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Screening of anabolic steroids in horse urine by liquid chromatography–tandem mass spectrometry

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Abstract

Anabolic steroids have the capability of improving athletic performance and are banned substances in the Olympic games as well as in horseracing and equestrian competitions. The control of their abuse in racehorses is traditionally performed by detecting the presence of anabolic steroids and/or their metabolite(s) in urine samples using gas chromatography–mass spectrometry (GC–MS). However, this approach usually requires tedious sample processing and chemical derivatisation steps and could be very insensitive in detecting certain steroids. This paper describes a high performance liquid chromatography–tandem mass spectrometry (HPLC–MS–MS) method for the detection of anabolic steroids that are poorly covered by GC–MS. Enzyme-treated urine was processed by solid-phase extraction (SPE) using a Bond Elut Certify® cartridge, followed by a base wash for further cleanup. Separation of the steroids was carried out on a reversed-phase DB-8 column using 0.1% acetic acid and methanol as the mobile phase in a gradient elution programme. The mass spectrometer for the detection of the steroids was operated in the positive electrospray ionisation (ESI) mode with multiple reaction monitoring (MRM). Urine samples fortified with 15 anabolic steroids (namely, androstadienone, 1-androstenedione, bolasterone, boldione, 4-estrenedione, gestrinone, methandrostenolone, methenolone, 17α-methyltestosterone, norbolethone, normethandrolone, oxandrolone, stenbolone, trenbolone and turinabol) at low ng/mL levels were consistently detected. No significant matrix interference was observed at the retention times of the targeted ion masses in blank urine samples. The method specificity, sensitivity, precision, recoveries, and the performance of the enzyme hydrolysis step were evaluated. The successful application of the method to analyse methenolone acetate administration urine samples demonstrated that the method could be effective in detecting anabolic steroids and their metabolites in horse urine.

Keywords: Anabolic steroids; Racehorse; Urine; Liquid chromatography–mass spectrometry

1. Introduction

Anabolic steroids are substances that can be used to promote muscle growth and therefore have the capability to improve athletic performance. The International Olympic Committee has banned the use of anabolic steroids since 1976. Anabolic steroids are also prohibited in the sport of horseracing by most horseracing authorities and by national equestrian federations and the Federation Equestre Internationale. The current method for testing anabolic steroids used in the authors' laboratory involves the isolation of the steroid sulphate and glucuronide conjugates using a C18 solid-phase extraction (SPE) cartridge followed by hydrolysis using anhydrous methanolic hydrogen chloride, or generally referred to as methanolysis [1]. The freed steroids are detected by GC–MS as their pentafluoropropionyl derivatives. Although this method is sensitive and robust, some steroids and their metabolites, such as bolasterone, 17α-methyltestosterone, normethandrolone, and trenbolone, cannot be effectively covered because they do not derivatize well with pentafluoropropionic acid anhydride. This is a major drawback of using GC–MS for detecting anabolic steroids, as derivatization is necessary to improve their GC properties; however, there is

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no universal derivatizing reagent that could work for all anabolic steroids.

HPLC–MS is well suited for the analysis of polar, non-volatile and heat-labile compounds. It is an attractive technique for the testing of anabolic steroids. Over the past decade, HPLC–MS has evolved into a mature technique and has been successfully applied to the analyses of anabolic steroids in various biological samples including cell culture media [2], human [3–6], and bovine [7,8]. In comparison, reported applications of HPLC–MS for the analyses of anabolic steroids in equine biological samples has been scarce and the scopes of testing in all cases were limited to a few or only one steroid [9–11]. This study describes a relatively simple sample preparation procedure for detecting 15 anabolic steroids using enzyme hydrolysis and solid-phase extraction, and subsequent detection by HPLC–MS–MS. This method has found to work well on anabolic steroids that are not adequately covered by our current GC/MS method.

2. Experimental

2.1. Materials

Androstadienone, 1-androstenedione, 4-estrenedione, boldione, normethandrolone and trenbolone were obtained from Steraloids (Newport, RI, USA). Bolasterone, testosterone glucuronide and d_3-testosterone were acquired from Sigma (St. Louis, MO, USA). Gestrinone was purchased from ShenZhen Konsaichem (ShenZhen, China). Methandrostenolone was obtained from Alltech (Deerfield, IL, USA) and methenolone was from Alltech (State College, PA, USA). 17α-Methyltestosterone was obtained from British Pharmacopoeia Commission (London, UK). Norbolethone and turinabol were obtained from the National Analytical Reference Laboratory (Pymble, NSW, Australia). Oxandrolone and testosterone were purchased from United States Pharmacopeial Convention, Inc. (Rockville, MD, USA). Stenbolone was a gift from Roche Palo Alto LLC (Palo Alto, CA, USA). Primobolan® (methenolone acetate) was obtained from Schering AG (Berlin, Germany). β-Glucuronidase (from *Patella vulgata*, lyophilized powder), protease (from bovine pancreases, type I, 6.9 units/mg solid) and sodium hydroxide (pellets, analytical grade) were purchased from Sigma (St. Louis, MO, USA). Acetic acid (96%), hydrochloric acid (30%), potassium hydroxide (pellets), potassium phosphate and sodium chloride (GR grade) were obtained from Merck (Darmstadt, Germany). Sodium sulphate was purchased from Farco Chemical Supplies (Beijing, China). Dichloromethane (GR grade), ethyl acetate (GR grade) and methanol (LiChrosolv®; LC grade) were obtained from Merck (Darmstadt, Germany). Bond Elut Certify® cartridge (130 mg, 3 mL) was purchased from Varian (Harbor City, CA, USA). HPLC grade deionised water was obtained from an in-house water purification system (Milli-Q, Molsheim, France).

2.2. Instrumentation

HPLC–MS–MS analysis was performed on a Thermo Finnigan TSQ Quantum mass spectrometer equipped with a Surveyor Autosampler and a MS Pump system (Thermo Finnigan, San Jose, CA, USA). Solid-phase extraction was carried out using a RapidTrace® SPE workstation (Zymark Corporation, Hopkinton, MA, USA).

2.3. HPLC–MS–MS analyses

A reversed-phase Supelcosil™ LC-8-DB column (10 cm × 2.1 mm i.d., 3 μm; Supelco, Bellefonte, PA, USA) was used for the analyses. The mobile phase was composed of 0.1% acetic acid in deionised water as solvent A and methanol as solvent B. A linear gradient was run at 0.2 mL/min, consisting of 40% solvent B at the start ($t = 0$ min), increased to 100% solvent B at $t = 5$ min, and then held at 100% solvent B for 5 min until $t = 10$ min. The gradient was then returned to 40% solvent B at $t = 11$ min, and stabilised at 40% solvent B for 4 more minutes before the next injection. Injection volume was 5 μL each.

The atmospheric pressure ionisation (API) source was operated in positive ESI mode. A capillary temperature at 300 °C was employed. The nitrogen sheath and auxiliary gas flow rates were set at 30 and 10 arbitrary TSQ Quantum units, respectively. Detection of the drugs was performed in the MRM mode with a single time segment containing 16 events. The peak widths for the selection of the precursor and the corresponding product ions in Q_1 (FWHM) and Q_3 (FWHM) were both at 0.7 amu. The scan width for the selected product-ions was set at 1 amu and the scan time at 50 ms per scan. The collision-induced dissociation (CID) energy was set at 15 eV at the source and 30 eV in the collision cell for all compounds. Argon was used in the collision cell and was set at 1.2 mTorr for all experiments. Data processing was performed using the Finnigan Xcalibur Version 1.3 software.

2.4. Semi-quantification of target compounds

For each batch of urine samples, a calibrator, containing the 15 anabolic steroids tested spiked in negative horse urine, was processed in parallel. A one-point calibration curve was prepared for each analyte using the Thermo Xcalibur quantification software. The target compound concentrations in urine samples were calculated automatically by the software after each run.

2.5. Primobolan® administration

Two thoroughbred geldings (castrated horses) were each administered with a single oral dose (400 mg from 5 mg × 80 tablets) of Primobolan® (methenolone acetate) by stomach tubing. Urine samples were collected before administration

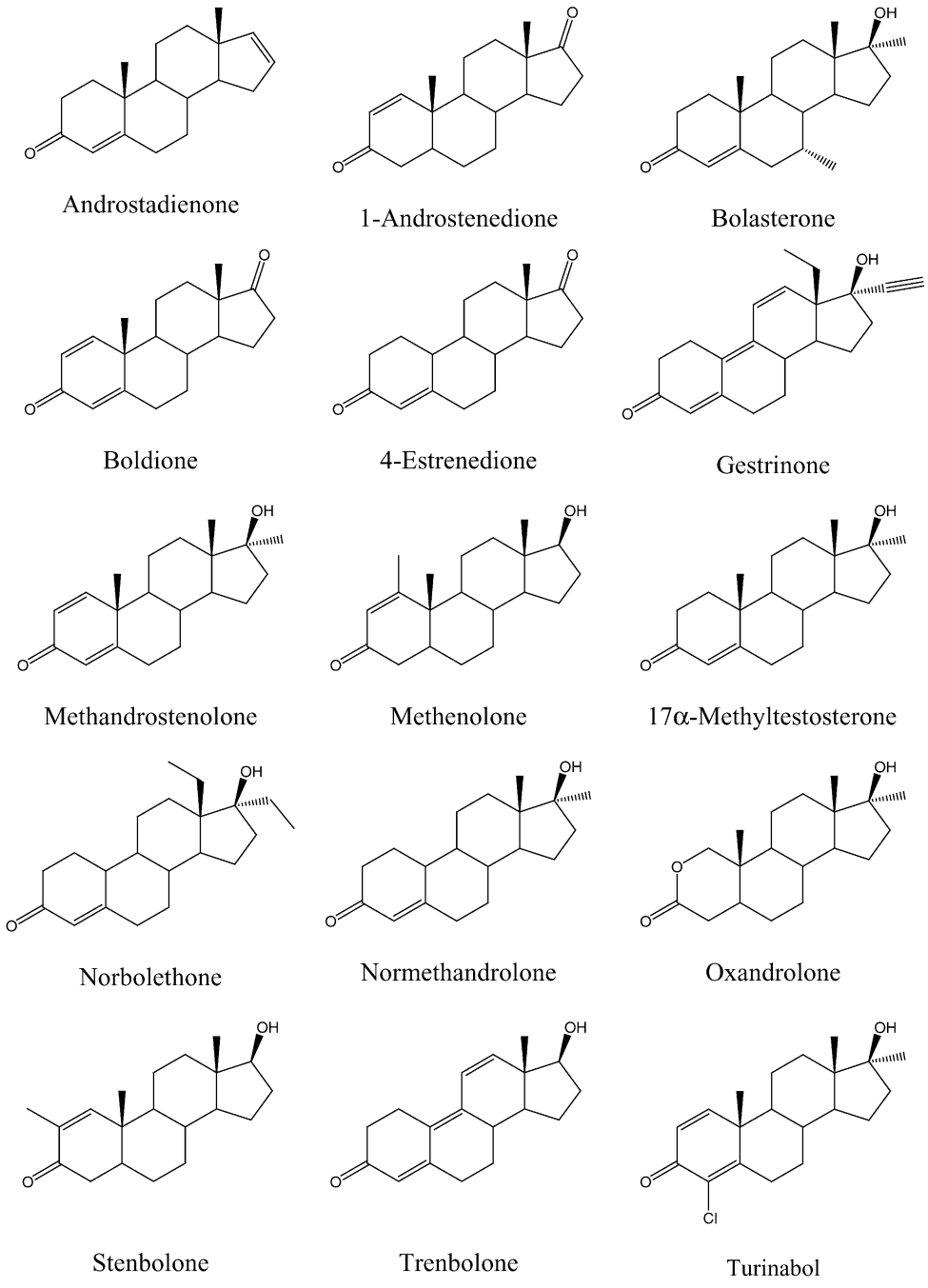

Fig. 1. Structures of the 15 targeted anabolic steroids.

and then at least two samples per day for up to 7 days post-administration.

2.6. Sample preparation and extraction procedures

Urine (3 mL) was spiked with d_3-testosterone (60 ng) as the internal standard and diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1 mL) and the pH was adjusted to 6.0 using either KOH (0.1 M) or HCl (0.1 M). A solution of protease (5 mg/mL, 60 μL) and β-glucuronidase (18,000 U/mL, 360 μL) was added and the urine sample was incubated at 65 °C for 3.5 h. The enzyme treated urine was then diluted with potassium phosphate buffer (pH 6.0, 0.1 M, 1.6 mL) before loading onto a Bond Elut Certify® cartridge, which had been pre-conditioned with methanol (2 mL), deionised water (2 mL), and potassium phosphate buffer (pH 6.0, 0.1 M, 2 mL). The cartridge was then washed with phosphate buffer (pH 6.0, 0.1 M, 2 mL) followed by acetic acid (1.0 M, 2 mL), and then eluted with dichloromethane/ethyl acetate (4:1, v/v, 3 mL). The eluate was further washed with NaOH/NaCl (1 M

Table 1
Precision data (% RSD) on peak area ratios and relative retention times for the 15 anabolic steroids obtained from the analysis of a spiked urine sample using d_3-testosterone as the internal standard

	Spike concentration (ng/mL)	Intra-day % RSD ($n = 6$)				Inter-day % RSD
		Day 1	Day 2	Day 3	Day 4	
Peak area ratio						
Androstadienone	10	14	11	12	8	11
1-Androstenedione	50	13	2	3	6	8
Bolasterone	5	7	3	4	4	5
Boldione	5	7	5	5	5	5
4-Estrenedione	50	10	7	9	4	8
Gestrinone	5	6	3	4	4	4
Methandrostenolone	5	4	2	4	3	3
Methenolone	10	5	4	4	3	4
17α-Methyltestosterone	10	6	3	3	4	4
Norbolethone	50	8	9	4	4	7
Normethandrolone	50	5	2	2	4	4
Oxandrolone	100	7	5	5	8	6
Stenbolone	20	12	6	3	4	7
Trenbolone	20	2	3	5	6	4
Turinabol	5	6	4	4	5	5
Relative retention time						
Androstadienone		0.19	0.12	0.12	0.14	0.14
1-Androstenedione		0.26	0.01	0.15	0.01	0.15
Bolasterone		0.21	0.11	0.15	0.13	0.15
Boldione		0.36	0.03	0.17	0.16	0.21
4-Estrenedione		0.35	0.14	0.21	0.05	0.22
Gestrinone		0.34	0.01	0.19	0.15	0.21
Methandrostenolone		0.21	0.16	0.16	0.13	0.16
Methenolone		0.27	0.14	0.16	0.02	0.17
17α-Methyltestosterone		0.26	0.15	0.16	0.02	0.17
Norbolethone		0.26	0.14	0.14	0.05	0.16
Normethandrolone		0.26	0.12	0.16	0.01	0.16
Oxandrolone		0.35	0.02	0.13	0.03	0.18
Stenbolone		0.26	0.15	0.14	0.05	0.17
Trenbolone		0.32	0.02	0.16	0.04	0.18
Turinabol		0.21	0.12	0.16	0.11	0.16

/0.15 M, 2 mL). The organic extract was filtered through an anhydrous sodium sulphate drying tube and evaporated to dryness under nitrogen at 60 °C. The dried residue was then reconstituted with methanol (50 μL) and transferred to a conical insert in a Chrompack autosampler vial for HPLC–MS–MS analysis.

3. Results and discussion

3.1. Method sensitivity and specificity

The structures of the 15 anabolic steroids under investigation are shown in Fig. 1. Using the method described in this study, all 15 anabolic steroids with the spiked concentrations listed in Table 1 could be easily detected from different urine matrices ($n = 15$) within a 10-min HPLC–MS–MS run. Fig. 2 shows the typical selected product-ion chromatograms of the 15 anabolic steroids and the internal standard together with their spiked concentrations and the corresponding MRM transitions in a spiked urine sample. All steroids could be clearly detected with good signal-to-noise (S/N) ratios. Fig. 3 shows the selected product-ion chromatograms of the 15 anabolic steroids spiked in a urine sample at one-twentieth of those concentrations listed in Fig. 2. At this level, good S/N (>3) could still be obtained for all steroids, indicating that the method is sensitive in detecting all target steroids at low ng/mL to high pg/mL levels.

The method specificity was assessed with different negative post-race urine samples collected on different days ($n = 45$) using the described method. No significant interferences from the matrices at the targeted ion masses and retention times were observed.

3.2. Method precision

The precision and reproducibility of the analyte-to-internal standard peak area ratios and the relative retention times for the 15 anabolic steroids were examined by extracting and analysing replicates ($n = 6$) of a spiked urine sample on four different days. The internal standard used was d_3-testosterone (20 ng/mL). The steroid spiking concentrations and the results of the precision study are summarised in Table 1. The intra-day and inter-day precisions for the peak

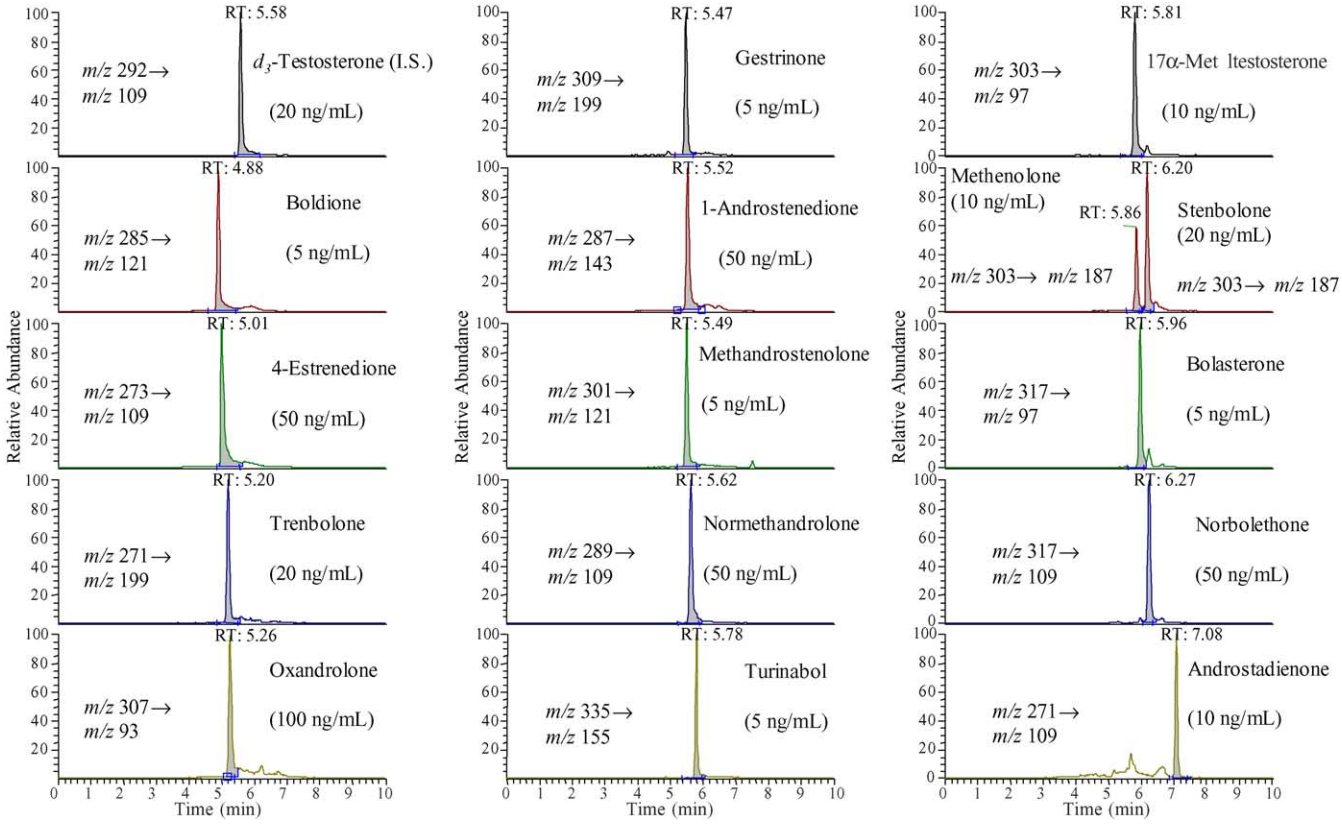

Fig. 2. Typical selected product-ion chromatograms of the targeted anabolic steroids obtained from the analysis of a spiked urine sample.

area ratios ranged from 2 to 14% and 3 to 11%, respectively, and those for the relative retention times were, respectively, 0.01–0.36% and 0.14–0.22%. These results indicated that the method has acceptable precision to be used on a routine basis.

3.3. Extraction recovery

Extraction recovery was investigated by analysing six different urine samples which had been spiked with the 15 anabolic steroids either before or after sample extraction. The corresponding concentrations in urine used for the recovery study are listed in Table 2. The internal standard (d_3-testorsterone, 20 ng/mL) was added to each sample after extraction. The area ratios of the recovered target steroids to the internal standard were compared with those obtained from the corresponding blank extracts spiked with the same amount of steroids and internal standard after extraction (taken as 100% recovery). The results are summarised in Table 2. The mean recoveries of the targeted drugs ranged from 66 to 89%, except for the relatively non-polar steroid, androstadienone, which gave a lower recovery of 37%.

3.4. Performance of the enzyme hydrolysis

The metabolism of several anabolic steroids in the horse have been well studied by several workers since the early 1980s [12–19]. It is generally well accepted that anabolic steroids and their metabolites are mainly excreted in horse urine as both sulphate and glucuronide conjugates. Attempts had been made initially to prepare urine samples using our routine methanolysis procedure to free both glucuronide and sulphate conjugates. Unfortunately, the resulting extracts showed unacceptably high background under HPLC–MS–MS conditions. Subsequent development work was therefore concentrated on the detection of the steroids that are glucuronide conjugated using enzyme hydrolysis. Ideally, steroid glucuronides, and not their free counterparts, should be used for the recovery study in order to provide more meaningful recovery data. Since glucuronides of the 15 anabolic steroids investigated in this study were not available commercially, testosterone glucuronide was therefore used as a model compound to evaluate the performance of the enzyme hydrolysis step. Six gelding (castrated horse) urine samples were spiked with testosterone glucuronide at 80.5 ng/mL (equivalent to 50 ng/mL of free testosterone) and processed with the method described in this study. The area ratios of testosterone to the internal standard were compared to those obtained from the corresponding urine blank spiked with 50 ng/mL of free testosterone (taken to be 100% hydrolysis efficiency). The results showed that the enzyme hydrolysis step had an average efficiency of 86% (% RSD = 13.9, $n = 6$). The recovery data in Table 2, being from spiked free steroids, did not make allowance for variations in the efficiency of enzyme hydrolysis for the various glucuronide con-

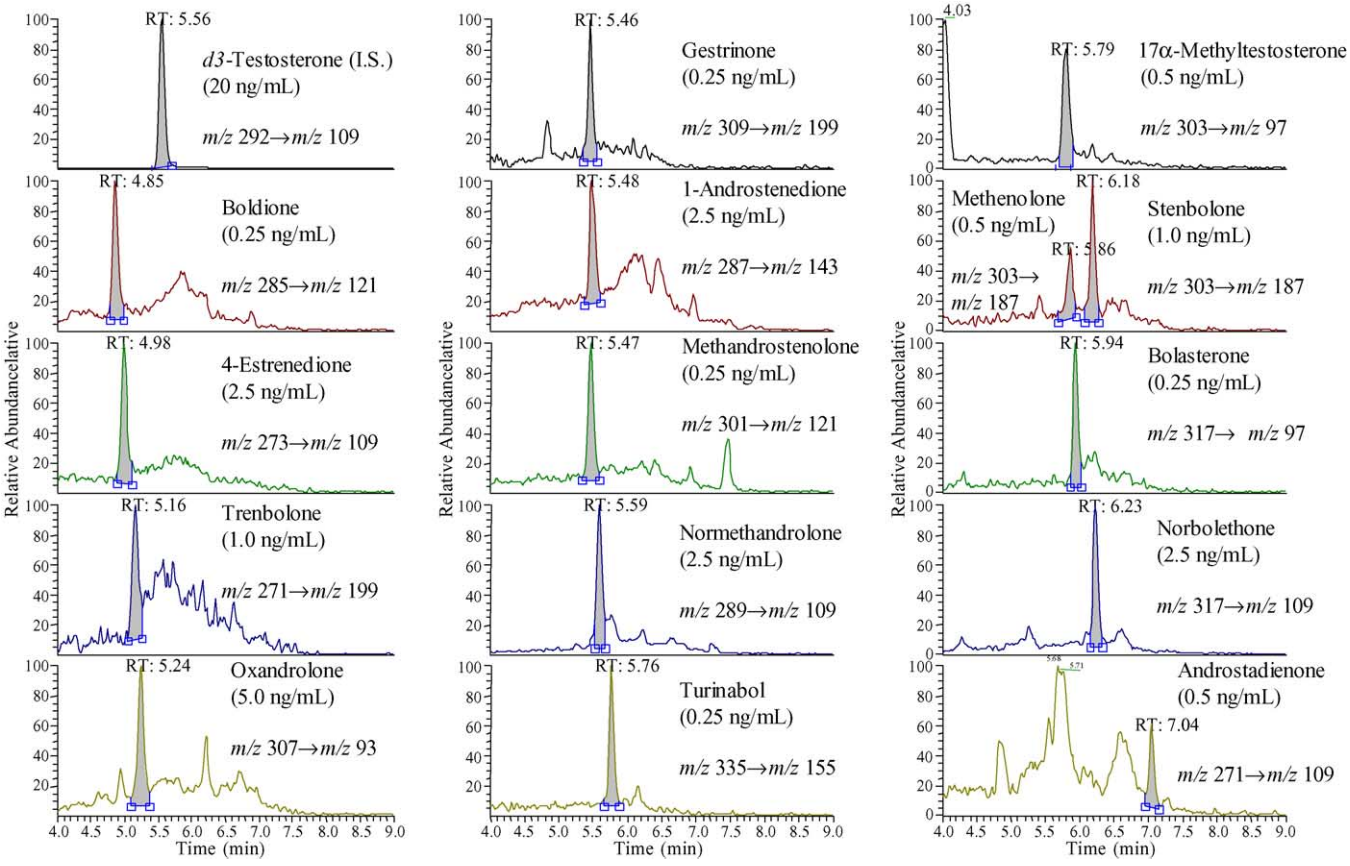

Fig. 3. Selected product-ion chromatograms of the targeted anabolic steroids obtained from the analysis of a urine sample spiked at low concentrations.

jugates. The actual extraction recovery for the steroids may therefore be lower.

3.5. Application of the method to administration samples

Methenolone acetate is an oral anabolic steroid that has both anabolic and androgenic properties. Administration studies of the drug to man have been reported [20] whereas no similar study was performed on racehorses. Fig. 4 shows the selected product-ion chromatograms obtained from the analysis of the pre-administration and the 23-h post-administration urine samples collected from one of the gelding after the administration of Primobolan®, compared to that of the methenolone standard. Detection of the product-ion (*m/z* 187) around 6 min in the pre-administration sample was minimal. The peak detected at 6.06 min of the 23-h post-administration sample was methenolone, which matched well with the retention time of the methenolone standard at

Table 2
Recovery results of the 15 anabolic steroids obtained from the analysis of different urine spikes

Drug	Spike concentration (ng/mL)	Average recovery (%) ($n = 6$)	% RSD
Androstadienone	40	37	51.9
1-Androstenedione	200	67	22.5
Bolasterone	20	74	6.4
Boldione	20	75	14.8
4-Estrenedione	200	76	19.4
Gestrinone	20	71	6.8
Methandrostenolone	20	76	4.5
Methenolone	40	73	4.4
17α-Methyltestosterone	40	74	3.2
Norbolethone	200	69	8.8
Normethandrolone	200	76	4.6
Oxandrolone	400	89	9.5
Stenbolone	80	66	10.4
Trenbolone	80	79	11.4
Turinabol	20	73	6.6

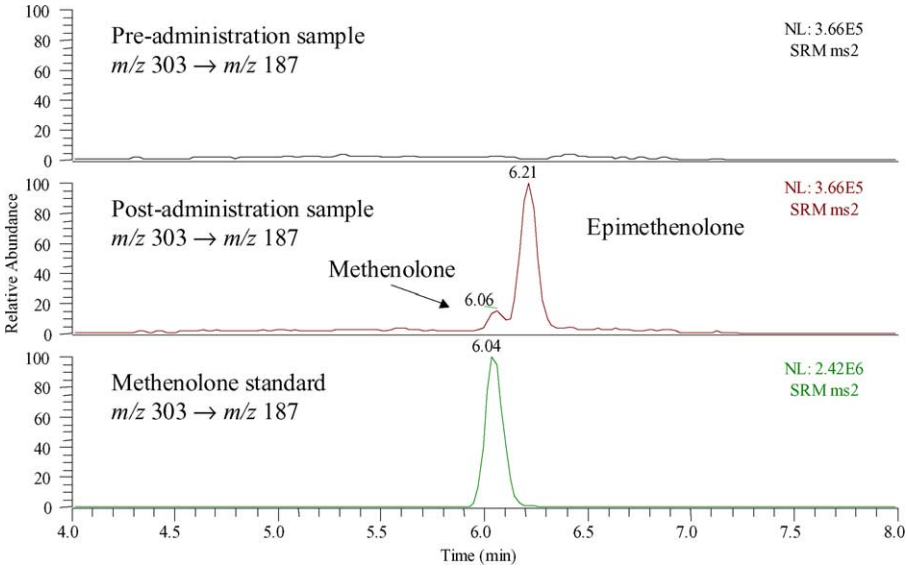

Fig. 4. The selected product-ion chromatograms obtained from the analysis of the pre-administration and the 23-h post-administration urine samples collected from one of the horse administered with 400 mg of methenolone acetate, compared to that of the methenolone standard.

6.04 min. The amount of methenolone in this sample was determined to be 18 ng/mL. Confirmation of methenolone in the administration sample was performed using full scan product-ion acquisition with *m/z* 303 as the precursor ion. The large peak at 6.21 min, which eluted immediately after the methenolone peak, was found to have product-ion spectrum similar to that of methenolone. This peak was assigned as the 17-epimer of methenolone, which had been reported as one of the major metabolites of methenolone in humans. Assuming the response factor for 17-epimethenolone was the same as methenolone, its concentration was estimated to be 130 ng/mL by comparing to the methenolone spiked calibrator. The corresponding product-ion spectra for the 23-h post-administration sample and the methenolone standard are shown in Fig. 5. Using the HPLC–MS–MS method developed in this study, the administration of methenolone acetate could be detected for 25 h in Horse A and 30 h in Horse B by monitoring the methenolone peak, or for 47 h in both Horse A and Horse B by monitoring 17-epimethenolone. More detailed results on the in vitro and in vivo metabolism studies on methenolone in horses using GC–MS are reported in another publication [21]. The results showed that epi-methenolone

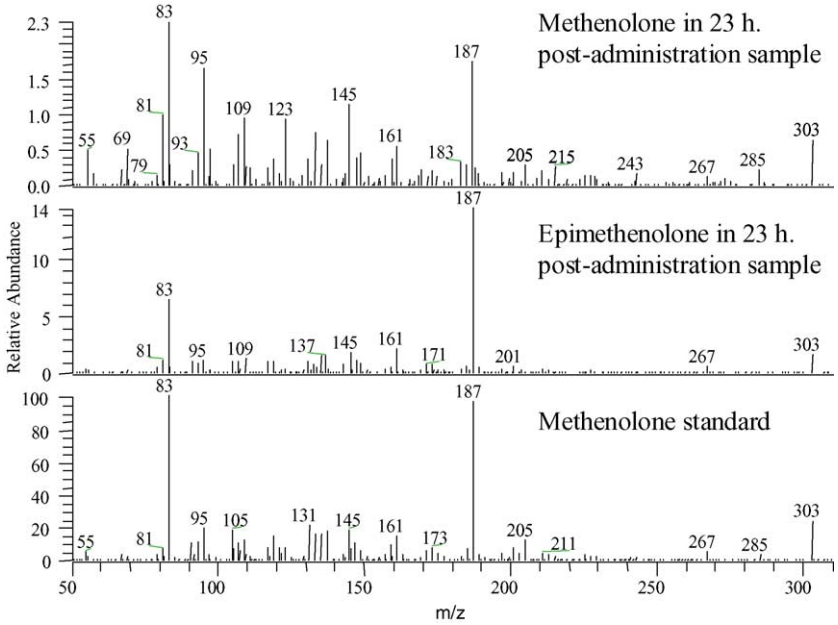

Fig. 5. The product-ion spectra obtained from the analysis of the 23-h post-administration urine sample collected from one of the horse administered with 400 mg of methenolone acetate, compared to that of the methenolone standard.

was a major glucuronic acid conjugated metabolite and is therefore the analyte of choice for detecting the administration of methenolone acetate using the HPLC–MS–MS method in this study.

4. Conclusion

An HPLC–MS–MS method for the screening of multiple anabolic steroids in racehorse urine was developed. All 15 anabolic steroids could be detected simultaneously within a single 10-min HPLC–MS–MS run at low ng/mL to high pg/mL levels. No significant interference to the targeted mass ions at the expected retention times was observed from the analysis of 45 different negative post-trace urine samples. The application of the method to drug administration samples was evaluated using two sets of methenolone acetate administration urine samples collected from two geldings. Methenolone could be detected for 25 h in Horse A and 30 h in Horse B. The 17-epimethenolone was detected up to 47 h in both horses. For samples that are flagged by this method, the presence of the anabolic steroids can be confirmed by matching the retention time and full-scan product-ion spectra with those of the corresponding authentic standards. It should be noted that this method uses β-glucuronidase to liberate the steroid conjugates, as such; its ability to detect steroid sulphate conjugates is limited. Nevertheless, it can still serve as a good complementary method to GC–MS for detecting anabolic steroids in horse urine as many anabolic steroids and their Phase-I metabolites are known to excret as glucuronide conjugates.

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