Targeted Metabolomics Approach to Detect the Misuse of Steroidal Aromatase Inhibitors in Equine Sports by Biomarkers Profiling

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Supporting Information

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EXPERIMENTAL

Materials

Progesterone and nandrolone were obtained from British Pharmacopoeia (Middlesex, UK). 19-Norepiandrosterone, estrone and androsterone were purchased from Alltech (Derrfield, IL, USA). 17β-estradiol was obtained from United States Pharmacopeia (Rockville, MD, USA). Testosterone glucuronide, 4-estrene-3,17-dione, 19-norandrosterone, 5α-estrane-3β,17β–diol, 17α-estradiol, 5β-dihydrotestosterone, 5α-androstane-3α,17α-diol, 5α-androstane-3α,17β-diol, 5α-androstane-3β,17α-diol, 19-noretiocholanolone, androstanedione, etiocholanedione, 5-androstene-3β,17β-diol, boldione, 4,6-androstadien-3,17-dione and 4,6-androstadien-17β-ol-3-one were obtained from Steraloids (Newport, RI, USA). Testosterone, β-estradiol-3-(β-D-glucuronide)-17-sulfate, β-estradiol-3-sulfate-17-glucuronide, epitestosterone, boldenone, 5α-androstane-3β,17β-diol, androstenedione, epiandrosterone, dehydroepiandrosterone, and stanolone were purchased from Sigma-Aldrich (St. Louis, MO, USA). 5α-Estrane-3β,17α-diol and 5(10)-estrene-3β,17α-diol were custom synthesised by the Hong Kong University of Science and Technology.[1] Testosterone sulfate, d_3-testosterone sulfate and d_3-nandrolone were obtained from National Measurement Institute (Sydney, Australia). d_3-Androstane-3α,17β-diol (d_3-androstanediol) was synthesised and characterised in-house.

Chloroform (GR grade), diisopropyl ether, *n*-hexane (GR grade), *n*-heptane (LC grade), ammonium sulfate (≥99.5 %), sodium chloride (≥99.5 %) and methanol (LiChrosolv grade) were obtained from Merck (Darmstadt, Germany). Ethyl acetate (GR grade), acetonitrile (LC Grade), sodium hydroxide (≥98 %) were obtained from Sigma-Aldrich. Pentafluoropropionic acid anhydride (PFPA) was obtained from Pierce (Rockford, IL, USA). β-Glucuronidase from *E. Coli.* K12 was obtained from Roche (Indianapolis, IN, USA). Anhydrous methanolic hydrogen chloride used for methanolysis was prepared according to the procedures reported previously.[2] Deionised water was generated from an in-house water purification system (Milli-Q, Mosheim, France). Sep-Pak Vac C18 solid phase

extraction (SPE) cartridge (3 mL, 500 mg) was supplied by Waters Corporation (Milford, MA, USA). ABS Elut NEXUS SPE cartridge (3 mL, 60 mg) was supplied by Agilent Technologies (Santa Clara, CA, USA).

In-house drug administration and control samples

Control (untreated) urine samples were collected from twenty-four healthy castrated male (gelding) thoroughbreds (imported from different countries in both hemispheres and aged between 3 and 9 years old) over a period from December 2013 to April 2014. Sampling was conducted under pre-race conditions on the morning of a raceday.

The administration protocols have already been published by the authors' laboratory.[3, 4] Briefly, a dose of 500 mg of androst-4-ene-3,6,17-tione (6-OXO) was administered orally to each of two thoroughbred geldings (aged 7 at 0.97 mg/kg and 11 at 0.98 mg/kg) by stomach tubing. Pre- and post-administration urine samples were collected at least twice daily for up to 6 days post administration.[3] For ATD, a dose of 800 mg of androsta-1,4,6-triene-3,17-dione (ATD) was administered orally to each of two thoroughbred geldings (aged 7 at 1.58 mg/kg and 8 at 1.63 mg/kg) by stomach tubing. Pre- and post-administration urine samples were collected at least twice daily for the first three days, and then once every day up to 9 days.[4] In this study, pre-administration urine samples were taken as untreated samples and were included in the control group. All samples were aliquoted as soon as possible upon receipt, and the aliquots were stored at around -80°C before analysis. Aliquots of administration urine samples as collected in the previous study were retrieved and analysed in this study. The administration experiments were approved in accordance with the Animals (Control of Experiments) Ordinance by the Licensing Authority of the Department of Health, the Government of the Hong Kong Special Administrative Region.

Deconjugation study

The efficiency of the deconjugation procedure was evaluated using testosterone sulfate (TS), testosterone glucuronide (TG), β-estradiol-3-(β-D-glucuronide)-17-sulfate (E2-3G17S) and β-estradiol-3-sulfate-17-glucuronide (E2-3S17G) as model compounds. Each of the conjugated steroids (equivalent to 50 ng/mL of free steroid) was spiked into a blank horse urine sample in duplicate and processed using the method described above. The concentrations of the deconjugated steroids and recoveries were estimated using a calibrator spiked with a standard mixture of testosterone (50 ng/mL) and 17β-estradiol (50 ng/mL) in blank urine analysed in parallel.

It is well known that many AAS undergo extensive phase II metabolism in the mammalian body.[5-7] In this study, urine samples were subjected to both enzyme hydrolysis and methanolysis in order to maximise the recoveries of free steroids from their glucuronide and/or sulfate conjugates, and hence provide a better estimate of the total amount of target steroids in the samples. The deconjugation efficiency of the method was evaluated using TS, TG, E2-3G17S, and E2-3S17G as model compounds. Our results showed that both TS and TG had very high recoveries at 126 % and 106 % respectively whereas the mixed conjugates E2-3G17S and E2-3S17G were lower at 76 % and 78 % respectively. Herein, the recoveries of TS and TG were found to be greater than 100 %, which could be attributed to the slight difference in extraction efficiency between free testosterone in the calibrator and conjugated testosterone in the hydrolysed spiked urine samples. On the other hand, the lower recoveries of the mixed conjugates steroids could be attributed to their possible loss during the SPE steps, or else the deconjugation steps used might be less effective on mixed conjugates. However, no attempt was made to verify the possible causes.

Table S1 GC-MS/MS experimental parameters of target steroids and their corresponding internal standards (IS).

Analyte	Abbr.	RT (min)	Quantitative transition	CE (eV)	Qualitative transition	CE (eV)	IS
5α-Androstane-3α,17α-diol	ααα	8.54	420.1 > 148.2	8	420.1 > 149.2	12	d_3-A
5(10)-Estrene-3β,17α-diol	EED	8.60	404.1 > 225.2	14	404.1 > 240.2	6	d_3-A
5α-Estrane-3β,17α-diol	EAD (αβα)	8.82	406.1 > 391.2	6	406.1 > 242.2	6	d_3-A
***d*$_3$-Androstanediol**	d_3-A	9.14	423.2 > 244.2	12	Internal Standard		
5α-Androstane-3α,17β-diol	ααβ	9.15	420.2 > 148.2	6	420.2 > 149.2	12	d_3-A
19-Norandrosterone	19-NA	9.16	422.1 > 378.2	6	422.1 > 199.2	18	d_3-A
Boldenone	B	9.17	578.1 > 414.1	6	578.1 > 251.2	14	d_3-T
Epitestosterone	E	9.18	580.1 > 253.2	10	580.1 > 146.2	8	d_3-T
5α-Estrane-3β,17β-diol	EAD (αββ)	9.20	406.1 > 201.2	8	406.1 > 159.1	12	d_3-A
17α-Estradiol	E2α	9.22	564.1 > 237.2	10	564.1 > 400.1	6	d_3-T
5α-Androstane-3β,17α-diol	αβα	9.27	420.1 > 148.2	8	420.1 > 149.2	12	d_3-A
***d*$_3$-Nandrolone**	d_3-N	9.36	569.1 > 148.1	10	Internal Standard		
Nandrolone	19-NT	9.38	566.1 > 133.1	16	566.1 > 146.2	8	d_3-N
4,6-Androstadien-17β-ol-3-one	6-T	9.39	578.1 > 133.2	16	578.1 > 280.1	8	d_3-T
19-Noretiocholanolone	19-NE	9.43	422.1 > 378.1	6	422.1 > 199.2	20	d_3-A
***d*$_3$-Testosterone**	d_3-T	9.51	583.1 > 568.2	10	Internal Standard		
Testosterone	T	9.52	580.1 > 270.1	8	580.1 > 146.2	10	d_3-T
5-Androstene-3β,17β-diol	AED	9.54	418.1 > 183.1	10	418.1 > 121.1	10	d_3-A
17β-Estradiol	E2	9.57	564.1 > 237.2	8	564.1 > 401.2	6	d_3-T
Androsterone	A	9.61	436.1 > 392.1	6	436.1 > 213.2	22	d_3-A
19-Norepiandrosterone	19-NEA	9.63	422.1 > 378.1	6	422.1 > 199.2	22	d_3-A
5α-Androstane-3β,17β-diol	αββ	9.67	420.2 > 148.2	6	420.2 > 149.2	10	d_3-A
4-Estrene-3,17-dione	NOR	9.80	418.1 > 149.1	12	418.1 > 105.1	26	d_3-T
4,6-Androstadien-3,17-dione	6-ADD	9.83	430.1 > 135.1	10	430.1 > 107.1	30	d_3-T
Boldione	1-ADD	9.85	430.1 > 264.1	8	430.1 > 235.1	20	d_3-T
Androstenedione	ADIONE	9.98	432.1 > 149.1	12	432.1 > 417.1	8	d_3-T
Estrone	E1	9.99	416.1 > 372.1	8	416.1 > 253.2	6	d_3-T
Dehydroepiandrosterone	DHEA	10.00	270.1 > 121.1	10	270.1 > 199.1	6	d_3-A
5β-Dihydrotestosterone	5β-DHT	10.08	436.1 > 366.2	8	436.1 > 201.2	16	d_3-A
Epiandrosterone	EA	10.15	436.2 > 213.2	22	436.2 > 239.2	16	d_3-A
Stanolone	5α-DHT	10.33	436.2 > 364.1	8	436.2 > 201.2	16	d_3-A
Etiocholanedione	5β-DIONE	10.60	288.2 > 244.2	6	288.2 > 255.2	8	d_3-A
Androstanedione	5α-DIONE	10.86	288.2 > 244.2	6	288.2 > 255.2	8	d_3-A
Progesterone	P4	10.88	460.1 > 147.1	10	460.1 > 455.2	8	d_3-T

Table S2 Inter-day precision, accuracy, extraction recovery of spiked quality control samples.

Analyte	5 ng/mL		20 ng/mL		40 ng/mL		Extraction recovery (%)
	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	
ααα	101	7.4	106	8.7	114	8.8	45.4
EED	94	2.8	100	6.8	100	6.9	43.6
EAD (αβα)	96	7.3	101	4.4	104	4.1	64.2
ααβ	91	5.7	96	3.5	102	6.2	51.5
19-NA	90	2.6	97	4.3	99	5.0	51.7
B	110	6.7	111	7.7	107	7.0	65.0
E	106	4.7	108	6.1	106	4.5	62.6
EAD (αββ)	87	5.9	93	6.9	95	4.7	59.0
E2α	108	11.1	108	10.2	109	14.1	19.8
αβα	97	2.9	100	4.5	104	3.0	53.3
19-NT	97	4.2	102	5.7	103	6.0	61.2
6-T	108	30.7	91	34.9	93	43.2	59.2
19-NE	93	2.8	98	2.6	103	2.5	60.4
T	105	4.2	105	5.7	103	1.6	62.7
AED	94	5.9	99	7.7	101	5.2	62.4
E2	104	12.3	103	9.5	101	11.9	17.6
A	95	5.8	97	4.9	101	4.2	47.5
19-NEA	92	6.2	99	5.4	101	2.9	55.3
αββ	93	4.5	96	6.5	99	2.7	56.7
NOR	99	6.9	101	8.6	97	2.7	58.2
6-ADD	105	18.5	90	21.6	91	25.5	58.1
1-ADD	100	8.1	101	8.0	99	6.0	64.1
ADIONE	102	5.4	103	8.7	99	3.5	60.6
E1	92	12.1	97	11.2	97	13.7	17.4
DHEA	91	5.5	96	8.4	98	7.3	57.0
5β-DHT	101	6.9	110	6.6	115	9.7	54.0
EA	99	8.6	100	9.3	95	12.9	51.1
5α-DHT	113	10.7	125	11.9	128	12.4	49.6
5β-DIONE	105	14.4	110	11.1	115	11.3	52.4
5α-DIONE	109	12.3	119	11.3	120	10.3	49.2
P4	96	6.2	99	13.1	97	5.8	30.9

Table S3 Intra-day precision, limit of detection (LoD) and limit of quantification (LoQ) of spiked quality control samples (n=6).

Analyte	5 ng/mL Intra-day precision (%RSD)	LoD (ng/mL)	LoQ (ng/mL)
ααα	3.3	0.5	1.7
EED	7.6	1.1	3.5
EAD (αβα)	2.5	0.4	1.2
ααβ	2.7	0.4	1.2
19-NA	4.0	0.6	1.9
B	2.9	0.5	1.7
E	4.8	0.9	2.9
EAD (αββ)	2.8	0.4	1.2
E2α	2.3	0.4	1.4
αβα	2.1	0.3	1.1
19-NT	2.1	0.3	1.1
6-T	11.4	1.9	6.2
19-NE	4.0	0.5	1.8
T	3.3	0.5	1.8
AED	2.0	0.3	0.9
E2	2.5	0.4	1.4
A	4.6	0.6	1.8
19-NEA	4.4	0.6	2.0
αββ	3.1	0.4	1.4
NOR	3.8	0.6	1.9
6-ADD	5.6	1.0	3.2
1-ADD	2.2	0.4	1.2
ADIONE	2.2	0.3	1.0
E1	2.6	0.4	1.4
DHEA	2.6	0.4	1.3
5β-DHT	5.6	0.7	2.5
EA	5.1	0.8	2.8
5α-DHT	5.1	0.7	2.4
5β-DIONE	4.3	0.6	2.0
5α-DIONE	4.4	0.6	2.1
P4	6.9	1.2	4.1

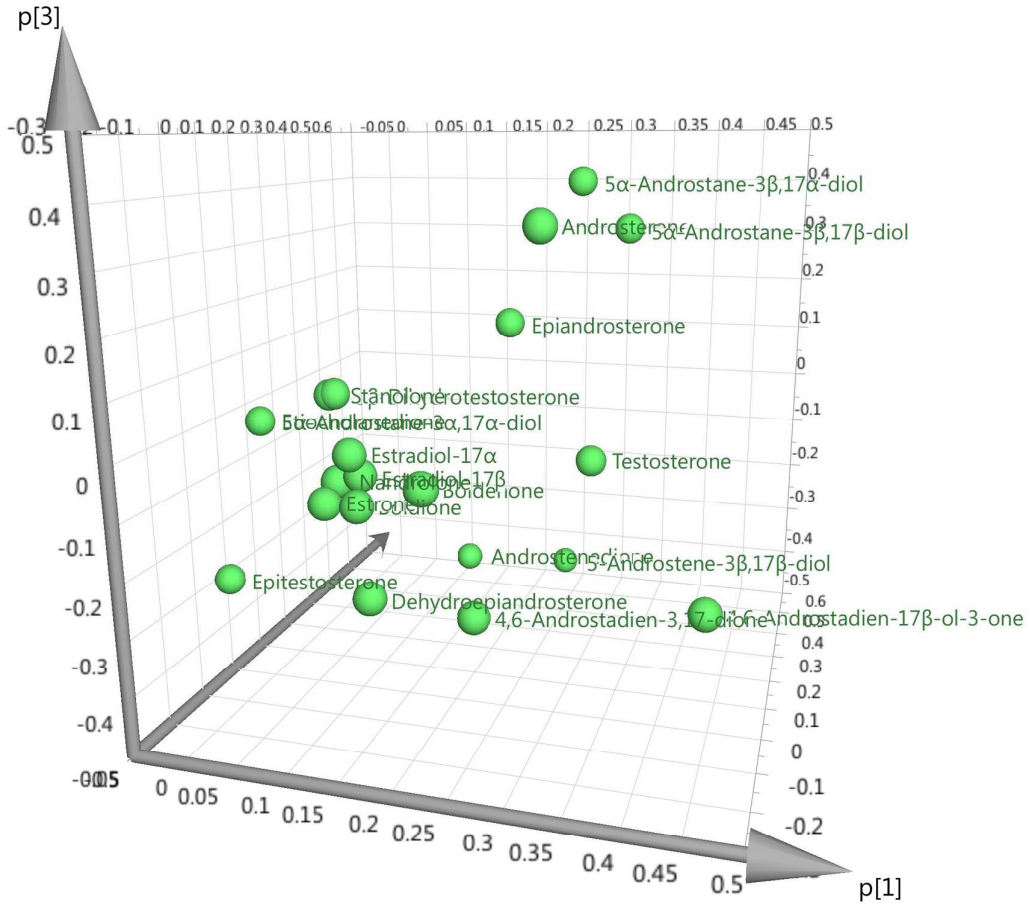

Figure S1 Three dimensional PCA loading plot with respect to the treatment classification and post administration time profile.

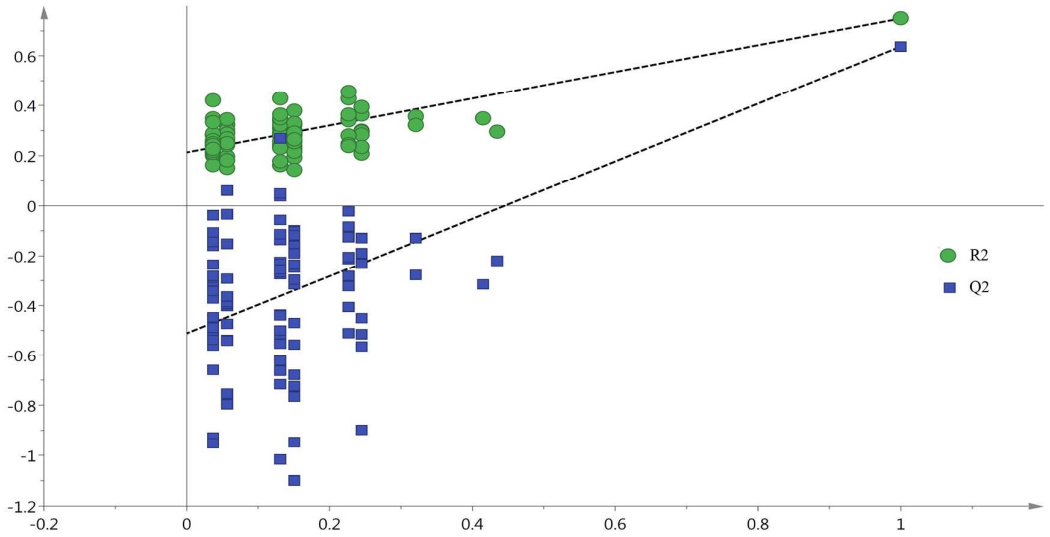

Figure S2 Validation plot obtained from 100 permutation tests of the OPLS-DA model.

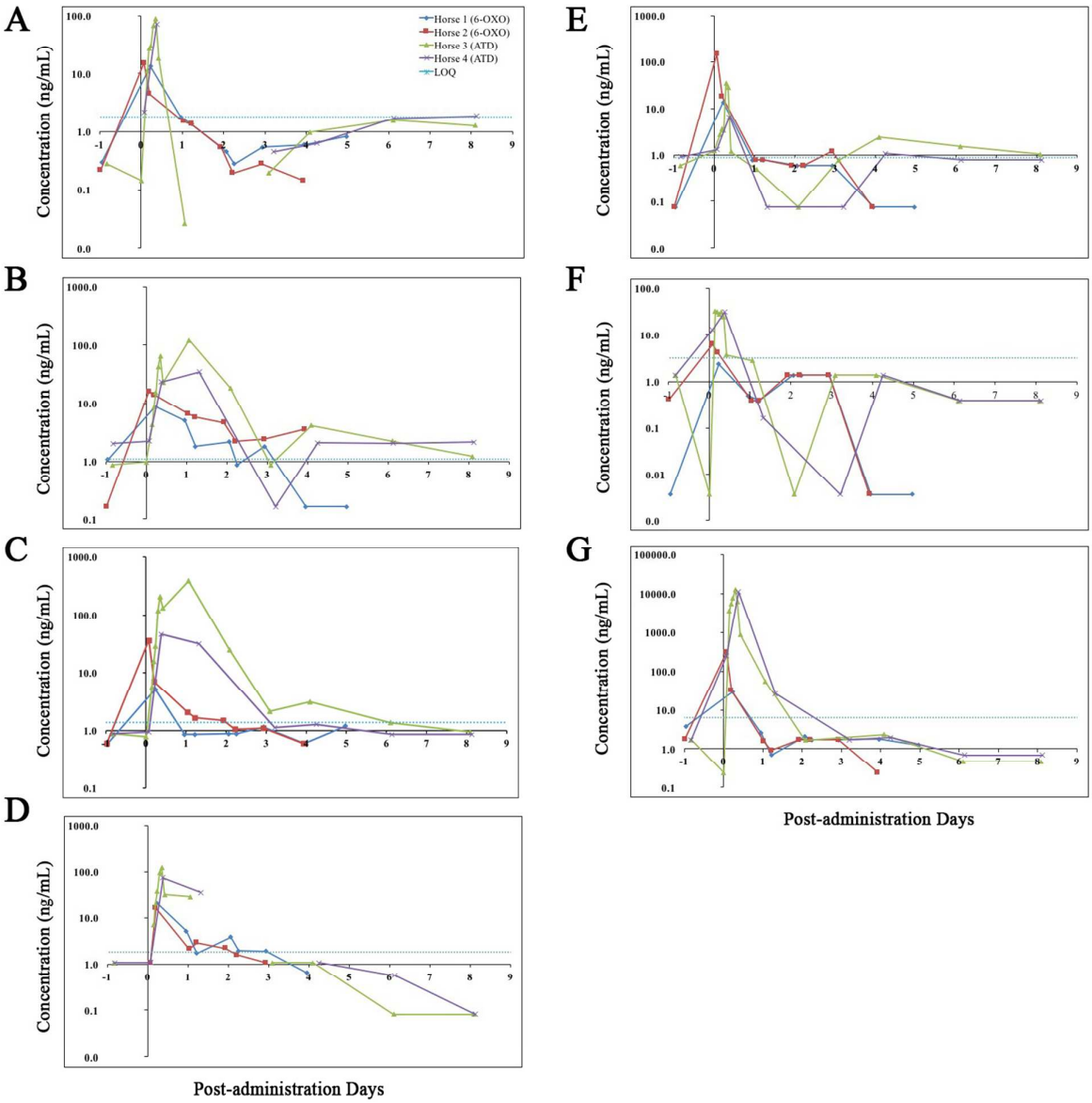

Figure S3 Urinary elimination profiles of (A) testosterone; (B) 5α-androstane-3β,17α-diol; (C) 5α-androstane-3β,17β-diol; (D) androsterone; (E) 5-androstene-3β,17β-diol; (F) 4,6-androstadien-3,17-dione; and (G) 4,6-androstadien-17β-ol-3-one following oral treatment with respectively 6-OXO (2 geldings, Horses 1 and 2, blue and red lines) and ATD (2 geldings, Horses 3 and 4, green and purple lines).

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