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Quantification of Three Prohibited Anabolic-Androgenic Steroids in Equine Urine using Gas Chromatography-Tandem Mass Spectrometry

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Abstract : Anabolic-androgenic steroids (AAS) are used illegally to enhance muscle development and increase strength and power. In this study, a reliable, and sensitive quantitative method was developed and validated using heptafluorobutyric acid anhydride (HFPA) derivatives for the simultaneous detection of prohibited AAS (testosterone [TS], boldenone [BD], 5α-estrane-3β,17α-diol [EAD]) using gas chromatography-tandem mass spectrometry (GC-MS/MS). For processing the samples, solid phase extraction, methanolic hydrolysis, and liquid-liquid extraction were used. For detection using mass spectrometry, the multiple reaction monitoring (MRM) mode was used with the electron ionization (EI) positive mode. The method was evaluated for selectivity, linearity, lower limit of quantification, intra- and inter-day precision, accuracy, and stability. The results showed that the method was accurate and reproducible for the quantitation of the three steroids. The developed method was finally applied to the analysis of a suspect gelding urine sample received from the Asian Quality Assurance Program (AQAP).

Keywords : Anabolic androgen steroid, GC-MS/MS, Doping control, Equine urine

Introduction

Anabolic-androgenic steroids (AAS) are hormones related to masculinity that exhibit both anabolic (protein-synthesizing) and androgenic (masculinizing) effects.[1] AAS enhance the development of muscles due to their anabolic effects that in turn help to increase strength and power.[2] For this reason, the use of natural or synthetic AAS in horses has been prohibited by the International Federation of Horseracing Authorities (IFHA).[3]

In horses, testosterone (TS), and its metabolites boldenone (BD) and 5α-estrane-3β,17α-diol (EAD) are the representative endogenous AAS (Figure 1).[4] TS (17β-hydroxyandrost-4-en-3-one) is a potent sex steroid hormone, produced endogenously in varying degrees by both intact male and female horses.[5] TS is often recommended in veterinary medicine to improve physical appearance.[6] BD (androsta-1,4-dien-17β-ol-3-one) is an anabolic steroid with a low androgenic potency that is used illegally as a growth promoter and performance enhancer in race horses.[7-8] Originally, it was developed primarily for veterinary use and is well known under the trade names Equipoise, Ganabol, Equigan, and Ultragan.[1] EAD is a metabolite of nandrolone and is produced naturally in pregnant animals or upon the administration of nandrolone.[9] For this reason, IFHA prohibits the use of three substances (TS, BD, EAD) and stipulates that the concentration should be distinguished when administered externally.[3] And for this, analytical evaluation for quantitative analysis in the laboratory is required.

Liquid chromatography-mass spectrometry (LC-MS) is a sensitive analytical technique used in simultaneous drug analysis. It is sometimes necessary to use LC-MS to analyze AAS to find an appropriate derivatization method. Also, atmospheric pressure chemical ionization (APCI) is used to improve sensitivity for AAS analysis due to unsuitable proton affinity for several steroids under electrospray ionization (ESI) conditions.[10-12] However, compared to gas chromatography-mass spectrometry (GC-MS), LC-MS lacks universal standardized derivatization that satisfies all the functional groups (hydroxyls, phenols, and carbonyls) of steroids.[10,12] GC-MS has traditionally been used in steroid analysis. By reducing the polarity of the steroid polar functional groups with various derivative methods, chroma-

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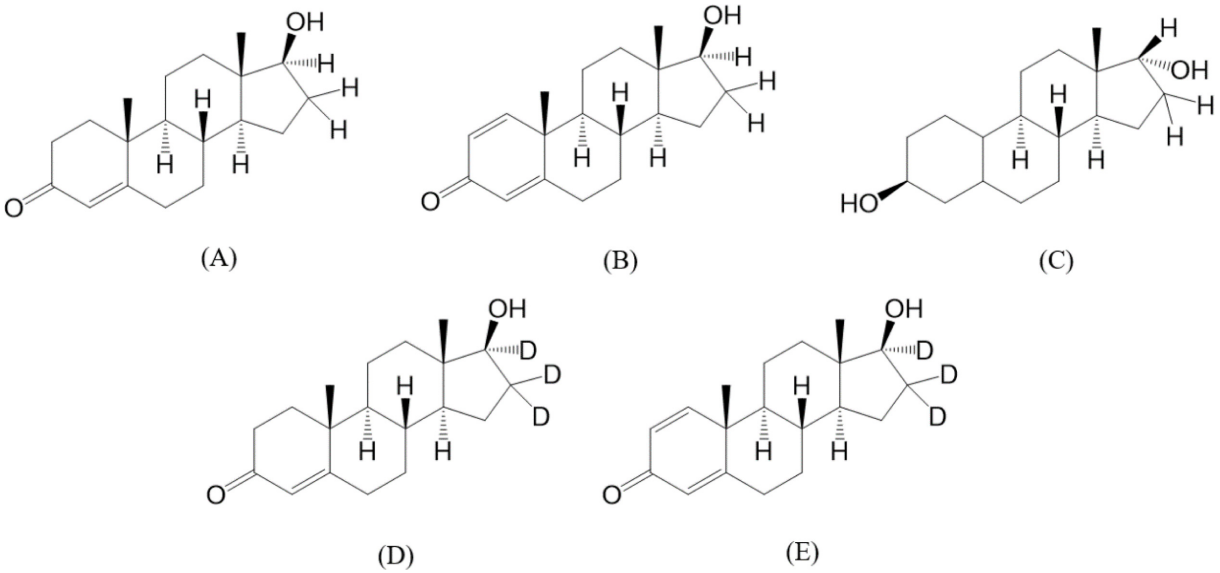

Figure 1. Chemical structure of (A) testosterone (TS), (B) boldenone (BD), (C) 5α-estrane-3β,17α-diol (EAD), (D) 16,16,17-*d3*-testosterone (TS-d3, ISTD), (E) 16,16,17-*d3*-boldenone (BD-d3, ISTD).

tography and peak resolution can be increased. Another option to increase selectivity is to use tandem mass spectrometers with a triple quadrupole. Gas chromatography-tandem mass spectrometry (GC-MS/MS) may reduce the matrix effect and provide higher sensitivity and accuracy than traditional GC-MS systems.[13] A method to detect steroids using GC-MS/MS on horse urine has been previously published by Wong et al..[14] This method which was used to qualitatively analyze more than 50 AAS analytes using pentafluoropropionic acid anhydride (PFPA) derivatives, showed satisfactory results in horse urine.

The purpose of this study was to develop and validate a reliable, and sensitive quantitative method using heptafluorobutyric acid anhydride (HFPA) derivatives for the simultaneous detection of prohibited endogenous AAS using GC-MS/MS. This method was also used for the confirmation test after the screening test.

Experimental

Materials

TS, BD, 16,16,17-*d3*-testosterone (TS-d3), and 16,16,17-*d3*-boldenone (BD-d3) were purchased from the National Measurement Institute (Sydney, Australia). EAD was purchased from the Hong Kong Jockey Club (Sha Tin, Hong Kong). The ABS Elut-NEXUS cartridge (60 mg, 3 mL) was purchased from Agilent Technologies (Harbor City, CA, USA). Acetonitrile, methanol, and chloroform were purchased from J.T. Baker (Phillipsburg, NJ, USA). Formic acid, ammonium sulfate, diisopropyl ether, ethyl acetate, n-hexane, n-heptane, sodium chloride, and sodium hydroxide were purchased from Junsei Chem (Chou-ku, Japan). Heptafluorobutyric acid anhydride (HFPA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Acetyl chloride was purchased from Sigma Aldrich (St Louis, MO, USA). Anhydrous methanolic hydrogen chloride was prepared by slowly stirring methanol and acetyl chloride in an ice-water bath. Deionized water was generated from an in-house water purification system (Milli-Q, Molsheim, France).

Sample preparation

Blank and negative urine samples were regarded as urine samples showing no signal at the relative retention time (RRT) in the transition chromatogram of each target material taken from mares and geldings post-race. A calibrator and quality control (QC) were prepared by spiking the target analytes and the internal standard (IS) into a blank urine sample.

Extraction of the urine sample

Ammonium sulfate (0.5 g) was added to 4 mL of equine urine and spiked with BD-d3 and TS-d3, according to the internal standards (15 ng/mL). The mixture was mixed using a vortexer and was centrifuged at 3,000 rpm for 5 min. The supernatant (3 mL) was loaded onto an ABS Elut-NEXUS cartridge. After the loading, the cartridge was continually washed using 3 mL of diluted water, and 3 mL of n-hexane was eluted with 2 mL of chloroform and methanol/ethyl acetate (5:95, v/v, 3 mL). The solvent was evaporated at 70°C under nitrogen. Anhydrous methanolic hydrogen chloride (1 M, 0.5 mL) was added and heated for 15 min at 70°C. After heating NaOH/NaCl (1 M/0.15 M, 2 mL), 3 mL of diisopropyl ether was added and vortex mixed for 30 sec. The water layer was iced using an ice

bath, and the upper organic layer was transferred to a sodium sulfate drying tube. The extract was evaporated at 80°C under nitrogen. Acetonitrile (100 μL) and 40 μL of HFPA were added to the extracted residue of heptafluorobutyric derivatives, following heating at 80°C for 18 min. After drying under nitrogen at 80°C, the residue was reconstituted with 50 μL of n-heptane for the GC-MS/MS analysis.

Instrumentation

The GC-MS/MS analyses were performed on a Thermo Scientific TRACE 1310 gas chromatograph with a Thermo Scientific TSQ 8000 Evo triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). The sample injection was performed with a Thermo Scientific TriPlus RSH autosampler. A DB-1ms (25 m×0.25 mm, 0.25 μm film thickness) column (J&W Scientific, Folsom, CA, USA) was used for the gas chromatography. The injector temperature was set at 260°C and 1 μL of the sample was injected in the splitless mode. Sample washing was performed once before injection and three times after injection, using methanol and ethyl acetate for each solvent. Helium flow at 1.2 mL/min was used for all analyses. The oven temperature was set initially at 80°C and held at this temperature for 1 min. This was increased to 200°C at 40°C/min and held for 1 min, followed by an additional increase to 250°C at 10°C/min and held for 1 min, then finally to 280°C at 30°C/min and held for 4 min. The GC-MS/MS analyses were performed in the EI (positive) mode. The mass transfer line and ion source temperature were set to 280°C. The acquisition mode was set to multiple reaction monitoring (MRM) for the analyses. The transitions monitored in detail are summarized in Table 1. Data processing was performed using TraceFinder (version 3.3).

Selectivity

The selectivity of the method was tested using blank urine, a sample-spiked with only IS (TS-d3, BD-d3) in the blank urine, and a positive sample-spiked ISs (TS-d3, BD-d3), and target analytes (TS, BD, EAD) in the blank urine (n=10, each).

Linearity

The Lower Limit of Quantification (LLOQ) was decided through the lowest concentration of QC samples with an acceptable intra- and inter-day precision and accuracy and S/N ratio greater than 9. Calibration standard concentrations at six levels (LLOQ, 1, 5, 10, 50, 100 ng/mL for TS; LLOQ, 0.5, 2, 10, 50, 100 ng/mL for BD; LLOQ, 1, 5, 10, 50, 100 ng/mL for EAD) were prepared in the blank urine samples and analyzed. TS-d3 was the IS for the TS and BD-d3 for the BD and EAD.

Intra- and inter-day precision and accuracy

Precision and accuracy were estimated using four-point internal concentration quality controls (LLOQ, low QC, middle QC, and high QC). The accuracy (%) and relative standard deviation (RSD) as precision values obtained for five replicates of the QC samples were targeted to be within 20% at the LLOQ and 15% at the other QCs. The inter-assay was performed for 5 days in terms of precision and accuracy for all QC levels, like the intra-day study (n=5).

Stability

Stability studies were evaluated by analyzing the QC samples under various conditions based on working days for post-race doping control. For post-preparative stability, the samples were analyzed after being set in the auto-sampler (room temperature) for 12 h. For short-term stability, the samples were stored at room temperature for 3 h. For long-term stability, they were stored at -2°C for two weeks. For freeze-thaw stability, they were stored at -20°C and thawed at room temperature three times (n=5, each).

Uncertainty

The combined standard uncertainty (u_c) was evaluated by the positive square root of the inter-day precision at concentrations of the threshold set by IFHA (at 20 and 50 ng/mL for TS, 15 ng/mL for BD, 45 ng/mL for EAD) and the bias of the analytical method, which comprises the uncertainty of the purity of the used standards ($U[C_{ref}]$), the accuracy of the bias (S_{bias}) and the root mean square of the bias (RMS_{bias}) (n=10). The combined expanded measurement uncertainty (U) was obtained by multiplying the standard measurement uncertainty (u_c) by a coverage factor (k=2) with 95% confidence.

Results and discussion

Chromatographic selectivity and stability of internal standards in urine sample

The selectivity results are summarized in Figure 2. As per

Table 1. Analytical parameters for the analytes.

Compounds	RRT	Precursor ion	Product ion	CE (V)
TS	1	680.2	320.1	10
BD	0.92	678.2	464.1	8
EAD	0.89	456.2	242.3	5
TS-d3 (IS)	1	683.2	320.1	10
BD-d3 (IS)	0.92	681.2	467.1	10

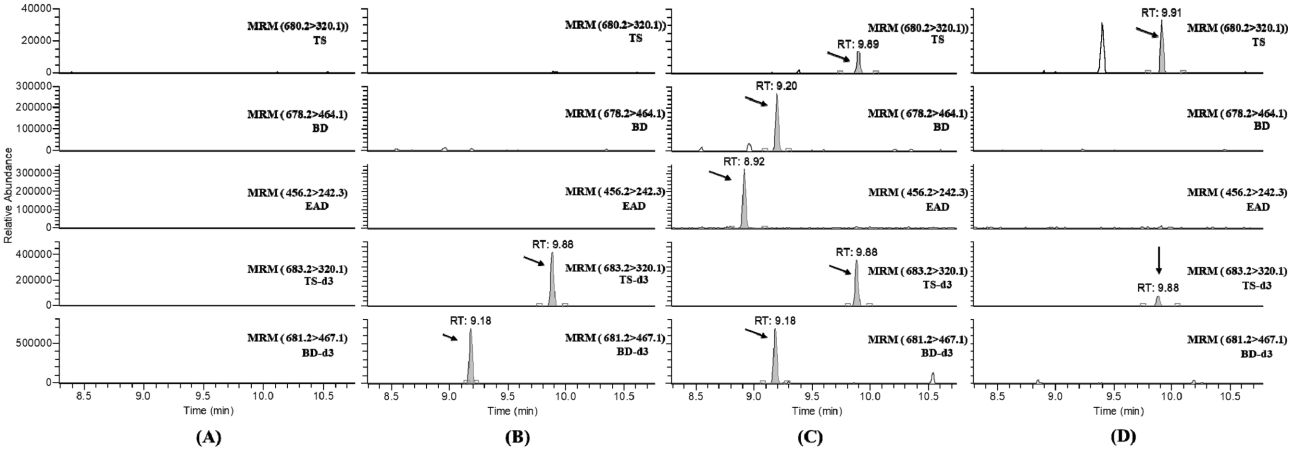

Figure 2. The representative MRM chromatograms for TS, BD, EAD, TS-d3 (ISTD), BD-d3 (ISTD) (A) Blank equine urine; (B) Blank equine urine spiked with TS-d3 (ISTD, 15 ng/mL) and BD-d3 (ISTD, 15 ng/mL); (C) Equine urine sample with TS (LLOQ, 0.2 ng/mL), BD (LLOQ, 0.5 ng/mL) and EAD (LLOQ, 0.2 ng/mL), TS-d3 (ISTD, 15 ng/mL) and BD-d3 (ISTD, 15 ng/mL); (D) Suspect urine sample of a gelding from the Asian Quality Assurance Program (AQAP).

the results, no interference from the matrix was observed during the retention time in blank samples. However, in the chromatographic analysis of TS and BD, slight peaks were observed due to decomposition by the deuterium atoms of internal standards (ISs) in the sample-spiked only ISs (16,16,17-d3-testoterone [TS-d3], 16,16,17-d3-boldenone [BD-d3]) in the blank urine (Figure 2B); a pure standard can be generated by decomposing deuterium atoms in unstable positions during the methanolysis in the extraction process.[15] However, in this study, similar to the result of Choi and Chung,[15] TS-d3 showed relatively low degradation (0.67% of the TS peak area compared to the TS-d3 peak area (n=5)) after using it as an IS. BD-d3 also showed low degradation (0.52% of the BD peak area compared to BD-d3 peak area (n=5)). This result showed a signal sufficient to be used as an IS at the corresponding IS concentration and did not affect the accuracy and precision at quality controls (QC)s.

Analytical performance and stability assessment of equine urine analysis for TS, BD, and EAD quantification

The lower limits of quantitation (LLOQs) were estimated by analyzing the target analytes in the blank equine urine samples. The LLOQ concentration for the target analytes was evaluated to be the point that displayed a signal/noise (S/N) ratio greater than 9 and an acceptable intra- and inter-day accuracy and precision. The calibration standard curves were linear and reliable over the standard concentrations across the calibration range. The regression coefficients were between 0.9990 to 0.9999 for TS, BD, and EAD. Thus, the calibration curves exhibited good linearity. The LLOQs of TS, BD, and EAD in equine urine were 0.2, 0.5, and 0.2 ng/mL, respectively (Table 2). This showed that analytical sensitivity was better in TS and EAD but not for BD when compared to the results of Wong et al.,[14] developed using PFPA derivatization (LOD, TS; 0.5 ng/mL, BD; 0.5 ng/mL, EAD; 0.5 ng/mL) and LLOQ at S/N ratio 9 or higher in this study. The intra-day precisions were between 1.7 and 10.5%, and the accuracies were 88.2% to 105.9% at all QC levels in the urine samples. The inter-day precisions and accuracies were within the range of 2.3% to 17.5% and 95.1% to 109.0%, respectively. These parameters were acceptable for quantitative analysis (Table 3). The stability of TS, BD, and EAD in the urine samples was investigated at all QC levels. The urine QC samples were stable at room temperature for at least 12 h and 2 weeks when stored frozen at -2°C, even after three freeze–thaw cycles. The autosampler QC samples were stable in the autosampler at room temperature for at least 12 h (n=5 each) (Table 4). These results showed that TS, BD, and EAD were stable throughout the preparation and analysis steps. As a result, this method is considered suitable for routine use. The measurement uncertainty (U) of target analytes at the threshold level was estimated to be within 1.7 to 5.0%. The decision limit at the threshold level was determined (Table 5).

Table 2. The low limits of quantification (LLOQ), calibration range, and linearity for analytes in equine urine.

Compounds	IS	LLOQ (ng/mL)	Range (ng/mL)	Linearity (r^2)
TS	TS-d3	0.2	0.2-100	0.9990
BD	BD-d3	0.5	0.5-100	0.9999
EAD	BD-d3	0.2	0.2-100	0.9991

Table 3. Intra-, Inter-day precision and accuracy for the determination of each target analytes in equine urine.

Compounds		LLOQ		Low QC[a]		Middle QC[b]		High QC[b]	
		Accuracy (%)	Precision (RSD)	Accuracy (%)	Precision (RSD)	Accuracy (%)	Precision (RSD)	Accuracy (%)	Precision (RSD)
TS	Intra-day (n = 5)	88.2	9.1	99.4	10	100.3	2.5	103.9	3.1
BD		105	7.6	93.6	3.3	102.6	1.7	98.1	1.7
EAD		90.9	10.5	105.9	5.3	105.9	3.4	95.2	6.4
TS	Inter-day (n = 5)	98.6	17.5	105.6	8.4	102.9	2.3	109	4.7
BD		99.8	7.9	95.1	3.4	103.5	3.9	103.3	4.9
EAD		106.6	12.8	97.6	8.1	102.8	5.8	105.1	6.6

[a] Low QCs of TS, BD, and EAD were tested at 2, 5, and 2 ng/mL, respectively.
[b] Middle QC and high QC were tested at 20 and 80 ng/mL, respectively

Table 4. Stability of the target analytes in equine urine samples under different conditions (n = 5).

Stability	QC level	% of control		
		TS	BD	EAD
Post-preparative stability (Room temperature, 12 h)	LLOQ	107.8±16.1	98.4±4.6	113.4±4.9
	Low QC[a]	105.2±13.0	93.7±3.0	106.6±8.6
	Middle QC[b]	100.0±3.2	98.9±1.2	108.4±6.1
	High QC[b]	102.7±3.3	97.0±2.5	102.2±4.8
Short-term (Room temperature, 3 h)	LLOQ	98.6±18.3	103.9±8.32	119.2±11.0
	Low QC[a]	112.7±13.2	91.7±10.1	110.3±10.6
	Middle QC[b]	100.6±14.3	100.7±3.6	99.1±7.4
	High QC[b]	106.7±10.3	108.2±5.8	108.8±6.8
Long-term (-2°C, 2 weeks)	LLOQ	110.0±14.7	108.5±8.9	103.2±5.2
	Low QC[a]	103.1±6.5	96.0±8.2	108.6±7.4
	Middle QC[b]	95.8±3.0	89.2±2.8	113.5±4.0
	High QC[b]	105.8±3.9	91.6±4.1	107.6±6.3
Freeze and thaw (-20°C, 3 cycle)	LLOQ	97.7±16.8	110.1±5.7	105.2±20.0
	Low QC[a]	95.6±7.6	87.2±4.2	85.5±4.2
	Middle QC[b]	103.7±2.2	100.7±1.8	105.3±2.9
	High QC[b]	103.0±2.5	92.1±3.1	100.3±6.7

[a] Low QCs of TS, BD, and EAD were tested at 2, 5, and 2 ng/mL, respectively.
[b] Middle QC and high QC were tested at 20 and 80 ng/mL, respectively.

Table 5. Estimation of measurement uncertainty of target analytes in equine urine.

Compound	Gender	Threshold (ng/mL)	Measurement uncertainty (%)	*Decision limit (ng/mL)
TS	Mare/filly	55.0	1.7	56.9
	Gelding	20.0	5.0	22.0
BD	Male	15.0	3.0	15.9
EAD	Male	45.0	2.4	47.2

* The coverage factor (k = 2.0) to ensure 95% confidence interval

Application of developed method: confirmation of testosterone presence in suspect gelding urine

The developed method was applied to the analysis of suspect gelding urine received from the Asian Quality Assurance Program (AQAP). In the primary screening test of the AQAP sample, peak of TS was detected, and to confirm this in a secondary quantification, only TS-d3 corresponding to the IS of TS was spiked into the AQAP sample and analyzed. The measurement was performed in triplicate and the measured concentrations were 21.4, 19.4, and 20.1 ng/

mL, all below the decision limit (22.0 ng/mL), respectively (Figure 2D and Table 5).

Conclusions

In this study, a method for quantifying endogenous steroids (TS, BD, EAD) in equine urine using GC-MS/MS was developed and validated. This method not only detected endogenous steroids accurately but was also sensitive and selective.

Abbreviations

TS: Testosterone; BD: Boldenone; EAD: 5α-estrane-3β,17α-diol; TS-d3:16,16,17-d3-testoterone; BD-d3: 16,16,17-d3-boldenone; LLOQ: Lower limit of quantitation; RRT: Relative retention time; CE: Collision energy; IS: Internal standard; QC: Quality control; MRM: Multiple reaction monitoring; TS: Testosterone; BD: Boldenone; EAD: 5α-estrane-3β,17α-diol; TS-d3:16,16,17-d3-testoterone; BD-d3: 16,16,17-d3-boldenone; LLOQ: Lower limit of quantitation.

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Conflict of Interest

The authors declare no conflict of interest.

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