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Simultaneous separation and determination of 16 testosterone and nandrolone esters in equine plasma using ultra high performance liquid chromatography–tandem mass spectrometry for doping control☆

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ABSTRACT

The potential for using testosterone and nandrolone esters in racehorses to boost the biological concentrations of these steroids and enhance athletic performance is very compelling and should be seriously considered in formulating regulatory policies for doping control. In order to regulate the use of these esters in racehorses, a sensitive and validated method is needed. In this paper, we report such a method for simultaneous separation, screening, quantification and confirmation of 16 testosterone and nandrolone esters in equine plasma by ultra high performance liquid chromatography–tandem mass spectrometry (UHPLC–MS/MS). Analytes were extracted from equine plasma by liquid–liquid extraction using a mixture of methyl *tert*-butyl ether and ethyl acetate (50:50, v/v) and separated on a sub-2 micron C_{18} column. Detection of analytes was achieved on a triple-quadrupole mass spectrometer by positive electrospray ionization mode with selected reaction monitoring (SRM). Mobile phase comprised 2 mM ammonium formate and methanol. Deuterium-labeled testosterone enanthate and testosterone undecanoate were used as dual-internal standards for quantification. Limits of detection (LOD) and quantification (LOQ) were 25–100 pg/mL and 100–200 pg/mL, respectively. The linear dynamic range of quantification was 100–10,000 pg/mL. For confirmation of the presence of these analytes in equine plasma, matching of the retention time with mass spectrometric ion ratios from MS/MS product ions was used. The limit of confirmation (LOC) was 100–500 pg/mL. The method is sensitive, robust, selective and reliably reproducible.

1. Introduction

In 2008, Pennsylvania (PA) became the first state in the USA to enforce the ban on the use of anabolic and androgenic steroids (AAS) in plasma of racehorses during competition. In order to enforce the ban, a high-throughput UHPLC–MS/MS method was developed and is routinely used for screening, quantification and confirmation of the eight most commonly abused AAS in equine plasma [1]. As the result of the limitation posed by the method for only eight AAS, the list of AAS was expanded to fifty-five by taking advantage of advances in mass spectrometry without compromising sensitivity [2,3]. These methods have provided incessant enforcement of the ban on the use of AAS in racehorses competing in PA.

Among the list of banned AAS in racehorses, testosterone (TES) and nandrolone (NAN) are often detected in plasma samples of male racehorses. Based on our investigation of more than 2000 plasma samples collected from intact male horses actively racing in PA, all of the samples contained TES. TES is considered an endogenous compound that is biologically produced in male horses [4,5]. For NAN, it was detected (>25 pg/mL) in 64.6% of the same plasma samples [6]. Whether or not NAN is an endogenous compound is still an unresolved issue but based on our investigation, NAN has been detected and quantified in post race plasma samples collected from intact male horses. Since TES and NAN were present in plasma samples of intact male horses, the previous methods [1–3] used for the detection of TES and NAN had limitations because the methods did not differentiate exogenous from endogenous TES and NAN in equine plasma. Differentiation of exogenous TES and NAN

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from their endogenous counterparts remains a challenge in equine doping control analysis.

In human doping control analysis, the ratio of testosterone glucuronide (TG) concentration to that of epitestosterone glucuronide (EpiTG) in urine is used to differentiate exogenous from endogenous TES [7]. Other potential indirect markers for identifying the administration of TES are reported as the ratio of urinary TG concentration to urinary luteinizing hormone and the ratio of plasma testosterone to that of 17α-hydroxyprogesterone [8,9]. In the past ten years, the use of gas chromatography/combustion/isotope ratio mass spectrometry (GC/C/IRMS) in doping control has steadily grown and the $^{13}C/^{12}C$ ratio of urinary steroids is used to distinguish endogenous from exogenous administration of anabolic steroids in human athletes [10–12].

In racehorse doping control analysis, due to lack of statistically validated reference data, the threshold of TG/EpiTG is difficult to establish for horse, therefore TG/EpiTG value is not adopted in the racehorse industry for doping control analysis. Although the ratio of $^{13}C/^{12}C$ has been demonstrated to be a useful and effective technique for differentiating exogenous from endogenous nandrolone in racehorses [13], its applicability in real world situations is still pending because the method requires sophisticated GC/C/IRMS instrument for which the cost is prohibitory to most equine doping control laboratories. Currently in equine forensics, there is no applicable method for differentiating exogenous TES and NAN from their endogenously produced counterparts by the horse.

Testosterone and NAN are available as the synthetic short-chain steroid esters for administration to horses. Following administration they are rapidly hydrolyzed by plasma esterases to release active TES and NAN. However, small quantities of the administered synthetic esters remain unchanged in the horse. Unlike long-chain fatty acid steroid esters, short-chain steroid esters that are used in drug formulations are not naturally produced in the body [14,15]. Thus, detection and confirmation of the presence of intact short-chain TES and/or NAN esters in equine plasma offer an unequivocal confirmation of the exogenous administration of these steroids, rather than use of the conventional detection and confirmation of the presence of the free steroids, TES and NAN.

Detection of steroid esters using gas chromatography–mass spectrometry (GC–MS) has been reported [16–20]. Most of the methods cited were developed for the detection of steroid esters in hair because hair has longer storage time window and is, therefore, used for retrospective analysis compared to urine and plasma [16,18–20]. Steroid esters are readily hydrolyzed, therefore concentrations of intact steroid esters in plasma are low, posing a problem for the analyst. Detection of TES esters at 1 ng/mL in human plasma by GC/MS has been reported [21]. With advanced features in high-performance liquid chromatography–mass spectrometry (HPLC–MS), HPLC–MS was used for analyzing steroid esters in plasma with greater sensitivity than GC–MS [22].

Taking advantage of the capabilities of UHPLC–MS, we have developed a method for detection, quantification and confirmation of 16 TES and NAN esters in equine plasma. This method was fully validated for specificity, matrix effect, linearity, limits of detection, accuracy, and precision. The method is routinely used in screening for these steroid esters in post-race plasma samples obtained from non-castrated male horses competing in PA. The method is sensitive, robust, selective and reliably reproducible.

2. Experimental

2.1. Chemicals and materials

The 16 steroid esters in this study include testosterone acetate (TESACE, Cat #: A6951-000; CAS #: 1045-69-8), testosterone caproate (TESCAP, Cat #: A6955-000; CAS #: 10312-45-5), testosterone cypionate (TESCYP, Cat #: A6960-000; CAS #: 58-20-8), testosterone decanoate (TESDECA, Cat #: A6963-000; CAS #:5721-91-5), testosterone enanthate (TESENA; Cat #: T-163; CAS #:315-37-7), tesosterone isocaproate (TESISO, Cat #: A6991-100; CAS #: 15262-86-9), testosterone laurate (TESLAURA, Cat #: A6991-500; CAS #:59232-78-9), testosterone phenylpropionate (TESPHENPRO, Cat #: A6992-000; CAS #: 1255-49-8), testosterone propionate (TESPRO; Cat #: A7000-000; CAS #:57-85-2), testosterone undecanoate (TESUNDECA, Cat #: A7030-000; CAS #: 5949-44-0), nandrolone acetate (NANACE, Cat #: E4051-000; CAS #:1425-10-1), nandrolone cypionate (NANCYP, Cat #: E4056-000; CAS #: 601-63-8), nandrolone decanoate (NANDECA, Cat #: E4057-000; CAS:360-70-3), nandrolone phenylpropionate (NANPHENPRO, Cat #: E4080-000; CAS #: 62-90-8), nandrolone undecanoate (NANUNDECA, Cat #: E4120-000; CAS #: 862-85-5), nandrolone laurate (NANLAUR, Cat #: E4076-000; CAS #: 26490-31-3) (Fig. 1). In addition to the catalog number, we have included the CAS # for each reference standard to avoid error when placing an order for any of these reference standards because of the similarity in chemical structure and name.

All the reference standards of steroid esters studied were purchased from Steraloids (Newport, RI, USA), except testosterone enanthate which was from Sigma-Aldrich (St. Louis, MO, USA). Testosterone enanthate-d_5 and testosterone undecanoate-d_5 were obtained from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada) and used as dual internal standards in the quantification of TES- and NAN-esters. Methyl *tert*-butyl ether (MTBE), formic acid, and ammonium hydroxide were obtained from EMD Chemical Inc. (Gibbstown, NJ, USA). Water (Optima grade) was purchased from Honeywell Burdick & Jackson (Muskegon, MI, USA) whereas methanol (Optima grade) and methylene chloride (HPLC grade) were purchased from Thermo Fisher Scientific (Fair Lawn, NJ, USA). Ethyl acetate (EA) was obtained from J.T. Baker (Philipsburg, NJ, USA).

2.2. Standard solutions

All stock solutions of steroid esters were prepared in methanol to avoid hydrolysis in aqueous solution. Each stock solution (1 mg/mL) was individually prepared by dissolving the weighed compound in methanol to yield 1 mg/mL and was stored at 4 °C. An aliquot (100 μL) of each stock solution (1 mg/mL) was added to 8.4 mL of methanol to prepare a mixture solution of the 16 steroid esters, with 10 μg/mL/ester in 10 mL. This stock solution was monitored for stability over a period of time (>12 months) and the results obtained indicated that the steroid esters were stable in methanol at 4 °C. Working standard solutions at 2.5, 5.0, 10, 25, 50, 100, 250, 500 ng/mL were prepared by serial dilution of the 10 μg/mL mixture in methanol and stored at 4 °C. Testosterone enanthate-d_5 and testosterone undecanoate-d_5 working solutions were prepared by dilution of the relevant stock solution (1 mg/mL) to100 ng/mL each in methanol.

Stock ammonium formate buffer comprising 1.0 mol/mL ammonium formate and 1.0 mol/mL formic acid was prepared by adding 15.4 mL formic acid and 13.5 mL ammonium hydroxide to 171 mL water (Optima grade). The unadjusted pH of the buffer was 3.4–3.5. A 2-mmol/L formate buffer was prepared by dilution of the stock formate buffer in water (Optima grade).

2.3. Preparation of calibration samples

Blank equine plasma was collected from healthy but retired female or gelded racehorses now resident at the PA Racing Commission Equine Facility at the University of Pennsylvania, School of Veterinary Medicine and was demonstrated to be free of all the

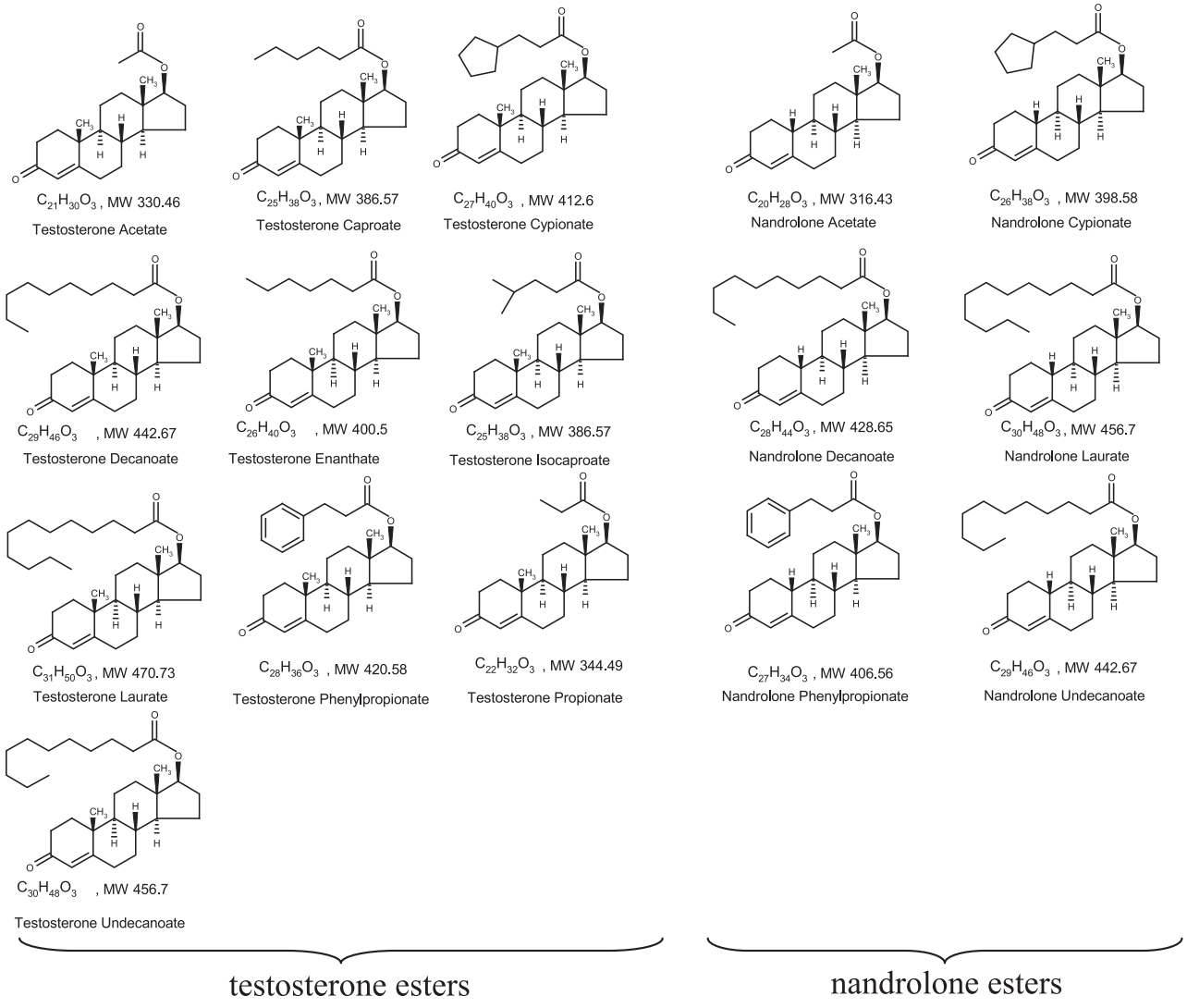

Fig. 1. Chemical structures, molecular formulae and weights of testosterone and nandrolone esters.

analytes using the same UHPLC–MS/MS method described in this paper. An aliquot (10 μL) of each working standard solution of 2.5, 5.0, 10, 25, 50, 100, 250, 500 ng/mL was spiked to 0.5 mL blank plasma to prepare calibration samples, with calibrators of 50, 100, 200, 500, 2000, 5000, 10,000 pg/mL. Calibration samples were processed in the exact same way as were plasma samples collected from racehorses.

2.4. Sample preparation

Analytes were recovered from plasma samples (0.5 mL) by liquid–liquid extraction (LLE) using a mixture of methyl *tert*-butyl ether and ethyl acetate (MTBE/EA, 50:50, v/v). A 0.5 mL aliquot of plasma samples was added to a pre-labeled 16 × 125 mm screw cap culture tubes, followed by 10 μL of each IS solution and mixed by vortex. A 5 mL aliquot of the above solvent mixture (50:50, v/v) was added to each test tube and the tubes were capped and mixed on a rotorack (Thermolyne, Dubuque, IA, USA) for 10 min prior to centrifugation at 3000 rpm (1610 × *g*) for 10 min. The upper organic layer was transferred to another pre-labeled culture tube and evaporated to dryness at 60 °C on a hot block (TechniDri-Block DB-3, Duxford, Cambridge, UK) under a steady stream of air. The extract was reconstituted in 120 μL 2 mM ammonium formate buffer:MeOH (20:80, v/v) and 100 μL was transferred into a 200 μL insert (Target PP Polyspring, National Scientific Company, Rockwood, TN, USA) from which 20 μL aliquot was used for LC–MS analysis.

2.5. Liquid chromatography/mass spectrometry

An Accela LC system (Thermo Fisher Scientific., San Jose, CA, USA), equipped with a sub-2 micron Hypersil Gold C_{18} analytical column (50 × 2.1 mm i.d., 1.9 μm particle size; Thermo Scientific, Waltham, MA, USA), was used for LC separation. Column temperature was set to 45 °C to reduce back pressure and speed analyte elution. Mobile phase A comprised 2 mM ammonium formate buffer, while that of B was MeOH. The following mobile phase gradient was employed for analyte separation: 0 min, 40/60 (A/B); 5.00 min, 28/72 (A/B); 11.00 min, 28/72 (A/B); 11.45 min, 15/85 (A/B); 17.00 min, 15/85 (A/B); 17.01 min, 40/60 (A/B); 18.00 min, 40/60 (A/B). Mobile phase flow was 500 μL/min. Total analysis time was 18 min.

A Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a Heated Electrospray Ionization (H-ESI) source was used in mass analyses. Xcaliber software v 2.0.7 (Thermo Fisher Scientific) was used for data acquisition and processing. Selected-reaction monitoring (SRM) mode was used for data acquisition. H-ESI source

parameters were optimized by syringe infusion of testosterone enanthate-d_5 into concurrent mobile phase flow of 20:80 (2 mM ammonium buffer:MeOH, v/v) at 500 μL/min. Collision energy (CE) and Tube Lens (TB) were optimized individually by injecting each analyte to generate three most intense SRM ion transitions for each ester. H-ESI source and mass spectrometer parameters common to all analytes were as follows: spray voltage, 1000 v; vaporizer temperature, 350 °C; sheath gas, 50 arbitrary unit; ion sweep gas, 15 arbitrary unit; auxiliary gas, 30 arbitrary unit; ion transfer capillary temperature, 300 °C; peak width relating to resolution (FWHM), 0.7 for Q1 and Q3; collision gas pressure, 1.5 mTorr (1 Torr = 133 pa); scan width (m/z), 0.5; scan time, 100–200 ms for each SRM.

2.6. Method validation

The method was validated for specificity, sensitivity, linearity, matrix effect, recovery, accuracy, precision and stability according to the FDA guidelines for validation of bioanalytical methods [23]. Specificity was assessed by comparing the chromatograms of six different lots of blank plasma to that spiked with a concentration of the reference standard that was reflective of the limit of quantification (LOQ). Sensitivity was evaluated by spiking varying concentrations of the reference standard to six different batches of plasma, and limit of detection was determined. Calibration curve was generated by plotting the ratio of peak area of the analyte to that of IS (y-axis) against analyte concentration on the x-axis. Linear regression model with $1/x$ weighting factor was used in describing the regression relationship. Matrix effect was determined by analyzing six replicate samples at three different concentrations (500, 2000, and 5000 pg/mL). Matrix effect was calculated by comparing the peak area of analytes-spiked blank plasma extract with that of the same analytes spiked in blank solvent. Analyte recovery from plasma by LLE was determined by comparing the peak areas of analytes in spiked plasma samples and then extracted with those of the analytes spiked in blank plasma extracts.

Intra-day accuracy and precision were determined by analyzing twenty-four validation samples at four different concentrations (200, 500, 2000, and 5000 pg/mL; $n = 6$ each) in one batch in a day. Inter-day accuracy and precision were measured in three consecutive batches in three separate days with the same concentration in validation samples. The concentrations of the analytes used for determining accuracy and precision corresponded to LOQ, low, medium and high concentrations used in constructing the calibration curves. Stability of the analytes in equine plasma under different temperature conditions and time-periods, as well as the effect of freeze–thaw conditions were evaluated. Stability studies were conducted at three different concentrations (500, 2000, and 5000 pg/mL) at room temperature, 4, −20 and −70 °C for different storage periods. Freshly prepared calibration curve was used to quantify stability study samples.

3. Results and discussion

3.1. Method development

3.1.1. Extraction solvent

MTBE is considered a "universal" solvent for solvating a wide variety of organic compounds and is widely used in our laboratory to extract analytes from aqueous matrices such as urine, serum and plasma [1–3,24,26,30]. Results of previous study showed that MTBE is a good extraction solvent for the recovery of anabolic steroids from equine plasma, with extraction efficiency >80% [24]. However, in this study, preliminary study results indicated that MTBE was not as good a solvent for the extraction of steroid esters from equine plasma as it was for anabolic steroids. The recovery efficiencies, for some of the esters, were as low as 5%, which could not assure sufficient analyte recovered to produce the desired level of sensitivity. To improve analyte recovery from plasma, different solvents and solvent combinations such as MTBE/EA (50:50, v/v), MTBE, ethyl acetate (EA), hexane/EA (50:50, v/v), hexane, and methylene chloride (DCM), were evaluated. Recovery of the analytes by various solvents or their combinations was conducted by extracting analytes at 2000 pg/mL from six batches of plasma samples. Relative extraction efficiency was calculated by comparing the average peak area from different solvents to that from MTBE/EA (Table 1). Results obtained indicated that MTBE/EA mixture (50:50, v/v) resulted in the highest extraction efficiency (defined as 100%) for all analytes. The average relative extraction efficiency from different solvents was ranked as MTBE/EA > MTBE > EA > hexane/EA > hexane > DCM (Table 1). Since MTBE/EA resulted in the highest extraction efficiency, it was selected as the extraction solvent for use in this study.

3.1.2. Liquid chromatographic gradient

Out of the 16 esters in the study, three pairs (TESCAP/TESISO, TESDECA/NANUNDECA and TESUNDECA/NANLAUR) have the same molecular weight and similar chemical structures. For those esters, their precursor ions are the same and their SRM transitions are very similar. Thus, mass spectrometry alone was unable to differentiate one from the other. To identify these pairs of analytes, it was necessary to resort to chromatographic separation. A two-step gradient program was developed to provide baseline separation for all the esters. The first step was from 0 to 5 min, the organic phase was increased from 60% to 72%; the mobile phase was isocratically maintained for the next 6 min. The second step was from 11 to 11.45 min, with organic phase augmented from 72% to 85%, then isocratically held for the next 6.55 min. As shown in Fig. 2, the two-step gradient programming generated sharp and asymmetric peaks of all 16 esters with base line separation. All analytes eluted within the 18 min analysis time (Table 2).

By parallel comparison of testosterone esters with nandrolone esters which have the same fatty acid group, such as TESACE vs NANACE, TESPHENPRO vs NANPHENPRO, TESCYP vs NANCYP, and so on, testosterone esters eluted later than nandrolone esters (Fig. 2, Table 2), suggesting that testosterone esters are more lipophilic than nandrolone esters. This behavior is due to the additional methyl group on the C-10 atom, which could function as a modifier to increase compound lipohilicity [25]. In general, compounds with longer hydrocarbon chain possess stronger lipophilicity than those with shorter carbon chain [25], as a result they can be retained in a reverse phase column longer and thus, are eluted later. In this study, the retention time increases as the number of carbon atoms increases in the fatty acid moiety of steroid esters, with the exception of phenylpropionate esters. For phenylpropionate esters, the benzene-ring could behave as does a methyl group, so both testosterone phenylpropionate ($t_R = 7.8$ min) and nandrolone phenylpropionate ($t_R = 6.8$ min) eluted earlier than most of the esters except nandrolone acetate ($t_R = 2.7$), testosterone propionate ($t_R = 4.5$) and testosterone acetate ($t_R = 3.3$) (Fig. 2).

3.1.3. Mass spectra

During method development, MS spectra were obtained to determine the precursor ion for each analyte. The MS spectra obtained (not shown) indicated that significant solvent adduct ions were formed in H-ESI source. These solvent adduct ions were 23 and 55 mass units higher than the protonated molecular weight of each analyte, suggesting that the solvent adduct ions were sodium $[M+Na]^+$ and sodium-methylhydroxide adducts $[M+Na+CH_3OH]^+$. In addition to solvent adduct ions, strong $[M+H]^+$ ions were detected and employed as precursor ions for further mass analysis using collision-induced dissociation (CID).

Table 1
Relative extraction efficiencies[a] of various solvents for steroid esters.

	MTBE/EA	MTBE	EA	Hexane/EA	Hexane	DCM
NANACE[b]	100.0 ± 16.1[c]	99.4 ± 9.0	98.5 ± 10.6	63.9 ± 15.4	64.5 ± 16.3	76.6 ± 17.5
TESACE	100.0 ± 17.2	110.9 ± 10.3	73.1 ± 11.0	67.4 ± 20.1	67.2 ± 16.0	68.3 ± 15.9
TESPRO	100.0 ± 15.6	102.5 ± 7.7	76.1 ± 26.1	69.7 ± 22.8	67.1 ± 15.4	35.5 ± 9.9
NANPHENPRO	100.0 ± 30.3	100.3 ± 17.4	57.0 ± 18.6	70.2 ± 26.3	61 ± 17.6	4.4 ± 1.7
TESPHENPRO	100.0 ± 33.3	92.0 ± 13.4	54.2 ± 17.9	70.7 ± 26.9	54.9 ± 16.7	3.0 ± 1.1
TESISO	100.0 ± 25.0	84.9 ± 11.9	53.9 ± 15.5	67.4 ± 25.0	54.0 ± 15.1	5.0 ± 1.7
TESCAP	100.0 ± 24.7	83.2 ± 13.7	55.6 ± 17.6	65.1 ± 22.6	52.4 ± 14.7	4.1 ± 1.7
NANCYP	100.0 ± 31.4	71.4 ± 8.5	55.7 ± 20.1	61.0 ± 23.9	35.7 ± 9.4	2.7 ± 0.7
TESENA	100.0 ± 48.4	40.9 ± 14.0	43.4 ± 8.5	70.1 ± 31.6	34.2 ± 12.8	3.0 ± 0.8
TESCYP	100.0 ± 44.5	43.3 ± 11.6	40.7 ± 12.8	47.7 ± 20.6	27.1 ± 13.6	1.6 ± 0.6
NANDECA	100.0 ± 45.6	20.1 ± 7.6	35.9 ± 7.9	17.8 ± 5.7	5.7 ± 4.1	4.4 ± 3.1
TESDECA	100.0 ± 49.1	17.3 ± 8.0	31.4 ± 6.0	12.2 ± 4.1	3.9 ± 1.9	1.5 ± 0.5
NANUNDECA	100.0 ± 51.7	15.8 ± 6.9	31.9 ± 5.0	10.1 ± 5.0	3.7 ± 1.8	2.5 ± 1.2
TESUNDECA	100.0 ± 61.6	25.4 ± 12.1	53.4 ± 12.7	16.7 ± 8.9	4.3 ± 1.7	2.4 ± 0.3
NANLAURA	100.0 ± 44.1	35.9 ± 13.6	80.2 ± 16.2	26.7 ± ± 13.5	NF[d]	4.8 ± 1.1
TESLAURA	100.0 ± 54.9	16.6 ± 6.5	47.8 ± 6.6	12.5 ± 5.4	1.4 ± 0.2	1.7 ± 0.6
Average of all	100.0	60.0	55.6	46.8	35.8	13.8

[a] Relative extraction efficiency = $A_{solvent}/A_{MTBE/EA} \times 100$, where $A_{solvent}$ is peak area from solvents and $A_{MTBE/EA}$ is peak area from MTBE/EA.
[b] NANACE = nandrolone acetate; TESACE = testosterone acetate; TESPRO = testosterone propionate; NANPHENPRO = nandrolone phenylpropionate; TESPHENPRO = testosterone phenylpropionate; TESISO = tesosterone isocaproate; TESCAP = testosterone caproate; NANCYP = nandrolone cypionate; TESENA = testosterone enanthate; TESCYP = testosterone cypionate; NANDECA = nandrolone decanoate; TESDECA = testosterone decanoate; NANUNDECA = nandrolone undecanoate; TESUNDECA = testosterone undecanotate; NANLAUR = nandrolone laurate; TESLAURA = testosterone laurate
[c] Mean ± RSD %.
[d] Not found.

MS/MS spectra of $[M+H]^+$ in all the analytes are shown in Fig. 3. All the testosterone esters produced similar spectra and product ions, suggesting that they followed similar CID fragmentation pathway (Fig. 3). This observation was also true for nandrolone esters. The most common fragmentation pattern for all the esters was the loss of the fatty acid portion of the molecule (R-CO). This loss was significant as evidenced by the generation of the relevant steroid ions (*m/z* 289 for testosterone and *m/z* 275 for nandrolone) from the respective esters.

Both testosterone and nandrolone esters produced steroid-specific product ions by CID fragmentation. The steroid-specific product ions are *m/z* 271, 253, 123, 109, 97 for testosterone derived from testosterone esters and *m/z* 257, 239 199, 145, 109 for nandrolone from nandrolone esters (Fig. 3). These product ions are similar to those generated from the relevant steroid [26,27]. Fragmentation pathways for generating these product ions have been proposed [26–29].

A typical product ion of *m/z* 175 was observed for all testosterone esters but absent in nandrolone counterparts and in testosterone itself, indicating that *m/z* 175 product ion is unique to CID fragmentation of testosterone esters. The fragmentation pathway for generating the ion of *m/z* 175 is unknown. Esters with phenyl fatty

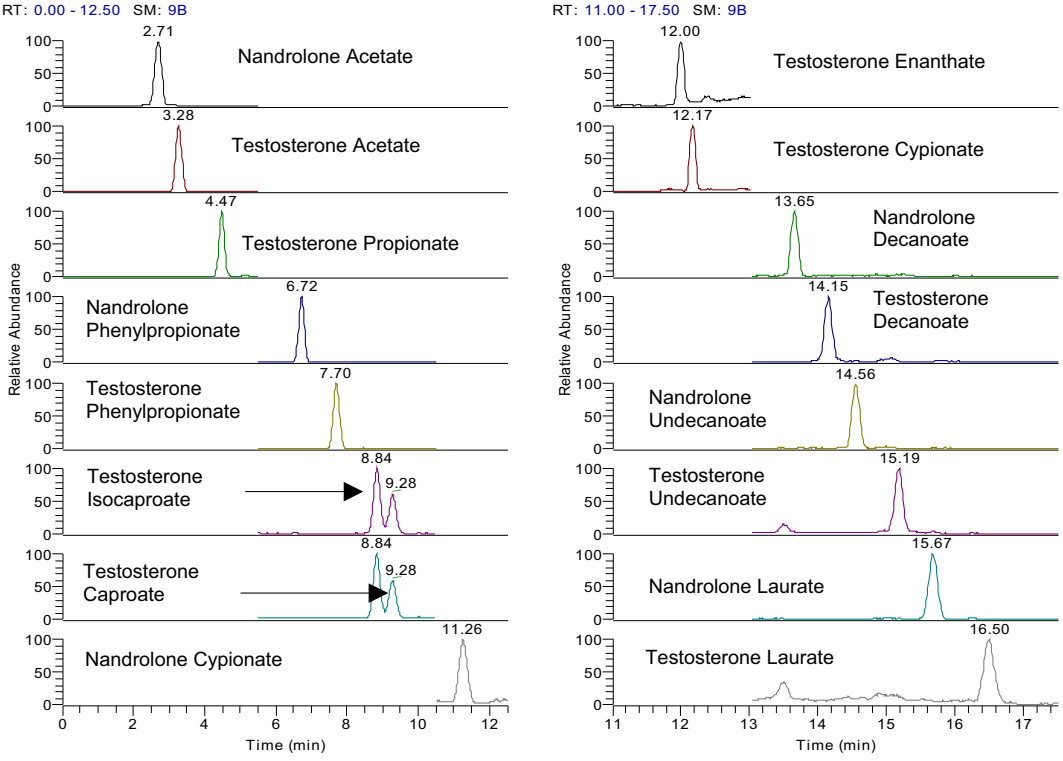

Fig. 2. HPLC–ESI(+)-MS/SRM chromatograms of the 16 steroid esters studied (1000 pg/mL each).

Table 2
Retention times and ion transitions for determination of steroid esters.

	Retention time (min)	Ion transition for screening and quantification	Ion transition for confirmation
NANACE[b]	2.7	317 → 257 (16[a])	317 → 239 (18), 257 (16), 275 (18)
TESACE	3.3	331 → 97 (22)	331 → 97 (22), 109 (30), 253 (17)
TESPRO	4.5	345 → 97 (23)	345 → 97 (23), 253 (17), 271 (16)
NANPHENPRO	6.8	407 → 105 (32)	407 → 105 (32), 239 (18), 257 (17)
TESPHENPRO	7.8	421 → 105 (32)	421 → 105 (32), 271 (16), 97 (31)
TESISO	8.9	387 → 97 (30)	387 → 97 (30), 253 (18), 271 (16)
TESCAP	9.4	387 → 97 (30)	387 → 97 (30), 253 (18), 271 (16)
NANCYP	11.4	399 → 107 (23)	399 → 107 (23), 257 (16), 239 (19)
TESENA	12.0	401 → 271 (16)	401 → 97 (32), 253 (18), 271 (16)
TESCYP	12.2	413 → 79 (35)	413 → 79 (35), 271 (17), 253 (19)
NANDECA	13.7	429 → 257 (17)	429 → 239 (19), 257 (17), 275 (18)
TESDECA	14.2	443 → 97 (32)	443 → 97 (32), 253 (19), 271 (17)
NANUNDECA	14.6	443 → 257 (17)	443 → 239 (20), 257 (17), 275 (19)
TESUNDECA	15.3	457 → 97 (33)	457 → 97 (33), 253 (19), 271 (17)
NANLAURA	15.8	457 → 257 (17)	457 → 239 (20), 257 (17), 275 (21)
TESLAURA	16.6	471 → 253 (20)	471 → 97 (31), 253 (20), 271 (18)

[a] Collision energy (*V*) in parentheses.
[b] Same abbreviations and definitions as in Table 1 footnote.

acid group (TESPHENPRO and NANPHENPRO) have a very intense base peak of *m/z* 105 which refers to a phenylethyl group contributed by its cleavage from the fatty acid portion of the molecule [29].

Based on MS/MS spectra, three SRM transitions for each analyte were selected for screening, quantification and confirmation of the 16 analytes. The choice of SRM transitions was based on the specificity of the analytes and sensitivity of the method. In the present method, one SRM transition was used for screening and quantification whereas three SRM transitions were used for confirmation of the presence of each analyte in a test sample (Table 2).

3.1.4. Scan segments

By using the two-step gradient program for LC separation, chromatographic peak widths of the analytes were between 0.1 and 0.3 min (Fig. 2). With 100 ms per ester scan speed, a complete screening scan cycle for the 16 esters plus two internal standards was ~1.8 s scan time. Thus, a chromatographic peak of 0.1 min could only produce ~3–4 data points across the peak, which would be insufficient for reconstructing the peak shape for accurate quantification. To increase data points across each peak, segment scan function was employed (X-Caliber Software, Thermo Fisher Scientific). The segment scan function allows grouping of analytes into

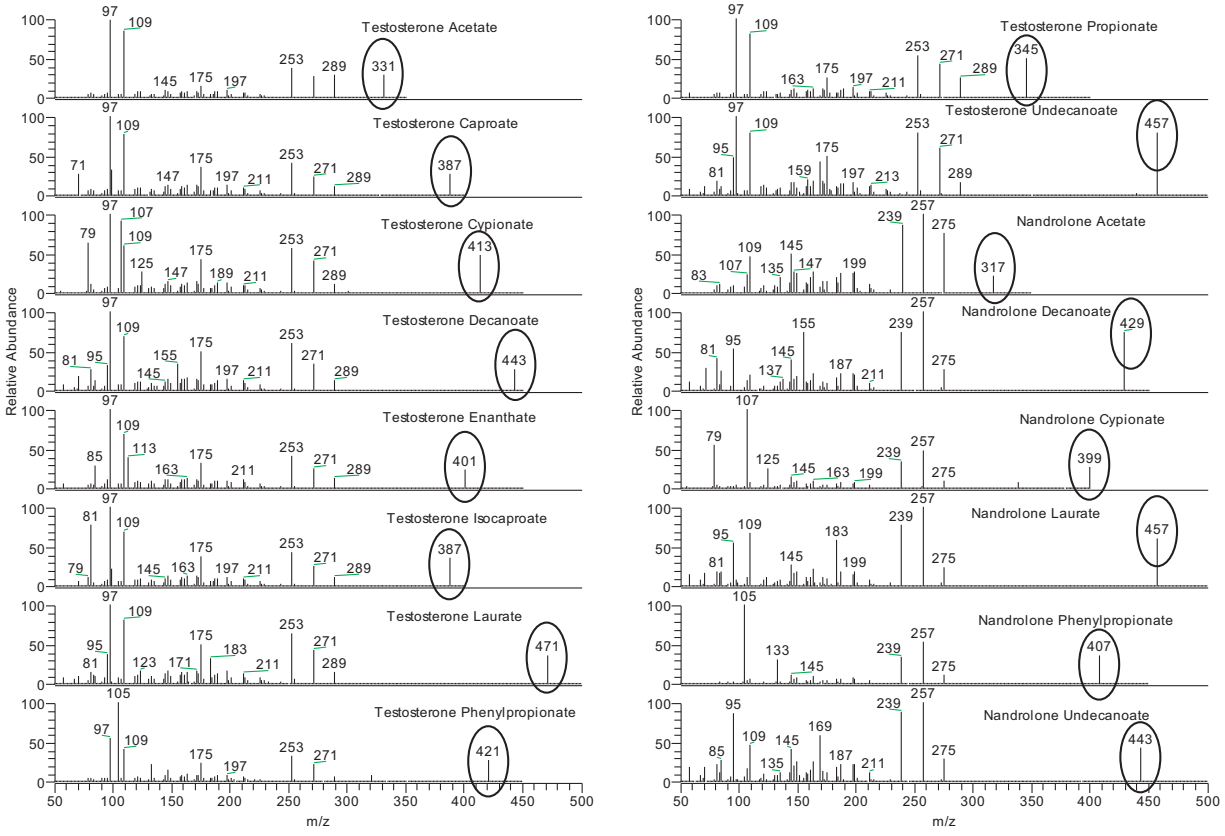

Fig. 3. H-ESI(+)-MS/MS spectra of $[M+H]^+$ for all steroid esters studied (circled ions are $[M+H]^+$, infusion conditions: ester concentration 10 ug/mL, injection speed 5 μL/min, mobile phase 500 μL/min of 2 mM ammonium buffer:MeOH (20:80, v/v)).

Table 3
Time sequence for scan segments for 16 steroid esters and 2 internal standards (IS).

Scan segment (min)	Esters scanned in segment	Scan time (ms)
0.0–5.5	NANACE[a], TESACE, TESPRO	200
5.5–10.5	NANPHENPRO, TESPHENPRO, TESISO, TESCAP	200
10.5–13.0	NANCYP, TESENA, TESCYP, TESENA-d_5	100
13.0–18.0	NANDECA, TESDECA, NANUNDECA, TESUNDECA, NANLAURA, TESLAURA, TESUNDECA-d_5	100

[a] Same abbreviations and definitions as in Table 1 footnote.

segments based on retention time. Within a segment, only the analytes placed in the segment were scanned. By using segment scan function, data points for each peak could be significantly increased. Another advantage of using segment scan was that a longer scan time produced stable ion intensity. Table 3 lists the details of segment scan and scan time used in each segment. Employing these segment scan functions, we were able to obtain 15–20 data points for each ester, which were sufficient for peak area integration and accurate quantification.

3.2. Method validation

3.2.1. Specificity

Method specificity measures the potential impurities eluted at the same or close to the retention time of the analyte. For doping control analysis, high specificity could ensure method sensitivity and quantification accuracy, reduce false positive and increase high-throughput screening. Fig. 4 shows the method specificity by comparing the chromatograms of blank plasma with that spiked with LOQ. There was no direct interference in the blank plasma caused by endogenous substances at the same retention time of each of the analytes, suggesting that sample preparation by LLE yielded clean samples and that liquid chromatographic gradient used was able to separate endogenous impurities from the analytes. Thus, the method is highly specific.

3.2.2. Sensitivity and linearity

Limits of detection (LOD) and quantification (LOQ) were estimated at a signal-to-noise ratio of 3:1 and 10:1, respectively. LOD and LOQ were 25–100 pg/mL and 100–200 pg/mL, respectively. Linear calibration curves were obtained over a range of 100–10,000 pg/mL. The coefficient of determination (r^2) was ≥0.99 for most of the analytes, except NANUNDECA, TESUNDECA, NANLAURA and TESLAURA with $r^2 \geq 0.98$.

3.2.3. Matrix effects and extraction recovery

In mass spectrometry, co-elution of residual components from the sample matrix could suppress or enhance ionization of target analytes, resulting in diminished precision and accuracy for quantification. In this study, matrix effect was evaluated and the results indicated that matrix effect was within ±20% range (Table 4). Negative and positive values in Table 4 indicate that by using LLE extraction, residual components from equine plasma could induce ion suppression or enhancement effects on the analysis of these esters. Since matrix effect of <10% was obtained for most of the analytes, additional steps to reduce matrix effects on the analysis of the analytes were not necessary.

MTBE/EA mixture had the highest extraction efficiency among all the solvents evaluated (Table 5). The study results indicate that extraction recovery ratio for most of the esters by MTBE/EA was >50%, except for NANDECA, TESDECA, NANUNDECA, TESUNDECA, NANLAURA at low concentrations (Table 5). For these 5 esters, recovery ratio was between 30% and 50%. The over all average recovery ratio of the analytes was 58%.

Table 4
Matrix effect[a] of plasma on steroid ester analysis.

	500 pg/mL	2000 pg/mL	5000 pg/mL
NANACE[b]	5.43	6.05	7.38
TESACE	2.30	6.94	−1.18
TESPRO	4.29	3.01	0.83
NANPHENPRO	−2.70	−0.38	−5.15
TESPHENPRO	−3.45	−0.95	−4.97
TESISO	−2.25	2.85	−1.44
TESCAP	−5.49	0.33	−4.00
NANCYP	6.72	13.95	11.83
TESENA	7.81	9.38	−1.38
TESCYP	−3.55	−0.38	−11.35
NANDECA	3.56	−3.92	18.56
TESDECA	8.37	4.84	18.97
NANUNDECA	1.05	7.18	14.84
TESUNDECA	8.07	12.87	14.41
NANLAURA	1.04	8.33	−3.12
TESLAURA	−16.57	−14.01	−20.17

[a] Matrix effect (ion suppression or enhancement %) = $(A_{extract} - A_{solvent})/A_{solvent} \times 100$, where $A_{solvent}$ is the peak area of an analyte spiked in reconstitution solvent and $A_{extract}$ is the peak area of an analyte spiked in blank equine plasma extract.
[b] Same abbreviations and definitions as in Table 1 footnote.

3.2.4. Accuracy and precision

Precision and accuracy results indicated that for NANACE, TESACE, TESPRO, NANPHENPRO, TESPHENPRO, TESISO, TESCAP, NANCYP, TESENA, TESCYP, NANDECA, the method was accurate with an acceptance limit of ±20% of the theoretical values, and RSD around the mean value did not exceed ±20% (Table 6). The result obtained suggested that the method used is capable of providing accurate quantification for each of the analytes. However, for TESDECA, NANUNDECA, TESUNDECA, NANLAURA, TESLAURA, the precision and accuracy were out of the 20% acceptable variation range, especially at low concentrations (200 pg/mL and 500 pg/mL). For this reason, this method only provides semi-quantified results for these 5 TES- and NAN-derived esters. Since the acceptable plasma concentration of TES and NAN-esters in racehorse during competition in PA is zero, accurate quantification is not required for reporting a positive finding. In the US, many jurisdictions have adopted the biological concentrations of TES (2000 pg/mL) and NAN

Table 5
Extraction efficiency[a] of MTBE/EA on various concentrations of steroid esters.

	500 pg/mL	2000 pg/mL	5000 pg/mL
NANACE[b]	77.4 ± 14.0[c]	84.3 ± 8.4	84.1 ± 12.3
TESACE	63.8 ± 15.0	69.0 ± 10.5	74.2 ± 11.7
TESPRO	72.8 ± 15.7	78.0 ± 9.1	79.8 ± 12.8
NANPHENPRO	57.1 ± 15.7	66.8 ± 10.7	72.9 ± 13.4
TESPHENPRO	53.9 ± 13.7	63.5 ± 11.9	71.1 ± 12.9
TESISO	55.7 ± 13.6	65.2 ± 9.9	70.8 ± 12.8
TESCAP	55.5 ± 17.9	62.8 ± 13.4	69.7 ± 13.3
NANCYP	50.7 ± 17.4	60.0 ± 17.1	67.5 ± 13.9
TESENA	53.8 ± 8.3	61.3 ± 14.9	69.3 ± 14.2
TESCYP	50.9 ± 17.0	58.3 ± 15.6	66.8 ± 12.5
NANDECA	43.1 ± 18.1	50.4 ± 23.2	57.0 ± 17.8
TESDECA	37.3 ± 19.4	45.5 ± 30.1	53.5 ± 16.0
NANUNDECA	39.6 ± 21.1	45.2 ± 31.9	57.5 ± 14.7
TESUNDECA	35.6 ± 18.2	41.7 ± 30.7	50.8 ± 17.2
NANLAURA	31.8 ± 24.5	40.6 ± 31.1	52.9 ± 17.7
TESLAURA	44.6 ± 17.0	47.2 ± 30.3	68.8 ± 15.7

[a] Extraction ratio (%) = $A_{processed}/A_{extract} \times 100$, where $A_{processed}$ is the peak area of analyte spiked in blank equine plasma and processed and $A_{standard}$ is the peak area of analytes fortified blank plasma extract.
[b] Same abbreviations and definitions as in Table 1 footnote.
[c] Mean ± RSD %.

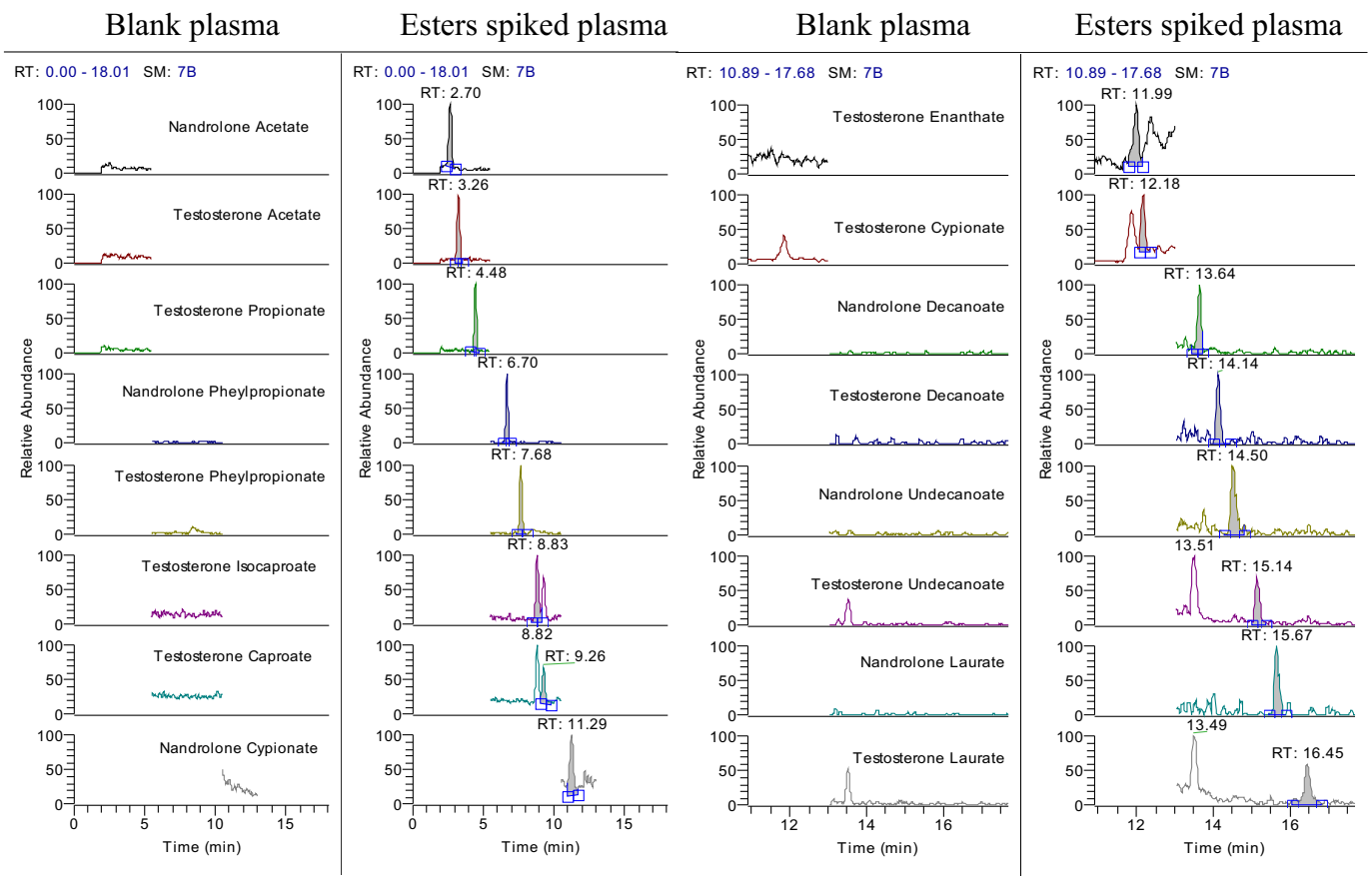

Fig. 4. HPLC/ESI(+)-MS/SRM chromatograms of blank plasma and blank plasma spiked with steroid esters indicating specificity of the method (spiked concentration: 50 pg/mL for nandrolone acetate, testosterone acetate, testosterone propionate, nandrolone phenylpropionate, and testosterone phenylpropionate; 100 pg/mL for tesosterone isocaproate, and testosterone caproate; 200 pg/mL for nandrolone cypionate, testosterone enanthate, testosterone cypionate, nandrolone decanoate, testosterone decanoate, nandrolone undecanoate, testosterone undecanotate, nandrolone laurate, and testosterone laurate).

(500 pg/mL) used by PA in the intact male horse [30]. However, the difference between the ceiling biological plasma concentration established for TES and NAN and the actual concentration detected in actively competing intact male horse is very wide [6]. The tendency to augment plasma TES and NAN concentrations by using the respective ester without breaking the rules is very compelling. Thus, regulatory policy against the use of TES and NAN should include TES and NAN-derived esters as the indirect markers in steroid control program to monitor the abuse of esters to augment biological concentrations of TES and NAN in racehorses.

3.2.5. *Stability of esters in equine plasma*

In PA, racehorse samples are collected at race tracks and transported to the laboratory for analysis. During transportation, the samples are not refrigerated in the delivery truck for 1–6 h. Upon receipt of samples at the laboratory, they are normally stored at 4 °C during the holding period for screening, quantification and confirmation analyses. The holding period may last 72–168 h. Following the holding period, positive samples required for long-term storage are transferred to −20 °C or −70 °C. For re-analysis of frozen sample, the sample may undergo a few freeze–thaw cycles. The effect of storage conditions on stability of the analytes is an important factor in maintaining the integrity of the sample, and therefore, needs to be evaluated as part of the validation process. In this study, stability was evaluated by storing spiked samples (500, 2000, and 5000 pg/mL, $n = 3$) under different temperature conditions (25 °C, 4 °C, −20 °C and −70 °C) and monitoring analyte concentration at different storage time periods. Stability was expressed as the percent change of the concentration of the analytes at different storage time periods compared with that of 0 h (control). Effect of freeze–thaw cycle on analyte stability was estimated by freezing sample at −20 °C for 21 h and thawing at ambient temperature for 3 h prior to analysis. Concentrations of the analytes in the freeze–thaw samples were determined using daily calibration curves.

Stability study results are presented in Tables 7–11. Table 7 indicates that at ambient temperature, steroid esters were stable during a 6 h storage period, except TESCAP, which exhibited instability after 2 h storage period. Upon storage for 24 h, concentrations of NANPHENPRO, TESPHENPRO, TESCAP, TESISO, NANCYP, TESENA, TESCYP significantly decreased to 70–30% (Table 7). TESCAP was extremely unstable as its concentration decreased to 30% of the initial concentration after storage at room temperature for 24 h (Table 7). At 4 °C, steroid esters were stable for the first 3 days storage only (Table 8). After storage for 7 days at 4 °C, concentrations decreased to 10–80% for NANPHENPRO, TESPHENPRO, TESCAP, TESISO, NANCYP, TESENA, TESCYP (Table 8). For samples stored at −20 and −70 °C, the concentration did not show significant decrease after storage for 60 days, suggesting that these analytes are stable at low temperature conditions (Tables 9 and 10). Freeze–thaw study results indicated that these esters were stable through five freeze–thaw cycle treatments, except NANUNDECA, NANLAURA and TESLAURA were unstable at low concentration. For

Table 6
Intra-day and inter-day precision and accuracy.

	QC 200 pg/mL		QC 500 pg/mL		QC 2000 pg/mL		QC 5000 pg/mL	
	Precision[a]	Accuracy[b]	Precision	Accuracy	Precision	Accuracy	Precision	Accuracy
Intra-day (n = 6)								
NANACE[c]	5.7	88.7	4.8	86.4	7.9	85.3	8.7	84.1
TESACE	7.7	99.6	5.2	87.9	8.4	86.5	8.8	84.1
TESPRO	10.3	97.4	5.6	88.4	6.9	91.3	8.4	87.0
NANPHENPRO	6.2	103.8	4.4	94.5	6.3	95.6	6.4	93.3
TESPHENPRO	5.1	102.8	3.6	91.3	8.2	96.0	6.7	93.4
TESISO	13.8	107.2	5.2	90.8	7.8	95.7	7.8	91.3
TESCAP	9.1	116.4	5.4	96.4	8.3	98.7	7.5	95.0
NANCYP	13.8	120.5	8.5	101.7	4.0	98.7	5.7	96.7
TESENA	7.1	105.4	4.6	98.1	5.2	94.9	5.3	92.3
TESCYP	12.2	97.7	7.0	97.0	3.3	96.2	2.4	94.6
NANDECA	4.6	85.9	9.7	81.4	9.1	92.7	7.3	89.0
TESDECA	20.2	67.6	28.2	69.2	9.8	92.9	11.1	81.9
NANUNDECA	30.6	68.1	29.9	68.7	11.6	91.8	13.5	79.0
TESUNDECA	20.3	71.3	15.3	62.7	5.9	95.9	13.1	78.0
NANLAURA	10.6	42.1	10.9	55.8	9.1	100.7	7.9	80.8
TESLAURA	27.2	87.0	17.5	81.6	20.7	87.8	5.5	96.6
Inter-day (n = 6)								
NANACE	14.6	101.1	9.3	96.3	9.0	92.3	9.2	84.2
TESACE	10.9	99.4	6.9	96.3	9.3	92.3	9.6	83.7
TESPRO	10.0	100.6	8.0	98.1	11.0	94.5	10.1	85.9
NANPHENPRO	8.6	103.5	6.6	100.5	10.8	96.6	10.8	88.8
TESPHENPRO	7.4	102.8	6.3	98.9	11.5	96.7	10.9	88.8
TESISO	20.1	113.9	10.4	89.6	18.3	85.8	24.9	78.2
TESCAP	18.0	108.9	9.4	100.5	13.1	98.5	12.8	89.3
NANCYP	10.7	105.4	9.4	103.4	8.5	97.6	11.1	91.3
TESENA	9.8	100.4	9.1	96.7	4.6	96.6	5.0	96.7
TESCYP	4.7	97.0	10.5	98.7	5.7	96.7	5.0	98.7
NANDECA	14.6	99.0	15.9	90.6	6.7	89.4	7.9	82.8
TESDECA	27.3	92.6	32.6	96.8	11.8	87.6	13.2	76.9
NANUNDECA	39.6	91.9	36.3	89.8	21.5	85.1	16.6	71.6
TESUNDECA	33.8	96.6	38.0	91.4	19.9	89.7	23.7	83.3
NANLAURA	45.0	82.6	44.1	95.1	19.7	93.9	28.9	90.7
TESLAURA	35.2	94.3	33.7	89.6	17.5	106.2	30.0	108.8

[a] Precision (RSD %) = standard deviation of conc. measured/conc. measured × 100.
[b] Accuracy (bias %) = Conc. measured/conc. spiked × 100.
[c] Same abbreviations definitions as in Table 1 footnote.

these three esters, their concentrations dropped to ~30% after five freeze–thaw cycle treatments (Table 11).

3.3. Confirmation

In doping control analysis, analyte suspects are confirmed by matching the "chemical fingerprints" of an unknown compound to those of an authentic reference standard. LC–MS/MS technology provides unique "chemical fingerprints" for each analyte, i.e. mass spectrum and retention time. SRM scan achieved with the use of a triple-quadrupole mass spectrometer produced multiple product ions. The ion intensity among these product ions is unique for each molecule, therefore, it is used as part of the 'chemical fingerprints" for confirmation. According to guidance from the American Society

Table 7
Stability[a] of steroid esters in equine plasma at ambient temperature.

	500 pg/mL				2000 pg/mL				5000 pg/mL			
	2 h	4 h	6 h	24 h	2 h	4 h	6 h	24 h	2 h	4 h	6 h	24 h
NANACE[b]	86	92	87	112	96	99	102	95	76	104	115	122
TESACE	86	95	92	119	98	105	109	123	86	102	117	116
TESPRO	90	93	93	108	96	100	101	110	84	99	108	110
NANPHENPRO	93	93	87	76	94	94	96	76	87	92	99	79
TESPHENPRO	91	86	80	48	95	88	87	51	86	87	91	52
TESISO	92	89	90	76	95	92	92	76	91	94	95	75
TESCAP	87	77	70	24	80	75	71	27	84	78	78	31
NANCYP	95	88	87	73	103	114	104	75	95	100	102	93
TESENA	95	89	82	51	99	88	87	53	99	88	87	52
TESCYP	97	99	84	64	98	92	86	55	99	98	96	62
NANDECA	94	88	83	80	100	87	91	89	103	90	106	105
TESDECA	99	92	96	121	118	108	85	94	94	101	101	106
NANUNDECA	106	87	93	118	118	91	112	112	112	96	114	126
TESUNDECA	99	95	99	101	93	93	95	87	87	91	94	95
NANLAURA	99	80	88	82	71	64	105	91	82	77	88	82
TESLAURA	88	97	82	82	87	80	107	65	98	77	101	100

[a] Stability = Conc. in stored sample/conc. in initial 0 h sample × 100.
[b] Same abbreviations and definitions as in Table 1 footnote.

Table 8
Stability[a] of steroid esters in equine plasma at 4 °C.

	500 pg/mL			2000 pg/mL			5000 pg/mL		
	1 day	3 days	7 days	1 day	3 days	7 days	1 day	3 days	7 days
NANACE[b]	101	101	124	89	72	92	119	97	102
TESACE	114	100	115	110	76	101	119	103	107
TESPRO	107	99	112	101	82	96	114	99	98
NANPHENPRO	91	98	83	89	73	68	110	76	58
TESPHENPRO	75	96	57	81	56	34	98	57	28
TESISO	90	100	80	89	76	56	102	82	49
TESCAP	63	103	41	68	31	12	81	34	11
NANCYP	83	91	86	84	92	78	122	93	73
TESENA	75	98	56	84	68	43	83	66	39
TESCYP	75	103	66	92	81	47	87	72	50
NANDECA	96	107	112	99	76	81	130	111	91
TESDECA	79	87	94	83	84	88	115	94	74
NANUNDECA	107	102	121	122	91	74	111	123	69
TESUNDECA	98	98	103	101	113	96	93	108	101
NANLAURA	145	122	121	94	67	55	90	88	40
TESLAURA	103	110	119	76	79	130	117	106	63

[a] Stability = Conc. in stored sample/conc. in initial 0 h sample × 100.
[b] Same abbreviations and definitions as in Table 1 footnote.

Table 9
Stability[a] of steroid esters in equine plasma at −20 °C.

	500 pg/mL			2000 pg/mL			5000 pg/mL		
	7 days	1 month	2 months	7 days	1 month	2 months	7 days	1 month	2 months
NANACE[b]	98	98	103	99	122	102	106	126	111
TESACE	94	101	89	106	123	91	107	123	93
TESPRO	91	109	86	100	117	97	97	127	97
NANPHENPRO	102	101	87	90	105	86	101	116	87
TESPHENPRO	97	99	84	89	100	85	98	103	87
TESISO	97	98	96	80	96	100	96	99	98
TESCAP	78	59	78	70	101	77	91	109	82
NANCYP	91	92	84	90	94	91	92	101	93
TESENA	85	87	91	95	95	92	91	89	97
TESCYP	83	95	85	90	88	82	97	98	91
NANDECA	59	81	67	84	82	107	85	103	81
TESDECA	81	104	88	79	99	101	57	95	96
NANUNDECA	53	103	94	76	123	116	79	102	121
TESUNDECA	90	108	108	86	80	94	106	85	97
NANLAURA	41	75	106	47	55	81	44	64	91
TESLAURA	70	65	92	93	58	72	78	52	76

[a] Stability = Conc. in stored sample/conc. in initial 0 h sample × 100.
[b] Same abbreviations and definitions as in Table 1 footnote.

Table 10
Stability[a] of steroid esters in equine plasma at −70 °C.

	500 pg/mL			2000 pg/mL			5000 pg/mL		
	7 days	1 month	2 months	7 days	1 month	2 months	7 days	1 month	2 months
NANACE[b]	75	133	105	102	138	112	109	127	113
TESACE	70	125	91	107	137	95	110	122	95
TESPRO	64	127	119	102	132	105	100	123	97
NANPHENPRO	123	121	97	99	128	91	109	122	89
TESPHENPRO	124	117	95	97	126	90	106	119	88
TESISO	104	107	105	83	116	101	99	100	97
TESCAP	109	104	93	73	113	83	96	107	85
NANCYP	106	124	91	99	134	95	87	129	95
TESENA	101	93	104	90	96	97	96	87	103
TESCYP	81	91	103	87	88	89	98	87	95
NANDECA	53	75	114	72	86	75	84	84	95
TESDECA	74	99	99	60	94	91	61	74	98
NANUNDECA	51	119	105	76	139	129	75	78	133
TESUNDECA	73	99	118	116	89	96	125	91	100
NANLAURA	88	76	120	76	42	66	85	46	91
TESLAURA	69	45	116	54	45	64	64	45	78

[a] Stability = Conc. in stored sample/conc. in initial 0 h sample × 100.
[b] Same abbreviations and definitions as in Table 1 footnote.

Table 11
Effect of freeze–thaw cycle on stability[a] of ester in equine plasma.

Freeze–thaw	500 pg/mL			2000 pg/mL			5000 pg/mL		
	1 cycle	3 cycles	5 cycles	1 cycle	3 cycles	5 cycles	1 cycle	3 cycles	5 cycles
NANACE [b]	116	102	107	105	84	102	123	85	111
TESACE	110	106	98	106	91	103	118	90	106
TESPRO	104	98	88	99	87	98	117	89	99
NANPHENPRO	99	97	109	94	93	117	110	96	106
TESPHENPRO	96	87	96	92	92	108	108	95	99
TESISO	106	96	104	92	91	101	104	96	105
TESCAP	93	55	69	85	73	80	105	81	87
NANCYP	76	95	99	96	108	118	120	109	115
TESENA	98	74	101	89	92	84	97	89	90
TESCYP	87	83	80	90	101	79	100	95	87
NANDECA	78	88	81	93	110	112	124	100	109
TESDECA	87	122	66	103	130	108	133	118	114
NANUNDECA	111	83	37	121	135	142	125	127	125
TESUNDECA	99	60	72	88	108	96	94	121	102
NANLAURA	66	83	28	60	80	75	97	92	87
TESLAURA	108	96	39	79	106	120	81	81	92

[a] Stability = Conc. in freeze–thaw treated sample/conc. in initial 0 h sample × 100.
[b] Same abbreviations and definitions as in Table 1 footnote.

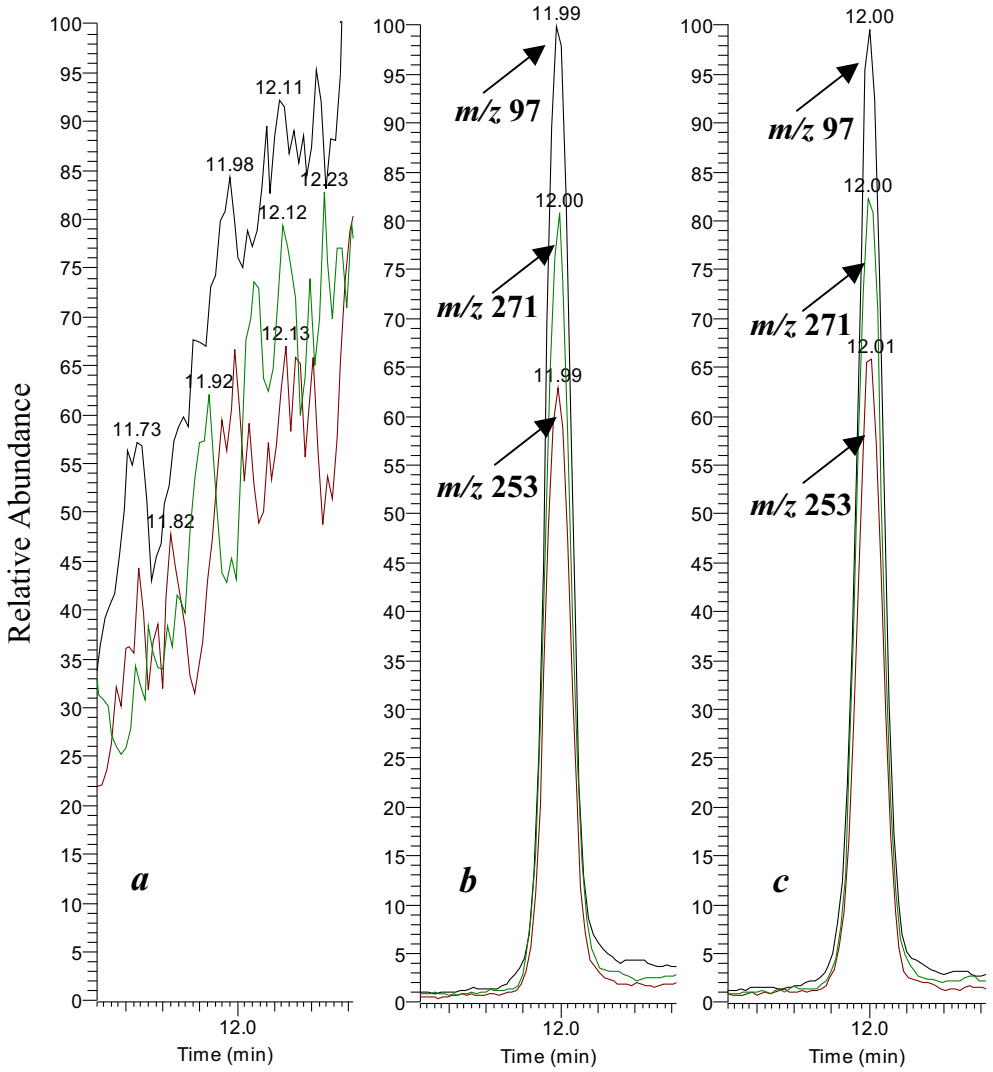

Fig. 5. LC–MS/MS chromatographic ion ratio confirmation for testosterone enanthate. (a) Blank plasma showing absence of testosterone enanthate; (b) 2000 pg/mL testosterone enanthate QC sample showing three ion transitions (*m/z* 401 → 97, *m/z* 401 → 271, *m/z* 401 → 253) were detected; (c) research horse plasma (after 24 h testosterone enanthate administration; dose: 2 mg/kg intramuscular) showing three ion transitions were detected and the ion ratios matched those of QC sample, which confirmed the presence of testosterone enanthate in the (c) sample.

for Mass Spectrometry [31], three product ions are required for confirmation and the guidelines were met in this study (Table 2). Ion ratios were calculated from the three product ions using peak height. Based on ion ratio, the similarity between unknown sample and calibration/QC samples can be calculated and used for analyte confirmation [1].

To confirm the presence of any of these esters in equine plasma, the ion intensity ratio similarity between unknown samples and the calibration/QC samples must be within 80–120%. In addition to ion ratio, retention time was also used as a criterion for confirmation of the analyte. The retention time of any of the esters in an unknown sample must be within ±0.2 min window of those in calibration/QC samples. A representative TESENA ion ratio confirmation graph indicates that the ion ratios of the research horse sample collected post TESENA administration matched those of an authentic QC sample (Fig. 5). The retention time of TESENA administration was the same as that of the authentic QC sample used (Fig. 5). Results indicated that the presence of TESENA was confirmed in TESENA administration sample. LOC was defined as the lowest concentration at which three product ions could be detected and the ion ratios between these ions are stable was 500 pg/mL for all 16 analytes.

4. Conclusions

A sensitive screening, quantification and confirmation method for 16 steroid esters using UHPLC–MS/MS was developed and validated. To our knowledge, this is the first report for simultaneous analysis of 16 steroid esters in equine plasma. This method covers almost all major testosterone and nandrolone esters that would be particularly valuable for regulating the use of these drugs in racehorses during competition in PA. This method is sensitive, reproducible, and reliable. It is complimentary to the previous steroid methods [1–3] used in PA to continue to enforce the ban on the use of anabolic and androgenic steroids in racehorses during competition.

References

[1] Y.W. You, C.E. Uboh, L.R. Soma, F.Y. Guna, X.Q. Li, J.A. Rudy, Y. Liu, J.W. Chen, Rapid Commun. Mass Spectrom. 23 (2009) 2035.
[2] Y. Liu, C.E. Uboh, L.R. Soma, X.Q. Li, F. Guan, Y. You, J.A. Rudy, J.W. Chen, Drug Test. Anal. 3 (2011) 56.
[3] F. Guan, C.E. Uboh, L.R. Soma, Y. You, Y. Liu, X.Q. Li, J. Mass Spectrom. 45 (2010) 1270.
[4] J.E. Cox, J.H. Williams, P.H. Rowe, J.A. Smith, Equine Vet. J. 5 (1973) (1973) 85.
[5] V.K. Ganjam, R.M. Kenney, J. Reprod. Fertil. Suppl. 23 (1975) 67.
[6] L.R. Soma, C.E. Uboh, Y. You, F. Guan, D. Tsang, S. McDonnell, J. Vet. Pharmacol. Ther., 34 (2011), doi:10.1111/j.1365-288.2011.01295.x.
[7] World Anti-Doping Agency: The World Anti-Doping Code. The 2008 prohibited list. International Standard. Available from: http://www.wada-ama.org/rtecontent/document/2008_List_en.pdf (accessed 25.01.11).
[8] A.T. Kickman, R.V. Brooks, S.C. Collyer, D.A. Cowan, M.N. Nanjee, G.J. Southan, M.J. Wheeler, Br. J. Sports Med. 24 (1990) 253.
[9] K. Carlström, E. Palonek, M. Garle, H. Oftebro, J. Stanghelle, I. Björkhem, Clin. Chem. 38 (1992) 1779.
[10] T. Piper, U. Mareck, H. Geyer, U. Flenker, M. Thevis, P. Platen, W. Schänzer, Rapid Commun. Mass Spectrom. 22 (2008) 2161.
[11] R. Aguilera, T.E. Chapman, B. Starcevic, C.K. Hatton, D.H. Catlin, Clin. Chem. 47 (2001) 292.
[12] M. Ueki, M. Okano, Rapid Commun. Mass Spectrom. 13 (1999) 2237.
[13] M. Yamada, K. Kinoshita, M. Kurosawa, K. Saito, H. Nakazawa, J. Pharmaceut. Biomed. Anal. 45 (2007) 654.
[14] J.M. Larner, S.L. Pahuja, C.H. Shackleton, W.J. McMurray, G. Giordano, R.B. Hochberg, J. Biol. Chem. 268 (1993) 13893.
[15] W. Borg, C.H.L. Shackleton, S.L. Pahuja, R.B. Hochberg, Proc. Natl. Acad. Sci. 92 (1995) 1545.
[16] K. Höld, C.R. Borges, D.G. Wilkins, D.E. Rollins, R.E. Joseph, J. Anal. Toxicol. 23 (1999) 416.
[17] S.H. Peng, J. Segura, M. Farré, J.C. González, X.D.L. Torre, Steroids 67 (2001) 39.
[18] S. Boyer, P. Garcia, M.A. Popot, V. Steiner, M. Lesieur, J. Chromatogr. B 852 (2007) 684.
[19] J. Segura, S. Pichini, S.H. Peng, X.D.L. Torre, Forensic Sci. Int. 107 (2000) 347.
[20] Y. Gaillard, F. Vayssette, A. Balland, G. Pépin, J. Chromatogr. B 735 (1999) 189.
[21] X. de la Torre, J. Segura, A. Polettini, M. Montagna, J. Mass Spectrom. 30 (1995) 1393.
[22] C.H.L. Shackleton, H. Chuang, J. Kim, X. de la Torre, J. Segura, Steroids 625 (1997) 523.
[23] U.S. Food and Drug Administration. Guidance for industry-Bioanalytical method validation. Available from: http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107.pdf (accessed 14.04.10).
[24] F.Y. Guan, C.E. Uboh, L.R. Soma, Y. Luo, J. Rudy, T. Tobin, J. Chromatogr. B 829 (2005) 56.
[25] E. PoP, D. Oniciu, M.E. Pape, C.T. Cramer, J.H. Dasseux, Croat. Chem. Acta 77 (2004) 301.
[26] F.Y. Guan, L.R. Soma, Y. Luo, C.E.S. Uboh, J. Am. Soc. Mass Spectrom. 17 (2006) 477.
[27] T.M. Williams, A.J. Kind, E. Houghton, D.W. Hill, J. Mass Spectrom. 34 (1999) 206.
[28] M.W.F. Nielen, J.J.P. Lasaroms, P.P.J. Mulder, J.V. Hende, J.A. van Rhijn, M.J. Groot, J. Chromatogr. B 830 (2006) 126.
[29] C.H.L. Shackleton, H. Chuang, J. Kim, X. de la Torre, J. Segura, Steroids 62 (1997) 523.
[30] L.R. Soma, C.E. Uboh, F. Guan, S. Mcdonnell, J. Vet. Pharmacol. Ther. 6 (2008) 587.
[31] R. Bethem, J. Boison, J. Gale, D. Heller, S. Lehotay, J. Loo, S. Musser, P. Price, S. Stein, J. Am. Soc. Mass Spectrom. 14 (2003) 528.