor were determined for each analyte at each of the QC levels ($n = 6$ per level) within each compounds quantitation range. % Recovery = (analyte peak area with TFC)/(analyte peak area without TFC) × 100. Matrix factor = (analyte peak area in serum)/(analyte peak area without serum) × 100.

Analyte	ISTD	LOD (ng mL^{-1})	LLOQ (ng mL^{-1})	ULOQ (ng mL^{-1})	QC levels	% Recovery		Matrix factor	
						Average	%CV	Average	%CV
6αA4	1	0.500	2.50	500	1–4	78.2	13.5	99.0	17.2
BS	2	0.250	0.500	25.0	2–4	37.2	27.1	140	30.9
E2S	2	0.150	0.250	25.0	2–4	39.0	25.1	122	28.6
NS	2	0.250	0.500	50.0	1–3	17.2	40.2	155	51.2
NG	3	0.750	2.50	500	1–4	87.5	10.9	79.2	17.3
TS	2	0.250	0.500	25.0	2–4	35.5	26.2	130	40.0
ADD	1	0.125	0.125	50.0	1–4	89.8	8.4	103	9.4
TG	3	0.150	0.250	50.0	1–4	86.0	40.5	112	78.2
19dione	1	0.250	1.25	250	1–3	84.3	14.2	101	25.1
DHEAS	2	0.150	0.500	50.0	2–4	38.4	35.1	101	61.0
Bold	4	0.100	0.125	25.0	1–3	89.7	9.4	103	12.1
A4	1	0.075	0.125	25.0	1–3	87.8	6.1	105	8.8
Nan	1	0.150	0.250	25.0	1–3	88.2	9.2	100	15.1
E1	1	0.150	0.250	10.0	1–3	80.0	14.5	114	14.1
βE2	3	2.50	5.00	500	2–4	85.6	8.3	110	10.2
αE2	3	1.00	5.00	50.0	2–4	85.7	6.1	113	8.1
Test	3	0.050	0.125	25.0	1–3	85.6	7.5	106	9.5
ENan	3	0.150	0.250	25.0	1–3	89.4	5.9	106	11.0
17P4	3	0.050	0.125	25.0	1–3	84.1	12.4	120	16.8
19EA	3	0.500	1.25	500	1–4	88.0	6.1	114	7.0
DHEA	4	1.25	2.50	250	1–3	86.1	11.5	115	11.3
17P5	3	2.50	5.00	500	2–4	90.0	8.6	111	17.8
5αDHN	3	0.750	1.25	500	1–4	86.7	5.0	104	11.0
ETest	3	0.025	0.125	25.0	1–3	84.3	10.8	108	16.1
5αE2	3	0.750	1.25	250	1–3	88.0	13.4	107	13.5
5αDHT	3	0.500	1.25	250	1–3	85.3	6.8	98	15.1
19A	3	0.500	1.25	250	1–3	87.4	3.8	110	5.7
5βDHT	3	0.250	1.25	250	1–3	85.7	6.2	88.0	17.9
P4	3	0.050	0.125	25.0	1–4	72.2	7.1	86.3	14.8
Adiol	3	2.50	10.0	500	3–4	84.7	6.3	49.9	25.7
Ediol	3	10.0	25.0	500	3–4	83.5	6.5	58.8	10.1
P5	3	1.00	2.50	500	1–4	68.7	12.3	96.7	14.7
5αDHP	3	2.50	5.00	500	2–4	65.6	18.6	95.9	13.7
AP	3	2.50	5.00	500	2–4	63.3	13.2	81.3	15.6
Pdiol	3	5.00	10.0	500	3–4	69.6	10.3	57.4	12.6

(Franklin, MA) composed of two online degassers, four Shimadzu LC-10AD (Columbia, MD) HPLC pumps (two quaternary pumps, two binary pumps), and a temperature controlled CTC Leap autosampler. The instrument was controlled using Aria software (Version 1.6.1). A Thermo Cyclone P extraction column (0.5 mm × 50 mm, 60 μm particle size, Franklin, MA) was utilized for online sample extraction of diluted serum in the first dimension. A ACE C18 analytical column (2.1 mm × 100 mm, 3 μm particle size, Chadds Ford, PA) with a guard column (2.1 mm × 10 mm, 3 μm particle size) maintained at 30 °C was used for reverse-phase gradient separation in the second dimension prior to introduction to the MS. Mobile phases A, B, C, and D were comprised of: A – water with 0.2% formic acid, B – methanol, C – acetonitrile/isopropyl alcohol/acetone (60/30/10, v/v/v), and D – water/acetonitrile (98/2, v/v) with 0.1% ammonium hydroxide. The quaternary pumps were used for online sample extraction while the binary pumps were used for reverse-phase gradient separation of analytes over a 24.15-min chromatographic method (Table S-1). Following injection, the analytes were extracted from serum by TFC, transferred to the second dimension to be chromatographically separated, and then introduced to the mass spectrometer and column re-equilibrated in a series of steps (Table S-1).

Tandem mass spectral detection was accomplished using a Thermo TSQ Vantage (San Jose, CA) triple quadrupole mass spectrometer with a second-generation heated electrospray (HESI) source operating at room temperature in both the positive and negative modes. The mass spectrometer was controlled using Xcalibur (version 2.0.7) and data processed using LCquan (version 2.5.6) software. HESI source conditions for sheath gas, ion sweep and aux gases were held at 45, 0.5, 30 arbitrary units of dry nitrogen, respectively. Spray voltage was set to 4000 V in positive mode and 5000 V in the negative mode. The ion transfer tube temperature was set to 350 °C. The skimmer offset was set at 0 V. Argon was used as a collision gas and set to 1.5 arbitrary units. Resolution parameters were set with $Q1 = 0.1$ and $Q3 = 0.7$ m/z at full width at half-maximum height. Detection and quantitation of all analytes was accomplished using SRM with a minimum of three transitions monitored per analyte, except 17P5 where SIM was used. The MS method was split into six segments over a 12.25-min period with polarity switching in the first and second segments (Table 2). The MS was triggered to acquire data 6.25 min into the chromatographic run to allow for multiplexing of the chromatographic system.

2.6. Method validation

The method was validated over four days with the following assessed: limit of detection (LOD), lower limit of quantitation (LLOQ), upper limit of quantitation (ULOQ), assay linearity, inter/intra-assay precision and accuracy, analyte recovery, matrix effects, and assay specificity. The reproducibility of chromatography and mass spectrometry was assessed and any deviations investigated. A series of experiments was undertaken to determine recovery and matrix effects using injections with and without TFC.

Table 2
Method parameters. The MS segment, ESI polarity, chemical formula, retention time (T_R), and S-lens setting are given for each compound. The monitored precursor ion along with the corresponding product ion, collision energy (eV), and relative abundance are also shown. The MS acquired data between 6.25 and 18.5 min. Italics indicate the transition used for quantitation.

Compound	Segment/ESI mode	Formula	T_R	S-lens	Precursor ion	Product ions	Collision energy	Relative abundance
6αA4	1/+	$C_{19}H_{26}O_3$	6.60	83	303.1	*227.1*, 209.1, 105.1	*19*, 20, 40	*100*, 90, 75
E2S	1/−	$C_{18}H_{24}O_5S$	7.15	161	351.1	*271.1*, 80.0, 144.9	*36*, 40, 55	*100*, 2, 5
NS	1/−	$C_{18}H_{25}O_5S$	7.24	147	353.1	*96.7*, 80.2, 250.9	*45*, 72, 61	*100*, 18, 1
BS	1/−	$C_{19}H_{25}O_5S$	7.25	80	365.1	*96.9*, 95.9, 350.1	*51*, 52, 31	*100*, 52, 47
NG	1,2/+	$C_{24}H_{33}O_8$	7.72	99	451.3	*85*, 109.0, 145.0	*34*, 33, 28	*100*, 70, 90
TS	1,2/−	$C_{19}H_{28}O_5S$	7.98	80	367.1	*96.9*, 80.1, 191.5	*45*, 80, 79	*100*, 10, 1
TS-d3	1,2/−	$C_{19}H_{24}D_3O_5S$	7.92	213	370.1	*98.0*	*50*	*100*
ADD	2/+	$C_{19}H_{24}O_2$	8.25	56	285.2	*121.0*, 77.0, 91.1	*23*, 49, 39	*100*, 25, 31
TG	2/+	$C_{25}H_{36}O_8$	8.50	120	465.2	*289.3*, 97.1, 109.0, 253.2	*18*, 34, 34, 19	*100*, 25, 25, 26
DHEAS	2/−	$C_{19}H_{28}O_5S$	8.55	138	367.1	*96.9*, 80.1, 191.5	*45*, 80, 79	*100*, 15, 1
19dione	2/+	$C_{18}H_{24}O_2$	8.85	82	273.2	*79.1*, 109.1, 197.1	*41*, 25, 16	*25*, 85, 100
Bold-d3	2,3/+	$C_{19}D_3H_{23}O_2$	9.44	50	290.2	*121.0*	*25*	*100*
Bold	2,3/+	$C_{19}H_{26}O_2$	9.47	50	287.2	*121.0*, 91.1, 77.0	*23*, 43, 52	*100*, 30, 38
A4-d7	2,3/+	$C_{19}H_{19}D_7O_2$	9.84	75	294.3	*100.1*	*21*	*100*
A4	2,3/+	$C_{19}H_{26}O_2$	9.93	72	287.2	*97.1*, 109.1, 79.1	*19*, 21, 40	*100*, 70, 22
Nan	3/+	$C_{18}H_{26}O_2$	10.09	72	275.2	*109.1*, 145.1, 91.1	*28*, 21, 42	*100*, 50, 62
E1	3/+	$C_{18}H_{22}O_2$	10.50	66	271.1	*159.1*, 157.0, 133.1	*22*, 19, 21	*82*, 100, 87
βE2	3/+	$C_{18}H_{24}O_2$	10.58	44	255.1	*159.1*, 133.1, 141.0	*17*, 18, 32	*100*, 30, 17
αE2	3/+	$C_{18}H_{24}O_2$	11.20	69	255.1	*159.1*, 133.1, 141.0	*17*, 18, 32	*100*, 30, 17
Test-d3	3/+	$C_{19}H_{25}D_3O_2$	11.35	83	292.2	*97.1*	*21*	*100*
Test	3/+	$C_{19}H_{28}O_2$	11.40	83	289.2	*97.1*, 109.0, 79.1, 81.1	*22*, 27, 43, 36	*100*, 90, 28, 16
ENan	3/+	$C_{18}H_{26}O_2$	11.68	72	275.2	*109.1*, 145.1, 91.1	*28*, 21, 42	*100*, 70, 60
17P4	3/+	$C_{21}H_{30}O_3$	12.06	91	331.2	*109.1*, 97.1 253.2	*29*, 28, 18	*100*, 85, 41
19EA	4/+	$C_{18}H_{28}O_2$	12.34	54	259.2	*241.2*, 145.1, 91.1	*10*, 18, 39	*100*, 38, 20
DHEA	4/+	$C_{19}H_{28}O_2$	12.45	53	271.2	*213.2*, 197.1, 253.2	*14*, 19, 11	*44*, 26, 100
17P5	4/+	$C_{21}H_{32}O_3$	12.61	99	355.2	*355.2*		
5αDHN	4/+	$C_{18}H_{28}O_2$	13.07	64	277.2	*241.2*, 91.1, 67.0	*14*, 43, 34	*100*, 28, 8
ETest	4/+	$C_{19}H_{28}O_2$	13.59	83	289.2	*97.1*, 109.0, 79.1, 81.1	*22*, 27, 43, 36	*100*, 92, 34, 18
5αE2	4/+	$C_{18}H_{30}O_2$	13.63	58	243.2	*147.1*, 91.1, 105.1	*16*, 38, 29	*100*, 65, 38
5αDHT	4/+	$C_{19}H_{30}O_2$	14.49	68	291.2	*255.1*, 273.2, 91.0	*10*, 7, 51	*100*, 18, 24
19A	4,5/+	$C_{18}H_{28}O_2$	15.19	60	259.2	*241.2*, 145.1, 91.1	*12*, 18, 42	*100*, 34, 22
5βDHT	4,5/+	$C_{19}H_{30}O_2$	15.41	68	291.2	*255.2*, 273.2, 91.0	*10*, 7, 51	*100*, 54, 20
P4	5/+	$C_{21}H_{30}O_2$	16.20	78	315.2	*97.1*, 109.1, 79.0	*22*, 26, 45	*100*, 95, 28
Adiol	5/+	$C_{19}H_{32}O_2$	16.43	74	257.2	*161.2*, 91.1, 175.2	*16*, 40, 13	*100*, 78, 94
Ediol	5/+	$C_{19}H_{32}O_2$	16.65	58	257.2	*161.2*, 91.1, 175.2	*15*, 43, 14	*100*, 78, 94
P5	6/+	$C_{21}H_{32}O_2$	17.47	75	299.2	*281.2*, 159.1, 105.1	*13*, 23, 35	*100*, 28, 24
5αDHP	6/+	$C_{21}H_{32}O_2$	17.68	65	317.2	*281.2*, 105.1, 159.1	*13*, 36, 22	*100*, 22, 24
AP	6/+	$C_{21}H_{34}O_2$	18.10	87	301.2	*189.1*, 91.1, 105.1	*21*, 43, 36	*100*, 92, 92
Pdiol	6/+	$C_{21}H_{36}O_2$	18.24	77	285.2	*189.1*, 203.1, 175.1	*16*, 15, 17	*100*, 90, 80

Two levels of standards were used in generating calibration curves for the analytes monitored depending on the ionization efficiency and physiological range of the steroids measured (Fig. S-1). The lower curve had a calibration range from 0.125–50 ng mL^{-1} and was composed of the following: ADD, A4, BS, E2S, Bold, DHEAS, E1, ENan, ETest, Nan, NS, 17P4, P4, TS, TG, and Test. The upper curve had a calibration range from 1.25–500 ng mL^{-1} and was composed of the following: 6αA4, 19A, Adiol, AP, 19dione, 5βDHT, 5αDHT, 5αDHN, 5αDHP, DHEA, Ediol, βE2, αE2, 5αE2, 19EA, NG, 17P5, P5, Pdiol. For both levels, nine matrix matched calibration samples were used in the generation of calibration curves along with matrix blanks both with and without the addition of the internal standard. The specific number of calibration samples used for each analyte depended on the limit of quantitation for that analyte (Table 1). Quality control (QC) samples were used to monitor accuracy and precision with four concentrations used for each level of standards. For the lower curve, QC concentrations at 0.4, 0.8, 4, and 30 ng mL^{-1} were used. The upper curve used QC concentrations at 4, 8, 40 and 300 ng mL^{-1}. An internal standard (IS) solution comprised of test-d3, boldenone-d3, A4-d7 at 4 ng mL^{-1} and TS-d3 at 40 ng mL^{-1} in HPLC grade water was used in all test samples.

3. Results/discussion

A method employing 2D-LC and tandem mass spectrometry for the detection of 35 steroids was developed and validated for the analysis of equine serum. Detection and quantitation by triple quadrupole mass spectrometry using electrospray ionization allowed for good precursor ion formation prior to detection by SRM or SIM, which allowed for low-level quantitation. The method was validated over four days and was able to provide reproducible low-level quantitation for all analytes studied. The method was then applied to analyze >2000 equine serum samples in order to determine the endogenous steroid profile in serum [22].

3.1. Online sample extraction and 2-dimensional liquid chromatography

The 2D-LC separation of the analytes was optimized to provide maximized analyte coverage and low detection levels. 2D-LC using TFC in the first dimension allowed for online sample extraction with good recoveries for most analytes and reverse phase chromatography in the second dimension efficiently separated analytes prior to introduction to the mass spectrometer. The 75 μL (50 μL serum + 25 μL IS solution) injection volume used in the method presented here uses less serum and does not require protein precipitation unlike many of the previously published methods [13–15,23]. While protein precipitation may allow for the monitoring of total steroid levels, the use of strong acids commonly used in protein precipitation may result in cleavage of 19-carboxylic acids into Nan causing false positives [9]. The use of online sample extraction provided by TFC allows for the analysis of small sample

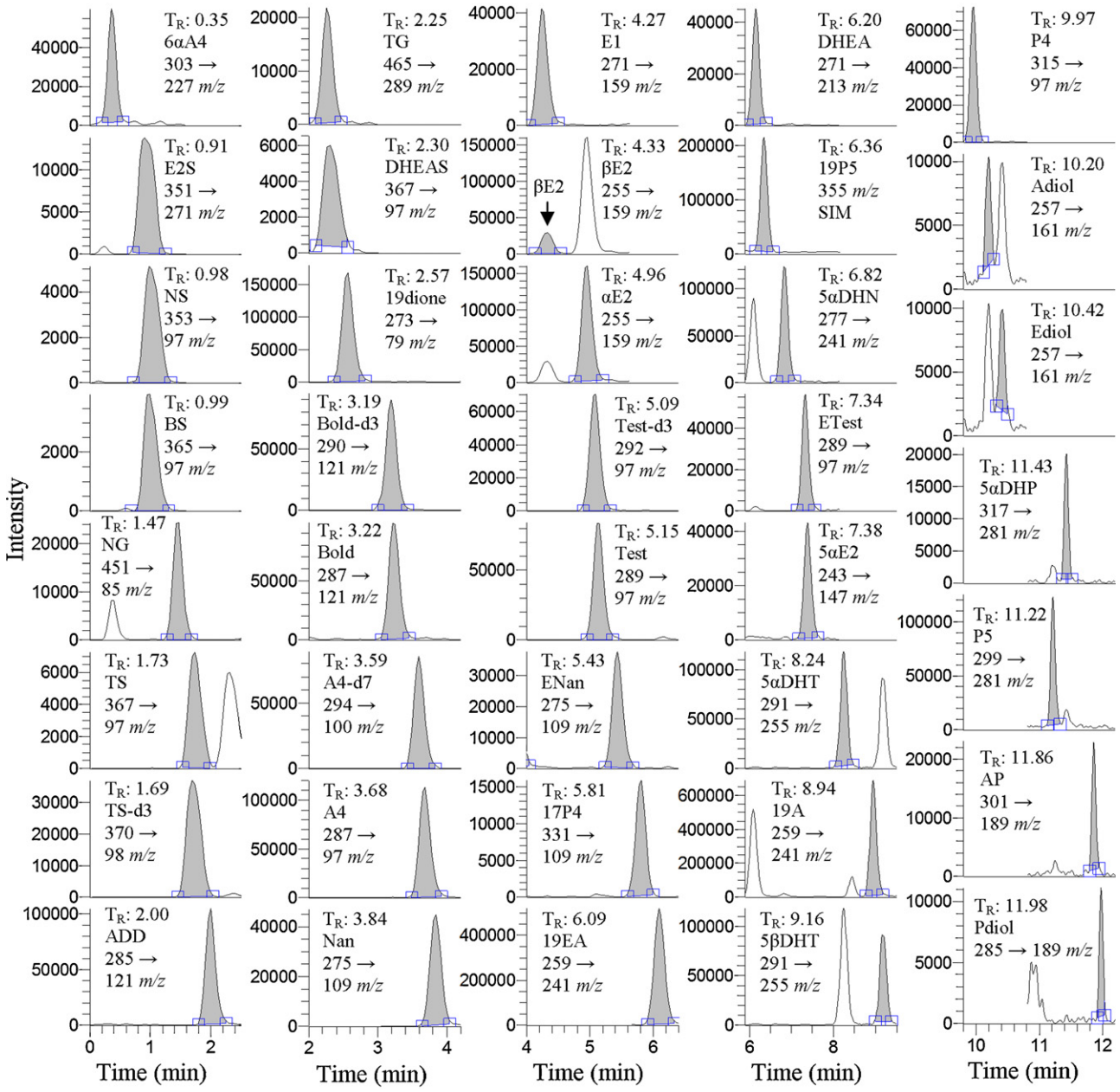

Fig. 1. Chromatograms of all steroids at 2.5 and 25 ng mL^{-1} for the low and high concentration groups, respectively.

volumes while still achieving parts-per-trillion detection of many analytes.

The 2.1 mm × 100 mm C18 column used for reverse phase gradient analysis of the analytes effectively retained and chromatographically resolved both polar conjugated and non-polar steroids over a 24.15 minute run (Fig. 1). To maintain adequate chromatographic performance the guard column was changed after ~300 samples. The impact of temperature on the chromatography of the analytes was investigated and increasing the temperature above 30 °C decreased resolution between the isomers Adiol and Ediol. Temperature was maintained at 30 °C throughout the study, which decreased the HPLC system backpressure and allowed for reproducible retention times for analytes throughout the analysis (Table 2). Methanol was chosen as the organic mobile phase due to higher sensitivity and lower solvent adduct formation compared to acetonitrile [24,25]. However, use of methanol did result in higher backpressure (~280 bar at 50:50 methanol:water) on the TFC system as compared to acetonitrile. Slight differences in retention times (~0.1–0.2 min) between the two systems were observed when the system was operated in multiplexed mode (data not shown). All targeted compounds eluted in the 12.25 min MS scan window allowing multiplexing of the TFC system, doubling of sample throughput. The polar conjugated compounds (sulfate and glucuronide metabolites) eluted within the first 2.5 min. The isomers T/ET, Nan/ENan, 5βDHT/5αDHT, βE2/αE2, and Adiol/Ediol were also well resolved.

3.2. Mass spectrometry

The ionization of steroids using atmospheric pressure ionization (API) coupled with liquid chromatography has been well studied with various API sources being more sensitive for each individual steroid [24,26–29]. The three API sources commonly used in steroid analysis are ESI, APCI and atmospheric

pressure photoionization (APPI). Ionization of steroids by API sources can lead the formation of several different precursor ions depending upon the specific steroid with $[M+H]^+$, $[M\text{-}H_2O+H]^+$, $[M\text{-}2H_2O+H]^+$, $[M+Na]^+$, [M+solvent]$^+$, and $[M-H]^-$ commonly observed [26,28,30–32]. These multiple ions can make steroid analysis problematic due to several ions being formed from a single compound which may have an identical mass as another compound.

APCI was compared to ESI in both positive and negative modes for all analytes and ESI was selected to give the optimal response, confirming results previously shown by several authors [24,25]. As was previously noted, APCI was found to be effective at ionization of most compounds studied but was limited by the thermal degradation of analytes resulting the formation of $[M\text{-}H_2O+H]^+$ and $[M\text{-}2H_2O+H]^+$ ions rather than the $[M+H]^+$ ion [27,29]. For the analysis of conjugated (sulfate and glucuronide) steroids APCI resulted in the in-source loss of the glucuronide or sulfate moieties along with water losses prior to the generation of precursor ions. The impact of thermal degradation and in-source CID using APCI caused many steroids to have identical precursor ions, which made identification difficult with steroids with similar retention times. APPI has been shown to be a good choice for steroid profiling and while it was not assessed in this study may offer additional improvements on detection limits for several analytes [13,33]. Thus, ESI was chosen as the preferred ionization source due to its reduction in water losses from precursor ions and its sensitivity in ionization of previously mentioned target androgens.

For each studied analyte, post-column infusion was used to determine precursor ion formation with each ion selected to maximize product ion formation and allow for low detection limits (Table 2). For most ions monitored in the positive ESI mode the $[M+H]^+$ ion was chosen, though in-source water loss was observed for several analytes including α/βE2, 19EA, DHEA, 19A, 5αDHP, AP and two water losses were observed for 5αE2, Adiol, Ediol and Pdiol (Fig. 2). A dominant sodium adduct was seen in the formation of the $[M+Na]^+$ ion for 17P5. For the compounds monitored in negative mode ESI the $[M-H]^-$ was the most abundant ion observed (Fig. 2). The most abundant ion was chosen as the precursor ion, thus a $[M\text{-}H_2O+H]^+$ ion was chosen if it dominated over the $[M+H]^+$ ion following post-column infusion. While most of the analytes could be monitored at low levels, some compounds such as the estrogens, 5α-reduced steroids and steroids lacking either a 3- or 17-keto group were not. Additional gains in steroid ionization efficiency for these problematic steroids may be achieved using APPI, APCI or by derivatization which would require additional sample processing steps [34]. Improvements in detection limits of estrogens may be gained using ESI in negative mode without the addition of an acidic modifier such as formic acid [23,32]. This was not investigated, acidic modifiers are necessary for ionization in ESI positive mode and the removal of formic acid from the method would have necessitated two separate chromatographic methods.

Tandem mass spectrometry spectra for many of the steroids studied have already been investigated in detail with proposed mechanisms for product ions of various steroids shown in the literature [28,30,35–38]. Product ions used as SRM transitions were determined by increasing collision energy following post-column infusion of standards with a large difference in the number of product ions generated depending on the specific analyte monitored. Product ions were chosen and SRM settings were optimized for each transition. Water losses were seen in the MS/MS spectra of several compounds including 19A, 19EA, DHEA, 5αDHT, 5αDHP, 5αDHN, and P5. While monitoring product ions from water losses is not preferred in quantitation, they were used for some analytes as they provided the highest signal to noise (S/N). All analytes could be quantitated using SRM except for 17P5, which upon fragmentation by CID did not produce an abundant amount of product ions.

3.3. Method validation

The method was validated over four days with the following assessed: LOD, LLOQ, ULOQ, assay linearity, inter/intra-assay precision and accuracy, analyte recovery, matrix effects, and assay specificity. The method was developed using the U.S. DSHHS FDA's "Guidance for Industry, Bioanalytical Method Validation", however not all analytes were able to meet these strict criteria.

The LLOQ, ULOQ and LOD of each compound were experimentally determined by fortifying a series of known concentrations of drug into charcoal stripped serum until the analyte was no longer detectable (Table 1). The LOD for each analyte was defined as a chromatographic peak giving a S/N of 3 to 1 when compared with chromatographic separation from negative control serum. The LLOQ was defined as a chromatographic peak giving a S/N of at least 10:1 with no more than 20% deviation from expected concentration. LODs and LLOQs varied considerably depending on the specific analyte monitored, its ionization by ESI and product ions monitored. The ULOQs were determined as the highest calibrator that allowed for quantitation without deviations from linearity.

Assay linearity was assessed for each analyte over the validation period and all analytes (other than DHEAS) monitored had correlation co-efficient of $R^2 > 0.98$ with most analytes being >0.99. Sample runs with $R^2 < 0.98$ were repeated. The lower group and upper groups had linear calibration ranges between 0.125–50 ng mL^{-1} and 1.25–500 ng mL^{-1}, respectively (Table 1).

Both inter-day (Table 3) and intra-day (Table S-2) accuracy and precision were assessed at four QC levels for each group of compounds at: 0.4, 0.8, 4 and 30 ng mL^{-1} for the lower curve and at 4, 8, 40 and 300 ng mL^{-1} for the upper curve. A goal of less than 20% deviation from expected concentration (%Acc) and no more than a 20% coefficient of variation (%CV) for the lowest QC concentration used for each analyte was set. For inter-day validation, most compounds were able to meet these goals at all QC levels except with Adiol, DHEA, DHEAS, E2S, Ediol, NG, TG, and TS that had larger %CV despite acceptable %Acc. αE2 had only 3 days of inter-day validation due to an incorrect time on the scan segment which was corrected for the subsequent days of validation. In contrast, the intra-day accuracy and precision were closer to the 20% targets for all analytes other than DHEAS at its lowest QC value (0.8 ng mL^{-1}). A goal of each analyte being monitored at a minimum of 3 QC concentrations was established and was met for all analytes other than Pdiol, Adiol and Ediol due to the LOQ being >8 ng mL^{-1}.

Analyte recovery was assessed by comparing analyte peak areas from injections (20 μL) of equivalent amounts of analyte with and without turbulent flow extraction at the four QC levels (Table 1). Each sample was fortified with internal standards of Test-d3, Bold-d3, A4-d7, TS-d3 at 0.1, 0.1, 0.1 and 1 ng on-column, respectively. The high group levels had 0.2, 0.4, 2, and 15 ng on-column while the low group had 0.02, 0.04, 0.2, and 1.5 ng on-column. The recovery was good (>80%) for most of the analytes monitored. The recovery of the sulfated steroids was lower (17–39%) which was most likely due to poor extraction and analyte breakthrough during TFC. Matrix effects were assessed by matrix factor determination comparing analyte area of charcoal stripped negative control serum ($n = 6$) and analyte standards ($n = 6$) at the four QC levels for both the high and low concentration groups (Table 1) [39]. The matrix factor (MF) for most compounds was between 80 and 120% with 100% MF having no difference between serum and standards. The conjugated steroids were the most impacted by matrix effects, which may be explained by competition for ionization between co-eluting analytes. Better results may be seen with a change in mobile phase conditions to favor negative mode ionization of acidic compounds

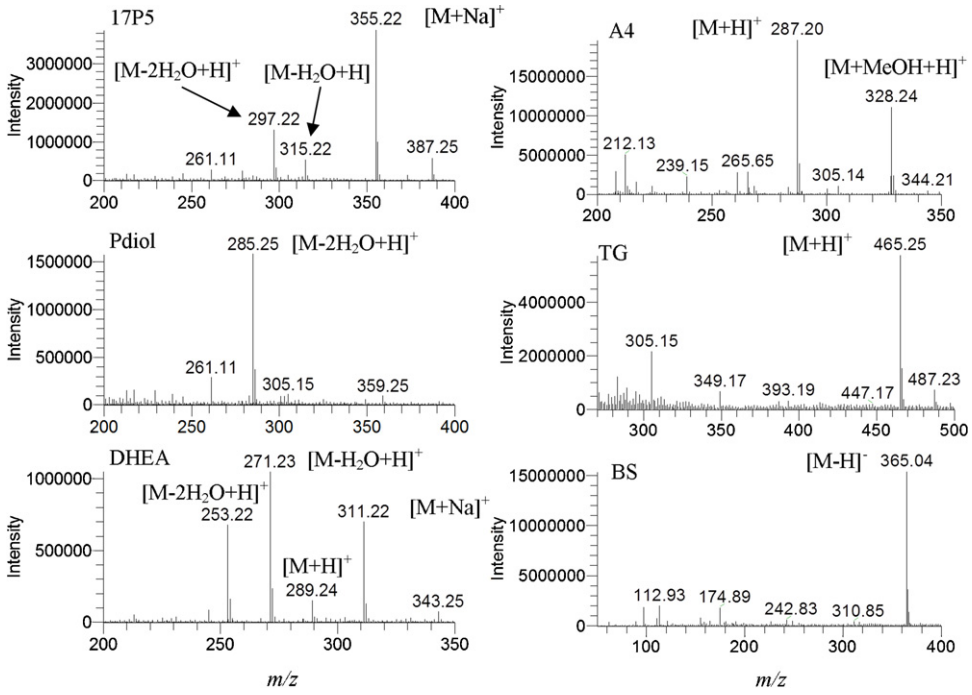

Fig. 2. MS spectra. Full scan MS spectra were generated following infusion of 10^3 ng mL^{-1} of (A) 17P5, (B) Pdiol, (C) DHEA, (D) A4, (E) TG, and (F) BS at 5 μL min^{-1} with 0.350 mL min^{-1} of 50:50 mobile phases A:B.

Table 3
Inter-assay accuracy and precision. The accuracy (% Acc) and precision (% CV) was determined at each QC level (n = 24 per level over 4 days). %Acc and %CV are not given for values less than the lower limit of quantitation (<LLOQ) or greater than the upper limit of quantitation (<ULOQ). *n = 18 per level due to data being collected over 3 rather than 4 days.

Analyte	Avg	% CV	% Acc	Avg	% CV	% Acc	Avg	% CV	% Acc	Avg	% CV	% Acc
Low level	QC 1–0.400 ng mL^{-1}			QC 2–0.800 ng mL^{-1}			QC 3–4.00 ng mL^{-1}			QC 4–30.0 ng mL^{-1}		
ADD	0.386	11.0	104	0.790	7.8	101	3.88	7.6	103	29.6	5.6	101
17P4	0.410	19.6	97.7	0.826	17.2	96.8	3.99	9.2	100	>ULLOQ	>ULLOQ	>ULLOQ
A4	0.395	9.0	101	0.799	7.2	100	3.93	6.3	102	>ULLOQ	>ULLOQ	>ULLOQ
Bold	0.397	11.8	101	0.798	6.7	100	4.01	7.1	99.8	30.6	4.8	98.1
BS	<LLOQ	<LLOQ	<LLOQ	0.788	17.6	102	3.87	10.9	103	29.4	6.1	102
DHEAS	<LLOQ	<LLOQ	<LLOQ	0.713	35.3	112	4.13	45.5	96.9	28.1	34.9	107
ENan	0.389	17.7	103	0.806	9.0	99.3	4.02	7.6	99.6	>ULLOQ	>ULLOQ	>ULLOQ
E2S	<LLOQ	<LLOQ	<LLOQ	0.761	23.8	105	3.94	9.6	102	30.5	7.7	98.5
ETest	0.394	11.7	101	0.769	7.7	104	3.93	7.2	102	>ULLOQ	>ULLOQ	>ULLOQ
E1	0.379	14.7	105	0.767	10.3	104	3.88	5.9	103	>ULLOQ	>ULLOQ	>ULLOQ
Nan	0.377	11.4	106	0.819	11.7	97.7	3.89	5.7	103	>ULLOQ	>ULLOQ	>ULLOQ
NS	0.374	20.7	107	0.759	14.9	105	3.86	8.7	104	>ULLOQ	>ULLOQ	>ULLOQ
P4	0.400	8.5	99.9	0.755	7.9	106	3.96	9.1	101	29.8	7.9	101
Test	0.405	10.8	98.8	0.790	7.2	101	4.07	8.5	98.2	>ULLOQ	>ULLOQ	>ULLOQ
TG	0.336	28.4	119	0.689	20.1	116	3.87	9.9	103	28.3	7.5	106
TS	<LLOQ	<LLOQ	<LLOQ	0.742	34.2	108	3.78	14.7	106	26.9	21.2	111
High level	QC 1–4.00 ng mL^{-1}			QC 2–8.00 ng mL^{-1}			QC 3–40.0 ng mL^{-1}			QC 4–300 ng mL^{-1}		
Adiol	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	40.3	27.2	99.3	298	16.8	101
17P5	<LLOQ	<LLOQ	<LLOQ	6.81	12.6	118	46.6	10.2	85.8	297	6.8	101
αE2*	<LLOQ	<LLOQ	<LLOQ	7.35	9.58	91.9	42.9	5.42	107	309	4.98	103
βE2	<LLOQ	<LLOQ	<LLOQ	6.96	11.2	115	43.9	7.2	91.0	301	8.1	99.5
Ediol	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	43.4	17.9	92.1	285	12.3	105
19A	3.98	8.7	101	8.00	6.8	100	40.9	8.7	97.7	>ULLOQ	>ULLOQ	>ULLOQ
19dione	4.03	5.9	99.2	8.21	6.4	97.5	40.1	6.3	99.6	>ULLOQ	>ULLOQ	>ULLOQ
19EA	3.92	8.2	102	8.03	6.0	99.6	41.4	8.1	96.5	>ULLOQ	>ULLOQ	>ULLOQ
5αDHP	<LLOQ	<LLOQ	<LLOQ	8.28	18.4	96.7	38.8	10.5	103	289	9.7	104
5αDHT	3.94	8.6	102	7.62	7.0	105	38.9	7.6	102	>ULLOQ	>ULLOQ	>ULLOQ
5αE2	3.71	12.5	108	8.09	14.1	98.9	41.7	9.7	96.0	>ULLOQ	>ULLOQ	>ULLOQ
5αDHN	3.90	7.5	103	7.75	7.7	103	39.5	8.5	101	291	7.5	103
5βDHT	4.05	8.0	98.9	7.86	6.2	102	40.3	7.8	99.3	>ULLOQ	>ULLOQ	>ULLOQ
6αA4	3.55	19.6	113	8.08	13.7	99.0	42.0	9.9	95.3	298	6.8	100
AP	<LLOQ	<LLOQ	<LLOQ	7.69	18.0	104	39.3	11.2	101	285	10.5	105
DHEA	3.42	23.9	117	7.92	9.6	101	41.0	7.3	97.6	>ULLOQ	>ULLOQ	>ULLOQ
NG	3.45	22.1	116	7.73	10.3	103	42.1	11.9	94.9	294	7.9	102
Pdiol	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	41.2	11.5	97.1	288	11.3	104
P5	4.09	9.1	97.9	7.46	10.0	107	37.2	7.5	107	290	8.8	101

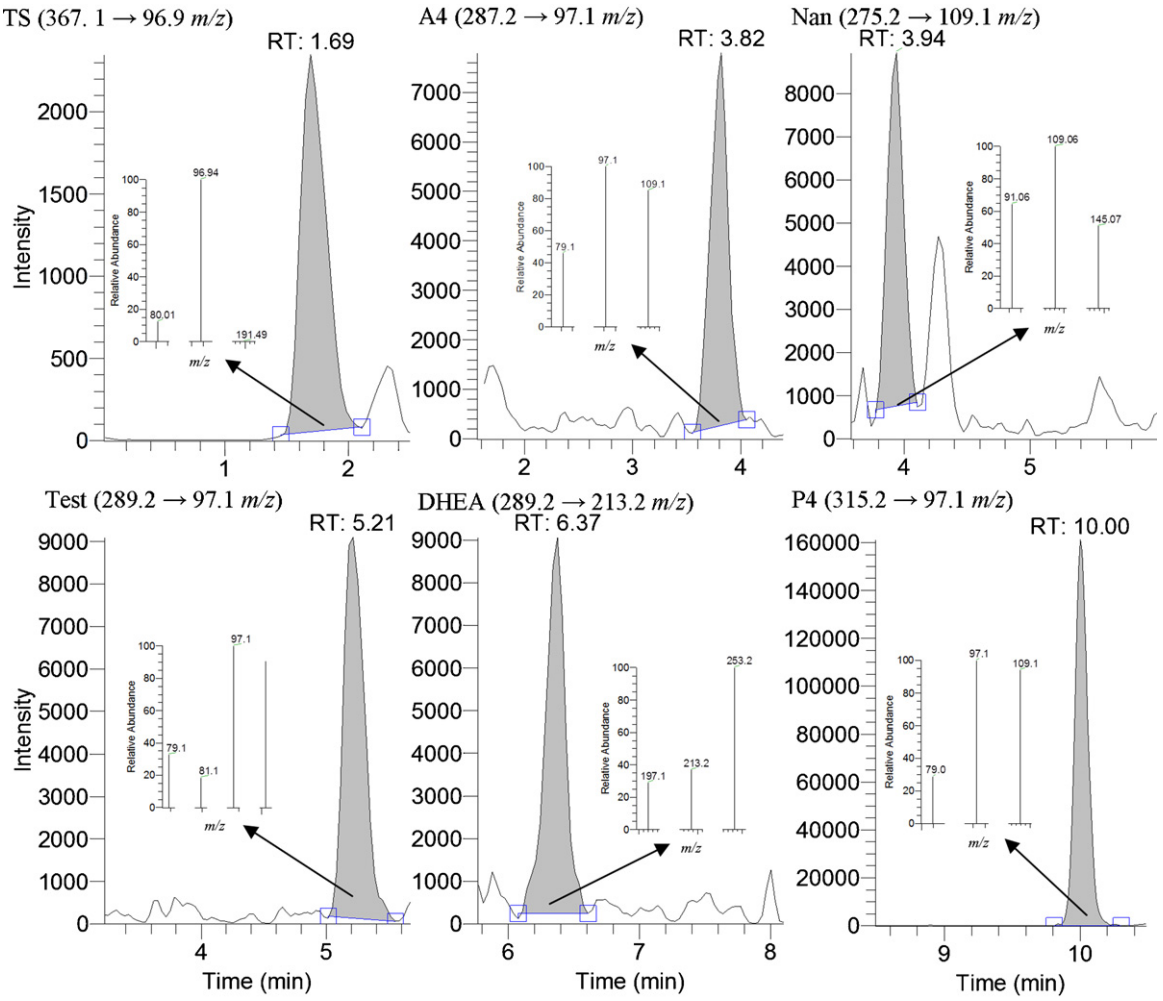

Fig. 3. Study samples. Extracted ion chromatograms along with SRM spectra from horses analyzed using 2D-LC–MS/MS. The following analytes TS, A4, Nan, Test, DHEA and P4 were quantitated in serum at 0.906, 0.216, 0.275, 0.205, 2.70, and 4.90 ng mL^{-1}.

and improved chromatographic resolution. Adiol, Ediol and Pdiol were potentially more impacted due to the monitoring of less specific water losses as quantitation ions.

Analyte specificity was assessed and no major interferences were noted other than for DHEAS and epitestosterone sulfate which are isobaric compounds with similar retention times both sharing the dominant 97 *m*/*z* product ion (Fig. S-2). P5 and 5αDHP are structurally similar isobaric steroids form different dominant precursor ions (299 and 317 *m*/*z*, respectively) by ESI that share similar product ions. These compounds were baseline resolved with ~0.2 min between peaks and as such were able to be differentiated and quantitated. Endogenous interferences in charcoal stripped serum were noted for estrone sulfate, cortisol and cortisone and thus these compounds were removed from the analysis. Singly charcoal stripped serum may be insufficient in eliminating these compounds and calibrators prepared in water containing albumin may be more appropriate [13].

Finding a suitable calibration matrix for endogenous compounds remains a large analytical challenge with no perfect solution currently available for high throughput quantitative analysis. The use of charcoal stripped serum/plasma or water with albumin may result in a more clean matrix as compared to the test specimen. This may lead to inaccuracies in quantitation of steroids, particularly if stable-isotope internal standards are not used for each target compound. It is preferable to have a calibration matrix that does not contain your target analyte and is as similar as possible to your test specimen to minimize potential differences. Serum from castrated male horses was used in calibration curves and quality control samples to minimize the presence of endogenous steroids, though steroid synthesis can occur in a number of non-gonadal tissues. In the method presented here, serum stripping was utilized to remove those endogenous steroids to obtain a clean blank matrix for calibration curves.

3.4. Steroid profiling of samples

This method has been successfully applied to monitor equine serum steroid profiles from over two thousand samples in horses from neonates to racing stallions [22,40]. The steroids detected in these samples depended on the age and sex of the animal. Several of the detected steroids from the different age groups included A4, Test, TS, DHEA and P4 (Fig. 3). Total versus free steroid concentrations were not determined using this methodology due to concerns with possible false positives resulting from the use of acids to free steroid/protein interactions [9,15]. The steroid concentrations determined using the methodology described in this method would likely result in an underestimation of total steroid concentrations in either serum or whole blood though the percentage of endogenous steroids bound to either albumin or steroid binding proteins is not well characterized in the horse.

The method has also been applied to the analysis of cell culture extracts with minimal changes to the method being necessary.

Due small the volume available, as little as 150 μL of cell culture media and 75 μL of the IS solution can be used with no observable problems. As culture media is a cleaner matrix than serum, LOQs were lowered for several analytes [41,42]. In addition, the method has also been adapted to analyze primate serum in the detection of androgens with similar results observed for primates as compared to equine serum [43,44].

The use of steroid profiles monitoring precursors, and phase I and II metabolites allows for a more complete understanding of endogenous steroid formation and the pathophysiology of various health conditions. A limitation of the methodology described in this manuscript, is the relatively small number of conjugated steroids monitored which reduces the ability to elucidate complete steroid profiles in biological specimens. One of the primary factors is the lack of availability of a large number of certified reference materials along with stable-isotope versions, this should decrease as additional commercially available standards become available. The second limitation was the relatively short elution window (2.5 min) for the conjugated steroids and their lower recovery which was more pronounced for the sulfate conjugates. A more targeted method focusing on conjugated steroids may be more useful in obtaining complete conjugated steroid profiles.

4. Conclusion

The combination of TFC with mass spectrometry allows for the detection and quantitation of 35 endogenous steroids in one method. The methodology described in this paper can collect steroid profiles from horses in a high throughput fashion with minimal sample preparation. It has been used to determine natural steroid profiles of non-treated horses and can be applied in quantitative analysis of steroids in a number of biological fluids.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2012.06.021.

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