Journal of Chromatography, 433 (1988) 9–21
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4427

SCREENING, CONFIRMATION AND QUANTIFICATION OF BOLDENONE SULFATE IN EQUINE URINE AFTER ADMINISTRATION OF BOLDENONE UNDECYLENATE (EQUIPOISE™)

LARS O.G. WEIDOLF*, TINA M.P. CHICHILA and JACK D. HENION*

Equine Drug Testing and Toxicology, New York State College of Veterinary Medicine, Cornell University, 925 Warren Drive, Ithaca, NY 14850 (U.S.A.)

(First received March 22nd, 1988; revised manuscript received August 8th, 1988)

SUMMARY

Methods for screening by thin-layer chromatography, quantification by high-performance liquid chromatography with ultraviolet detection and confirmation by gas chromatography–mass spectrometry of boldenone sulfate in equine urine after administration of boldenone undecylenate (Equipoise™) are presented. Sample work-up was done with C_{18} liquid–solid extraction followed by solvolytic cleavage of the sulfate ester. Confirmatory evidence of boldenone sulfate in equine urine was obtained from 2 h to 42 days following a therapeutic intramuscular dose of Equipoise. The use of 19-nortestosterone sulfate as the internal standard for quantification of boldenone sulfate is discussed.

INTRODUCTION

Anabolic steroids are widely used in horses throughout the world for both therapeutic and non-therapeutic purposes [1]. The therapeutic value of these steroids in the treatment of diseased and debilitated horses has been demonstrated, but reports are contradictory as to whether improvement in the racing performance horse may be achieved by using these drugs [1,2]. The anabolic steroids are lipophilic compounds, rendering them susceptible to extensive metabolism and conjugation prior to excretion, and identification of their major metabolites is essential for forensic purposes [3,4]. Analytical methods for confirmation of the unlawful use of anabolic steroids are most commonly based on isolation and determination of metabolites of these steroids in urine or blood. The presence of highly polar conjugates makes liquid–solid extraction (LSE) a suitable means for isolation of these compounds and the extract is usually subjected to enzymatic

*Present address: AB Hässle, S-431 83 Mölndal, Sweden.

0378-4347/88/$03.50

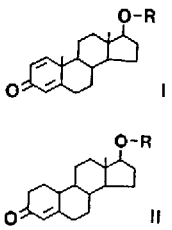

Fig. 1. Structures of the compounds studied. (I) R = H: boldenone; R = SO_3^-: boldenone sulfate. (II) R = H: 19-nortestosterone; R = SO_3^-: 19-nortestosterone sulfate.

or acidic hydrolysis prior to derivatization and analysis by gas chromatography–mass spectrometry (GC–MS) [5].

Boldenone (BO, Fig. 1) belongs to a group of synthetic anabolic steroids in which the ratio of the androgenic activity, which causes unwanted side-effects, has been decreased as compared to the anabolic activity through introduction of the 1,2-double bond not present in endogenous steroids, e.g. testosterone [6]. Studies on the metabolism of testosterone, 19-nortestosterone (NT) and BO have shown that an important metabolic pathway for these compounds is the direct formation of a sulfo-conjugate of the 17β-hydroxy group, followed by rapid renal excretion [3,4,7]. The study on BO [7] showed that the sulfo-conjugated parent compound accounted for 18–25% of the amount recovered in urine over the first 24 h post-dose of [^{3}H]BO to three gelded horses. The structure of the sulfo-conjugate was determined directly by fast atom bombardment MS and by GC–MS after solvolytic cleavage of the sulfate ester. These steroids, however, are frequently administered intramuscularly as long-chain fatty acid 17β-ester derivatives dissolved in a vegetable oil to obtain a slow distribution of the compound from the site of injection into the systemic circulation over a period of several weeks [2]. Once in the systemic circulation, the ester is rapidly hydrolysed to give the pharmacologically active compound. The metabolic profile of these steroid esters, however, is qualitatively not different from that obtained after administration of the unesterified drug [3]. Accordingly, in our previous study [8], boldenone sulfate was identified unambiguously in horse urine by high-performance liquid chromatography (HPLC)–tandem MS after intramuscular administration of boldenone undecylenate (Equipoise^{TM}).

This paper presents methods for the selective isolation, screening, confirmation and quantification of boldenone sulfate, a major metabolite of BO excreted in horse urine, as evidence for the administration of Equipoise.

EXPERIMENTAL

Chemicals

Structural formulae of the compounds used in this study are given in Fig. 1. BO (1,4-androstadien-17β-ol-3-one) and NT (4-estren-17β-ol-3-one) were purchased from Sigma (St. Louis, MO, U.S.A.). Boldenone sodium sulfate (BOS, 1,4-androstadien-17β-ol-3-one sodium sulfate) and 19-nortestosterone sodium sulfate (NTS, 4-estren-17β-ol-3-one sodium sulfate) were obtained from Stera-

loids (Wilton, NH, U.S.A.). The purity of each compound was verified by HPLC before use. HPLC-grade acetonitrile, ethyl acetate, methanol and ammonium acetate and reagent-grade glacial acetic acid, pyridine, sulfuric acid and sodium carbonate were purchased from Fisher Scientific (Rochester, NY, U.S.A.). HPLC-grade water was obtained from an in-house water purification system (Nanopure II, Barnstead, Boston, MA, U.S.A.). LSE cartridges (J.T. Baker, Phillipsburg, NJ, U.S.A.) packed with 200 mg C_{18}-bonded silica (Model 7020-2, 40 μm, 60 Å) were used with a vacuum manifold equipped to hold twelve cartridges (Supelco, Bellefonte, PA, U.S.A.). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Pierce (Rockford, IL, U.S.A.) and O-methoxyamine hydrochloride from Supelco.

Animal experiments

Equine urine samples were obtained from experimental horses housed within the Cornell Equine Drug Testing and Toxicology Program. Standardbred mares (horse A: 590 kg; horse B: 480 kg) were injected intramuscularly with 1.1 and 1.7 mg/kg body weight of boldenone undecylenate (Equipoise, E.R. Squibb and Sons, Princeton, NJ, U.S.A.), respectively. The manufacturer's recommended therapeutic dose is 1.1 mg/kg body weight every three weeks. Urine samples were collected via an indwelling urethral catheter before injection of the drug and at 2, 4, 6, 8, 10, 12, 24, 32, 48 h and 3, 4, 5, 7, 9, 11, 13, 14, 17, 21, 24, 28, 31, 35, 42 days post-dose from horse A. Urine from horse B was collected in an identical manner up to 48 h post-dose and then only one sample at 35 days was obtained. The samples were stored at −18°C until analysis.

Sample preparation procedures

Development of the LSE conditions used for sample preparation has been described in detail elsewhere [9].

Thin-layer chromatography (TLC)

A 1-ml volume of equine urine was mixed with 1 ml methanol–0.65 *M* ammonium acetate pH 5.4 (20:80), placed in an ultrasonic bath for 15 min and centrifuged for 5 min at approximately 1000 *g*. The sample solution was applied to an LSE cartridge which had been conditioned with 2 ml of methanol and 1 ml of 25 m*M* ammonium acetate pH 5.5. The cartridge was washed with 1 ml of 25 m*M* ammonium acetate pH 5.5, 2 ml of methanol–25 m*M* ammonium acetate pH 5.5 (40:60) and 3 ml of water. The fraction containing BOS was eluted with 2 ml of methanol–water (35:65) and 2 ml of methanol were added to facilitate evaporation under a gentle stream of nitrogen at 60°C. The residue was dissolved in 0.1 ml of methanol, followed by 5 ml of ethyl acetate which had been saturated with 2.2 *M* sulfuric acid (one tenth volume). The sulfo-conjugates were solvolysed by heating the solution to 50°C for 30 min [10]. The organic layer was then washed with 1.5 ml of 0.94 *M* sodium carbonate pH 10.3 (pH adjusted with solid sodium bicarbonate) and 5 ml of water. The phases were separated and the organic layer was evaporated under a gentle stream of nitrogen at 60°C.

High-performance liquid chromatography

High-level samples (> 1.2 μg/ml; > 3.3 μM). A 1-ml urine volume was mixed with 1 ml of methanol–0.65 *M* amonium acetate pH 5.4 (20:80) and 200 μl of 25.3 μ*M* NTS in water as internal standard (giving 5.1 nmol NTS per ml urine). Calibration curves were prepared from control urine to which aliquots of 13.3 μ*M* BOS in water were added, giving concentrations ranging from 2.00 to 10.6 nmol BOS per ml urine.

Lower-level samples (< 1.2 μg/ml; < 3.3 μM). A 2-ml urine volume was mixed with 2 ml of methanol–0.65 *M* ammonium acetate pH 5.4 (20:80) and 75 μl of 25.3 μ*M* NTS in water as internal standard (giving 0.949 nmol NTS per ml urine). Calibration curves were prepared from control urine to which aliquots of 13.3 μ*M* BOS in water were added, giving concentrations ranging from 0.133 to 3.33 nmol BOS per ml urine.

After preparation of the solutions, the high-level and lower-level samples were treated identically. The solutions were sonicated, centrifuged and purified by LSE as described above for the TLC screening samples. Solvolysis was performed at 50°C for 50 min, and the organic layer was washed twice with 2 ml of 0.94 *M* sodium carbonate pH 10.3 and once with 3 ml of water. The phases were separated and the ethyl acetate was evaporated. The residue was transferred with three 0.2-ml portions of methanol–water (50:50) to LSE cartridges which had been conditioned with 2 ml methanol and 1 ml water. The cartridges were washed with 1 ml water and 2 ml methanol–water (55:45) after which elution was performed with 2 ml methanol–water (80:20). The eluate was evaporated under a gentle stream of nitrogen at 60°C, reconstituted in 100 μl mobile phase and analysed by HPLC.

Gas chromatography–mass spectrometry

The samples purified for confirmation by GC–MS were treated exactly as the lower-level samples for HPLC as given above, but without the addition of internal standard. The final residue was treated as described below.

Preparation of methyloxime-trimethylsilyl (MO-TMS) derivatives of BO and NT

The MO-TMS ether derivatives of BO or NT were prepared by dissolving the urine extract or 5 μg of standard in a 0.3-ml ReactiVial (Pierce) with 200 μl of a 50% (w/v) solution of O-methoxyamine hydrochloride in pyridine. The vial was sealed and heated for 1.25 h at 95°C. The sample was then taken to dryness under a gentle stream of nitrogen following addition of 25 μl ethyl acetate to facilitate the evaporation of the pyridine. The residue was dissolved in a 50% (v/v) solution of BSTFA in ethyl acetate (200 μl for standards or 25 μl for urine extract). This mixture was heated at 60°C for 15 min. A 1-μl aliquot of this reaction mixture was injected on-column for GC–MS analysis.

Thin-layer chromatography

Each sample residue was dissolved in 20–30 μl ethyl acetate and spotted on 10×20 cm Kieselgel 60 F_{254} TLC plates of 0.25 mm layer thickness (EM Science, Cherry Hill, NJ, U.S.A.). Each plate was also spotted with 1–2 nmol standard of

BO and NT, testosterone, 17β-estradiol and dehydroepiandrosterone as controls for possible interference. The plates were developed 5 cm with ethyl acetate–glacial acetic acid (39:1) and dried under warm air. The drugs were observed in short-wave UV light for fluorescence quenching and sprayed with vanillin–concentrated sulfuric acid (1:99) followed by heating on a hot plate for a few minutes to induce a color reaction.

Liquid chromatography

The HPLC system consisted of an M 6000A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7010 injector equipped with a 20-μl sample loop (Rheodyne, Cotati, CA, U.S.A.) and a Spectroflow 783 variable-wavelength UV detector at 254 nm (Kratos, Ramsey, NJ, U.S.A.). Chromatograms and peak areas were obtained with a Model 3390A reporting integrator (Hewlett-Packard, Avondale, PA, U.S.A.). The chromatographic column was 83 mm$\times$4.6 mm I.D. Pecosphere-3C C_{18} packed with 3-μm particles (Perkin-Elmer, Wilton, CT, U.S.A.). The mobile phase was acetonitrile–methanol–25 m*M* ammonium acetate pH 5.5 (7:50:43) maintained at a flow-rate of 1.1 ml/min. The mobile phases were degassed in an ultrasonic bath followed by sparging with helium and filtered through a 0.45-μm Nylon-66 filter (Rainin, Woburn, MA, U.S.A.) before use.

Before analysis of each set of samples a standard mixture of BO and NT was chromatographed repetitively to demonstrate reproducible retention times followed by a blank injection of mobile phase to demonstrate the absence of carry-over effects. Duplicate or triplicate injections of 20-μl aliquots of each sample were then performed with a solvent blank injection between each sample.

Gas chromatography–mass spectrometry

Confirmation of BOS as the MO-TMS derivative of BO after solvolysis was done on a Carlo Erba (Peabody, MA, U.S.A.) Mega Series Model 5300 high-resolution gas chromatograph interfaced to a Hewlett-Packard 5970 mass-selective detection (MSD) system. The capillary column used was an HP-Ultra 100% methyl silicone capillary, 24 m$\times$0.2 mm I.D., 0.11 μm film thickness (Hewlett-Packard, Avondale, PA, U.S.A.). The column was directly interfaced to the MSD system. Injector temperature was maintained at 275°C. Injections were made in the splitless mode while the oven temperature was programmed from 150 to 225°C at 15°C/min and then to 275°C at 4°C/min followed by a 10-min hold. The mass spectrometer was scanned repetitively from m/z 35 to m/z 425 at a rate of one scan per second.

Parameters for selected-ion monitoring (SIM). The MO-TMS derivatives of NT and BO were monitored under SIM conditions. The ions monitored for the MO-TMS derivative of NT were m/z 254.25, 344.35 and 360.25, and the molecular ion at m/z 375.35. The ions monitored for the MO-TMS derivative of BO were m/z 266.20, 340.20, 356.25 and 372.25, and the molecular ion at m/z 387.30 with a dwell time of 50 ms.

RESULTS AND DISCUSSION

Isolation of BOS and NTS

The isolation procedure for BOS and NTS includes LSE of the sulfo-conjugates followed by the mild acid-catalysed cleavage of the sulfate ester bond described by Vihko [10]. The solvolysis is selective towards sulfate esters and does not hydrolyse glucuronic acid conjugates, keeping the co-extracted endogenous matrix to a minimum. Samples prepared for HPLC and MS analysis were subjected to a second LSE clean-up prior to analysis to allow for quantification and confirmation of BOS in samples of low concentrations. The recovery of BOS and NTS determined as the released parent steroid was 78–88% at a urinary concentration of about 1 nmol/ml [9].

Screening by thin-layer chromatography

The sample preparation procedure for TLC screening was simplified compared to the procedure for HPLC and GC–MS analysis to allow for the samples to be processed in a shorter time (twelve samples in 5 h). This compromise could be made since the entire extract of 1 ml urine was spotted on the TLC plate. Most of the matrix components migrated faster ($R_F > 0.8$) than BO with the solvent system used, and BO was well resolved from the investigated standard compounds (Table I). The selected standard compounds (NT, 17β-estradiol, testosterone and dehydroepiandrosterone) may exist endogenously as sulfo-conjugates and would be partially co-extracted by the isolation procedure [8], thus posing a potential risk of interference with the TLC detection modes for BO. The TLC plates showed fluorescence quenching for BO in short-wave UV light for the urines collected between 6 h and 31 days with the exception of the 21-day sample corresponding to a detection limit of 110 ng/ml of urine (0.3 μM). The induced color reaction with vanillin–concentrated sulfuric acid (1:99) (Table I) was less sensitive but allowed for detection of BO between 8 h and 14 days post-dose [approximately 500 ng/ml of urine (1.4 μM)].

TABLE I

R_F VALUES, FLUORESCENCE QUENCHING PROPERTIES IN LOW-WAVELENGTH UV LIGHT AND COLOR REACTIONS INDUCED BY CONCENTRATED SULFURIC ACID-VANILLIN (99:1) AND HEATING

Solvent system: ethyl acetate–glacial acetic acid (39:1).

Compound	R_F	Fluorescence quenching	Color reaction
Boldenone	0.63	Yes	Purple
19-Nortestosterone	0.69	Yes	Red
Testosterone	0.72	Yes	Red
Dehydroepiandrosterone	0.80	No	Red/brown
17β-Estradiol	0.85	No	Green/brown

Quantification by HPLC

Choice of internal standard. The high selectivity of the sample preparation procedure is based upon the isolation of the sulfo-conjugate of BO by LSE, mild acid treatment for cleavage of the sulfate, liquid–liquid extraction of the released parent steroid and finally purification by LSE. The correct choice of internal standard (I.S.) for this procedure is important, since the I.S. must behave as the target compound in every step. A structural analogue of BOS, such as NTS, meets these requirements nicely. However, NT is an endogenous compound and has been detected in urine of the stallion at low levels after enzymatic hydrolysis [11]. In addition, sulfo-conjugation is a known route of metabolism of NT [4], increasing the possibility of endogenous interference for quantification of BOS according to our proposed procedure. Before attempting to use NTS as the I.S., control urines from three mares and two stallions were processed according to the sample pretreatment described herein. Only minor endogenous peaks due to the chemical

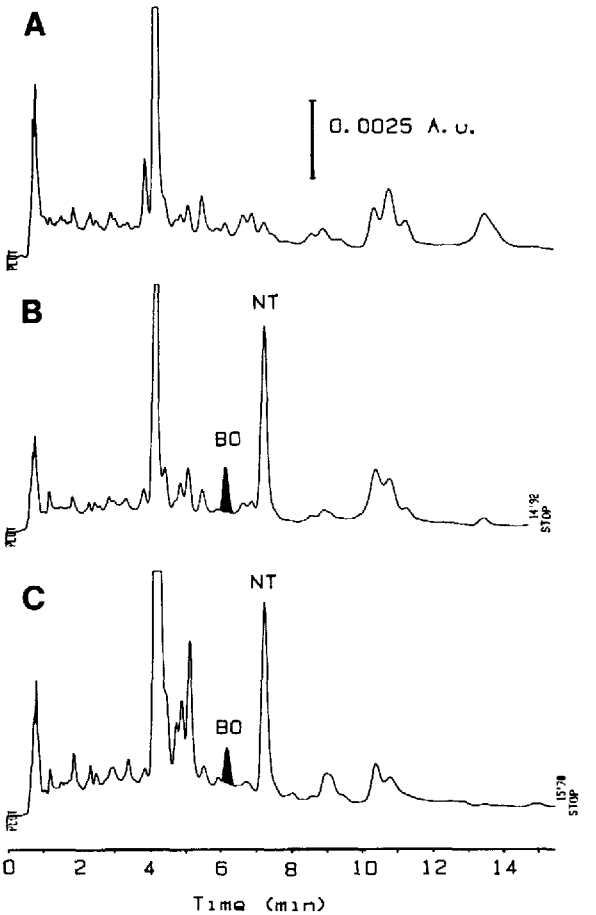

Fig. 2. HPLC separation of an extract from (A) 2 ml control equine urine, (B) 2 ml equine urine collected 2 h after an intramuscular dose of 610 mg boldenone undecylenate and (C) 2 ml equine urine collected 42 days after an intramuscular dose of 610 mg boldenone undecylenate. Internal standard: 19-nortestosterone sulfate (0.949 μM). Peaks: BO = boldenone; NT = 19-nortestosterone.

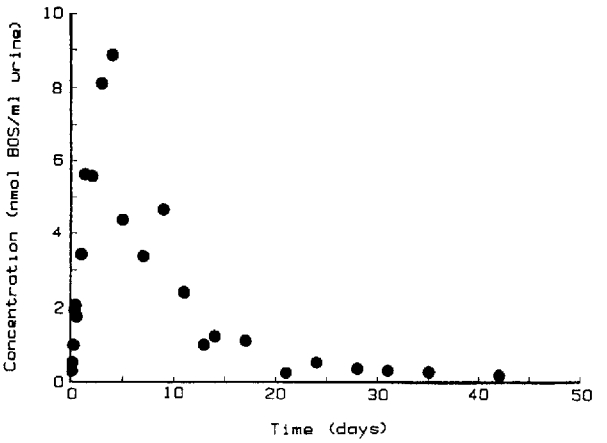

Fig. 3. Urinary concentrations of boldenone sulfate (BOS) determined by HPLC-UV after an intramuscular dose of boldenone undecylenate (610 mg) to a mare.

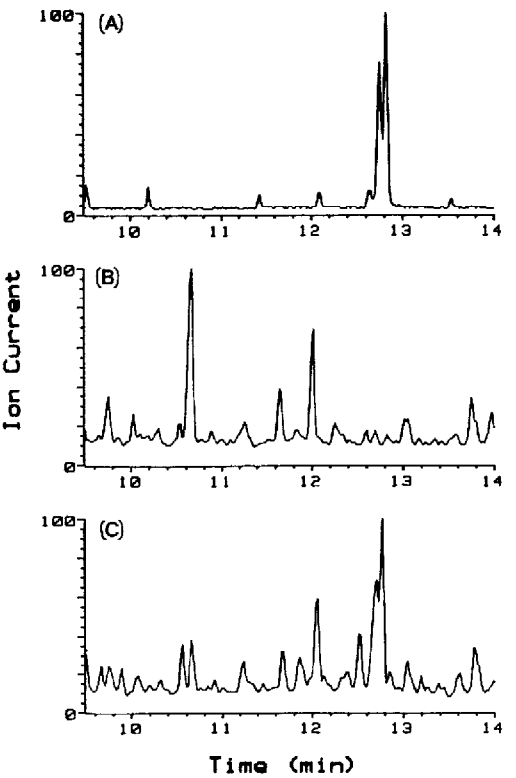

Fig. 4. Total ion current chromatograms from full-scan EI capillary GC-MS analyses of (A) 25 ng boldenone as its MO-TMS derivative, (B) control equine urine extract and (C) equine urine extract 32 h after boldenone undecylenate administration.

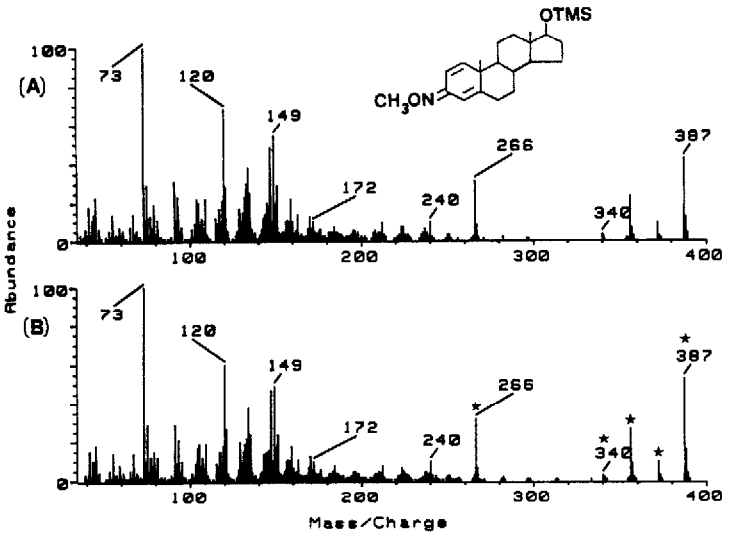

Fig. 5. EI mass spectra for the 12.8 min retention time peak observed in (A) Fig. 4A and (B) Fig. 4C. The ions with a star in (B) were monitored in SIM GC–MS experiments. The mass spectra for the *syn*- and *anti*-boldenone MO-TMS derivative were identical.

background close to the retention times corresponding to NT or BO were detected (Fig. 2A). The mobile phase was optimized for chromatography of the extracts from the two experimental mares used in this study with respect to the acetonitrile/methanol ratio as the organic modifiers. However, this ratio could be adjusted to obtain the necessary retention time window for BO and NT without endogenous interference for urine extracts from other horses. Furthermore, NT was not detected in samples from the boldenone undecylenate administration study which were monitored for NT by SIM GC–MS.

Quantification. The BOS excretion study included two mares of which one was sampled for urine according to a complete collection schedule for six weeks after a therapeutic dose of 1.1 mg/kg body weight (horse A). The other horse (B) was sampled during the first two days only and then once after five weeks following a dose of 1.7 mg/kg body weight. The discussion below concerns horse A, although similar results were obtained from horse B.

Standard curves were prepared daily including five to seven concentrations which were analysed in duplicate or triplicate by HPLC–UV. The linear regression coefficients were equal to or higher than 0.998. The limit of detection (LOD) was not determined from the signal-to-noise ratio (S/N) of the UV detector, but rather from the chemical noise from the endogenous matrix constituents. The 2-h and the 42-day samples (Fig. 2B and C) were both above the LOD ($S/N > 3$) which was estimated to be 0.175 nmol/ml of urine (64 ng/ml) from the control urine extract (Fig. 2A). The limit of quantification (LOQ) has been suggested to be obtained from levels at $S/N \geq 10$ [12] which corresponds to 0.583 nmol/ml of urine (212 ng/ml). Samples collected between 6 h (0.995 nmol/ml of urine) and 17 days (1.10 nmol/ml of urine) were above this concentration while the samples

of lower levels are considered to be in the region of detection [12] (Fig. 3). The excretion rate or half-life of BOS cannot be calculated from these data since urine samples were collected directly from the bladder and the total volume of voided urine was not measured. However, the peak concentration occurred at 96 h post-dose at 8.87 nmol/ml of urine (3.24 μg/ml).

Confirmation by GC–MS

The MO-TMS derivatives of BO and NT gave an abundant molecular ion (i.e. 45% relative abundance) with structurally significant high-mass fragment ions, which are important features for SIM GC–MS. Massé et al. [13] have defined the characteristic fragmentation patterns of several steroids as their MO-TMS

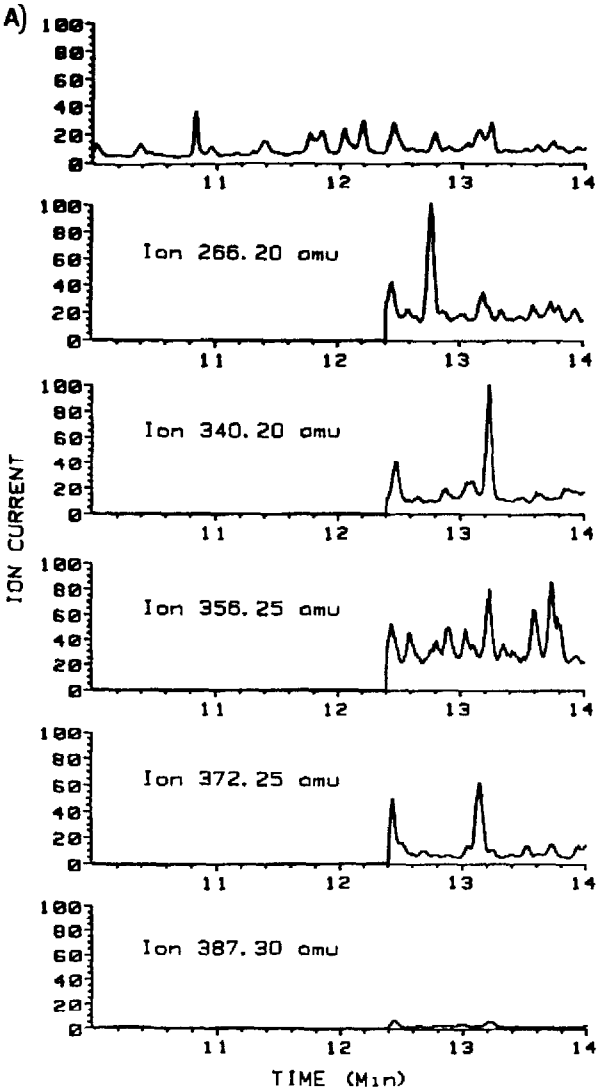

Fig. 6.

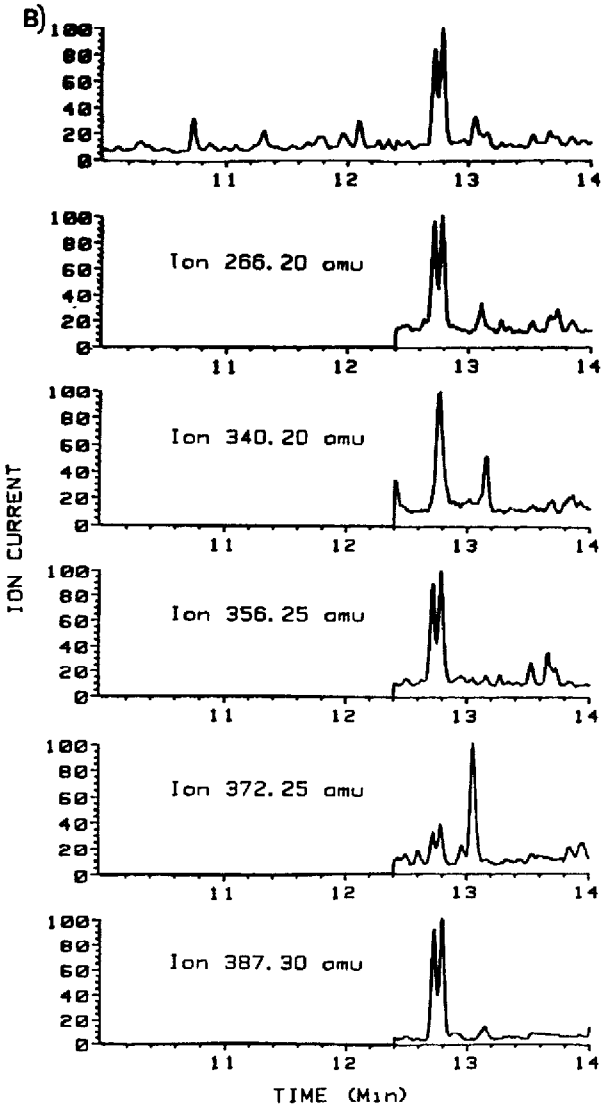

Fig. 6. Total and selected ion current chromatograms from the capillary SIM GC–MS analysis of (A) an MO-TMS-derivatized control and (B) an equine urine extract 1008 h after boldenone administration.

derivatives. The mass spectra of these compounds have intense $[M-31]^+$ and $[M-(31-90)]^+$ ions. The MO-TMS derivative of BO gives these fragments at m/z 356 and 266, respectively. Full-scan mass spectral confirmation of BOS as the MO-TMS derivative of BO after solvolysis was possible in samples as early as 4 h post-administration. Fig. 4A is a full-scan total ion current chromatogram of a 25-ng standard of the BO MO-TMS derivative. Partial separation of the *syn*- and *anti*-isomers of the methoxyamine derivatives is evident, but efforts to better resolve these isomers through different temperature programming schemes were

not successful. Even though the isomers were not chromatographically well resolved, their corresponding retention times provided additional evidence for the presence of the MO-TMS derivative of BO.

The total ion current chromatograms of control and 32 h post-administration urine extracts, in the full-scan capillary GC–MS mode, are shown in Fig. 4B and C, respectively. Unequivocal confirmation of BO was possible by comparing the mass spectra of BO from this extract with that from the standard (Fig. 5A and B). The abundant molecular ion and high-mass fragments of the MO-TMS derivative of BO, used in the SIM GC–MS mode, are indicated in the full-scan mass spectrum of the BO derivative shown in Fig. 5B. BOS as its MO-TMS derivative after solvolysis was confirmed by capillary SIM GC-MS in urine for as long as 1008 h (42 days) post-dose. For comparison, a study of the excretion of metabolites of 400 mg 19-nortestosterone phenylpropionate given intramuscularly to geldings and fillies showed that three or four urinary metabolites could be confirmed as the MO-TMS derivatives after conjugate cleavage for up to 21 days post-dose [4].

The total and selected ion current chromatograms for a control urine extract and a urine extract 1008 h post-administration are shown in Fig. 6A and B, respectively. There was some interference with the m/z 266 ion in this control, yet ion ratios for m/z 266 and the molecular ion at m/z 387 in the 1008-h sample corresponded to those for standards. On the other hand, the m/z 340/387 ratio for this extract was less reliable due to greater interference at this mass.

CONCLUSION

It has been suggested that the reliability of an analytical method for confirmation of an administered drug increases when the method focusses on several metabolites when it is impossible to monitor the parent compound for excretory or metabolic reasons [3,4]. Nevertheless, we have allowed our proposed method for screening, confirmation and quantification of boldenone undecylenate administrations to horses to focus on the excretion of BOS only. This is due to the fact that BO is a synthetic anabolic steroid and the risk of interference from endogenous compounds is minimal. The work-up procedure for BOS is extremely selective and only sulfo-conjugates of similar physicochemical properties, both as conjugate and as free aglycone, will be isolated [9]. Further, the method focuses on the sulfo-conjugate of the parent compound, which is re-formed during solvolysis. Thus, confirmation is performed on the parent compound, and the spectroscopic data obtained can easily be compared with the reference compound for increased reliability.

Quantification of BOS with the use of NTS as the internal standard might be debated for two reasons: NT is an endogenous compound with sulfo-conjugation as a prominent metabolic route and different esters of NT are used for doping of racehorses [4]. However, as in any quantitative analysis where an internal standard is to be used, one has to minimize matrix interferences through chromatographic optimization before attempting the proposed procedure. If such experiments demonstrate that neither the analyte(s) nor the selected internal

standard are present in the control sample, then the latter is a suitable choice. In this work NT was not observed in pre-administration urine samples by HPLC with UV detection or capillary SIM GC–MS. As for the presence of endogenous NTS in equine urine, it would appear that the concentrations of added NTS as internal standard in this study (1–5 μM) must be much higher than the endogenous level and will not jeopardize quantification of BOS in the concentration range obtained after a therapeutic dose of boldenone undecylenate.

ACKNOWLEDGEMENTS

Dr. E. Dewey is acknowledged for assistance with the animal administration experiments. Financial support was obtained from International Minerals and Chemicals Corporation (Terra Haute, IN, U.S.A.), the Harry M. Zweig Memorial Fund and the New York State Racing and Wagering Board.

REFERENCES

1 T. Tobin, Drugs and The Performance Horse, Charles C. Thomas Publisher, Springfield, IL, 1981, p. 149.
2 D.H. Snow, C.D. Munro and M. Nimmo, in F.J. Mine (Editor), Proceedings of the 23rd Annual Convention, American Association of Equine Practitioners, Vancouver, December 1977, p. 411.
3 M.C. Dumasia, E. Houghton and S. Sinkins, J. Chromatogr., 377 (1986) 23.
4 E. Houghton, M.C. Dumasia, P. Teale, M.S. Moss and S. Sinkins, J. Chromatogr., 383 (1986) 1.
5 C.H.L. Shackleton, J. Chromatogr., 379 (1986) 91.
6 E. Houghton, Xenobiotica, 7 (1977) 683.
7 M.C. Dumasia, E. Houghton, C.V. Bradley and D.H. Williams, Biomed. Mass Spectrom., 19 (1983) 434.
8 L.O.G. Weidolf, E.D. Lee and J.D. Henion, Biomed. Environ. Mass Spectrom., 15 (1988) 283.
9 L.O.G. Weidolf and J.D. Henion, Anal. Chem., 59 (1987) 1980.
10 R. Vihko, Acta Endocrinol., 109 (Suppl.) (1966) 29.
11 E. Houghton, J. Copsey, M.C. Dumasia, P.E. Haywood, M.S Moss and P. Teale, Biomed. Mass Spectrom., 11 (1984) 96.
12 G.L. Long and J.D. Weinfordner, Anal. Chem., 55 (1983) 712A.
13 R. Massé, C. Laliberté and L. Tremblay, J. Chromatogr., 339 (1985) 11.