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Direct detection of boldenone sulfate and glucuronide conjugates in horse urine by ion trap liquid chromatography–mass spectrometry

Fan Pu[a], Andrew R. McKinney[b], Allen M. Stenhouse[b], Craig J. Suann[c], Malcolm D. McLeod[a,*]

[a] *School of Chemistry, F11, University of Sydney, Camperdown, NSW 2006, Australia*
[b] *Australian Racing Forensic Laboratory, P.O. Box 528, Kensington, NSW 1465, Australia*
[c] *New South Wales Thoroughbred Racing Board, P.O. Box 528, Kensington, NSW 1465, Australia*

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Abstract

A study of the equine phase II metabolism of the anabolic agent boldenone is reported. Boldenone sulfate, boldenone glucuronide and their C17-epimers were synthesised as reference standards in our lab and a method was developed for their detection in a horse urine matrix. Solid phase extraction was used to purify the analytes, which were then detected by ion trap LC/MS. Negative and positive ionisation mode MS^2 were used for the detection of sulfate and glucuronide conjugates, respectively. Boldenone sulfate and 17-epiboldenone glucuronide were detected as the major and minor phase II metabolites, respectively, in horse urine samples collected following the administration of boldenone undecylenate by intramuscular injection.

Keywords: Boldenone sulfate; Boldenone; Glucuronide

1. Introduction

Anabolic steroids are an important class of performance enhancing drugs with potential for misuse in horse racing and other sports. With the increasing number of known agents of abuse comes a mounting need for efficient, rapid and reliable methods for their detection. A significant problem associated with this analysis is that administration of anabolic steroids frequently results in little or no excretion of the parent steroid in the urine and instead, the drug is metabolised into more hydrophilic phases I and II metabolites. Phase II metabolites, arising from the conjugation of hydroxyl groups as either sulfates or β-glucuronides, may make up to 90% of the excreted metabolites [1] and are therefore an important class of compounds for drug screening in sports.

Due to the lack of authentic standards and the low volatility and thermal instability of these conjugates, they are typically hydrolysed by enzymatic or chemical means and the resulting steroid further derivatised prior to GC/MS analysis. This procedure is slow, labour intensive, and moreover the need for extensive sample handling and chemical transformation may lead to unreliable results due to factors such as incomplete hydrolysis [2,3]. For these reasons, the introduction of efficient, rapid and reliable methods for the direct detection of these phase II metabolites is of great interest for doping control. Recent developments such as liquid chromatography/mass spectrometry (LC/MS) allow for the direct detection of phase II metabolites, leading to increased speed and efficiency of analysis and even the potential for track-side drug testing. The first step in the development of these methods is to identify the phase II metabolites, or directly confirm their identity where they have been previously inferred from indirect methods of analysis.

In this study, the equine phase II metabolism of the anabolic agent boldenone was investigated. Four reference standards derived from the anabolic steroid boldenone, which are the sulfate and glucuronide conjugates of the parent and their

* Corresponding author. Fax: +61 2 93516650.
E-mail address: m.mcleod@chem.usyd.edu.au (M.D. McLeod).

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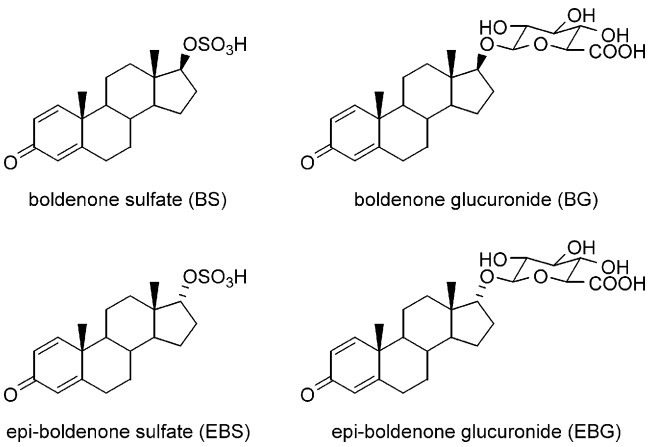

Fig. 1. The structures of boldenone reference standards.

C17-epimers (Fig. 1), were used as standards for the direct detection of these metabolites in horse urine using ion trap LC/MS. Solid phase extraction was used for the purification and concentration of these analytes from the horse urine matrix before LC/MS analysis. Method validation for this study, including precision, extraction efficiency, and sensitivity were all assessed.

2. Experimental

2.1. Chemicals

Boldenone was obtained from Steraloids (Newport, RI, USA). 17-Epiboldenone was synthesised from boldenone by a Mitsunobu reaction [4] and subsequent ester hydrolysis. Boldenone sulfate (BS) and 17-epiboldenone sulfate (EBS) were formed by sulfation with sulphur trioxide pyridine complex [5]. Boldenone glucuronide (BG), and 17-epiboldenone glucuronide (EBG) were synthesised using the Koenigs–Knorr method [6]. All reference standards were characterized by ^{1}H and ^{13}C NMR, LC, MS, IR and optical rotation and full experimental details will be reported elsewhere. Reference compounds d_3-testosterone sulfate and d_3-testosterone glucuronide were obtained from National Analytical Reference Laboratory (Pymble, NSW, Australia). C18 (500 mg, 3 cc) and Oasis HLB (30 mg, 1 cc) solid phase extraction cartridges were purchased from Waters (Rydalmere, NSW, Australia), NH_2 (500 mg, 3 cc), DEA (500 mg, 3 cc), PBA (100 mg, 3 cc) and Certify II (200 mg, 10 cc) solid phase extraction cartridges from Varian (Mulgrave, Vic., Australia) and SAX (500 mg, 3 cc) solid phase extraction cartridges from IST (Hengoed, UK).

2.2. Instrumentation

An Agilent (North Ryde, NSW, Australia) 1100 Series LC/MSD Ion Trap equipped with an electrospray ionisation (ESI) interface was used for all LC/MS analyses. A Phenomenex (Pennant Hills, NSW, Australia) Synergi Hydro-RP column (4 μm, 1 mm × 150 mm i.d.) protected with an Optimize Technologies (Oregon City, OR, USA) Opti-Guard C18 guard column (40 μm, 1.0 mm × 15 mm i.d.) was connected directly to the ion source. The capillary voltage was set to +3.5 or −3.5 kV, drying temperature to 350 °C, drying gas flow to 8 l min^{-1} and nebuliser pressure to 25 p.s.i.

BS and EBS were detected in negative ionisation mode. Tandem mass spectrometry (MS^2) experiments were performed using ion trap collision induced dissociation using *m/z* 365 ($[M - H]^-$) as the precursor ion with an isolation width of *m/z* 1.0, fragmentation amplitude of 1.2 V and a low mass cut-off of *m/z* 120. MS^3 of BS used the *m/z* 350 ($[M - H–CH_3]^-$) fragment as the second precursor ion. Sulfate conjugates were quantified in full scan product ion MS^2 mode, with integration performed on the precursor ion. Gradient elution was used throughout under the following conditions: (A) 100%, (B) 0% hold for 2 min; then ramp to (A) 50%, (B) 50% at 12 min; then ramp to (A) 20%, (B) 80% at 14 min; where solvent A was aqueous ammonium acetate (25 mM) and solvent B was acetonitrile.

BG and EBG were detected in positive ionisation mode. Tandem mass spectrometry (MS^2) experiments were performed using ion trap collision induced dissociation using *m/z* 463 ($[M + H]^+$) as the precursor ion with an isolation width of *m/z* 2.0, fragmentation amplitude of 1.2 V and a low mass cut-off of *m/z* 130. Glucuronide conjugates were quantified in full scan product ion MS^2 mode, with integration performed on the combined *m/z* 287 ($[M - C_6H_7O_6]^+$) and *m/z* 269 ($[M - C_6H_9O_7]^+$) fragments. Gradient elution was used throughout under the following conditions: (A) 100%, (B) 0% hold for 1 min; ramp to (A) 45%, (B) 55% at 12 min; ramp to (A) 20%, (B) 80% at 14 min; where solvent A was aqueous formic acid (25 mM) and solvent B was acetonitrile. The flow rate was set at 70 μl min^{-1} in each case.

The high resolution measurement was performed by skimmer collision induced dissociation (CID) of BS on a Bruker (Billerica, MA, USA) BioAPEX II Fourier transform ion cyclotron resonance mass spectrometer (FTICRMS) equipped with a 7 T superconducting magnet and a Unix O2 work station. This instrument was coupled to an external Analytica (Branford, CT, USA) electrospray ion source with a capillary voltage of −4 kV. A capillary-skimmer voltage of +180 V and a skimmer with a potential of +5 V was used to give CID.

2.3. Solid phase extraction (SPE)

Solid phase extraction of BS and EBS used the following procedure. A C18 cartridge was conditioned with methanol (3 ml), then water (3 ml) and the urine sample (1 ml) was loaded onto the cartridge. The cartridge was washed with water (3 ml), dried briefly with air and washed with 70% ethyl acetate/hexane (3 ml). The sulfate conjugate was eluted with

methanol (3 ml) and the eluate was evaporated to dryness below 60 °C under a stream of nitrogen. The dried eluate was reconstituted in sodium dihydrogenphosphate buffer (0.1 M, pH 7.5, 1 ml). A DEA cartridge (500 mg, 3 cc) was conditioned with methanol (3 ml), formic acid (0.1 M, 3 ml) and sodium dihydrogenphosphate buffer (0.1 M, pH 7.5, 5 ml) and the reconstituted extract was loaded onto the cartridge. The cartridge was washed with water (3 ml) and methanol (3 ml), dried briefly with air and washed with ethyl acetate containing 2% diethylamine (3 ml). The sulfate was eluted with 70% ethyl acetate/methanol containing 2% diethylamine (3 ml) and the eluate was dried under a stream of nitrogen at 60 °C. The dried eluate was reconstituted in methanol (10 μl) and water (90 μl) for LC/MS analysis.

Solid phase extraction of BG and EBG used the following procedure. A Certify II cartridge was conditioned with methanol (3 ml), water (3 ml), formic acid (0.1 M, 3 ml) and sodium dihydrogenphosphate buffer (0.1 M, pH 7.5, 5 ml). Urine (2 ml) was diluted with water (3 ml), adjusted to pH 9.5 using ammonium hydroxide and loaded onto the cartridge. The cartridge was washed with water containing 2% ammonium hydroxide (5 ml), then methanol containing 2% ammonium hydroxide (3 ml) and the glucuronide conjugates were eluted using methanol containing 2% acetic acid (4 ml). The eluate was evaporated to dryness and reconstituted in methanol (10 μl) and water (90 μl) for LC/MS analysis.

2.4. Extraction recovery and precision

Extraction recovery was estimated against d_3-testosterone sulfate (50 ng ml^{-1}) for sulfate conjugates and d_3-testosterone glucuronide (40 ng ml^{-1}) for glucuronide conjugates as internal standards spiked in drug free horse urine. To one set of samples containing the internal standard, three concentrations of BS (10, 50 and 150 ng ml^{-1}), EBS (20, 50 and 150 ng ml^{-1}) or glucuronide (20, 40 and 100 ng ml^{-1}) conjugates were added prior to SPE, while to a second control set, the internal standards were added before extraction with sulfate and glucuronide conjugates added after the extraction but before evaporating the extract to dryness. Extraction recovery was then estimated by comparing peak area ratios between the analytes and the internal standards. Precision was assessed by comparing the peak area ratios between analyte and internal standard at each concentration analysed. The results were obtained from triplicate analysis for each conjugate at each concentration given.

2.5. Standard calibration curve for quantitation and accuracy

Calibration curves were determined against d_3-testosterone sulfate (100 ng ml^{-1}) for sulfate conjugates and d_3-testosterone glucuronide (40 ng ml^{-1}) for glucuronide conjugates as internal standards spiked in drug free urine. A range of concentrations of BS (10, 25, 50, 100, 200 and 400 ng ml^{-1}), BG and EBG (10, 20, 40, 80, 150 and 300 ng ml^{-1}) were used for calibration. The calibration curves were linear from 25 to 200 ng ml^{-1} for BS and 20 to 150 ng ml^{-1} for BG and EBG. Each concentration was analysed in duplicate and the mean peak area ratio of analyte to internal standard was plotted against concentration. In the linear dynamic range, the regression coefficient indicated a good fit for BS [four points, $y = 289.93(\pm 9.15)x - 25.58(\pm 7.51)$, r 0.9835], BG [four points, $y = 8.07(\pm 2.02)x - 9.31(\pm 1.95)$, r 0.9945] and EBG [four points, $y = 66.89(\pm 7.24)x - 2.91(\pm 3.57)$, r 0.9904]. The accuracy of the test was determined for BS, BG and EBG. Three known and independently prepared concentrations of BS (20, 100 and 200 ng ml^{-1}) and BG and EBG (20, 40 and 100 ng ml^{-1}) were spiked into drug free horse urine and quantitated using the constructed calibration curves. Accuracy was expressed as a percentage based on the ratio of assayed analyte concentration to known spiked concentration.

2.6. Animal administration

A registered proprietary veterinary preparation containing boldenone undecylenate in oil (Boldebal-H®, Ilium Veterinary Products, Smithfield, NSW, Australia; 4 ml = 200 mg boldenone undecylenate) was administered by intramuscular injection to an aged standardbred gelding (565 kg). Urine samples were collected by conditioned spontaneous voiding daily for 7 days post-administration, then once weekly for 8 weeks. The samples were immediately frozen and stored at −20 °C until required for analysis. The administration was approved by the New South Wales Thoroughbred Racing Board Animal Care and Ethics Committee.

3. Results and discussion

A number of previous studies have reported the detection of BS as a phase II metabolite in horse urine [1,7–9]. The application of fast atom bombardment MS was used for the detection of BS isolated from horse urine, although this was complicated by matrix interference [1]. The use of LC/MS^2 has also been reported for BS detection using a C18 SPE method for sample cleanup [7]. BS was detected in the urine from 10 h to 17 days following the administration of boldenone undecylenate by intramuscular injection. No quantitation was performed by LC/MS, this being instead undertaken indirectly by HPLC/UV [8]. More recently, the direct detection of endogenous BS in the horse urine of entire male horses but not geldings has been reported. The study used a four-step extraction procedure of C18 and DEAE-Sephadex SPE, immunoaffinity column and C18 SPE, followed by detection using a high resolution Q-ToF LC/MS^2 analysis. Quantification of BS was not reported but was instead performed for total boldenone metabolites following acidic methanolysis and analysis of boldenone by GC/MS or LC/MS.

Little has been reported on the detection or analysis of glucuronide metabolites derived from boldenone. Dumasia et al. [1] reported that 40–60% of boldenone metabolites were glucuronide conjugates in a study involving the administration of radio-labelled drug and fractionation of the resulting conjugates, although no analysis of the glucuronide fraction was reported.

3.1. LC/MS

Under negative ion ESI conditions, BS and EBS both gave a pseudomolecular ion at *m/z* 365 ($[M-H]^-$) only. This ion in each case could be fragmented by ion trap CID and resulted in a single fragment at *m/z* 350 ($[M-H–CH_3]^-$). This fragmentation is unusual in that it must involve loss of a methyl radical, an uncommon occurrence for ions generated by a low energy ionisation source such as ESI. The composition of this fragment was confirmed by high resolution measurement performed by skimmer CID on an FTICRMS instrument and using the known composition of *m/z* 365 ($[M-H]^-$) as an internal reference (found: 350.1207; $C_{18}H_{22}O_5S$ ($[M-H–CH_3]^-$) requires 350.1188).

In the case of BS, the above result also stands in contrast to that reported for CID of BS in a triple quadrupole instrument under negative ion conditions, where loss of the sulfate function as hydrogensulfate gives rise to a strong base peak at *m/z* 97 [8,10] with no *m/z* 350 fragment at all. In the case of the ion trap, the former ion falls below the low mass cutoff value and is thus not observed. MS^3 treatment of the BS *m/z* 350 fragment did give a more complex mass spectrum, and this appears in Fig. 2a. Results for EBS were identical. The major fragmentation here gave a peak at *m/z* 177, deriving from a C-ring cleavage through C12/C13 and C8/C14 ($[C_6H_9O_4S]^-$), and this fragment is observed in both ion trap MS^3 and triple quadrupole MS^2 spectra [8,10]. Unfortunately, the fragmentations described above were relatively inefficient and resulted in poor sensitivity. A more effective approach in a urinary matrix was a slight reduction of the activation energy so as to retain the pseudomolecular ion while still eliminating substantial background interference through CID. This was the approach adopted for all urinary analyses for sulfate conjugates described here.

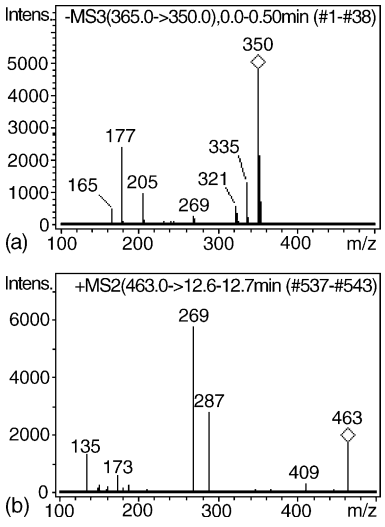

Fig. 2. Mass spectra of BS and BG: (a) BS, negative ion MS^3 obtained by direct infusion; (b) BG, positive ion MS^2 obtained by LC injection.

BG and EBG could be analysed by either negative or positive ion ESI. In negative ion mode, the pseudomolecular ion at *m/z* 461 ($[M-H]^-$) was observed as the base peak in each case and ion trap CID treatment gave *m/z* 461 ($[M-H]^-$) together with *m/z* 443 ($[M-H_3O]^-$) as the base peak and only significant fragment. In positive ion mode, ion trap CID of the pseudomolecular ion at *m/z* 463 ($[M+H]^+$) in each case gave a mass spectrum dominated by fragments at *m/z* 287 ($[M-C_6H_7O_6]^+$) and 269 ($[M-C_6H_9O_7]^+$) resulting from loss of the sugar moiety. It was found that positive ion mode afforded better sensitivity and so this was used in the following LC/MS analysis. The MS^2 spectrum for BG appears in Fig. 2b, that for EBG being identical.

BS and EBS eluted closely on the C18 column under the conditions reported. However, a partial separation allowed the two epimers to be distinguished on the basis of retention time (Fig. 3a). In contrast, good separation of the two epimeric glucuronides was achieved (Fig. 3d). In each case, the 17-epiboldenone conjugates eluted later than their respective boldenone counterparts. This has also been observed for the glucuronide and sulfate conjugates derived from testosterone [2,6].

3.2. Solid phase extraction

Steroidal sulfate and glucuronide conjugates are commonly isolated from horse urine by SPE using C18 sorbents [11] on the assumption of subsequent cleanup steps following chemical hydrolysis. This was obviously not feasible for a study of phase II metabolism and so more specific extraction procedures had to be developed. For the sulfates, a diethylaminopropyl (DEA) cartridge was introduced following C18 extraction. DEA is a weak anion exchange sorbent with a pK_a of 10.7 and was found to be suitable for extraction of sulfate conjugates. After the removal of most of the salts and pigments in the urine by the C18 cartridge, the sulfates were further purified using this sorbent. The two-stage extraction provided very clean samples for LC/MS analysis.

More limited information has been reported on the purification of glucuronide conjugates from horse urine [1]. The two-stage extraction procedure used for sulfate conjugates did not work for the glucuronide below a threshold of 100 ng ml^{-1} in urine. Initial investigations of other relatively new SPE sorbents also gave unsatisfactory results. Oasis HLB [12] and PBA [13] cartridges afforded no recovered glucuronide and NH_2 [14] and SAX cartridges gave poor extraction recovery. We found that the Certify II mixed mode sorbent combining C8 and DEA functionalities gave a better extraction.

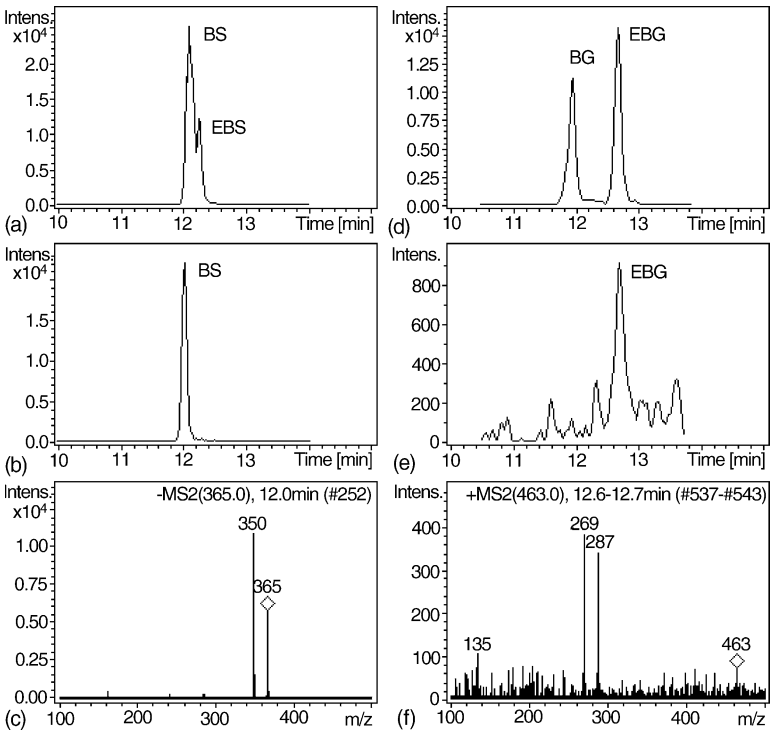

Fig. 3. Extracted ion chromatograms of sulfate (*m/z* 365) and glucuronide (*m/z* 287 and 269) conjugates: (a) standard of BS and EBS; (b) BS (201 ng ml^{-1}) in horse urine 7 days post-administration; (c) negative ion MS^2 of BS in horse urine 7 days post-administration; (d) standard of BG and EBG; (e) EBG (20 ng ml^{-1}) in horse urine 4 days post-administration; (f) positive ion MS^2 of EBG in horse urine 4 days post-administration.

The extraction recovery and precision ($n = 3$) were measured using spiked urine (Table 1). Recovery for sulfate conjugates ranged from 53 to 79% and the average recovery for both glucuronide conjugates was around 22%. The limit of detection for this extraction and analysis was estimated as the lowest concentration of conjugate spiked into drug free urine that gave a S/N > 3 in the LC/MS chromatogram. Based on this method, the limit of detection for both BS and EBS is 10 ng ml^{-1} and for BG and EBG 20 ng ml^{-1}.

Table 1
Extraction recovery and precision of BS, EBS, BG and EBG spiked in horse urine ($n = 3$)

Concentration [ng ml^{-1}]	Extraction recovery [% (% R.S.D.)]	Precision [analytes/IS (% R.S.D.)]
BS		
10	79.3 ± 9.1 (11.5)	0.37 ± 0.02 (5.4)
50	60.3 ± 1.0 (1.6)	0.81 ± 0.07 (8.6)
150	55.0 ± 2.6 (4.7)	2.13 ± 0.14 (6.5)
EBS		
20	65.1 ± 7.0 (10.7)	0.29 ± 0.01 (3.4)
50	64.5 ± 3.5 (5.4)	1.04 ± 0.02 (1.9)
150	53.0 ± 5.7 (10.7)	2.02 ± 0.06 (2.9)
BG		
20	20.6 ± 1.2 (5.8)	0.35 ± 0.04 (11.4)
40	25.3 ± 3.8 (15)	0.71 ± 0.06 (8.4)
100	19.1 ± 2.6 (13.6)	1.34 ± 0.11 (8.3)
EBG		
20	20.0 ± 1.1 (5.5)	0.29 ± 0.02 (6.8)
40	27.6 ± 5.0 (18)	0.65 ± 0.09 (13.8)
100	17.7 ± 2.0 (11.3)	1.20 ± 0.09 (7.5)

3.3. Linearity and accuracy

Calibration curves were linear over the range of 25–200 ng ml^{-1} for BS and 20–150 ng ml^{-1} for BG and EBG (Section 2.5). Accuracy was estimated across the range using the regression equation derived from these calibration curves (Table 2). Linearity and accuracy were not determined for EBS as this conjugate was not found in the administration samples.

Table 2
Accuracy for BS, BG and EBG ($n = 1$)

Spiked concentration (ng ml^{-1})	Accuracy (%)
BS	
20	78
100	105
200	98
BG	
20	104
40	107
100	95
EBG	
20	107
40	95
100	84

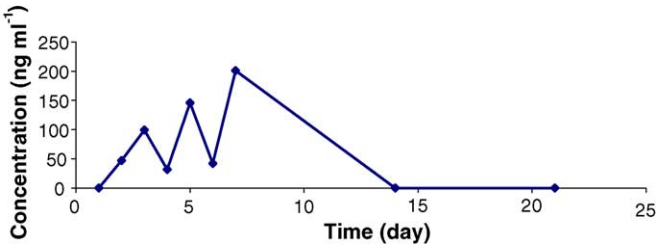

Fig. 4. Excretion curve of BS in horse urine post-administration.

3.4. Application to administration urine samples

The LC/MS method we developed in this study was used to determine the levels of equine phase II metabolites of boldenone in post-administration urine samples. BS was detected as the major metabolite with EBG also detected in trace amounts. No EBS or BG was detected in the 15 urine samples collected from the same horse. An excretion curve for BS was generated by analysis of urine samples collected from a horse for a period of 2 months after drug administration. Quantitation could be reliably performed from day 2 through to day 7 with the peak concentration (201 ng ml^{-1}) on day 7 (Figs. 3b, c and 4). The peak excretion of EBG was observed in the urine from day 4 (Fig. 3e, f) with an estimated concentration of 20 ng ml^{-1}, close to the limit of detection for this analyte. A possible trace of this compound was also detected on day 7.

4. Conclusion

This report describes the direct detection of boldenone phase II metabolites in equine post-administration urine by ion trap LC/MS. A rapid sample purification by SPE combined with ESI LC/MS^2 analysis provided the ability to simultaneously screen and confirm the identity of phase II metabolites derived from boldenone in the horse and eliminates the need for time consuming and tedious sample pretreatment procedures. The major phase II metabolite of boldenone in the horse was found to be BS, consistent with previous direct and indirect analyses [1,7–9]. No EBS was detected. In addition, EBG was identified as a minor phase II metabolite and the only glucuronide conjugate detected by this study.

Acknowledgements

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