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Detection and confirmation of 60 anabolic and androgenic steroids in equine plasma by liquid chromatography-tandem mass spectrometry with instant library searching

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In 2008, Pennsylvania (PA) became the first State in the USA to ban and enforce the ban on the use of anabolic and androgenic steroids (AAS) in equine athletes by using plasma for analysis. To enforce the ban, a rapid and high-throughput method for analysis of 60 AAS in equine plasma was developed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Analytes were recovered from plasma by liquid-liquid extraction (LLE) using methyl *tert*-butyl ether, separated on a reversed-phase C_{18} column and analyzed by electrospray ionization mass spectrometry. Multiple-reaction monitoring (MRM) scan was employed for screening. When the MRM signal of an analyte exceeded 1000 counts per second (cps), information-dependent acquisition (IDA) triggered generation of an enhanced product ion (EPI) scan of the analyte. A library for the analytes was simultaneously established using the EPI spectrum. Unambiguous identification of any of the 60 AAS in a test sample was based on both the presence of MRM response within the correct retention time (t_R) window and a qualitative match between EPI spectrum of the test sample and that of the reference drug standard stored in the library. Total analysis time was 7 min. The limit of detection (LOD) and limit of confirmation (LOC) for most of the analytes were 0.01–2 ng/mL and 0.1–10 ng/mL, respectively. Recovery of the analytes from plasma by LLE was 74–138%. The method was successfully verified and is routinely used in the screening of post-race equine plasma samples for the presence of these 60 AAS. The method is rapid, sensitive, reproducible, and reliable.
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Keywords: anabolic and androgenic steroids; LC-MS; MRM; plasma; racehorses

Introduction

Anabolic and androgenic steroids (AAS) are related to the male hormone, testosterone. These agents promote muscle growth and protein production by converting fat to protein.[1] Although these physiological and biochemical properties of AAS are beneficial under certain circumstances, they are, perhaps, the most compelling reasons for their extensive use and abuse to enhance performance in both human and equine athletes.[2] The use of AAS in human sports is prohibited by the World Anti-Doping Agency (WADA),[3] and the recent US Congressional Hearings on the use of AAS by baseball players has led to unprecedented actions by the Commissioner of baseball to enforce the ban on the use of AAS by baseball players.[4] In the horseracing industry, the Association of Racing Commissioners' International banned the use of AAS in equine athletes in 2008, whereas the International Federation of Horseracing Authorities (IFHA) in Asia and Europe had banned and enforced AAS in racehorses many decades earlier. Prior to the 2008 ban, AAS were freely employed in equine veterinary practice in the USA. Besides enhancing athletic performance, the use of AAS in horses induces aggressive behaviour which can threaten not only the safety and welfare of horses but also of the personnel working with and around equine athletes.[5]

To protect the safety and welfare of racehorses and their support personnel, Pennsylvania (PA) became the first jurisdiction in the USA to employ plasma concentration of AAS in enforcing the ban on their use in racehorses. Since testosterone and probably nandrolone, are naturally produced by colts, stallions, cryptochids, and monorchids, the PA Racing Commission established tolerance concentrations of testosterone and nandrolone in colts and stallions.[6] Although the ban on AAS in sports in European and Asian countries has steadfastly been enforced through doping control for some decades, the recent actions taken in the USA, though seemingly late, are encouraging.

Recently, the use of liquid chromatography-mass spectrometry (LC-MS) for the analysis of AAS in doping control has steadily gained popularity.[7–20] A screening method for the detection of up to 34 AAS in urine by LC-MS has been reported.[21] In a recent publication, an LC-MS method for screening of 66 drugs including 20 AAS in equine plasma was also described.[22] The first

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LC-MS method for the detection, quantification, and confirmation of eight AAS in equine plasma was published in 2005,[23] and updated in 2009 using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) in which the analysis time was greatly improved.[24] Based on the recognition that the abuse of AAS in racehorses is not limited to only 8 AAS, a more comprehensive and high-throughput method to monitor a larger number of AAS was needed. In recognition of the limitations of our previous study,[23] it was necessary, in the present study, to increase the number of AAS reported in our 2005 and 2009 publications,[23,24] to include some of the agents from the WADA list,[3] and are only those AAS that are considered readily ionizable and those non-ionizable AAS are the subject of our future work by gas chromatography-mass spectrometry (GC-MS).

Banning the use of these agents in competition is insufficient and unenforceable by any jurisdiction without the use of a sensitive, verifiable, and defensible method for screening, detection, and confirmation of the presence of AAS in a test sample. To address this issue, a rapid, high-throughput, and comprehensive method for analysis of 60 AAS (Figure 1) in equine plasma was developed.

Experimental

Chemicals and reagents

2α-Hydroxymethylethisterone, 6β-hydroxyturinabol, norbolethone, methyldienolone, 6β-hydroxyfluoxymesterone, turinabol, melengesterol, 16β-hydroxystanozolol, and 3-hydroxystanozolol were obtained from Cerilliant (Round Rock, Texas, USA). Tetrahydrogestrinone (THG) was kindly donated by Professor Thomas Tobin of the Maxwell H. Gluck Equine Research Center, University of Kentucky (Lexington, KY, USA).[25] The remaining AAS were purchased from Steraloids (New Port, RI, USA) (Table 1). All chemical solvents used were of high performance liquid chromatography (HPLC) grade purchased from ThermoFisher Scientific (Fair Lawn, NJ, USA). Ammonium formate (certified) was purchased from ThermoFisher Scientific (Pittsburgh, PA, USA). Formic acid was purchased from Merck KgaA (Darmstadt, Germany). HPLC-grade water was obtained from Burdick & Jackson (Muskegon, MI, USA).

Standard solutions

Stock solution (1.0 mg/mL) of each of the reference AAS standards was individually prepared from the powder by dissolving it in methanol. The working solutions of each analyte at 10, 1, 0.1 and 0.01 µg/mL were prepared by dilution of each stock solution with 10% acetonitrile in 5 mM ammonium formate (pH 3.51), and stored at 4 °C.

A stock solution of ammonium formate buffer (pH 3.51) was prepared from ammonium formate (0.5 mol/L) and formic acid (0.5 mol/L), and stored at 4 °C. A lower concentration (5 mmol/L) ammonium formate buffer was prepared by diluting the stock solution with water (HPLC grade) and transferred into a reagent bottle with a screw cap and stored at room temperature. All reagents were discarded after 180 days or if cloudiness occurred.

Sample preparation

Blood samples were centrifuged at 3000 rpm (~1409×g) for 10 min to harvest plasma. Analytes were extracted from plasma by liquid-liquid extraction (LLE). To each plasma aliquot (0.5 mL), 5 mL of methyl *tert*-butyl ether (MTBE) was added and gently mixed by rotorack for 10 min before centrifugation as described above. The organic layer (top) was carefully decanted into a prelabelled fresh

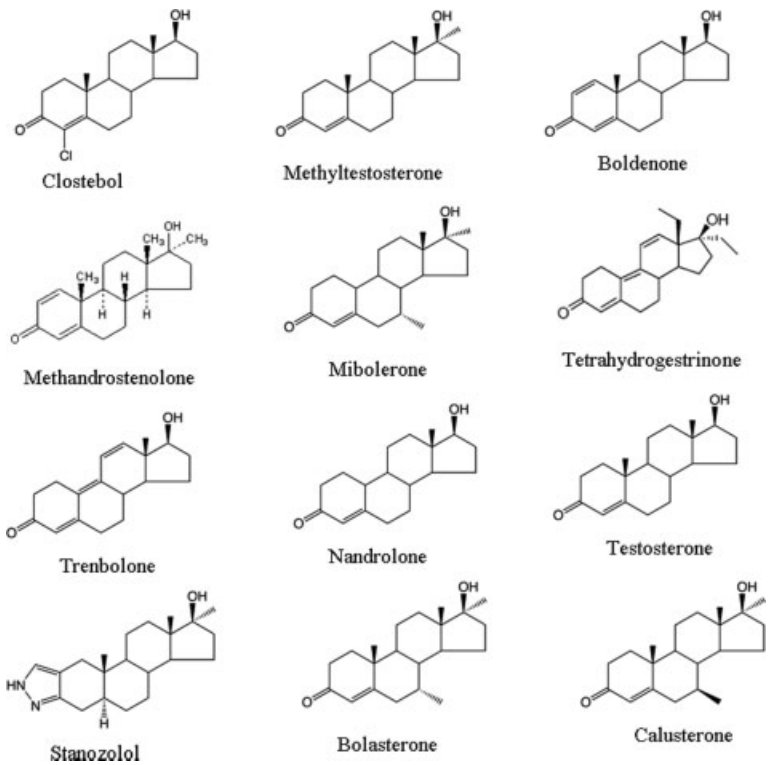

Figure 1. Chemical structures of the 60 AAS in this study.

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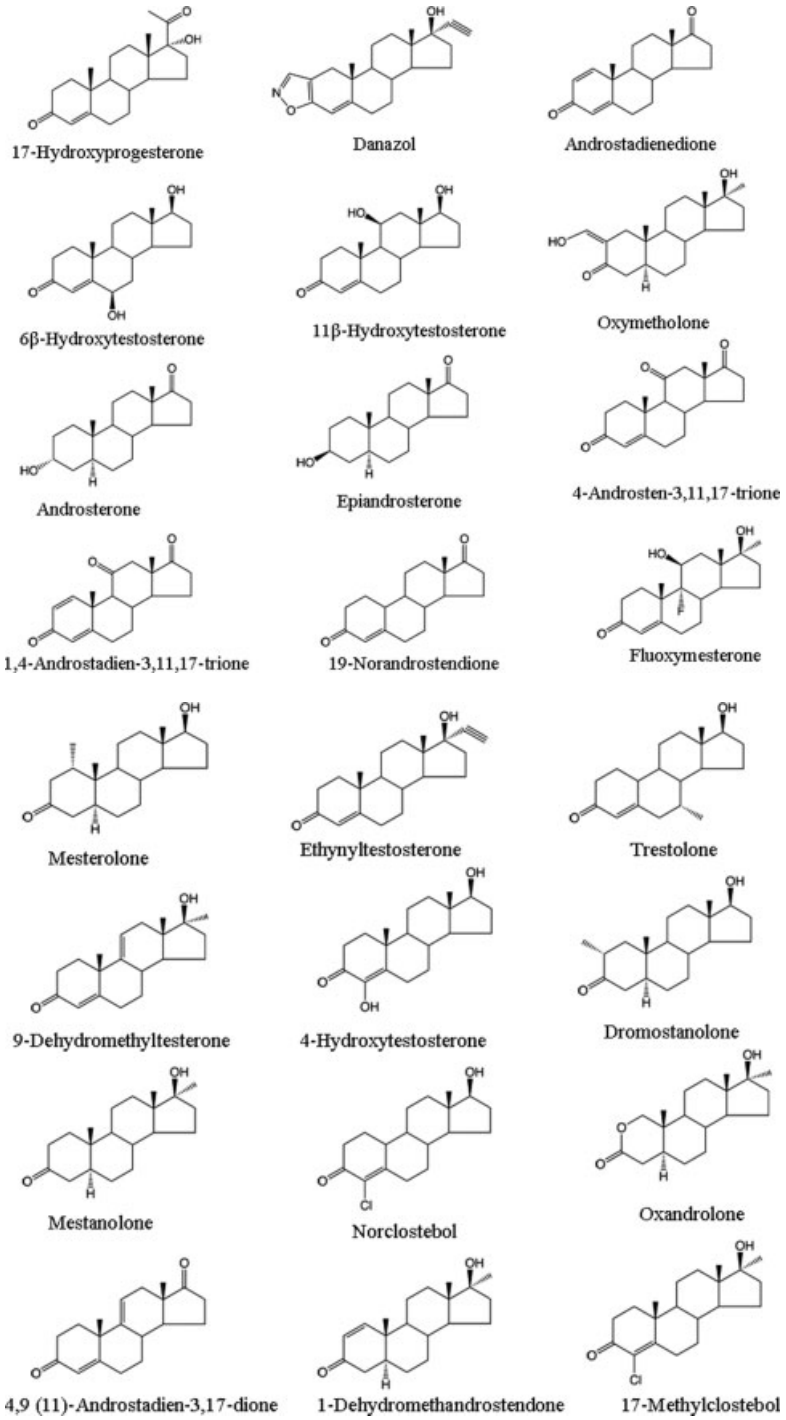

Figure 1. (*Continued*).

glass tube (16 mm × 100 mm) and dried at 50 °C on a hot block (Techni Dri-Block DB-3, Duxford, Cambridge, UK) under a steady stream of air or nitrogen.[23,24] The dried residue was reconstituted in 100 µL of 10% acetonitrile in 5 mM ammonium formate (pH 3.51) from which 20 µL was used for analyses by LC-MS.

LC-MS conditions

The HPLC system consisted of a 20A LC with SIL-HTc autosampler (Shimadzu Scientific Instruments, Columbia, MD, USA). Analyte separation was achieved on a reversed-phase ACE C_{18} column (7.5 cm × 2.1 mm ID, 5 µm particle size) with its guard column (1 cm × 2.1 mm ID, 5 µm particle size; Mac-Mod Analytical, Chadds Ford, PA, USA). Mobile phase comprised 5 mM ammonium formate (pH 3.51) as solvent A with acetonitrile as solvent B. Programmed mobile phase gradient was used for analyte separation: 90% solvent A and 10% solvent B for the first 0.1 min, solvent B was increased to 80% from 0.1 to 2 min and held for additional 2 min prior to returning to initial condition for 3 min. The mobile phase flow was at 300 µL/min. Column compartment temperature was maintained at 30 °C.

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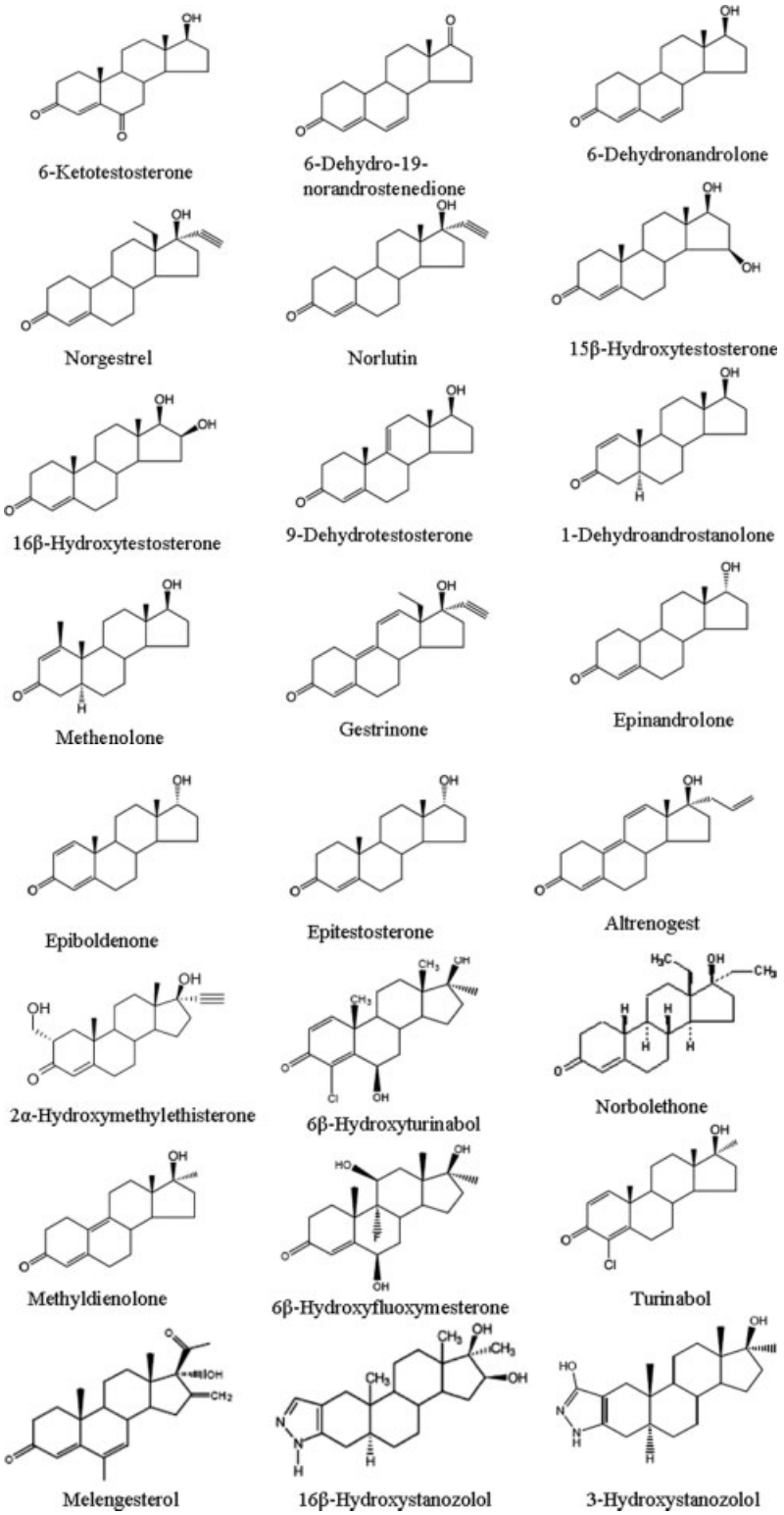

Figure 1. (*Continued*).

LC-MS analysis was performed on a 4000 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) with an electrospray ionization (ESI) source operated in positive ion mode. The source-dependent parameters were optimized using flow injection analysis (FIA). IonSpray voltage was set at 4000V, and source temperature was at 550 °C. Curtain gas, gas 1 and gas 2 were 25, 40 and 40 psi, respectively. In MRM scan mode, detection of the 60 AAS was achieved by monitoring the most intense product ion (PI) of each analyte. Declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) used for each analyte are listed in Table 1. Entrance potential (EP) for all analytes was 10 V. Dwell time for each ion transition

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Table 1. MRM parameters and retention time for each of the 60 AAS

NO	Drug	Precursor ion (*m/z*)	Selected Product ion (*m/z*)			Retention time (min)	DP (V)	CE (eV)	CXP (V)
1	Clostebol	323	143*	131	269	4.24	82	36	10
2	Methyltestosterone	303	109*	97	109	4.11	95	39	7
3	Boldenone	287	121*	135	173	3.82	56	30	9
4	Methandrostenolone	301	121*	149	173	3.93	80	36	8
5	Mibolerone	303	107*	267	245	4.11	70	42	8
6	Tetrahydrogestrinone	313	159*	241	199	4.41	120	33	13
7	Trenbolone	271	199*	107	165	3.8	84	33	16
8	Nandrolone	275	109*	239	145	3.89	49	38	8
9	Testosterone	289	97*	109	253	4.01	54	34	7
10	Stanozolol	329	81*	95	107	4.3	50	74	6
11	Bolasterone	317	97*	123	203	4.23	85	37	7
12	Calusterone	317	97*	123	203	4.29	72	36	7
13	17-Hydroxyprogesterone	331	109*	97	271	4.13	78	41	7
14	Danazol	338	148*	120	310	4.52	78	36	12
15	Androstadienedione	285	121*	147	151	3.95	52	33	8
16	6β-Hydroxytestosterone	305	269*	251	157	3.39	75	21	6
17	11β-Hydroxytestosterone	305	269*	121	147	3.58	77	23	6
18	Oxymetholone	333	99*	107	121	4.94	106	45	7
19	Androsterone	291	255*	147	199	4.44	60	21	6
20	Epiandrosterone	291	255*	147	161	4.22	60	21	6
21	4-Androsten-3,11,17-trione	301	257*	265	121	3.79	106	32	6
22	1,4-Androstadien-3,11,17-trione	299	147*	171	223	3.74	65	32	11
23	19-Norandrostendione	273	109*	197	83	4.01	73	36	8
24	Fluoxymesterone	337	131*	181	241	3.66	85	47	9
25	Mesterolone	305	269*	229	173	4.35	83	25	6
26	Ethynyltestosterone	313	97*	109	123	4.09	42	35	6
27	Trestolone	289	107*	109	177	3.99	87	41	8
28	9-Dehydromethyltesterone	301	147*	159	91	4.05	81	37	11
29	4-Hydroxytestosterone	305	125*	113	175	4.01	74	38	9
30	Dromostanolone	305	269*	215	173	4.57	80	23	6
31	Mestanolone	305	269*	229	159	4.41	88	24	6
32	Norclostebol	309	143*	213	237	4.16	79	41	10
33	Oxandrolone	307	271*	229	121	3.92	68	20	7
34	4,9(11)-Androstadien-3,17-dione	285	252*	147	227	4.11	79	35	6
35	1-Dehydromethandrostendone	303	201*	145	267	4.29	53	26	4
36	17-Methylclostebol	337	143*	131	157	4.37	83	40	10
37	6-Ketotestosterone	303	121*	133	105	3.65	77	32	9
38	6-Dehydro-19-norandrostenedione	271	149*	105	107	3.95	77	30	11
39	6-Dehydronandrolone	273	133*	151	79	3.82	85	32	10
40	Norgestrel	313	109*	245	133	4.23	76	39	8
41	Norlutin	299	109*	231	145	4.01	63	40	8
42	15β-Hydroxytestosterone	305	97*	109	269	3.33	80	36	7
43	16β-Hydroxytestosterone	305	97*	109	269	3.6	96	36	7
44	9-Dehydrotestosterone	287	147*	259	145	3.91	82	36	12
45	1-Dehydroandrostanolone	289	187*	205	91	4.13	78	29	14
46	Methenolone	303	83*	187	205	4.12	80	36	6
47	Gestrinone	309	241*	199	262	4.05	76	33	13
48	Epinandrolone	275	109*	239	145	4.01	77	39	8
49	Epiboldenone	287	121*	135	173	3.95	54	35	9
50	Epitestosterone	289	109*	97	253	4.14	80	37	8
51	Altrenogest	311	227*	269	159	4.22	81	33	5
52	2α-Hydroxymethylethisterone	343	139*	127	109	3.79	74	35	10
53	6β-Hydroxyturinabol	351	147*	315	155	3.64	66	28	7
54	Norbolethone	317	109*	281	245	4.61	69	41	8
55	Methyldienolone	287	159*	135	107	3.9	87	40	12
56	6β-hydroxyfluoxymesterone	353	315*	239	121	3.25	72	25	8

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Table 1. (*Continued*)

NO	Drug	Precursor ion (*m/z*)	Selected Product ion (*m/z*)			Retention time (min)	DP (V)	CE (eV)	CXP (V)
57	Turinabol	335	155*	149	107	4.2	56	41	12
58	Melengesterol	355	279*	237	187	4.21	72	30	7
59	16β-Hydroxystanozolol	345	81*	95	121	3.81	80	78	6
60	3-Hydroxystanozolol	345	97*	121	107	3.62	83	68	7

* is the most intense product ion used in MRM survey scan. CE and CXP are for the most intense product ion.

was 30 ms with 5 ms pause time. In enhanced product ion (EPI) scan mode, the scan rate was 4000 Da/s, linear ion trap (LIT) fill time was 20 ms, collision activated dissociation (CAD) was set to high, collision energy (CE) was 35 eV, and collision energy spread (CES) was 15 eV. Information dependent acquisition (IDA) was set to acquire EPI spectrum only when MRM signal of an analyte was >1000 cps. In cases involving co-eluted peaks, former target ions were always excluded after two occurrences in 30 sec. Data acquisition and analysis were accomplished with the help of Analyst 1.4.2 software (Applied Biosystems).

Creating library for identification of analytes

Two experiments were performed in order to create a search library for identification of analytes in a single analysis. The first involved MRM scans to obtain the most intense PI; the second was for sensitive EPI scans to obtain EPI spectra. The acquired EPI spectrum of a given analyte was then stored in the library for subsequent use during library search for analyte identification by spectral match comparison. The retention time (t_R) and EPI spectrum of each reference drug standard were obtained during a single analysis.

Extraction efficiency

MTBE was evaluated for its extraction efficiency of the 60 AAS from equine plasma. The analytes were divided into four groups to prepare a mixture of standards/group. Three sets of six duplicate plasma samples containing the analytes at three different concentrations (0.5, 1, 5 ng/mL) for each group were prepared and extracted using MTBE. Extraction efficiency was calculated according to the Equation (1):

$$\text{Extraction Efficiency (\%)} = A_{extract}/A_{non-extract} \times 100 \quad (1)$$

where $A_{non-extract}$ is the peak area of an analyte standard added to the extract of blank plasma, and $A_{extract}$ is that of the analyte standard added to blank plasma and extracted.

Matrix effect

Matrix effect of the 60 AAS was evaluated. Water instead of plasma was used to compare the matrix effect contributed by plasma with that by water. Matrix effect was evaluated in six replicates at 5 ng/mL and calculated by comparing chromatographic peak area of each of the AAS standards added to water extract with that of the analyte added to blank plasma extract and calculated according to the Equation (2):

$$\text{Matrix effect (\%)} = (A_{plasma\ extract} - A_{water\ extract})/A_{water\ extract} \times 100 \quad (2)$$

where $A_{water\ extract}$ is the peak area of an analyte standard in water extract, and $A_{plasma\ extract}$ is that of the analyte standard added to blank plasma extract.

Results and Discussions

Chromatography

LC-MS/MRM chromatograms of the 60 AAS each at 5 ng/mL spiked into blank equine plasma, previously determined to be free of AAS by this same method, extracted and analyzed are shown in Figure 2. The sensitivity of androsterone and epiandrosterone was lower than that of other AAS and thus, were spiked at 100 ng/mL, similarly extracted and analyzed. Some of the 60 AAS have very similar chemical structures, such as testosterone and epitestosterone, boldenone and epiboldenone, nandrolone and epinandrolone which are epimer pairs, respectively, whereas 15β-hydroxytestosterone and 16β-hydroxytestosterone are isomer pairs. Each pair of epimer or regio-isomer was completely resolved under the present LC conditions. The retention time of each of the 60 AAS is listed in Table 1.

Mass spectrometry

Optimization of the analyte-dependent parameters was performed by manually tuning for each analyte. Selection of protonated molecule, $[M+H]^+$, and optimization of DP were performed by infusing 1 µg/mL or 10 µg/mL of individual reference drug standard solution in full-scan mode. Identification of the three most abundant PIs (any PI resulting from loss of water molecule was excluded due to lack of specificity) and selection of the optimum CE and CXP for each analyte was achieved in PI and MRM scan modes. Three ion transitions from $[M+H]^+$ were selected for each analyte (Table 1), the most intense PI (diagnostic) was chosen for use in screening for the specific analyte, whereas the remaining two ions from the same precursor ion were used for confirmation of the analyte by comparison of ion intensity ratio. The MRM transitions, DP, CE, and CXP of each target analyte are listed in Table 1. It is important to note that in this method, only 51 MRM transitions were used to detect the 60 analytes, since some of the analytes share the same MRM transitions. For example, nandrolone and epinandrolone shared the same ion transition of their protonated molecule to specific PI (*m/z* 275 → 109); boldenone and epiboldenone shared *m/z* 287 → 121; androsterone and epiandrosterone shared *m/z* 291 → 255; bolasterone and calusterone, as diastereomers, shared *m/z* 317 → 97;

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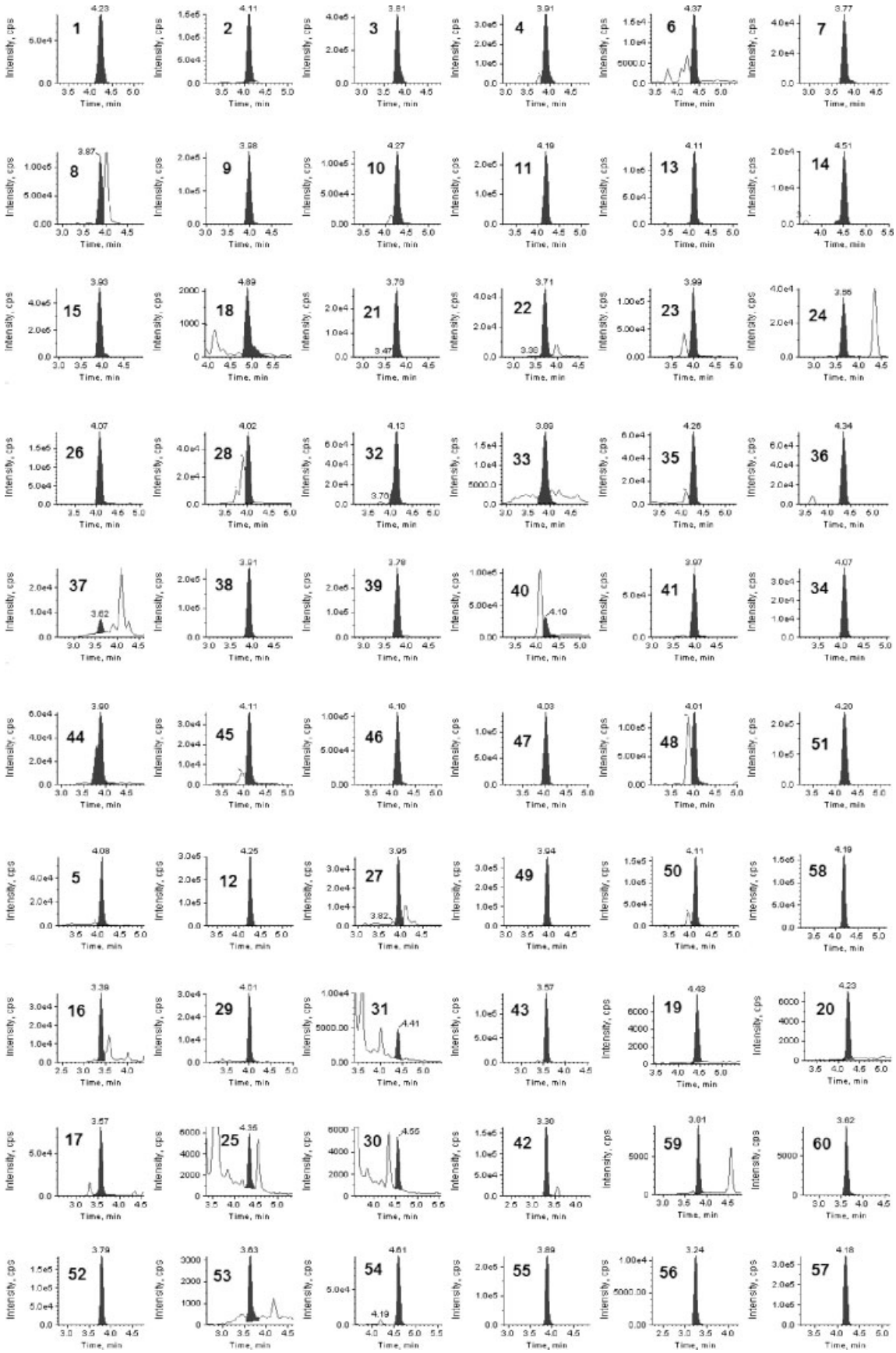

Figure 2. LC-MS/MRM chromatograms of the 60 AAS (5 ng/mL) spiked into blank equine plasma, extracted and analyzed. The numbering of each chromatogram is in the same order as in Table 1, which provides the names of the corresponding AAS. All blank plasma samples were collected from geldings and tested by this method to ensure that they were free of these analytes.

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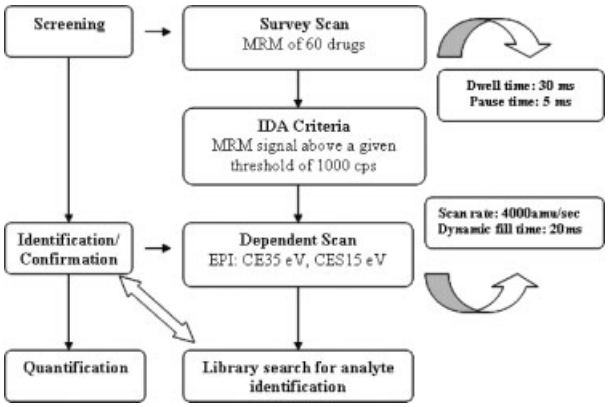

Figure 3. Schematic representation of the screening, identification, confirmation and quantification processes for the 60 AAS.

6β-hydroxytestosterone, 11β-hydroxytestosterone, mesterolone, dromostanolone and mestanolone shared *m/z* 305 → 269; 15β-hydroxytestosterone and 16β-hydroxytestosterone shared *m/z* 305 → 97 transition. The EPI spectrum of an unknown compound was readily obtained if its MRM transition were one of the 51 transitions (Table 1).

Screening and identification of 60 AAS in a single analysis

In this study, we employed the scan speed and hybrid functionalities of the mass spectrometer (MS), particularly its ability to switch the operational mode from quadrupole-quadrupole (QqQ) to quadrupole-linear ion trap (QqLIT) to acquire EPI spectra which allowed simultaneous acquisition of sensitive MRM scan and full scan information. The fast scan speed and the linear accelerator collision cell (LINAC) technology allowed acquisition of a large number of MRM scans within a short time period. The specificity of each MRM obtained remained free from cross-talk that is greatly dampened by LINAC even in very fast MRM scan mode without compromising sensitivity, specificity, and selectivity. In addition, it provides high duty cycle with decreased dwell time and wide *m/z* range even during multi-target analyses.[26] With these various capabilities of the MS, we were able to develop a high-throughput method for screening, detection, and identification of the 60 analytes within a single analysis.

During method verification using post-race equine plasma, an IDA experiment was conducted which allowed analysis of data during acquisition, and determined the masses on which to perform dependent scans. Predefined criterion of 1000 cps was used to activate an IDA experiment. The survey scan was an MRM scan for screening, monitoring only the most intense ion transitions for each analyte (Table 1). IDA was configured to initiate a sensitive EPI scan when the MRM survey scan signal was >1000 cps. Figure 3 is the schematic summary of the overall method used in this study.

The generation of PI was based on collision energy spread (CES) function, and the resulting EPI spectrum was the sum of the spectra acquired at three different CE values (CE-CES, CE, CE+CES). With CES, information for low and high mass fragments was obtained in a single spectrum. An example of the difference between fixed CE and CES is shown in Figure 4. Panels (a), (b), and (c) represent EPI spectrum of boldenone at different CE: 20, 35 and 50 eV,

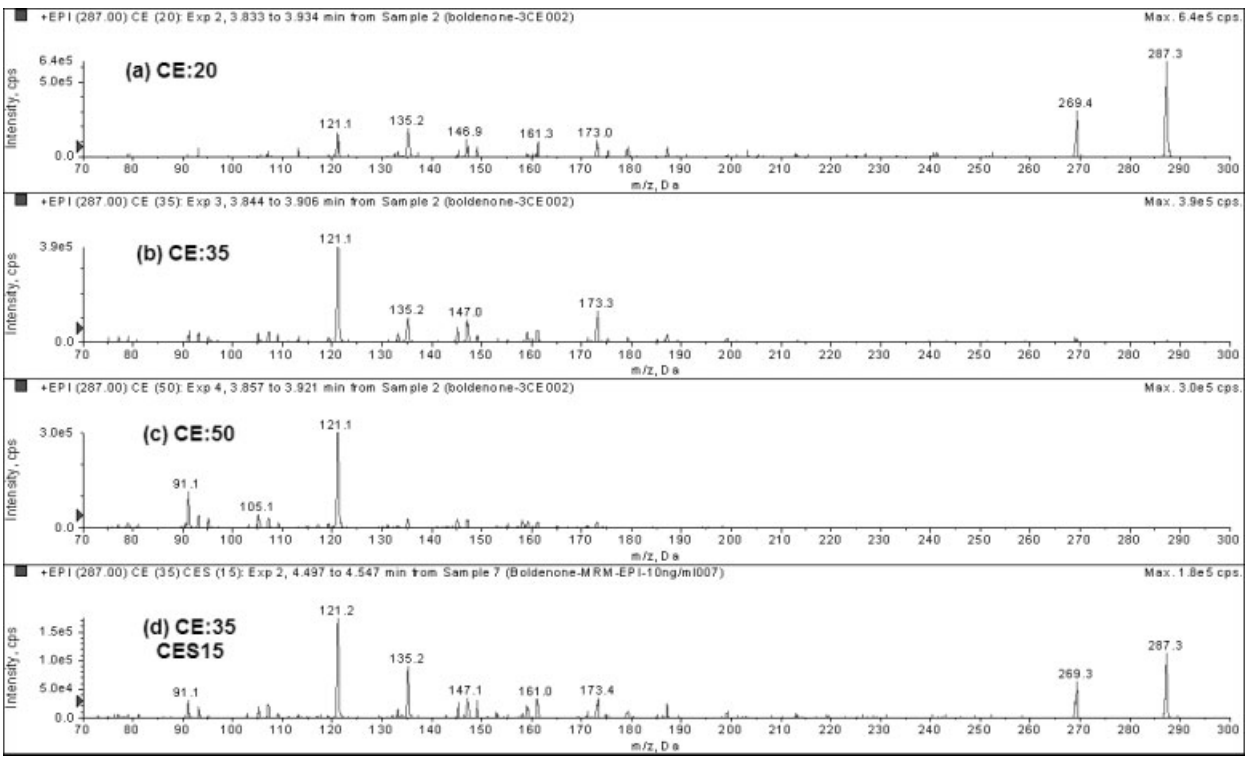

Figure 4. Comparison of EPI spectra of boldenone under different CEs (20, 35 and 50 eV). Panel (a) is the EPI spectrum of boldenone under CE of 20 eV, panel (b) is the EPI spectrum of boldenone under CE of 35 eV, panel (c) is the EPI spectrum of boldenone under CE of 50 eV, panel (d) is the EPI spectrum of boldenone under CE of 35 eV with CES of 15 eV, which is the sum of the spectra at three different CEs (20, 35, 50 eV). In comparing these EPI spectra of boldenone (panels (a), (b) and (c)), panel (d) under CE of 35 eV with CES of 15 eV provided richer information for low and high mass fragments in a single spectrum than those of CE 20, 35 or 50 eV alone.

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respectively. Panel (d) is the EPI spectrum of boldenone under CES function (CE 35 eV, Spread 15 eV). From this example, it was determined that the EPI spectrum that was obtained using CES function provided richer information on the target analyte than that of CE alone. Thus, the EPI spectrum of each reference drug standard at CE (35 eV) with CES (15 eV) was what was actually stored in the library.

When a 'real world' test sample was analyzed for AAS, the EPI spectrum that was obtained from IDA experiment was recorded at a specified CE (35 eV) with CES (15 eV), which represented the same collision energies used in creating the library. Thus, the EPI spectrum was used to perform an instant library search for an analyte based on the EPI spectral information that had already been determined and stored in the library for each of the 60 AAS during method development. The usefulness of an IDA experiment and instant library searching is that detection and identification of an analyte could be achieved at the same time to avoid false identification. In addition, the analysis time was greatly reduced. Total analysis time was 7 min. The criteria for identification of 60 AAS in equine plasma were defined as (1) library match indicating the similarity in EPI spectrum between an unknown sample and that stored in the library, the match of which must be higher than 70%; and (2) t_R of any of the AAS in an unknown sample must be within $\pm$0.1 min of that in Table 1.

Although AAS are very similar in chemical structure, most of them were completely resolved and identified by the combination of t_R and EPI spectrum. It should be noted that there were still some of the AAS that were not readily identified even after applying library searching. For example, bolasterone and calusterone as a diastereomer pair with the same protonated molecule, $[M + H]^+$, at *m/z* 317, and their EPI spectra in the library were identical and could not be completely resolved by t_R, so these two drugs were not readily discriminated from each other. In addition, mesterolone and mestanolone could not be discriminated from each other. The method for discriminating these four AAS from one another was developed in a separate study, and would readily be applied if the presence of mesterolone (t_R = 4.35 min), mestanolone (t_R = 4.41 min), bolasterone (t_R = 4.23 min) or clausterone (t_R = 4.29 min) were predicted by this method.

Confirmation of AAS

In doping control, confirmation of the presence of an analyte in a test sample is required to demonstrate that the 'chemical fingerprint' of the analyte in a sample is the same as that of an authentic drug standard.[23,24] In this study, due to the large number of AAS and the similarity in chemical structures, confirmation by only one criterion is unacceptable. Thus, retention time, product ion intensity ratio, and EPI spectrum were used as criteria for analyte confirmation. The three product ions employed in confirmation for calculating ion intensity ratio are listed in Table 1. The ion intensity ratio was obtained using peak height and was calculated relative to the most abundant product ion. Similarity in the ion intensity ratio was calculated according to Equation (3):

$$\text{Ion intensity ratio similarity (\%)} = R_{unknown}/R_{standard} \times 100 \quad (3)$$

where $R_{unknown}$ represents the ion intensity ratio for the unknown sample and $R_{standard}$ represents that for the drug standard. Thus, the criteria for confirmation of the presence of AAS in equine plasma were defined as (1) library match indicating the similarity in EPI spectrum between an unknown sample and that in the library, the match of which must be higher than 70%; (2) similarity in ion intensity ratio between unknown samples and corresponding standard samples must be within 90–110%; and (3) t_R of any of the AAS in an unknown sample must be within $\pm$0.1 min of the reference drug standard (Table 1).

Extraction efficiency and sensitivity

Extraction efficiency of MTBE achieved for all the target analytes was 74–138% with relative standard deviation of less than $\pm$25% (Table 2) indicating that the recovery of all the analytes from plasma by MTBE was acceptable.[27] With respect to sensitivity, the limit of detection (LOD) was defined as the lowest concentration of any of the AAS spiked into plasma resulting in MRM signal that was three times greater than noise. The limit of confirmation (LOC) was the lowest concentration at which the EPI spectrum could be obtained for confirmation of each analyte. LOD of most of the analytes was 0.01–2 ng/mL and LOC was 0.05–10 ng/mL, except 3-hydroxystanozolol, androsterone and epiandrosterone (Table 2).

Matrix effect

Endogenous components extracted from plasma may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes. It is for this reason that the matrix effect was evaluated under the experimental conditions used in this study to determine if plasma enhanced or suppressed response for any of the AAS. As shown in Table 2, the average ion suppression or enhancement for all these analytes was less than 20%, except for danazol, suggesting that matrix effect was negligible.

Method application

Applicability of the method was tested on 'real world' plasma samples collected from racehorses post-competition in PA. The samples were monitored for the 60 AAS using the most intense MRM transition and t_R for screening and EPI spectrum with library searching for identification. If the presence of any of the AAS were identified via library searching, then confirmation by ion intensity ratio comparison between the sample and the reference drug standard and quantification method would be separately initiated for the analyte as previously reported.[24] Quantification of all AAS listed below for which plasma threshold had been established is important to determine whether the result obtained is above or below the allowable plasma threshold established for boldenone, nandrolone, testosterone, and stanozolol in PA since all other AAS have zero tolerance.[6] An example of the result obtained from the analyses performed on a plasma sample collected from a racehorse post-competition in PA is shown in Figure 5. Panel (a) in Figure 5 represents the total ion chromatogram (TIC) of the unknown sample in which 51 MRM transitions of AAS were monitored. Panel (b) is the extracted ion chromatogram (XIC) of the unknown peak with *m/z* 311 $\rightarrow$ 227, and t_R of 4.27 min. Thus, the screening result indicated that the suspect analyte was altrenogest (Table 1). Panel (c) represents the EPI spectrum of the unknown analyte at CE (35 eV) with CES (15 eV), which was simultaneously obtained during IDA experiment. EPI spectrum was used in searching for a match in the established library of the target analytes (60 AAS). The spectrum of the unknown (panel (a) in Figure 6) and that of the first matched compound (panel (b) in Figure 6; altrenogest)

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Table 2. Recovery, LOD and LOC of the 60 AAS

NO	Drug	Recovery (%)/CV (%) 0.5 ng/mL (n=6)	1 ng/mL (n=6)	5 ng/mL (n=6)	LOD (ng/mL)	LOC (ng/mL)	Matrix effect
1	Clostebol	114 (12.5)	115 (13.5)	101 (14.6)	0.05	0.25	−9.1
2	Methyltestosterone	116 (7.7)	107 (7.4)	97 (7.2)	0.05	0.25	−8.2
3	Boldenone	107 (9.7)	113 (3.8)	100 (6.9)	0.03	0.25	−11.0
4	Methandrostenolone	108 (13.4)	115 (7.4)	98 (5.6)	0.02	0.1	−8.5
5	Mibolerone	120 (20.2)	81 (7.13)	113 (10.8)	0.05	0.25	−6.3
6	Tetrahydrogestrinone	–	100 (20.9)	106 (14.8)	0.5	2.5	−12.3
7	Trenbolone	112 (6.6)	106 (8.5)	93 (8.6)	0.1	0.5	−6.6
8	Nandrolone	92 (13.6)	116 (6.7)	99 (8.4)	0.1	0.5	−3.1
9	Testosterone	109 (4.9)	111 (6.6)	99 (10.3)	0.02	0.1	−9.0
10	Stanozolol	123 (13.3)	104 (18.9)	125 (19.3)	0.1	0.5	−18
11	Bolasterone	120 (11.2)	116 (11.6)	100 (14.6)	0.02	0.1	−11.6
12	Calusterone	113 (7.5)	101 (8.7)	122 (11.8)	0.02	0.1	−2.8
13	17-Hydroxyprogesterone	124 (15.3)	123 (10.9)	96 (12.3)	0.05	0.25	5.1
14	Danazol	100 (9.2)	95 (14.4)	101 (18.1)	0.05	0.25	−35.9
15	Androstadienedione	98 (9.9)	108 (3.3)	101 (6.0)	0.01	0.05	−7.5
16	6β-Hydroxytestosterone	78 (27.9)	96 (10.7)	96 (5.5)	0.1	0.5	−9.2
17	11β-Hydroxytestosterone	109 (11.3)	100 (6.5)	114 (3.9)	0.05	0.25	−8.6
18	Oxymetholone	–	–	111 (34.4)	1.7	10	−6.1
19	Androsterone	–	–	–	50	250	–
20	Epiandrosterone	–	–	–	50	250	–
21	4-Androsten-3,11,17-trione	100 (9.1)	92 (5.2)	102 (6.9)	0.1	0.5	−0.2
22	1,4-Androstadien-3,11,17-trione	92 (16.1)	95 (12.2)	104.3 (6.6)	0.1	0.5	−3.2
23	19-Norandrostendione	109 (13.5)	106 (4.0)	99 (5.4)	0.1	0.5	−3.5
24	Fluoxymesterone	84 (10.3)	85 (8.2)	102 (6.3)	0.05	0.25	−8.8
25	Mesterolone	–	93 (15.4)	138 (9.6)	1	5	−2.1
26	Ethynyltestosterone	112 (7.9)	114 (7.8)	95 (10.3)	0.03	0.25	−13.4
27	Trestolone	100 (9.4)	93 (7.0)	109 (8.7)	0.05	0.25	−5.3
28	9-Dehydromethyltesterone	113 (18.0)	116 (13.1)	96 (10.6)	0.1	0.5	−19.4
29	4-Hydroxytestosterone	116 (23.5)	103 (14.0)	112 (11.0)	0.1	0.5	−9.4
30	Dromostanolone	–	74 (33.4)	129 (12.4)	0.5	2.5	2.1
31	Mestanolone	–	–	116 (17.3)	1	5	−8.3
32	Norclostebol	102 (12.5)	107 (7.1)	98 (10.4)	0.05	0.25	−11.9
33	Oxandrolone	74 (40.0)	94 (25.1)	91 (12.6)	0.1	0.5	3.8
34	4,9(11)-Androstadien-3,17-dione	100 (18.3)	111 (10.6)	96 (8.4)	0.05	0.25	−9.8
35	1-Dehydromethandrostendone	113 (15.1)	125 (9.0)	100 (11.4)	0.1	0.5	−17.6
36	17-Methylclostebol	116 (13.8)	109 (15.4)	106 (14.8)	0.03	0.25	−9.7
37	6-Ketotestosterone	–	–	103 (15.9)	1	5	−3.1
38	6-Dehydro-19-norandrostenedione	104 (10.7)	106 (8.6)	98 (7.1)	0.02	0.1	3.2
39	6-Dehydronandrolone	101 (11.5)	114 (4.5)	97 (7.0)	0.02	0.1	−9.5
40	Norgestrel	–	–	89 (14.1)	1	5	−11.8
41	Norlutin	106 (9.8)	116 (8.3)	98 (9.8)	0.05	0.25	−5.0
42	15β-Hydroxytestosterone	110 (3.9)	122 (7.1)	117 (3.9)	0.02	0.1	−9.6
43	16β-Hydroxytestosterone	110 (8.4)	100 (5.7)	101 (7.3)	0.1	0.5	−9.1
44	9-Dehydrotestosterone	114 (21.4)	131 (8.6)	98 (8.7)	0.1	0.5	−9.7
45	1-Dehydroandrostanolone	118 (17.9)	115 (13.3)	96 (10.1)	0.1	0.5	−2.9
46	Methenolone	110 (10.9)	124 (10.6)	101 (11.5)	0.05	0.25	−8.5
47	Gestrinone	109 (8.7)	117 (10.9)	97 (12.1)	0.02	0.1	−11.2
48	Epinandrolone	93 (21.2)	114 (8.0)	100 (7.5)	0.1	0.5	−3.5
49	Epiboldenone	103 (10.2)	91 (7.8)	106 (9.6)	0.05	0.25	−11.3
50	Epitestosterone	109 (8.7)	89 (7.3)	116 (10.8)	0.02	0.1	−7.9
51	Altrenogest	120 (12.1)	118 (13.1)	100 (13.5)	0.01	0.05	−6.2
52	2α-Hydroxymethylethisterone	100 (5.8)	111 (6.3)	99 (4.4)	0.05	0.25	−6.3
53	6β-Hydroxyturinabol	–	–	94 (15.1)	2	10	−1.6
54	Norbolethone	82 (17.1)	122 (17.1)	83 (17.8)	0.05	0.25	3.2
55	Methyldienolone	101 (5.8)	118 (5.1)	103 (6.3)	0.02	0.1	−6.9
56	6β-hydroxyfluoxymesterone	93 (18.6)	98 (15.1)	112 (19.3)	0.5	2.5	−0.6

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Table 2. (*Continued*)

NO	Drug	Recovery (%)/CV (%) 0.5 ng/mL (n=6)	1 ng/mL (n=6)	5 ng/mL (n=6)	LOD (ng/mL)	LOC (ng/mL)	Matrix effect
57	Turinabol	93 (9.7)	113 (11.8)	92 (7.3)	0.02	0.1	0.7
58	Melengesterol	88 (10.2)	126 (10.9)	98 (7.9)	0.05	0.25	−11.3
59	16β-Hydroxystanozolol	111 (13.6)	112 (9.3)	97 (10.0)	0.1	0.5	−10.4
60	3-Hydroxystanozolol	–	–	102 (15.2)	5	50	−19.3

" – " not available at the concentrations indicated.

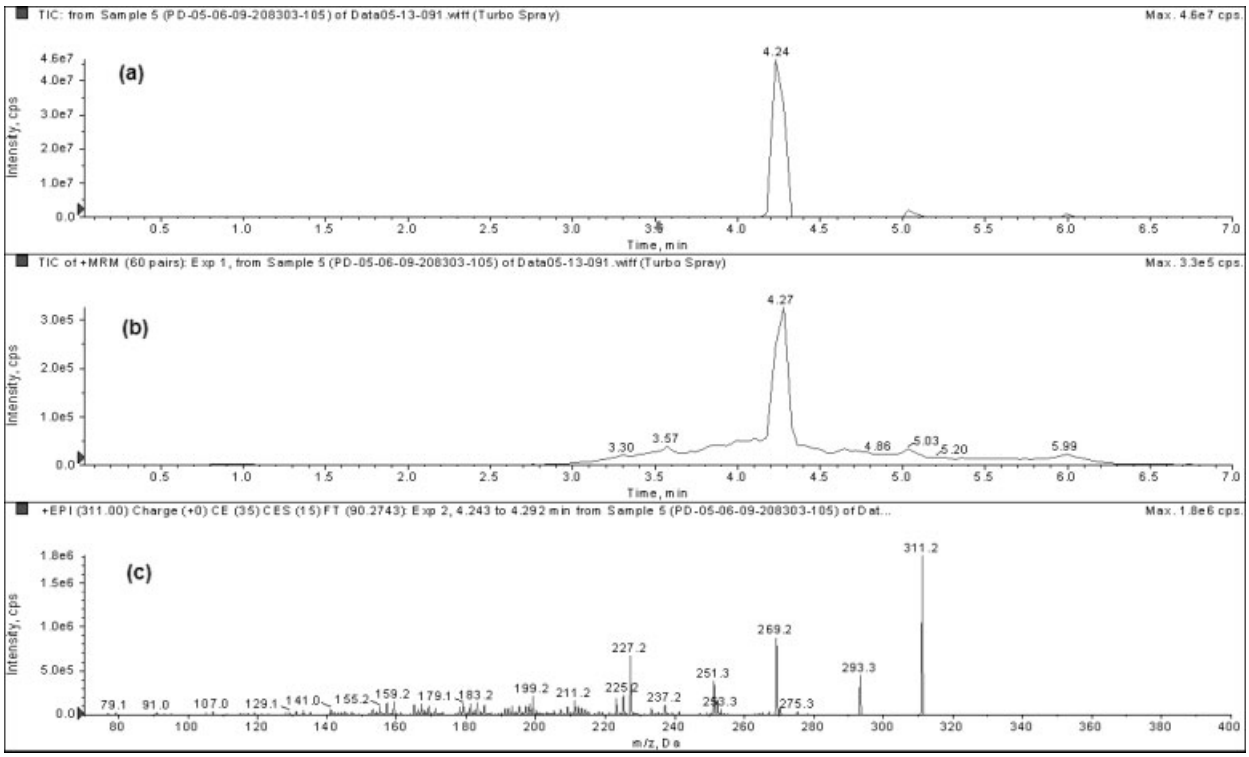

Figure 5. Example of IDA experiment for the identification of altrenogest in equine plasma. Panel (a) represents TIC of the unknown sample in which 51 transitions of 60 AAS were monitored. Panel (b) is the XIC for the unknown peak while panel (c) is its EPI spectrum.

by performing instant library search is shown in Figure 6. The fit and reverse fit values (%) were 93% and 91%, respectively (Analyst v 1.4.2; Applied Biosystem). These values indicate a very high match of the EPI spectrum for the unknown with that of the reference drug standard (altrenogest) in the library. A match of 70% or greater was considered an acceptable measure of similarity in the EPI spectrum of the unknown compared with that of the reference drug standard. From the result obtained, the presence of altrenogest in the test sample was identified. Ion intensity ratio comparison was also obtained as an additional criterion for confirmation of the presence of altrenogest in test sample #208303 to ensure accuracy and reliability of the result. The three selected MRM transition ions (*m/z* 311 → 227, 311 → 269 and 311 → 159) of altrenogest (Table 1) were used in performing ion intensity ratio comparison (Figure 7). The result indicated that the unknown compound in sample #208303 of panel (b) in Figure 7 had the same ion intensity ratios with those of the reference standard (altrenogest) in panel (a). Thus, the presence of altrenogest in sample #208303 was confirmed by t_R, similarity in ion intensity ratios, and EPI spectrum match. Finally, quantification was performed to determine the concentration of altrenogest in the plasma sample #208303. Standard response curve was generated ($r^2 = 0.9989$) using a weighted (1/x) linear regression model from which the concentration (4.88 ng/mL) of altrenogest in the plasma sample #208303 was determined. This concentration of altrenogest that was present in equine plasma during competition would ordinarily constitute a violation of the ban on AAS during competition in PA because the only acceptable plasma concentration of most AAS in racehorses at the time of competing in an official race is zero except endogenous testosterone and nandrolone in colts and stallions (male horses).[6] However, since altrenogest has an acceptable therapeutic value for blocking 'heat' cycle in mares, both the concentration of altrenogest in plasma and the sex of the horse must be reported to the regulating PA Racing Commission.

While the use of hair samples would provide information on retrospective use of AAS, its use in the racing industry is not practical. In fact, in some US jurisdictions, the type of sample

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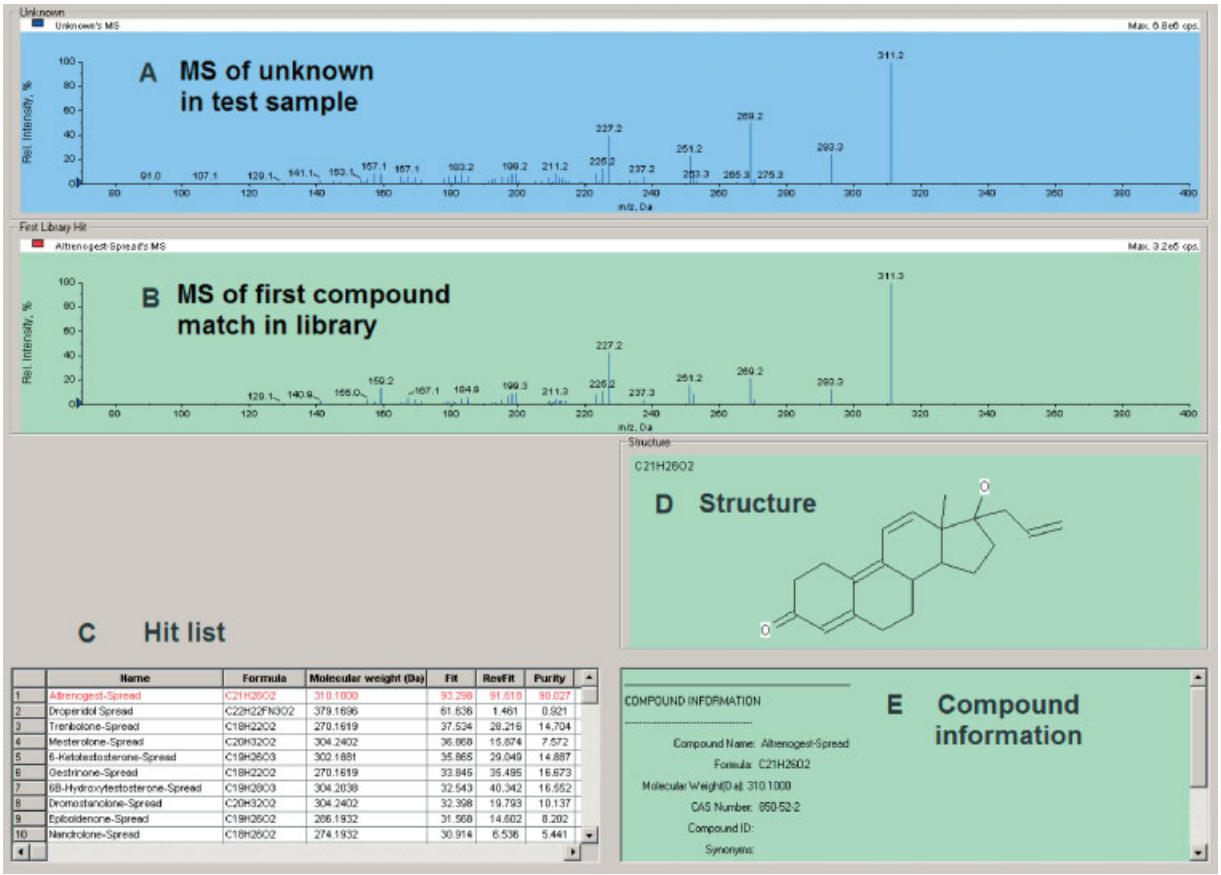

	Name	Formula	Molecular weight (Da)	Fit	RevFit	Purity
1	Altrenogest-Spread	C21H26O2	310.1000	93.298	91.618	90.027
2	Droperidol Spread	C22H22FN3O2	379.1696	61.636	1.461	0.921
3	Trenbolone-Spread	C18H22O2	270.1619	37.534	28.216	14.704
4	Mesterolone-Spread	C20H32O2	304.2402	36.868	15.874	7.572
5	6-Ketotestosterone-Spread	C19H26O3	302.1881	35.865	29.049	14.887
6	Gestrinone-Spread	C18H22O2	270.1619	33.845	35.495	16.673
7	6B-Hydroxytestosterone-Spread	C19H28O3	304.2038	32.543	40.342	16.552
8	Dromostanolone-Spread	C20H32O2	304.2402	32.398	19.793	10.137
9	Epiboldenone-Spread	C19H26O2	286.1932	31.568	14.602	8.202
10	Nandrolone-Spread	C18H26O2	274.1932	30.914	6.536	5.441

Figure 6. Identification of target compound by library searching. Panel A represents EPI spectrum of an unknown while B represents that of reference standard stored in the library. The fit (%) and reverse fit (%) values for the first compound in the hit list (panel C) indicated the similarity between the two spectra (panels A and B). The chemical structure of the first matched compound in the library is displayed in panel D. Panel E provides compound information such as compound name, formula, molecular weight and CAS #.

allowed for equine drug testing is legislated and hair is not one of the samples. Thus, under such circumstances, deviation from urine and plasma to using hair becomes difficult if not impossible to introduce without a legislative mandate.

Since urine and plasma are the two most popular matrices for doping control analyses in the horseracing industry, we chose plasma for this study because it is readily available and has greater relevance than urine to the biophase for the pharmacology of any drug. Some have raised the issue of persistent pharmacologic effect of AAS long after the plasma concentrations have declined to undetectable concentrations. This type of action is either related to the low rate of dissociation of AAS from the receptor or the ability to initiate a cascade of physiologic or biochemical events that proceed thereafter in the absence of AAS as the initiators. For example, in the androgenic effect of AAS, once protein synthesis is initiated, muscle-building processes would proceed in the absence of AAS from plasma. The administration of testosterone in a mare or gelding induces offensive sexual and aggressive behaviour which threatens the safety and welfare of the horse and personnel.[28] However, when it is withdrawn, the offensive behaviour is equally withdrawn. Again, following an intramuscular administration (IM) of a therapeutic dose (1.1 mg/kg) of boldenone to the horse, the range of plasma concentration was 679–1704 pg/mL within 48 to 192 h but slowly declined to 58 pg/mL on day 30.[29] The behavioural effects were noted in approximately one week of the IM which coincided with the peak plasma concentration and diminished within two weeks as the plasma concentration declined. Clinically, the dose is repeated approximately every two weeks to sustain maximal effect. These observations do not suggest highly sustained pharmacologic actions long after plasma concentrations of those AAS that were studied had been depleted or decreased below detection. Thus, the suggestion that the pharmacology of AAS persists long after the plasma concentrations had been depleted may be drug-selective and should not be generalized. In terms of detection, it should be noted that an inability to detect an analyte does not always mean total absence of the analyte from the medium or biophase; rather it is sometimes an indication of the LOD of the analyte by the instrument/method used.

The Commonwealth of PA regulates the use of AAS by monitoring plasma samples obtained from equine athletes post competition. If meaningful guidelines are to be established for withdrawal times or upper tolerance concentrations for AAS, plasma is the appropriate matrix to use. Penalties for violation of the rules on the use of AAS in equine athletes competing in PA are loss of purse, suspension, and fine.

Conclusion

A high-throughput LC-MS/MS method was developed for comprehensive and simultaneous analysis of 60 AAS in equine plasma. The

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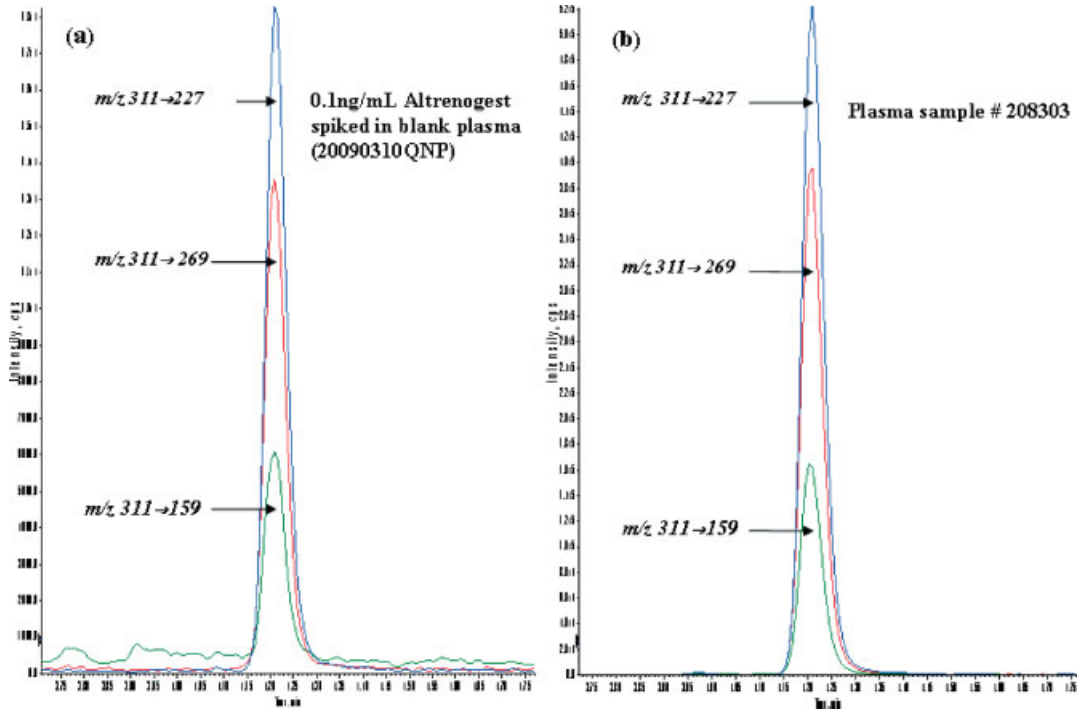

Figure 7. Comparison of the ion intensity ratios between the ''real world'' plasma sample (panel (b)) and altrenogest reference standard (0.1 ng/mL) spiked into blank equine plasma and similarly extracted and analyzed (panel (a)). Panel (a) shows three MRM transitions of altrenogest spiked in blank plasma and analyzed. Panel (b) shows the same three MRM transitions (m/z 311→269, 311→227 and 311→159) of altrenogest in equine plasma sample #208303 obtained from a racehorse post-competition in PA. The similarity in ion intensity ratios comparison indicated that plasma sample #208303 contained the same analyte, altrenogest, as shown in panel (a).

method was successfully verified and is routinely used in the analysis of plasma samples collected from racehorses post-competition in PA. The 60 AAS covered in this study were detected, identified, and confirmed with subsequent quantification for testosterone and nandrolone in non- castrated male horses, and boldenone and stanozolol for all horses regardless of sex.[6]

All other AAS in any racehorse competing in PA have zero regulatory tolerance concentration in plasma during competition. A library containing the EPI spectra of all 60 analytes was also established during the method development to permit unambiguous analyte identification by instant library searching and comparison of the 'fingerprint' of any of the 60 AAS in a suspect sample with that of the corresponding reference standard. With an IDA experiment which combines an MRM scan with that of EPI during the same analysis, screening and identification of the 60 AAS in a single analysis of 7 min was achieved. To our knowledge, this is the first report on the analysis of 60 AAS in a single analysis using equine plasma. This method has provided a new and defensible enforcement arm for banning the use of AAS in racehorses during competition in PA. The method is fast, sensitive, comprehensive, and reliably reproducible.

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