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Detection, quantification and confirmation of anabolic steroids in equine plasma by liquid chromatography and tandem mass spectrometry

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Abstract

Anabolic androgenic steroids are related to the male sex hormones and are abused in equine sports. In an effort to deter the abuse of anabolic steroids, a sensitive LC–MS/MS method was developed for detection, quantification and confirmation of eight major anabolic steroids (testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, tetrahydrogestrinone (THG), trenbolone, and stanozolol) in equine plasma. Formation of solvent adduct ions of the analytes was observed under electrospray ionization (ESI) conditions, and desolvation of the solvent adduct ions by source collision-induced decomposition (CID) increased the abundance of the $[M + H]^+$ ions as well as the multiple-reaction monitoring (MRM) signals. ESI (+) and APCI (+) were compared with respect to sensitivity for the analytes and the former provided better sensitivity. The matrix effect on ion suppression or enhancement was evaluated, and was negligible. Confirmation of the analytes was performed using criteria of three ion transitions and LC retention time of each analyte. The limit of detection (LOD) and quantification (LOQ) was 25 pg/mL. The limit of confirmation (LOC) was 25 pg/mL for boldenone; 50 pg/mL for normethandrolone, nandrolone, and methandrostenolone; and 100 pg/mL for testosterone, THG, trenbolone, and stanozolol. The analytes were evaluated for stability and found to be stable in plasma for 24 h at room temperature, 13 days at 4 °C, and 34 days at −20 and −70 °C. The method was successfully applied to analyses of equine plasma samples for pharmacokinetics study. This method is sensitive and useful for detection, quantification and confirmation of these anabolic steroids in equine plasma.

Keywords: Anabolic steroid; LC–MS; Plasma; Horse; Doping control; THG; Testosterone; Boldenone

1. Introduction

Anabolic androgenic steroids are synthetic substances related to the male sex hormones (androgens). These agents promote growth of skeletal muscle (anabolic effects) and development of male sexual characteristics (androgenic effects). They are used in treating delayed puberty, select impotence, and wasting of the body caused by some diseases [1]. However, anabolic steroids can be abused in human and animal sports [2–4], as evidenced by the latest reports on steroid abuse [5,6] and the recent discovery of "designer" steroids, tetrahydrogestrinone (THG) [7] and norbolethone [8]. The use of these agents in sports is prohibited by the International Olympic Committee via its anti-doping arm, the World Anti-Doping Agency [9]. They are also prohibited in the horseracing industry by the Association of Racing Commissioners International [10]. To enforce the prohibition on anabolic steroid abuse, effective monitoring, detection, identification and confirmation methods are required. For screening and confirmation of anabolic steroids, GC–MS is the technique commonly used for the analysis of urine samples by anti-doping or analytical toxicology laboratories [8,11–15].

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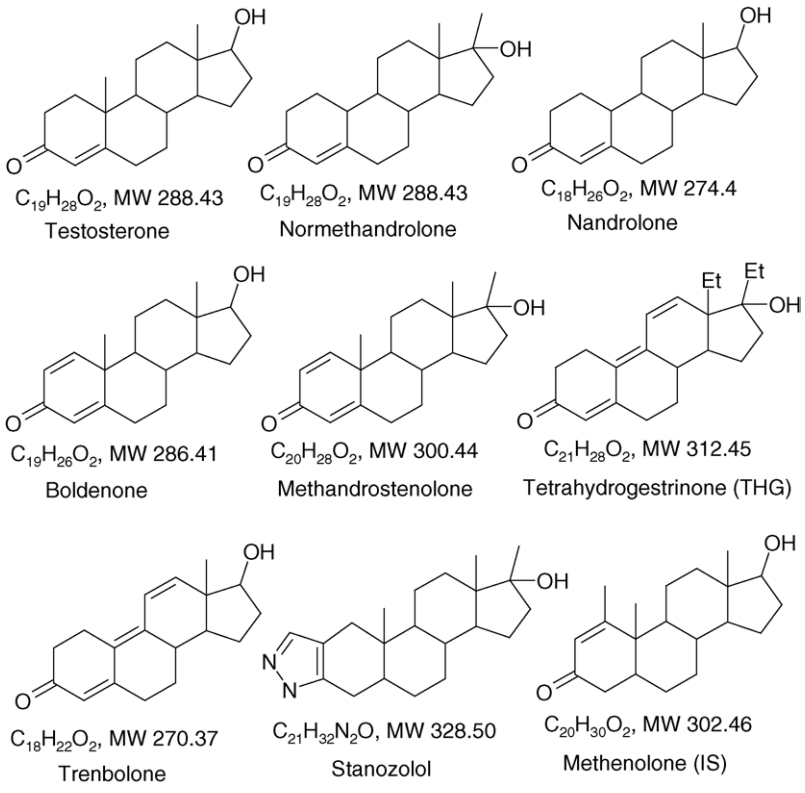

Fig. 1. Chemical structures of nine anabolic steroids used in the study.

With the advance of LC–MS technology, the emphasis has shifted in favor of this new technology. There are reports of LC–MS methods for quantitative determination of anabolic steroids such as testosterone, nandrolone, and trenbolone in human or bovine plasma or serum [16–19], in bovine or equine or human urine [20–28] and in human saliva [29–31]. To our knowledge, there is no report on LC–MS methods for qualitative and quantitative determination of anabolic steroids in equine plasma. The purpose of this paper was to develop a sensitive LC–MS/MS method for screening, quantification and confirmation of eight major anabolic steroids (testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, tetrahydrogestrinone (THG), trenbolone, and stanozolol, the chemical structures of which are shown in Fig. 1) in equine plasma. These agents were chosen for this study based on their potential for abuse in racehorses.

2. Experimental

2.1. Chemicals and reagents

All the steroids in this study, except THG, were purchased from Steraloids (Newport, Rhode Island, USA). THG was synthesized at the Maxwell H. Gluck Equine Research Center, University of Kentucky, Lexington, USA [32]. Ammonium formate (Certified), HPLC grade methanol and water and methyl *tert*-butyl ether (MTBE) were obtained from Fisher Scientific (Pittsburgh, Pennsylvania, USA). Formic acid was purchased from EM Science (Gibbstown, New Jersey, USA).

Stock solution (1.0 mg/mL) of all the steroids, and methenolone (internal standard) was individually prepared from dry chemical powder of each analyte standard by dissolving in methanol, and stored at 4 °C. An intermediate solution of mixture of the eight analytes each at 10 μg/mL was prepared by adding 50 μL of each stock solution (1.0 mg/mL) to 4600 μL of methanol in a 7 mL vial. Working solutions of the eight analytes mixed at 100, 10 and 1.0 ng/mL were prepared from the intermediate mixture solution of the analytes by dilution with methanol, and stored at 4 °C.

Stock formate buffer comprising 1.0 mol/mL of ammonium formate and 1.0 mol/mL of formic acid was prepared from the dry chemical powder and concentrated formic acid, and the pH of the buffer was 3.4. Working formate buffer solution containing 2 mmol/L of ammonium formate and formic acid, used as LC mobile phase, was prepared by diluting the stock formate buffer with HPLC grade water.

2.2. Drug administration and sample collection

Female and castrated male horses ranging in age from 4 to 10 years with an average weight of 550 ± 49 kg were used in the study. The University of Pennsylvania Institutional Animal Care and Use Committee approved the study protocol. A single dose (1.1 mg/kg) of boldenone undecylenate (Fort Dodge, Madison, NJ, USA) was intramuscularly administered to each of six horses. Blood samples were collected before drug administration and at various intervals post-drug administration. The samples were centrifuged (2500–3000 rpm or 776–1318 × *g*) at 4 °C for

15 min to obtain plasma. The plasma samples were transferred into separate tubes, frozen and stored at −70 °C pending analysis. A single dose (1.1 mg/kg) of nandrolone decanoate (Fort Dodge, Madison, NJ, USA) was intramuscularly administered to a horse, and plasma samples were collected and similarly treated. THG (15 mg) was intramuscularly administered to a horse (539 kg), and plasma samples were collected and treated as described above.

2.3. Preparation of plasma samples

Control equine plasma used in this study was collected from drug-free mares, and was determined to be free of the analytes, using the same LC–MS/MRM method described in this paper. Plasma calibrators for internal calibration quantification were prepared by spiking plasma aliquots of 1 mL each with the working solutions of the mixed eight analytes and the internal standard, containing each analyte at 25, 50, 100, 250, 500, 1000, 2500, 5000 and 10,000 pg/mL, and IS at 1000 pg/mL. Plasma calibrators were freshly prepared shortly before use. Quality control (QC) plasma samples (1.0 mL) containing IS at 1000 pg/mL and the eight analytes, each at 50, 500 and 5000 pg/mL, were similarly prepared. Test plasma samples (1.0 mL) from drug administration or racehorses were spiked with IS (1000 pg).

2.4. Extraction of the analytes from plasma samples

Analytes were extracted from plasma by liquid–liquid extraction (LLE). To each of the prepared plasma samples, MTBE (5 mL) was added and gently mixed by rotorack (Thermolyne, Dubuque, Iowa) for 5 min and centrifuged at ∼3000 rpm (∼1409 × *g*) for 5 min. The organic layer (top) was decanted into a fresh glass tube (16 mm × 100 mm) and dried at 50 °C (Techne Dri-Block DB·3, Duxford, Cambridge, UK) under a steady stream of nitrogen. The dried extract was reconstituted in 100 μL of 60% methanol in formate buffer (2 mmol/mL, pH 3.4) and a 20 μL aliquot was analyzed by LC–MS.

2.5. Intra- and inter-day precision and accuracy

For evaluation of intra-day precision and accuracy, three sets of six duplicate plasma samples containing IS (1000 pg/mL) and the eight analytes at the low (50 pg/mL), medium (500 pg/mL) and high (5000 pg/mL) concentrations were prepared, extracted, and analyzed on the same day. As for inter-day precision and accuracy, three sets of two duplicate plasma samples containing IS (1000 pg/mL) and the eight analytes at the same low, medium and high concentrations were prepared, extracted, and analyzed per day for 3 days (six samples in total for each concentration).

2.6. Evaluation of stability of the anabolic steroids in equine plasma

The eight anabolic steroids in equine plasma (1.0 mL) at three different concentrations of 50, 500 and 5000 pg/mL were prepared as described above, and stored at different temperatures (25, 4, −20 and −70 °C) for evaluation of stability of the analytes. These temperatures were chosen based on sample collection, handling, shipment and storage conditions to which the samples could be exposed before and after receipt at the laboratory. To assess stability of the analytes at ambient temperature (25 °C), the plasma samples in test tubes were allowed to remain on benchtop for 2, 4, 6 and 24 h, spiked with IS (1000 pg), extracted, and analyzed. For assessment of the stability at other temperatures, the plasma samples containing the analytes were stored at 4 °C for 13 days, and at −20 and −70 °C for 34 days, thawed, brought to ambient temperature, spiked with IS (1000 pg), extracted, and analyzed at different time intervals. The mean concentration of each analyte from triplicate samples at each temperature and time point was determined.

2.7. Instrumentation and operating parameters

Sample analyses were performed by LC–MS (Thermo Electron Corp., San Jose, CA, USA) consisting of a Surveyor LC pump with an on-line degasser, a Surveyor autosampler, and a TSQ Quantum triple stage quadrupole mass spectrometer equipped with an electrospray ionization (ESI) source operated in positive ion mode.

LC separations were performed on an Ace C_8 column (2.1 mm × 50 mm, 5 μm) (Mac-Mod Analytical, Chadds Ford, PA, USA) with an Ace C_8 guard column (2.1 mm × 12.5 mm) (Mac-Mod Analytical) that was at ambient temperature. LC mobile phase gradient in both composition and flow rate (Table 1) was used for resolution of the analytes.

TSQ Quantum mass spectrometer was calibrated with a solution of polytyrosine-1,3,6 according to the manufacturer. The ESI source parameters were optimized for nandrolone at LC flow of 300 μL/min based on preliminary results indicating that nandrolone was the analyte for which the method had the lowest sensitivity. The optimized source parameters were as follows: sheath gas pressure, 45 psi; auxiliary gas flow, 23 (arbitrary units); spray voltage, 4500 V; capillary temperature, 300 °C; tube lens offset, 178 V; and source collision-induced decomposition (CID), 20 V. For quantification, the mass spectrometer was set to the data acquisition mode of multiple-reaction monitoring (MRM), and the specified acquisition parameters are shown in Table 2. The acquisition parameters common to all analytes were: scan width (*m*/*z*) 0.70, scan time 0.4 s, peak width (FWHM) 0.7 for both Q1 and Q3, and collision gas pressure 1.5 mTorr. Data acquisition

Table 1
LC mobile phase gradient in composition and flow rate

LC run time (min)	Formate buffer[a] (%)	Methanol (%)	Flow rate (μL/min)
0	40	60	300
4.0	40	60	300
8.0	10	90	300
8.1	40	60	300
8.2	40	60	400
10.4	40	60	400
10.5	40	60	300

[a] Ammonium formate, 2 mM, pH 3.4.

Table 2
Parameters for MRM acquisition of the analytes

	Trenbolone	Boldenone	Nandrolone	Methandrostenolone	Testosterone	Normethandrolone	IS	THG	Stanozolol
Retention time (min)	2.98	2.99	3.39	3.92	4.46	4.57	6.00	6.93	7.89
Ion transition (*m/z*) for quantification (collision energy, V)	271 → 199 (20)	287 → 121 (15)	275 → 109 (17)	301 → 121 (15)	289 → 109 (16)	289 → 213 (18)	303 → 187 (15)	313 → 241 (15)	329 → 329 (35)
Ion transitions (*m/z*) for confirmation (collision energy, V)	271 → 227, 199, 183 (20)	287 → 173, 135, 121 (15)	275 → 239, 145, 109 (17)	301 → 173, 149, 121 (15)	289 → 253, 109, 97 (16)	289 → 253, 231, 213 (18)		313 → 266, 241, 239 (15)	329 → 329, 121, 107 (40)

and analysis were accomplished with Xcalibur software v.1.3 (Thermo Electron Corp., San Jose, CA, USA).

3. Results and discussion

3.1. ESI (+) MS spectra and solvent adduct ions

In the early phase of the method development, seven anabolic steroids were studied with flow injection into the LC with a flow of 300 μL/min of methanol/$HCOONH_4$ (2 mmol/L, pH 3.4) (60/40, v/v). Testosterone (Fig. 2a), normethandrolone (Fig. 2b), and nandrolone (Fig. 2c) formed $[M+H]^+$ ions at *m/z* 289, 289 and 275, respectively, as well as solvent adduct ions $[M+H+CH_3OH]^+$ with methanol at *m/z* 321, 321 and 307. In contrast, boldenone (Fig. 2d), methandrostenolone (Fig. 2e) and stanozolol (Fig. 2g) formed abundant $[M+H]^+$ ions at *m/z* 287, 301 and 329, respectively, but weak solvent adduct ions $[M+H+CH_3OH]^+$ at *m/z* 319, 333 and 361. Trenbolone (Fig. 2f) formed dominant $[M+H]^+$ ion at *m/z* 271 without noticeable solvent adduct ion $[M+H+CH_3OH]^+$ at *m/z* 303. The tendency of the analytes to form $[M+H]^+$ ion and solvent adduct ions is related to their chemical structures (Fig. 1). For example, trenbolone has four conjugated double bonds and boldenone and methandrostenolone have three such bonds, and these double bonds stabilize the $[M+H]^+$ ions formed under the ESI condition. Stanozolol has a pyrazole ring (a weak base) that stabilizes the $[M+H]^+$ ion. However, testosterone, normethandrolone and nandrolone have only two conjugated double bonds that may not sufficiently stabilize the $[M+H]^+$ ions, and thus, solvent adduct ions $[M+H+CH_3OH]^+$ were formed because solvent molecules can stabilize positive ions (a well known example is that metal ions in aqueous solution exist in a hydrated form). In short, the minor difference in chemical structures of the seven anabolic steroids mentioned above, specifically the number of conjugated double bonds, accounts for the difference in formation of the $[M+H]^+$ ions and solvent adduct ions $[M+H+CH_3OH]^+$ described above.

In addition to chemical properties of the anabolic steroids, the organic solvent used in LC mobile phase also had an effect on formation of solvent adduct ions and, thus, affected intensity of the $[M+H]^+$ ions. Compared with methanol, acetonitrile showed a stronger tendency to form solvent adduct ions with the anabolic steroids, as revealed by the MS spectra of the analytes acquired with acetonitrile in the LC flow (Fig. 3). Unlike methanol, acetonitrile formed dominant solvent adduct ions $[M+H+CH_3CN]^+$ with testosterone (Fig. 3a), normethandrolone (Fig. 3b), and nandrolone (Fig. 3c) at *m/z* 330, 330 and 316, respectively, but no $[M+H]^+$ ions at *m/z* 289, 289 and 275. Acetonitrile also formed abundant solvent adduct $[M+H+CH_3CN]^+$ ions with boldenone (Fig. 3d), methandrostenolone (Fig. 3e), trenbolone (Fig. 3f), and stanozolol (Fig. 3g) at *m/z* 328, 342, 312 and 370, respectively. The tendency of acetonitrile and methanol to form solvent adduct ions is related to their proton affinity (PA = 8.2 eV for acetonitrile, and 7.9 eV for methanol [33]): acetonitrile has a stronger proton affinity than methanol, and thus has higher tendency to form solvent adduct ions than methanol. This rela-

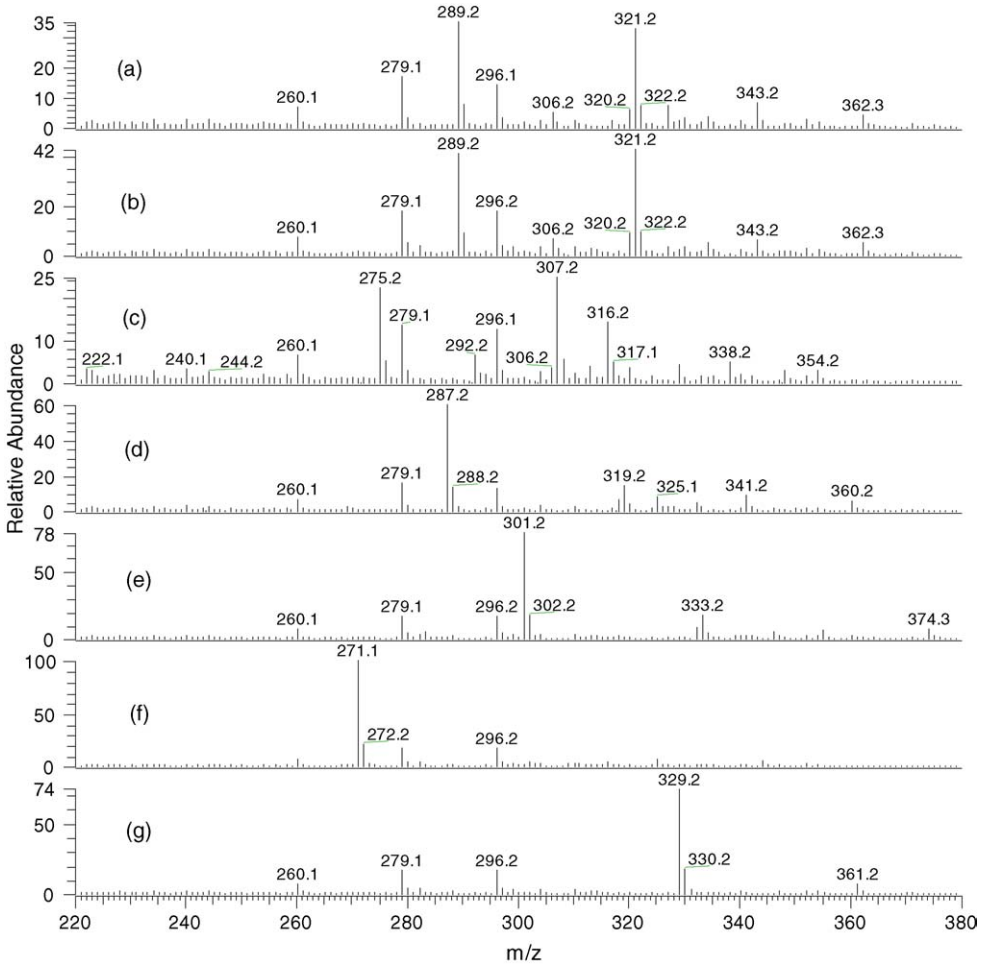

Fig. 2. ESI (+) MS spectra of testosterone (a), normethandrolone (b), nandrolone (c), boldenone (d), methandrostenolone (e), trenbolone (f), and stanozolol (g) showing formation of $[M + H]^+$ ions as well as solvent adduct $[M + H + CH_3OH]^+$ ions with methanol. Spectra a–g are from top to bottom. $[M + H]^+$ and $[M + H + CH_3OH]^+$ ions are *m/z* 289 and 321 for testosterone (a) and normethandrolone (b), *m/z* 275 and 307 for nandrolone (c), *m/z* 287 and 319 for boldenone (d), *m/z* 301 and 333 for methandrostenolone (e), *m/z* 271 for trenbolone (f), and *m/z* 329 and 361 for stanozolol (g), respectively. Each anabolic steroid (10 μg/mL) was introduced by syringe infusion at 20 μL/min into LC mobile phase (CH_3OH/$HCOONH_4$, 60/40, v/v) flow (300 μL/min) to the mass spectrometer. The temperature of the ion transfer capillary was 275 °C.

tionship is in agreement with Field's rule [34]. Understanding of the relationship between proton affinity of an organic solvent and its tendency to form solvent adduct ions is helpful in selecting LC solvent for LC–MS studies of analytes that may not form stable $[M + H]^+$ ions.

Abundant $[M + H]^+$ ions are always desired for sensitive, qualitative and quantitative methods. Conversely, solvent adduct ions are undesirable since they decrease abundance of $[M + H]^+$ ions. To minimize formation of solvent adduct ions in this study, methanol was chosen over acetonitrile as solvent for the LC mobile phase. To desolvate the undesired solvent adduct ions $[M + H + CH_3OH]^+$, temperature of the ion transfer capillary, the desolvation device of the mass spectrometer, was increased from 275 to 350 °C. The increase in temperature reduced the intensity but did not completely abolish formation of the solvent adduct ions. This result indicated that the increase in temperature alone was not sufficient for desolvation of the solvent adduct ions. However, careful selection of the source CID voltage was successful in desolvation of the solvent adduct ions but did not fragment the $[M + H]^+$ ions. For this reason, the source CID was used for desolvation to improve sensitivity for the analytes.

3.2. ESI (+) MS/MS spectra

Product ion spectra of the anabolic steroids are shown in Fig. 4. Testosterone and normethandrolone are different compounds with the same molecular formula and $[M + H]^+$ ion (*m/z* 289). However, their product ion spectra were different from each other (Fig. 4a and b). Testosterone had an abundant product ion at *m/z* 97 that was very weak in the product ion spectrum of normethandrolone; normethandrolone had two abundant product ions at *m/z* 231 and *m/z* 213 that were very weak in the product ion spectrum of testosterone. Testosterone, nandrolone, and boldenone are similar in chemical structure, but were different in product ion spectra (Fig. 4a, c and d). Normethandrolone and nandrolone, a homologous pair, resulted in spectra with different product ion profile (Fig. 4b and c). However, boldenone and methandrostenolone, another homologous pair, showed spectra with similar product ion profile (Fig. 4d and e). Though similar

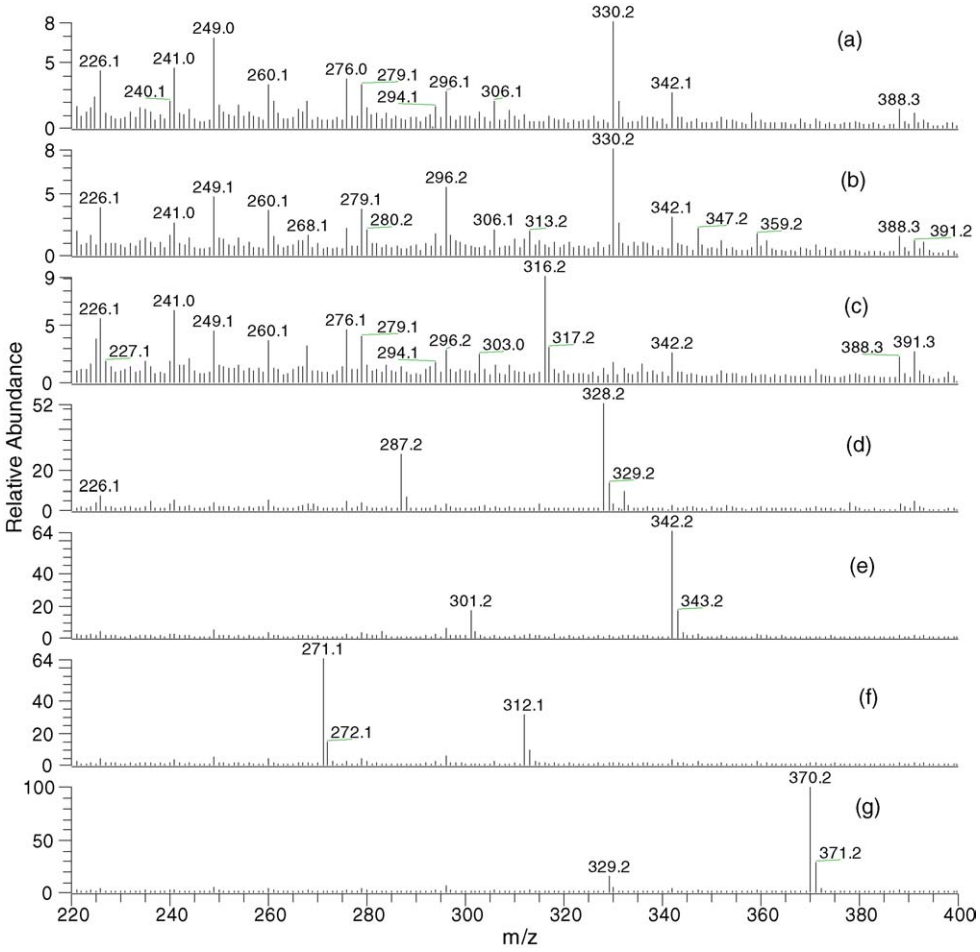

Fig. 3. ESI (+) MS spectra of testosterone (a), normethandrolone (b), nandrolone (c), boldenone (d), methandrostenolone (e), trenbolone (f), and stanozolol (g) showing formation of solvent adduct $[M + H + CH_3CN]^+$ ions with acetonitrile at *m/z* 330, 330, 316, 328, 342, 312 and 370, respectively. Spectra a–g are from top to bottom. Each anabolic steroid (10 μg/mL) was infused by syringe at 20 μL/min into LC mobile phase (CH_3CN/$HCOONH_4$, 60/40, v/v) flow (200 μL/min) to the mass spectrometer. The temperature of the ion transfer capillary was 275 °C.

in structure, THG and trenbolone produced different product ion profiles (Fig. 4f and g). In short, the differences in product ion spectra of structurally similar anabolic steroids or in *m/z* value of $[M + H]^+$ ions of the homologous pairs provide solid basis for differential detection and confirmation of the anabolic steroids. Based on the MS/MS spectra, specific product ions or ion transitions were chosen for detection, quantification and confirmation of the anabolic steroids by MRM. The selected ion transitions for MRM of the anabolic steroids are summarized in Table 2.

3.3. ESI versus APCI

The two commonly used atmospheric-pressure ionization (API) modes, electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), were evaluated for sensitivity in determining the analytes. With ESI, the lowest quantity of the injected analyte standards detectable by MRM was 5 pg each of normethandrolone, nandrolone, boldenone, methandrostenolone, trenbolone and stanozolol; whereas with APCI, that quantity was 20 pg each, which was four times as high as that by ESI. This result showed that ESI provided higher sensitivity for the analyte standards than APCI. The result is rather unexpected since APCI is considered suitable for neutral and small molecules without any amino or carboxyl group and is commonly used for determination of anabolic steroids [19,25,35]. However, our results agree with those of other investigators [22,36] showing that ESI is equally suitable for determination of anabolic steroids and thus, it was chosen for use in this study.

3.4. Extraction

The anabolic steroids were extracted from equine plasma by liquid–liquid extraction. These agents are neutral compounds and were extracted from equine plasma by MTBE without any adjustment in pH. MTBE was used, based on its usefulness in the extraction of beclomethasone, a synthetic corticosteroid, in a previous study by our laboratory [37]. The extraction efficiency of MTBE for the anabolic steroids was above 74% (Table 3).

3.5. Matrix effect—ion suppression or enhancement

Matrix effect is a special phenomenon associated with LC–MS/MS determination of drugs from biological fluids such as plasma and urine [38–40]. Endogenous components extracted

Table 3
Extraction efficiency, accuracy and reproducibility (coefficient of variation, CV) for quantification of testosterone, normethandrolone, nandrolone, boldenone, methandrostenolone, THG, trenbolone and stanozolol in equine plasma

Added (pg/mL)	Testosterone	Normethandrolone	Nandrolone	Boldenone	Methandrostenolone	THG	Trenbolone	Stanozolol
Extraction efficiency (%)[a]								
50	95	105	96	92	92	81	94	82
500	89	86	88	89	86	85	83	74
5000	83	84	84	84	84	80	86	77
Intra-day accuracy (%)[b]								
50	112	114	106	106	108	114	104	100
500	105	106	105	104	104	105	101	114
5000	93	97	94	93	93	93	95	111
Inter-day accuracy (%)[c]								
50	96	84	108	90	56	100	92	90
500	101	106	105	102	103	102	104	103
5000	98	100	99	98	98	100	97	99
Intra-day CV (%)[b]								
50	21	24	19	14	20	19	22	5.8
500	3.3	5.4	5.1	5.0	4.4	7.8	6.3	8.6
5000	4.7	4.3	4.3	4.6	4.4	5.4	4.5	4.4
Inter-day CV (%)[c]								
50	20	18	38	11	10	25	10	8.0
500	8.0	5.0	9.0	7.0	7.0	13	8.0	12
5000	4.2	5.6	3.8	4.8	4.7	5.8	4.6	8.4

[a] Extraction efficiency = peak area of an analyte extracted from plasma/peak area of the analyte standard added to the extract of blank plasma. The value was the result averaged from six duplicate samples analyzed on the same day.
[b] Intra-day accuracy and precision results were obtained from six duplicate samples ($n = 6$) for each concentration of the analyte analyzed on a single day.
[c] Inter-day accuracy and precision results were obtained by analyzing six duplicate samples for each concentration of the analyte on three separate days (two samples each day).

along with analytes from plasma or urine may suppress or enhance ionization of the analytes in electrospray source if they co-elute with the analytes from LC column. Matrix effect may impair accuracy and reproducibility. For this reason, matrix effect was evaluated under the experimental conditions used in this study, and the results are shown in Table 4. Negative values represent ion enhancement, and positive values represent ion suppression. Ion enhancement or suppression was less than 13%, which would be within acceptable random experimental errors, except in the case of THG at low concentration (50 pg/mL). In total, this result indicates that matrix effect caused by plasma was negligible.

3.6. LC–MS/MRM for detection, quantification and confirmation

The LC–MS/MRM chromatograms of blank plasma extract and the eight analytes spiked into equine plasma and extracted are presented in Fig. 5. All the analytes were well resolved either chromatographically (Fig. 5) or mass spectrometrically (Fig. 4). In the chromatograms of blank plasma extract, though there was a peak (at $t_R = 7.34$ min) in the ion transition channel for THG, it was well separated from that of THG and did not interfere with determination of THG. Quantification of the analytes was not interfered with by endogenous components in the plasma.

Confirmation of the anabolic steroids in equine plasma was performed using criteria of three ion transitions and LC retention time of each anabolic steroid (Table 2). Specifically, the relative intensities of three major product ions of a given analyte were unique (Table 5) and they did not appreciably change over the concentration range tested and so were used for confirmation of the presence of the analytes in plasma. Variation of the relative ion intensities within 20% is usually acceptable for confirmation purposes [28].

Internal standard calibration quantifications of the analytes were performed, and a plot of the ratio of the chromatographic

Table 4
Ion suppression or enhancement (%) showing matrix effect on ESI LC–MS/MS of the anabolic steroids[a]

Added (pg/mL)	Testosterone	Normethandrolone	Nandrolone	Boldenone	Methandrostenolone	THG	Trenbolone	Stanozolol
50	−4.0	8.8	12	0.3	−5.9	−27	−7.4	11
500	−5.8	−9.3	−9.2	−4.9	−8.3	−5.3	−12.3	4.9
5000	−6.1	−7.0	−4.6	−6.0	−6.9	0.6	−7.7	1.8

[a] Ion suppression (%) = $[1 - (A_{extr}/A_{st})] \times 100$, where A_{st} is the chromatographic peak area of a certain quantity of a drug standard and A_{extr} is the peak area of the same quantity of the drug standard added to the extract of 1.0 mL of equine plasma. Each value is the averaged result of three duplicate samples. The negative sign indicates ion enhancement, whereas the positive sign indicates ion suppression.

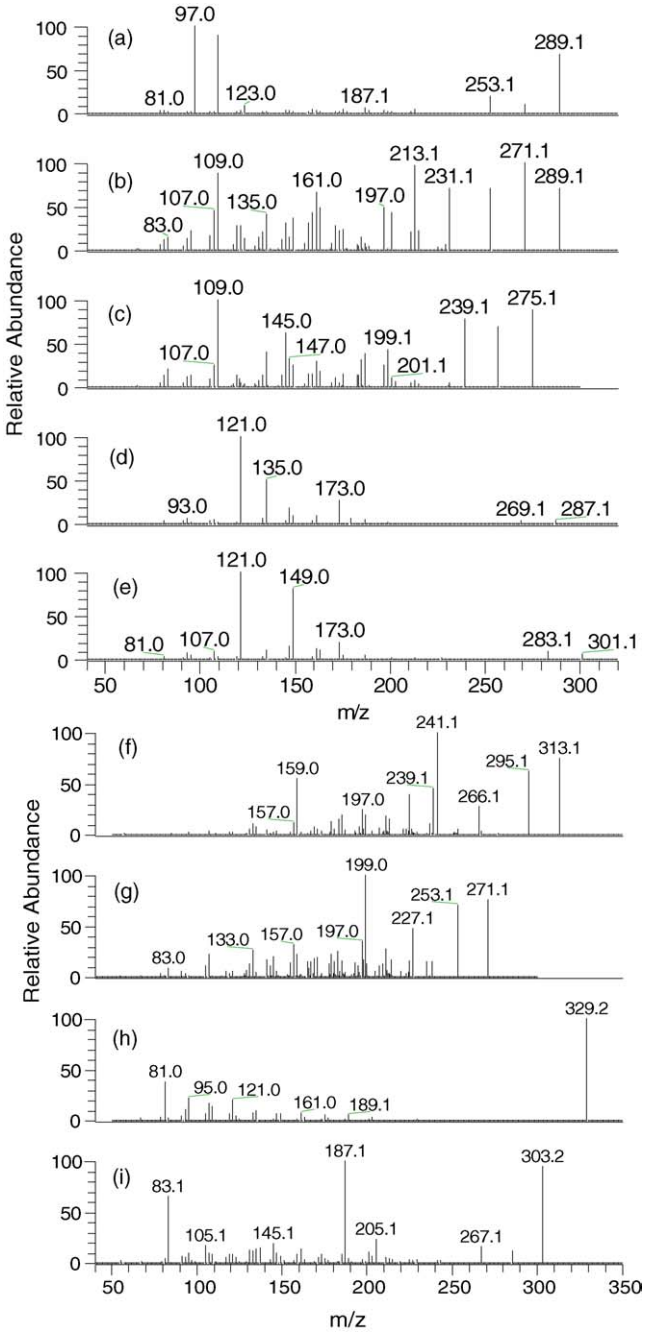

Fig. 4. MS/MS spectra of testosterone (a), normethandrolone (b), nandrolone (c), boldenone (d), methandrolone (e), THG (f), trenbolone (g), stanozolol (h), and methenolone (i). Each of the analytes at concentration of 1 μg/mL in $CH_3OH/HCOONH_4$ (2 mmol/L, pH 3.4) (50/50, v/v) was introduced into the ESI source by syringe infusion at 5 μL/min. Collision energy was 16 V for testosterone; 18 V for normethandrolone; 17 V for nandrolone; 15 V for boldenone, methandrostenolone, THG, and methenolone; and 40 V for stanozolol. Source CID voltage was 20 V for all the analytes.

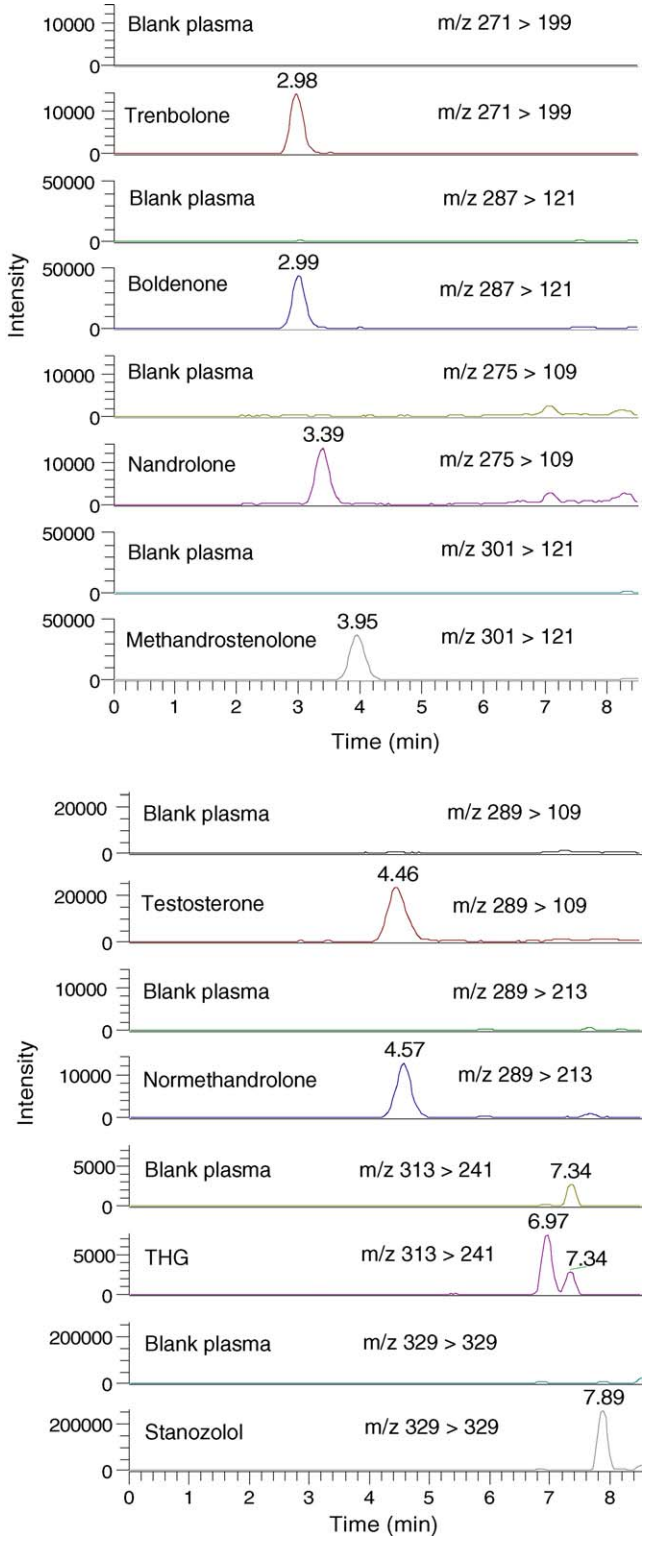

Fig. 5. LC–MS/MRM chromatograms of blank equine plasma extract and eight anabolic steroid analytes (250 pg each) spiked into equine plasma (1.0 mL) and extracted. The peak (t_R = 7.34 min) in the ion transition channel for THG was from an unknown endogenous plasma component but did not interfere with determination of the analyte (t_R = 6.97 min).

peak area of each analyte to that of IS against the analyte concentration was linear in the range of 25–10,000 pg/mL for each of the anabolic steroids spiked into equine plasma. In this paper, we define the limit of detection (LOD) as the lowest concentration of an anabolic steroid spiked into plasma that resulted in the selected reaction monitoring (SRM) signal five times greater

Table 5
Product ion intensity ratios (%) used for confirmation of the anabolic steroids[a]

	Concentration (pg/mL)									Mean	S.D.
	25	50	100	250	500	1000	2500	50000	100000		
Testosterone											
m/z 109/97			100	86	76	89	85	82	87	87	8
m/z 253/97			19	24	20	21	23	22	23	22	2
Normethandrolone											
m/z 231/213		77		76	70	67	77	77	77	74	4
m/z 253/213		61		71	68	71	82	75	77	72	7
Nandrolone											
m/z 145/109		48	59	59	56	53	51	53	55	54	4
m/z 239/109		45	43	52	57	53	54	53	54	51	5
Boldenone											
m/z 173/121	32	31	25	28	28	29	28	28	28	29	2
m/z 135/121	56	60	51	54	53	55	54	54	53	54	3
Methandrostenolone											
m/z 149/121		92	86	89	93	90	86	90	89	89	2
m/z 173/121		22	18	19	20	19	20	20	20	20	1
THG											
m/z 239/241			51	60	49	52	49	54	51	52	4
m/z 266/241			35	32	34	37	35	33	34	33	4
Trenbolone											
m/z 183/199			36	22	33	28	28	27	27	29	5
m/z 227/199			49	43	55	48	47	46	46	47	4
Stanozolol											
m/z 107/329			21	21	22	22	22	22	23	22	3
m/z 121/329			27	26	26	27	27	27	28	27	3

[a] The anabolic steroids were spiked into equine plasma and extracted prior to analysis.

than noise, the limit of quantification (LOQ) as the lowest quantifiable concentration of an anabolic steroid spiked into plasma, and the limit of confirmation (LOC) as the lowest concentration of an anabolic steroid spiked into plasma that was confirmed by the criteria of LC retention time (Table 2) and product ion intensity ratio (Table 5). LOD and LOQ were 25 pg/mL for all the analytes. LOC was 25 pg/mL for boldenone; 50 pg/mL for normethandrolone, nandrolone, and methandrostenolone; and 100 pg/mL for testosterone, THG, trenbolone, and stanozolol. The accuracy of this method was within 84–114% for all the analytes, and the precision expressed as coefficient of variation (CV) was within 13% (Table 3) except in the case of low concentration (50 pg/mL) where it was higher because variations were higher at the concentration close to the limit of quantification. It should be noted that the LOQ (25 pg/mL) for the analytes spiked into plasma was in agreement with that for the authentic analyte standards (5 pg injected onto column), given the fact that one fifth of the analytes spiked into and extracted from plasma (20 μL out of 100 μL of solution of the extracted analytes) was used for analysis by LC–MS.

Radio immunoassay (RIA) is a very sensitive technique and is widely used for quantitative determination of testosterone in plasma. The LC–MS/MRM method developed in this study is more sensitive for testosterone than RIA when the LOD (4 ng/dL = 40 pg/mL) and LOQ (20 ng/dL = 200 pg/mL) by RIA [41] are compared with the LOD (25 pg/mL) and LOQ (25 pg/mL) by this method. This method is also more sensitive than the LC–MS methods for determination of some of the anabolic steroids in human or bovine plasma reported by other investigators [16–19].

3.7. Stability of the anabolic steroids in equine plasma under storage conditions

The stability results showed that the anabolic steroids spiked into equine plasma were stable for 24 h at ambient temperature, for 13 days at 4 °C, and for 34 days at −20 and −70 °C (Table 6). The number of days chosen for stability study was based on the maximum number of days a sample was likely to be stored prior to analysis by the laboratory. Stability of the anabolic steroid extracts in the sample solvent on autosampler during overnight analysis of samples was also observed over a 24 h period, and the analytes were stable for the period of observation.

3.8. Preliminary applications for specificity of the method

The method described above was successfully applied to analyses of: (1) plasma samples collected from horses post administrations of boldenone, nandrolone, and THG for pharmacokinetic studies; (2) official racehorse samples for THG screening; and (3) stallion plasma samples for quantification of endogenous testosterone in research horses.

Table 6
Stability of the analytes under different storage conditions[a]

Added (pg/mL)	Time	Testosterone (pg/mL)	Normethandrolone (pg/mL)	Nandrolone (pg/mL)	Boldenone (pg/mL)	Methandrostenolone (pg/mL)	THG (pg/mL)	Trenbolone (pg/mL)	Stanozol (pg/mL)
Ambient temperature									
50	2 h	57	47	61	54	56	51	49	21
50	4 h	50	54	55	54	55	47	57	21
50	6 h	52	61	56	53	55	49	49	32
50	24 h	42	49	44	44	48	37	47	39
500	2 h	530	543	541	538	530	510	504	230
500	4 h	562	577	555	569	566	536	518	303
500	6 h	579	597	588	573	590	530	510	347
500	24 h	532	530	509	535	543	527	505	450
5000	2 h	5210	5165	5143	5176	5112	5026	4670	2284
5000	4 h	5199	5245	5166	5214	5173	4908	4705	2866
5000	6 h	5565	5669	5521	5598	5561	5182	4900	3446
5000	24 h	4955	5018	4637	4952	4951	4768	4538	4029
4 °C									
50	3 days	48	55	52	50	52	45	51	31
50	4 days	61	53	60	38	41	38	46	48
50	12 days	43	48	53	44	51	51	50	46
50	13 days	48	37	51	45	48	42	38	48
500	3 days	552	499	547	547	532	514	507	511
500	4 days	519	477	488	488	484	471	465	516
500	12 days	456	421	450	452	449	461	424	469
500	13 days	463	446	453	463	471	448	457	416
5000	3 days	5148	5010	5251	5251	5045	5092	4972	4822
5000	4 days	4939	4920	5110	5110	4968	4999	4838	5236
5000	12 days	4546	4585	4448	4603	4617	4438	4488	4696
5000	13 days	4734	4663	4738	4735	4735	4094	4523	3658
−20 °C									
50	13 days	44	46	46	44	44	35	45	55
50	20 days	46	52	50	45	42	46	46	50
50	27 days	47	45	50	52	48	54	46	43
50	34 days	52	47	43	47	49	50	47	50
500	13 days	465	482	475	469	465	451	445	461
500	20 days	475	491	492	477	483	458	464	527
500	27 days	457	462	461	467	466	435	456	453
500	34 days	438	450	425	438	435	418	397	456
5000	13 days	4821	4788	4781	4771	4751	4598	4599	4503
5000	20 days	4687	4723	4638	4674	4656	4581	4469	4729
5000	27 days	4387	4370	4377	4345	4370	4227	4041	4232
5000	34 days	4084	4103	4099	4071	4116	4214	3950	4243
−70 °C									
50	13 days	43	46	37	41	42	43	39	47
50	20 days	45	53	69	43	41	45	37	47
50	27 days	49	50	48	47	42	47	46	40
50	34 days	52	50	55	46	51	52	48	47
500	13 days	465	474	453	458	468	467	455	466
500	20 days	486	503	494	468	471	479	466	497
500	27 days	451	479	460	439	436	464	435	428
500	34 days	443	453	452	420	436	444	453	421
5000	13 days	4611	4716	4620	4488	4567	4585	4587	4697
5000	20 days	4478	4514	4379	4192	4387	4310	4135	4650
5000	27 days	4573	4763	4570	4422	4471	4467	4486	4418
5000	34 days	4124	4298	4158	4032	4093	4216	4387	4144

[a] Each value represents the result averaged from triplicate samples ($n = 3$).

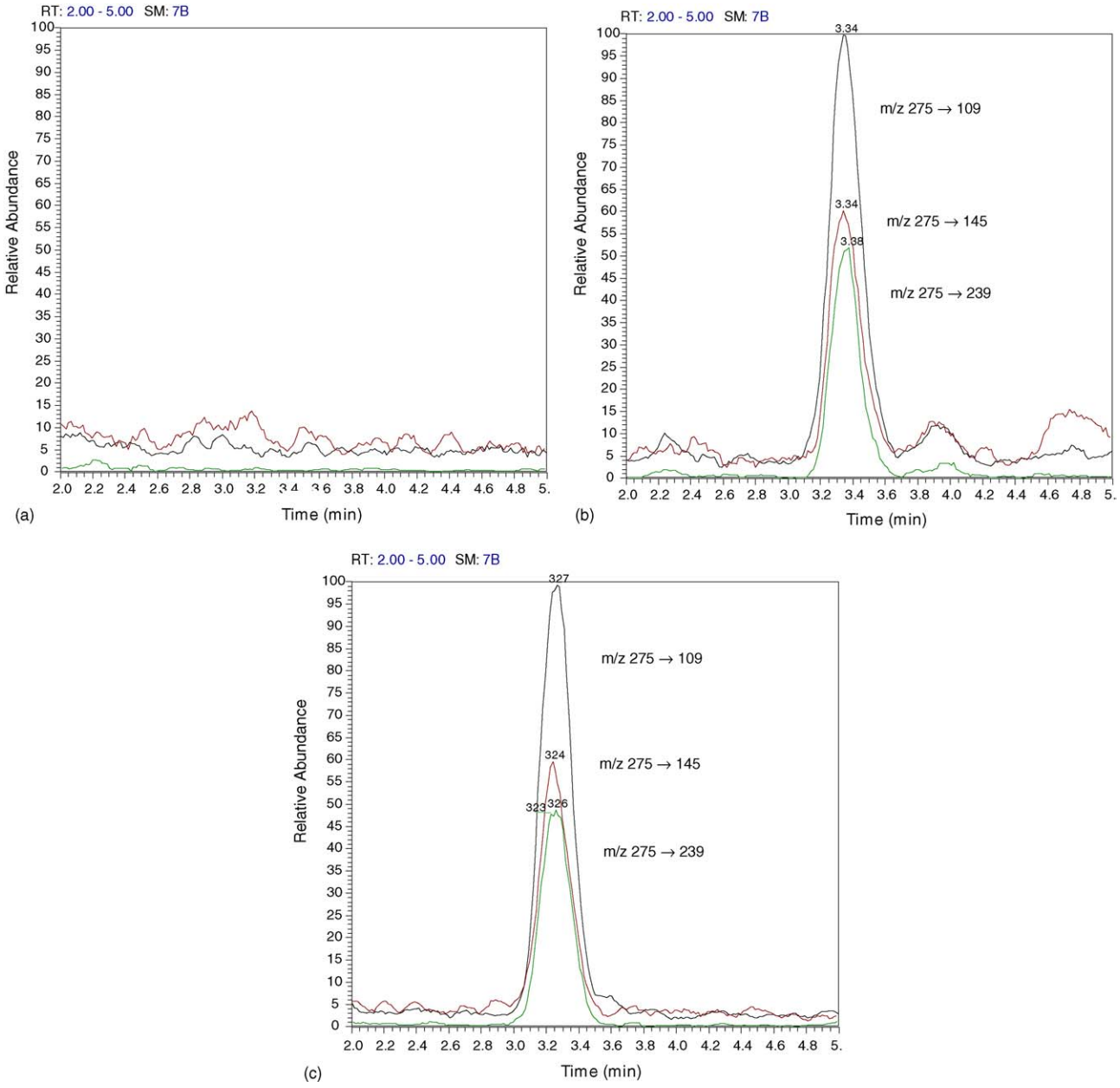

Fig. 6. LC–MS/MRM chromatograms of blank plasma extract (a), stallion plasma extract (b), and nandrolone (250 pg) spiked into blank plasma (1.0 mL) and extracted (c). Comparison of (a)–(c) above confirms endogenous nandrolone (136 pg/mL) in a plasma sample from stallion (b). The three MRM traces for confirmation were *m/z* 275 → 109, 145 and 239.

Boldenone was detected and quantified in plasma samples up to 29 days post intramuscular administration of boldenone undecylenate (1.1 mg/kg, IM), and nandrolone up to 62 days post administration of nandrolone decanoate (1.1 mg/kg, IM). The pharmacokinetic results will be published elsewhere. THG was detected and quantified in plasma samples up to 48 h following its administration (15 mg, IM) to a 539 kg horse (Table 7). In addition, a large number of official plasma samples collected from racehorses were routinely screened for THG for a period of greater than 2 months. This exercise was based on alleged use of THG at a particular racetrack, but THG was not detected in any of the official race samples whereas all QC samples were confirmed for the presence of THG.

As expected, endogenous testosterone was detected and quantified in most of the 216 stallion plasma samples from drug-free research horses (57 stallions), and its concentration varied, based on the breeding function of the stallions and the seasons when the plasma samples were collected. Testosterone was not detected in the gelding and mare plasma samples (from 4 geldings and 28 mares) tested. In addition, the stallion plasma samples were also screened for nandrolone and boldenone since endogenous boldenone conjugate in equine urine [21,42] and nandrolone metabolites in human urine had been reported [35,43,44]. Boldenone was not detected in any of the stallion plasma samples, though boldenone sulfate in stallion urine had been reported [21,42]. However, endogenous nan-

Table 7
Concentration of THG (pg/mL) in equine plasma samples[a]

Time (h)	Plasma
0	0
0.25	9818
0.5	11119
0.75	10625
1	9399
2	8403
4	4453
6	2350
8	1468
10	1026
24	363
48	40
72	N/D[b]

[a] From the horse dosed with THG (15 mg/539 kg, IM).
[b] Not detected.

drolone was detected, confirmed (Fig. 6) and quantified in 90 of the 216 stallion plasma samples (from 30 of the 57 stallions) from drug-free research horses. This result is in agreement with the report that endogenous nandrolone was detected by RIA in stallions [45]. The concentration of nandrolone quantified was much lower than that of testosterone, but there was no correlation between them. The results will be published elsewhere.

4. Conclusions

The anabolic steroids formed $[M+H]^+$ ions and solvent adduct $[M+H+\text{solvent}]^+$ ions with methanol or acetonitrile under the ESI (+) LC–MS conditions. Methanol formed less solvent adduct ions with the analytes than acetonitrile, and thus, it was chosen over acetonitrile as an LC solvent for this study. The undesired solvent adduct ions were desolvated with the source CID so that the abundance of the $[M+H]^+$ ions as well as the MRM signals were increased. As for chemical properties of the analytes, the more conjugated double bonds present in the molecule, the more stable its $[M+H]^+$ ion and the less its solvent adduct ion $[M+H+\text{solvent}]^+$ formation.

The LC–MS/MRM method was validated for accuracy, precision, LOD, LOQ, and LOC. The method was successfully applied to analyses of the anabolic steroids in plasma samples collected post drug administrations to horses for pharmacokinetics study, official racehorse samples for drug screening, and stallion plasma samples from research horses for quantification of endogenous testosterone concentration. Surprisingly, endogenous nandrolone was detected and confirmed in some of the 216 stallion plasma samples from research horses. Endogenous boldenone was not detected in any of the stallion plasma samples though there have been reports that endogenous boldenone sulfate conjugate was detected and confirmed in equine urine samples from stallion. This method is sensitive and useful for detection, quantification and confirmation of the presence of anabolic steroids in equine plasma and has been successfully applied in pharmacokinetic study and doping control in racehorses.

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