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Detection of endogenous boldenone in the entire male horses

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Abstract

Boldenone (1,2-dehydrotestosterone) is a common veterinary anabolic agent. Its structure is very similar to testosterone. Testosterone is endogenous in the horse, whereas there has been no report concerning the detection of endogenous boldenone. This paper reports the direct observation of sulphate conjugate of boldenone in equine urine from entires. The detection procedures involved solid-phase extraction, immunoaffinity column (IAC) purification, and then LC–MS–MS analysis on a Q-ToF instrument. The identification of boldenone sulphate has provided direct evidence for the endogenous nature of boldenone in entire male horses. Quantification data for the normal level of boldenone in Hong Kong racehorses will also be discussed.
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Keywords: Boldenone

1. Introduction

Boldenone (1,4-androstadien-17β-ol-3-one or 1,2-dehydrotetosterone) is a common anabolic steroid used in veterinary practice. Its structure is highly similar to testosterone with dehydrogenation at the C-1,2 position. Testosterone is endogenous in entire male horses, however there is no report concerning the detection of endogenous boldenone in equine biological fluid. Boldenone had been reported to be endogenous in other mammals, like humans [1] and cattle [2].

Boldenone was reported to be almost exclusively excreted in urine as the 17β-sulphate conjugate. The detection period of boldenone from hydrolysis of sulphoconjugates after administration of boldenone undecylenate in therapeutic doses was about 40 days by GC–MS [3]. This paper reports the direct detection of intact boldenone sulphate in equine urine from normal entire male horses and also free boldenone after methanolysis. The analysis procedures involve solid-phase extraction of steroids, immunoaffinity column (IAC) purification and LC–MS–MS analyses on a high-resolution Q-ToF.

Owing to the presence of boldenone in normal urine from entire male horses, the range of urinary boldenone levels should be determined for a large number of entire male horses with the objective of assessing a threshold value for boldenone. Quantification of absolute urinary concentration of free and conjugated boldenone was achieved using both GC–MS (selected-ion monitoring mode) and LC–MS (selected-reaction monitoring mode) analyses with extensive purification and specific extraction steps.

2. Experimental

2.1. Materials

Boldenone was purchased from Alltech (Pennsylvania, USA) and Sigma (Missouri, USA), testosterone from USP (Maryland, USA), boldenone sulphate and testosterone sulphate from Steraloids (Rhode Island, USA) and 16,16,17-d_3-boldenone from National Analytical Reference Laboratory (Pumble, Australia). Ammonium acetate, acetyl

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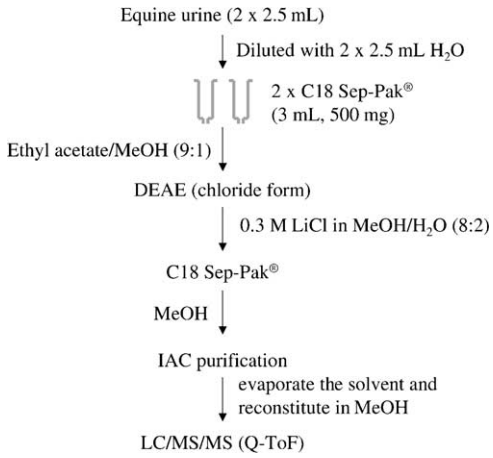
chloride and anhydrous sodium sulphate were obtained from Merck (Darmstadt, Germany). Sodium hydroxide, sodium chloride and lithium chloride were purchased from Riedel-de Haen (Seelze, Germany). Methanolic hydrogen chloride was prepared according to reported procedures [4]. Trimethylchlorosilane (TMCS) was obtained from Fluka (Missouri, USA). Heptafluorobutyric acid anhydride (HFBA), *N*-methyl-*N*-trimethylsilyltrifluoro-acetamide (MSTFA) and the AminoLink® Kit for preparation of immunoaffinity column for boldenone was purchased from Pierce (Illinois, USA). The C18 Sep-Pak® (3 mL, 500 mg) and Supelclean™ LC-Si (3 mL) solid-phase extraction cartridges were obtained from Waters (Massachusetts, USA) and Supelco (Pennsylvania, USA) respectively. DEAE-Sephadex A25 (chloride form) and phosphate buffered saline were obtained from Sigma (Missouri, USA) and Sephadex LH-20 from Pharmacia (Uppsala, Sweden). Polyclonal anti-boldenone antibody was raised against boldenone-17β-hemisuccinate conjugated to bovine serum albumin by male New Zealand white rabbits [5]. Acetic acid (100%; Suprapur®), acetonitrile (LC hypergrade; LiChrosolv®), ammonium acetate (extra pure grade), methanol (LC gradient grade; LiChrosolv®), *n*-heptane (LC gradient grade, LiChrosolv®), chloroform (LC gradient grade, LiChrosolv®) and ethyl acetate, diisopropylether, ethanol and *n*-hexane of analytical grade were obtained from Merck (Darmstadt, Germany). HPLC grade water was acquired from an in-house water purification system (Milli-Q, Molsheim, France). The HPLC mobile phases were filtered through a 0.45 μm Nylon-66 filter (Aglient Technologies, USA) before use.

2.2. Instrumentation

LC–MS qualitative analyses of boldenone and boldenone sulphate were conducted on an API QSTAR Pulsar (MDS Sciex, California, USA) interfaced with a Perkin-Elmer Series 200 MicroPump and Autosampler (Connecticut, USA). LC–MS quantitative analyses of boldenone were performed on a TSQ Quantum (Thermo Finnigan, San Jose, CA, USA) interfaced with a Surveyor Autosampler and MS Pump system (Thermo Finnigan, San Jose, CA, USA). GC–MS analyses of boldenone were carried out using an Agilent Technologies 6890N Network GC system with an Agilent 7683 series Injector coupled to an Agilent 5973 Network Mass Selective Detector (Agilent Technologies, California, USA).

2.3. Samples collection and preparation

Urine samples were obtained from horses immediately post race. Urine samples were stored at 4 °C prior to analyses. For storage studies, aliquots of an equine urine sample from an entire male horse were kept under the following conditions: (a) at 4 °C for 48 h, (b) at 37 °C for 24 h, and (c) at 65 °C for 24 h.



Scheme 1. Flow diagram for extraction procedures for confirmation of boldenone sulphate by LC–MS.

2.4. Extraction procedures for confirmation of boldenone sulphate by LC–MS

2.4.1. C18 solid-phase extraction (SPE)

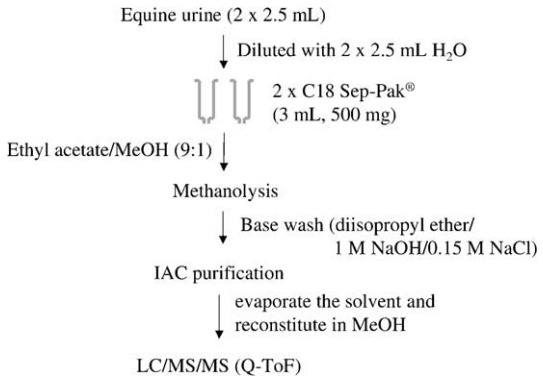
Urine (2 × 2.5 mL) was diluted with water (2 × 2.5 mL) and loaded onto two C18 Sep-Pak® cartridges, each pre-conditioned with methanol (5 mL) and deionised water (5 mL). Each cartridge was rinsed with deionised water (10 mL) and *n*-hexane (5 mL), then eluted with ethyl acetate/methanol (9:1, 3 mL). The combined eluate was evaporated to dryness at 60 °C under nitrogen (Scheme 1).

2.4.2. Extraction of sulphoconjugates by anion exchanger (DEAE-Sephadex chloride form)

The dried residue from C18 SPE was reconstituted in methanol/water (8:2, 0.5 mL) and loaded onto a disposable column (20 mm × 5 mm) packed with DEAE-Sephadex A25 chloride form in methanol/water (8:2). The column was washed with 0.03 M LiCl in methanol/water (8:2, 1.5 mL) and eluted with 0.3 M LiCl in methanol/water (8:2, 2 mL). The eluate was evaporated and re-dissolved in deionised water (4 mL). The solution was loaded onto a C18 Sep-Pak® cartridge pre-conditioned with methanol (5 mL) and deionised water (5 mL). The cartridge was washed with deionised water (10 mL) and *n*-hexane (5 mL), then eluted with methanol (3 mL). The eluate was evaporated to dryness.

2.4.3. Immunoaffinity column (IAC) extraction

The IACs were prepared according to the manufacturer's instructions with the anti-boldenone anti-serum 1:19 diluted by coupling buffer. The dried residue from anion-exchange purification was reconstituted in methanol (0.2 mL), and phosphate buffered saline (pH 7.4, 5 mL) was added. This was loaded onto an IAC equilibrated with phosphate buffered saline (pH 7.4, 10 mL). The IAC was washed with phosphate buffered saline (pH 7.4, 10 mL), deionised water (5 mL), then eluted with ethanol/deionised water (7:3, 5 mL) with the first 1 mL of eluate discarded. The collected eluate



Scheme 2. Flow diagram for extraction procedures for confirmation of boldenone by LC–MS.

was evaporated to dryness. The residue was reconstituted in 30 μL of methanol for LC–MS–MS (Q-ToF) analyses, see Scheme 1.

2.5. Extraction procedures for confirmation of boldenone by LC–MS

2.5.1. Methanolysis

The dried extract obtained from the C18 SPE as described above was hydrolysed with 1 M methanolic hydrogen chloride (0.5 mL) at 60 °C for 10 min. Diisopropyl ether (3 mL) was added and the mixture was base washed with 1 M NaOH/0.15 M NaCl (2 mL). The organic layer was passed through an anhydrous sodium sulphate drying tube, and evaporated to dryness.

The extract was then purified by immunoaffinity column extraction as described above and analysed by LC–MS–MS (Q-ToF) (Scheme 2).

2.6. Extraction procedures for quantification of boldenone by GC–MS

An aqueous methanol solution of internal standard, 16,16,17-d_3-boldenone, was added to 5 mL aliquots of all samples (calibrators, quality controls and unknowns) to give a concentration of 4 ng/mL. All samples were subject to C18 SPE and methanolysis as described above.

2.6.1. Removal of estrogen by anion exchanger (DEAE-Sephadex free base form)

The residue was reconstituted in methanol (0.5 mL) and loaded onto a disposable column (10 mm × 5 mm) packed with DEAE-Sephadex A25 free base form in methanol [6]. The column was eluted further with methanol (1.5 mL). The eluate was collected and evaporated to dryness.

2.6.2. Derivatisation

The dry extract was dissolved in acetonitrile (100 μL) and HFBA (30 μL). The mixture was vortexed and heated at 70 °C for 30 min and evaporated to dryness. The residue was reconstituted in chloroform (50 μL) and the solution was loaded onto a Supelclean™ LC-Si cartridge pre-conditioned with chloroform (2 mL). The cartridge was eluted with chloroform (2 mL) and the eluate was evaporated to dryness under nitrogen. Methanol/pyridine (9:1, 100 μL) was added and the mixture was allowed to stand at room temperature for 10 minutes. The solvent was then evaporated. The residue was incubated with MSTFA/TMCS (99:1, 40 μL) at 70 °C for 30 min, then evaporated to dryness, re-dissolved in *n*-heptane/ethyl acetate (3:1, 50 μL) and applied onto a disposable column (20 mm × 5 mm) packed with Sephadex LH-20 in *n*-heptane/ethyl acetate (3:1). The column was eluted with the same solvent (4 mL) and the eluate evaporated to dryness. The residue was reconstituted in 40 μL *n*-heptane for GC–MS analyses, see the flow diagram below (Scheme 3).

2.6.3. Preparation of calibrators and quality controls

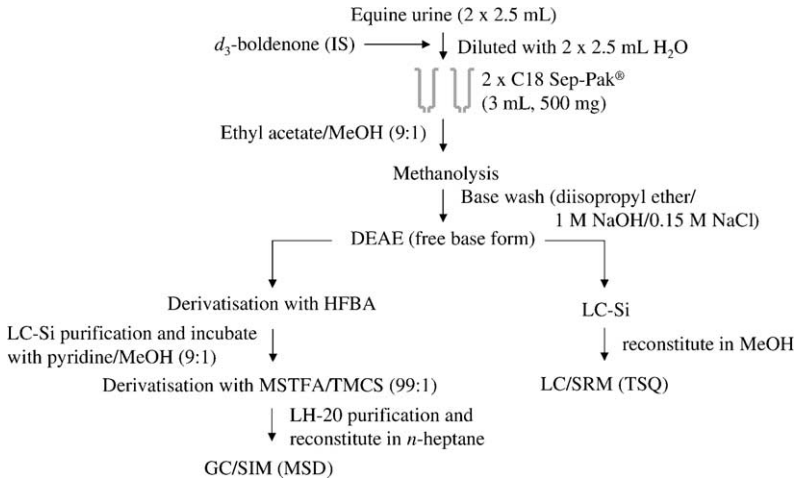
Calibrators of boldenone at 0, 1, 2, 4 and 6 ng/mL in gelding urine (with no detectable boldenone) were prepared. Three quality control samples, 2 ng/mL boldenone in gelding urine, 1 ng/mL boldenone and 1.2 ng/mL boldenone sulphate in entires urine, were prepared. Calibrators and quality controls were prepared independently from separate weighing of the drug standard. For each batch of unknown samples, the calibrators and quality controls were prepared freshly. All samples (calibrators, quality controls and unknowns) were processed in duplicate and analysed in the same run. The peak area ratios of boldenone to d_3-boldenone versus the concentration of the boldenone calibrators were fitted using linear regression to obtain the calibration curve.

2.7. Extraction procedures for quantification of boldenone by LC–MS

An aqueous methanol solution of internal standard, 16,16,17-d_3-boldenone, was added to 5 mL aliquots of all samples (calibrators, quality controls and unknowns) to give a concentration of 4 ng/mL. All samples were subject to C18 SPE, methanolysis and removal of estrogen by anion exchanger (DEAE-Sephadex free base form) as described above.

2.7.1. LC-Si purification

The residue was reconstituted in ethyl acetate (50 μL) and the solution was loaded onto a Supelclean™ LC-Si cartridge pre-conditioned with chloroform/ethyl acetate (1:1, 3 mL). The cartridge was eluted with chloroform/ethyl acetate (1:1, 3 mL) with the first 0.5 mL of eluate discarded, and the collected eluate was evaporated to dryness under nitrogen. The residue was reconstituted in 50 μL methanol for LC–MS analyses, see the flow diagram above for quantification by GC–MS (Scheme 3). Preparation of calibrators and quality controls were carried out as for GC–MS quantitative analyses.



Scheme 3. Flow diagram for extraction procedures for quantification of boldenone by GC–MS and LC–MS.

2.8. LC–MS analyses for confirmation of boldenone and boldenone sulphate by Q-ToF

LC was carried out using a Supelcosil™ LC-18-DB column (15 cm × 2.1 mm; 3 μm, Supelco, Bellefonte, PA, USA). The mobile phase was composed of water containing 10 mM ammonium acetate (pH 6.7) as solvent A and methanol as solvent B. A gradient was run at 0.2 mL/min, consisting of 2% solvent B for the first 0.5 min, increasing to 98% solvent B at 20.5 min, and then held at 98% solvent B for 10 min. Injection volumes were 10 μL each. The API source was operated in the positive ESI mode. An ESI Ion Spray Voltage of 5.5 kV was applied. The nitrogen nebuliser and auxiliary gas flow rate were set at 40 and 80 arbitrary Q-ToF units respectively. All TOF measurements were performed at high-resolution settings. The resolution of the quadrupole mass filter was set with the peak width of 0.7 mass units at half height. The precursor ions selected for boldenone and boldenone sulphate were their protonated molecular ions at respectively *m*/*z* 287.2 and 367.2. The collision energies were 23 eV for boldenone, 20 eV for d_3-boldenone and 23 eV for boldenone sulphate. Nitrogen was used as the collision gas. The ToF scan ranges were *m*/*z* 115–300 and *m*/*z* 115–380 for boldenone and boldenone sulphate, respectively. The pulse frequency and accumulation time were 14.980 kHz and 1.5 s, respectively. For accurate mass measurement, the instrument was calibrated prior to sample analyses in the MS–MS mode over the same acquisition mass ranges using two fragment ions, *m*/*z* 121.065339 and 269.190540, from the respective standards.

2.9. GC–MS analyses for quantification of boldenone by MSD

GC separation was performed on a DB-5 fused silica column (30 m × 0.25 mm, 0.25 μm film thickness) (Alltech, Illionis, USA) with a constant helium flow of 1.2 mL/min. The oven temperature was initially 130 °C for 0.5 min, ramped at 4.5 °C/min to 260 °C and then at 20 °C/min to a final temperature of 310 °C, and held there for 3 min. Extracts (2 μL) were injected at 275 °C in splitless mode. Boldenone was quantified in selected-ion monitoring mode with 70 eV electron energy, monitoring *m*/*z* 554.2 (for the HFB-TMS derivative of boldenone) and *m*/*z* 557.2 (for the HFB-TMS derivative of d_3-boldenone). The dwell time was 100 ms for each of the two ions, and the cycle time was 4.26 cycle/s.

2.10. LC–MS analyses for quantification of boldenone by TSQ quantum

LC separation was achieved using a Hypersil ODS column (10 cm × 2.1 mm; 5 μm, Aligent, USA). The mobile phase was composed of water containing 5 mM acetic acid as solvent A and methanol as solvent B. A gradient was run at 0.3 mL/min, consisting of 30% solvent B for the first 2 min, increasing to 70% solvent B at 22 min, to 100% solvent B at 26 min, and then held at 100% solvent B for 3 min. Injection volumes were 5 μL each. The API source was operated in the positive APCI mode. A discharge current of 4.0 μA, a capillary temperature at 200 °C, and a vaporizer temperature at 450 °C were employed. The nitrogen sheath and auxiliary gas flow rate were set at 60 and 40 arbitrary TSQ Quantum units respectively. Boldenone was quantified in selected-reaction monitoring mode, monitoring *m*/*z* 121.057 (for boldenone) and *m*/*z* 121.045 (for d_3-boldenone) from their corresponding precursors of *m*/*z* 287.245 and 290.307. The collision energies were 18 eV for boldenone and d_3-boldenone, and the collision gas pressure was 1 mTorr of argon.

2.11. Validation of quantification method for boldenone by GC–MS

Accuracy was assessed by spiking of boldenone to gelding urine (at 0.5, 2.0, 5.0 ng/mL), and boldenone and boldenone sulphate to entires urine (at respectively 1.0 and 1.2 ng/mL; the latter is equivalent to 0.88 ng/mL of free boldenone). The precisions were determined from the means and stan-

dard deviations of four replicates for gelding urine samples spiked with boldenone. Recovery studies were measured by comparing the response of boldenone from urine samples spiked before extraction with urine samples extracted and spiked post-extraction and derivatized (taken as 100% recovery), and d_3-boldenone (internal standard) was added just before the derivatisation process. The recovery is taken as the percentage ratio between them. The limit of quantification (LoQ) is established based on the normal background reading at the retention time of the analyte in negative gelding urine samples, and is set to be 10S.D. The possibility of production of boldenone from testosterone sulphate during the sample preparation process was assessed by spiking a gelding urine sample with testosterone sulphate.

3. Results and discussion

3.1. Confirmation of boldenone sulphate in urine

The detection of either boldenone or boldenone sulphate at low ppb levels in highly complicated entire male horse urine matrix required extensive clean-up and specific extraction of the target analytes. Equine urine contains high concentrations of polar substances, such as inorganic ions, which need to be removed by SPE C18 cartridges to lower the ionic strength of the sample before further purification by DEAE-Sephadex anion exchangers and immunoaffinity columns. The polyclonal antibody raised from rabbits immunised with boldenone-17β-hemisuccinate conjugated to bovine serum albumin cross-reacted with boldenone, boldenone sulphate, and slightly with testosterone, androstenedione and progesterone [5]. The highly specific IAC extraction is especially useful for the separation of boldenone and its conjugates from the highly complicated equine urine matrix, resulting in much less interference in the LC–MS–MS chromatograms. Confirmation of boldenone sulphate by LC–MS–MS in negative ESI mode, although having a lower limit of detection, did not provide useful structural information as compared to positive ESI mode. Only product ions of *m*/*z* 177 and 97 (HSO_4^-) were observed in the negative ESI MS–MS spectra. The *m*/*z* 177 fragment was formed by the cleavage of the C-ring, leaving D-ring with the 18-methyl and the sulphate moieties intact

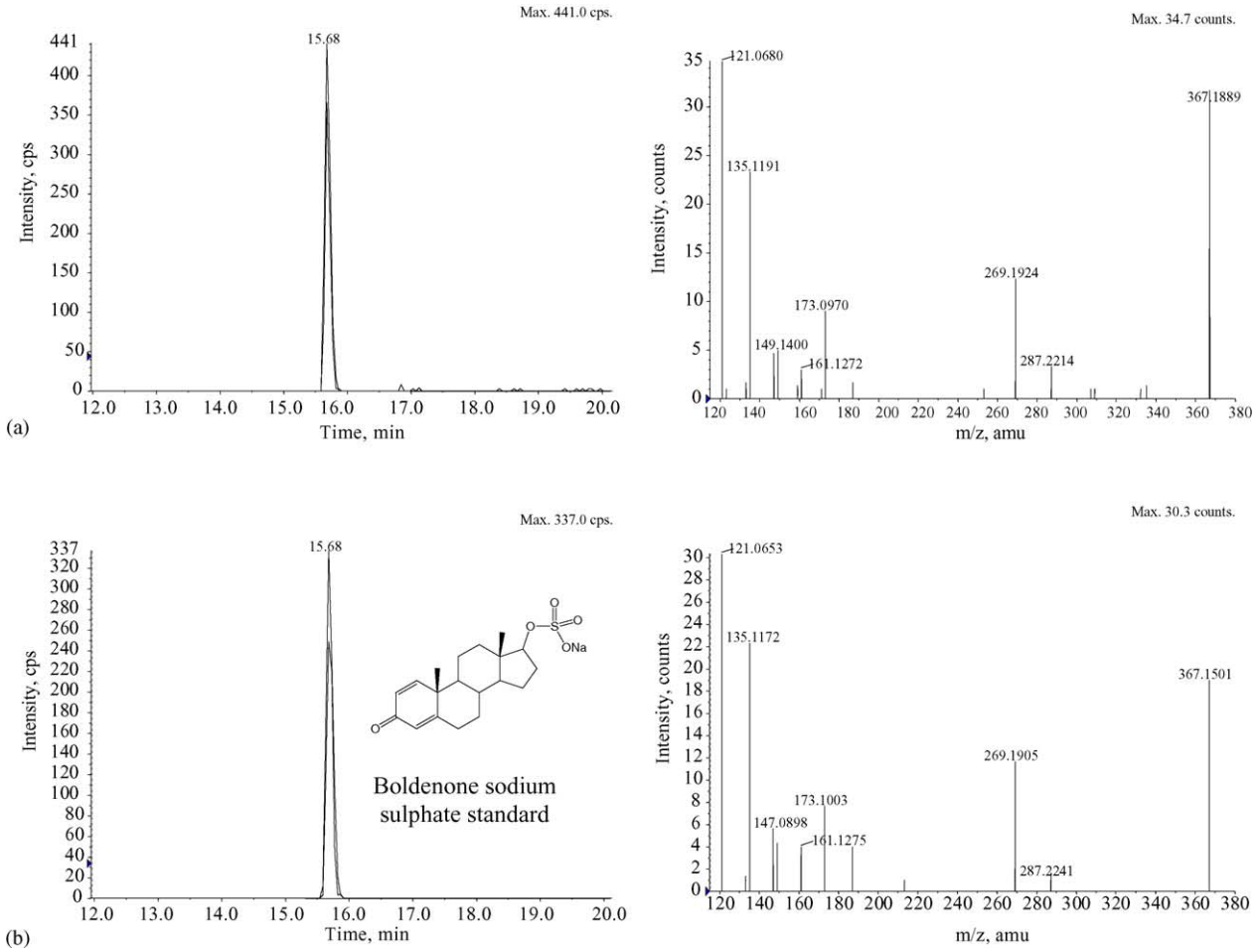
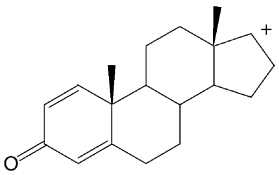
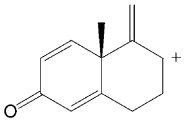
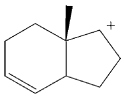
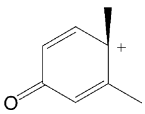


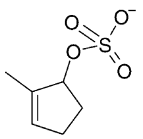
Fig. 1. Extracted-ion chromatogram of *m*/*z* 121 and 135 and product-ion scan of precursor ion *m*/*z* 367.2 of boldenone sulphate from: (a) an entire male horse urine sample, (b) boldenone sulphate standard.

Table 1
Accurate mass measurement of major boldenone sulphate and boldenone fragment ions

Structure of fragment	Formula	Calculated mass (amu)	Fragments from boldenone sulphate		Fragments from boldenone	
			Measured mass (amu)	Mass error (mDa)	Measured mass (amu)	Mass error (mDa)
	$C_{19}H_{25}O$	269.1905	269.1924	1.9	269.1861	−4.4
	$C_{12}H_{13}O$	173.0966	173.0970	0.4	173.0956	−1.0
	$C_{10}H_{15}$	135.1173	135.1191	1.6	135.1175	0.1
	C_8H_9O	121.0653	121.0680	2.7	121.0652	−0.1

($C_6H_9O_4S^-$). The possible structure of *m*/*z* 177 was shown below (Scheme 4).

Fig. 1 shows the confirmation of boldenone sulphate in an urine sample from an entire male horse by LC–MS–MS in positive ESI mode. Fig. 1(a) and (b) are the extracted-ion chromatograms of *m*/*z* 121 and 135 and product-ion scans of *m*/*z* 367.2 (MH^+ of boldenone sulphate) from an entire male horse urine sample and boldenone sulphate standard respectively. Both the retention time and the product-ion spectrum from boldenone sulphate in the sample gave a good match with those from the authentic standard. The ions *m*/*z* 269, 173, 135 and 121 corresponded well with each other in less than 4 mDa deviations. The product ion of *m*/*z* 269 was from the loss of H_2SO_4 and ions *m*/*z* 173, 135 and 121 from ring fragmentation. The elemental compositions of the postulated fragment ions were further confirmed using accurate mass measurement. The comparison of the re-calibrated fragment ion masses with the calculated values is summarised in Table 1. The accuracy obtained is acceptable considering the low level of boldenone sulphate in the sample. Boldenone sulphate was detected in 19 out of 23 entires urine samples, but not from any of the four samples from geldings. The confirmation of the presence of boldenone sulphate by LC–MS–MS provided a direct proof for the endogenous nature of boldenone in entire male horses.



Scheme 4. Structure of *m*/*z* 177 ($C_6H_9O_4S^-$).

3.2. Confirmation of boldenone in urine

Fig. 2 shows the confirmation of boldenone in an urine sample from an entire male horse. Fig. 2(a) and (b) are the extracted-ion chromatograms of *m*/*z* 121 and 135 and product-ion scans of *m*/*z* 287.2 (MH^+ of boldenone) from an entire male horse urine sample and boldenone standard respectively. Both the retention time and the product-ion spectrum from boldenone in the sample corresponded well with those from the authentic standard. The ions *m*/*z* 269, 173, 135 and 121 matched well with each other with less than 5 mDa deviations. The product-ion spectrum from *m*/*z* 287.2 shows *m*/*z* 269 (loss of H_2O) and a series of ions similar to those from boldenone sulphate. The elemental compositions of the proposed fragment ions were further confirmed using accurate mass measurement. The measured masses were not significantly deviated (<5 mDa) from the calculated masses (Table 1), confirming the identity of boldenone in the sample. Boldenone could be confirmed in seven out of nine urine samples from entire male horses but not from any of the four samples from geldings.

3.3. Validation of quantification method for boldenone by GC–MS

The accuracy was assessed from the observed values of the quality control samples of boldenone at 0.5, 2.0, 5.0 ng/mL spiked in gelding urine, and boldenone at 1.0 ng/mL and boldenone sulphate at 1.2 ng/mL (equivalent to 0.88 ng/mL of free boldenone) fortified in entires urine. The amount of boldenone quantified and their relative standard deviation for precision were summarised in Table 2. The recovery of

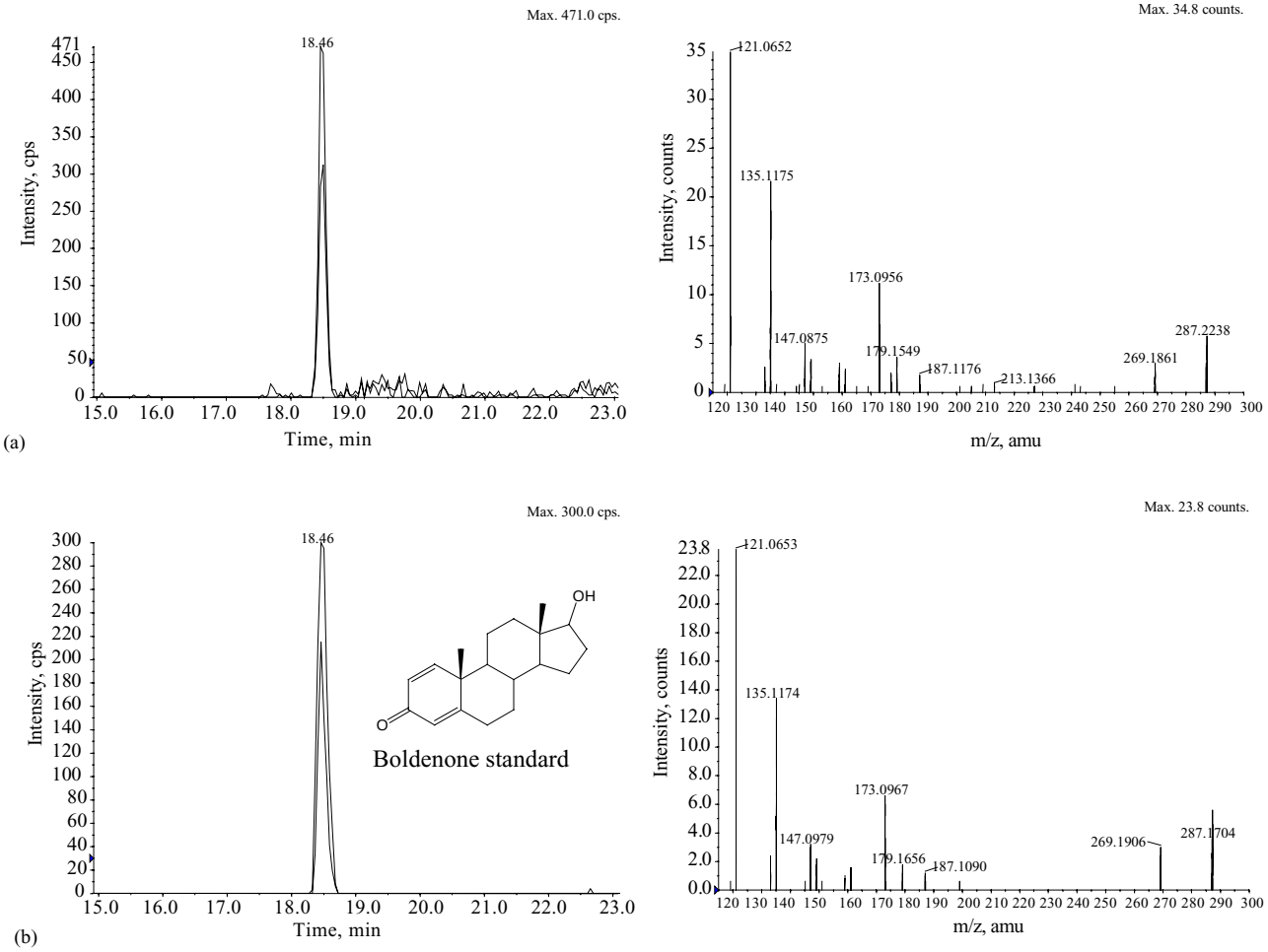


Fig. 2. Extracted-ion chromatogram of *m/z* 121 and 135 and product-ion scan of precursor ion *m/z* 287.2 of boldenone from: (a) an entire male horse urine sample, (b) boldenone standard.

boldenone was found to be 71 ± 6% at 2.0 ng/mL (n = 4). The LoQ of boldenone quantified by GC–MS, defined as 10S.D. of background reading in negative gelding urine samples (n = 3), was determined to be 0.10 ng/mL. Boldenone was not detected from a gelding urine sample spiked with testosterone sulphate at 200 ng/mL, therefore boldenone is not an artefact from testosterone sulphate. The extraction method for the quantification of urinary boldenone by GC–MS is specific and effective, allowing low levels of endogenous boldenone to be quantified accurately in entires urine. The high sensitivity and good S/N ratio observed may also be attributed to the use of a mixed derivative of trimethylsilyl ether (high thermal stability) and heptafluorobutyl ester (high mass increment) [7].

Table 2
Accuracy and precision (R.S.D. (%)) of boldenone detected in urine samples

Concentration (ng/mL)	Accuracy (ng/mL)	Precision, R.S.D. (%)
Boldenone in gelding urine (n = 4)		
0.5	0.53 ± 0.01	2.1
2.0	2.09 ± 0.11	5.5
5.0	5.23 ± 0.20	3.7
Boldneone in entires urine (n = 4)		
1.0	1.08 ± 0.13	11.7
Boldenone sulphate in entires urine (n = 2)		
1.2 (equivalent to 0.88 ng/mL of free boldenone)	0.92 ± 0.06	6.1

Based on isotopic calculation, the amount of "A+3" isotope contribution from the unlabelled boldenone to the internal standard (d_3-boldenone) is less than 0.012 ng/mL (or <0.6%) at the highest calibrator (6 ng/mL). The "A+3" isotope contribution to the lower calibrators will of course be even lower. It is therefore safe to assume that the "A+3" isotope contribution will not have significant impact on the quantification results. The accompanying certificate for d_3-boldenone states that the isotopic impurity is less than 0.5%. The maximum contribution to d_0-boldenone is therefore less than 0.02 ng/mL (based on an internal standard concentration of 4 ng/mL), which is significantly less that the LOQ (0.1 ng/mL). D_3-boldenone is therefore suitable as the internal standard for this quantification study.

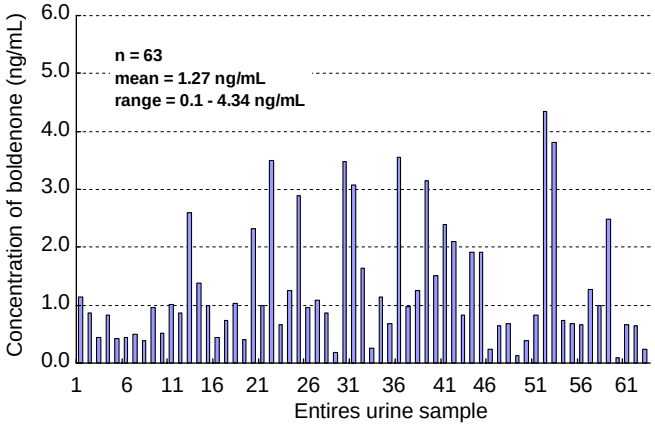


Fig. 3. Endogenous boldenone concentration in urine from entire male horses in Hong Kong by GC–MS.

3.4. Quantification of boldenone by GC–MS

Post race entires urine samples ($n = 63$, from 37 horses) and post race gelding urine samples ($n = 8$, from eight horses) were quantified for boldenone by GC–MS. Calibration curves for the quantification of boldenone were with correlation coefficients (r) greater than 0.99, and the quality control at 2 ng/mL were found to have less than 10% deviation.

The range of free and conjugated boldenone was determined to be between 0.1 and 4.34 ng/mL, and the mean was 1.27 ± 1.03 ng/mL. Boldenone was not detected in geldings. Fig. 3 shows the distribution of urinary boldenone levels in the 63 male horse samples by GC–MS.

3.5. Storage studies

The possibility of bacterial, enzymatic or chemical transformation of other precursors to produce boldenone in vitro was assessed through storage experiments of an urine sample from an entire male horse at 37 °C (for microbiological degradation) and 65 °C (for chemical transformation). No apparent change (< 5% variation) in total boldenone concentration was observed upon incubation at 37 °C or 65 °C for 24 h as compared to that of the control sample stored at 4 °C. Therefore it would be unlikely that boldenone was produced in vitro from other steroids present in entires urine.

3.6. Quantification of boldenone by LC–MS

Quantification of boldenone was also performed by LC–MS for comparison on nineteen urine samples (from 13 entire male horses) previously analysed by GC–MS. A correlation analysis using linear regression of boldenone concentrations ($n = 19$) quantified by both techniques was performed. Good correlation was observed, with slope of 1.094, y-intercept of -0.14 and correlation coefficient (r) of 0.98, indicating LC–MS might be used as an alternative method for quantifying boldenone.

4. Conclusion

Exogenous boldenone is known to be extensively conjugated in phase-II metabolism, and is excreted mainly as the sulphate conjugate in equine urine [2,8]. The identification of intact boldenone sulphoconjugate has provided a direct evidence for the endogenous nature of boldenone in entire male horses. The mean of free and conjugated boldenone in entires urine samples ($n = 63$, from 37 horses) was found to be 1.27 ± 1.03 ng/mL, with a range of 0.1–4.34 ng/mL. The endogenous origin of boldenone was also supported by sustained and similar levels of boldenone found in urine samples collected on different occasions from the same horses. For external administration, elevated urinary boldenone should decrease with time and eventually return to the normal level. The quantitative results showed that the GC–MS method is reliable for quantification of low levels of boldenone, and might also be extended to other endogenous steroids in horses.

An appropriate international threshold should be adopted by racing authorities for controlling the misuse of boldenone in entire male horses. To this end, a database of basal values of boldenone has to be established for a significant number of untreated entire male horses from different geographical locations. This work would require further international collaboration.

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