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Analysis of methyloxime derivatives of intact esters of testosterone and boldenone in equine plasma using ultra high performance liquid chromatography tandem mass spectrometry

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Analysis of equine plasma samples to detect the abuse of anabolic steroids can be complicated when the parent steroid is endogenous to the animal. Anabolic steroids are usually administered intramuscularly as synthetic esters and therefore detection of the exogenous esters provides unequivocal proof of illegal administration. An ultra high performance liquid chromatography tandem mass spectrometric (UPLC-MSMS) method for the analysis of esters of testosterone (propionate, phenylpropionate, isocaproate, and decanoate) and boldenone (undecylenate) in equine plasma has been developed. Esters were extracted from equine plasma using a mixture of hexane and ethyl acetate and treated with methoxyamine hydrochloride to form methyloxime derivatives. Metenolone enanthate was used as an internal standard. After chromatographic separation, the derivatized steroid esters were quantified using selected reaction monitoring (SRM). The limit of detection for all of the steroid esters, based on a signal to noise ratio (S/N) of 3 : 1, was 1–3 pg/mL. The lower limit of quantification (LLOQ) for the all of the steroid esters was 5 pg/mL when 2 mL of plasma was extracted. Recovery of the steroid esters was 85–97% for all esters except for testosterone decanoate which was recovered at 62%. The intra-day coefficient of variation (CV) for the analysis of plasma quality control (QC) samples was less than 9.2% at 40 pg/mL and less than 6.0% at 400 pg/mL. The developed assay was used to successfully confirm the presence of intact testosterone esters in equine plasma samples following intramuscular injection of Durateston® (mixed testosterone esters).

Keywords: steroids; esters; equine; plasma; methyloxime

Introduction

The analysis of equine plasma and urine samples for the detection of the abuse of androgenic anabolic steroids (AAS) has been performed at doping control laboratories around the world for many years and a number of methods have been developed to detect the parent steroids and their metabolites.[1–6] These methods provide excellent coverage for a wide range of steroids and have been used to successfully confirm the illegal use of such drugs on many occasions. Recent publications by McKinney[7] and Teale and Houghton[8] provide a comprehensive review and discussion of the metabolism and analysis of AAS in the equine. Determining abuse can, however, prove difficult when the parent steroid is endogenous to the animal.

The three AAS generally considered to be endogenous in the equine are testosterone, nandrolone, and boldenone. A comprehensive review of endogenous AAS in meat producing animals by Scarth *et al*.[9] reveals that testosterone is found in urine and plasma of both male and female horses at varying concentrations depending upon a number of factors. Nandrolone has been detected in the urine and plasma of intact male horses (colts) but not in geldings (castrated males) or mare/fillies. Boldenone has been detected in the urine of intact male horses but has not, to date, been detected in the urine or plasma of geldings and mare/fillies, or the plasma of colts.

Controlling the abuse of testosterone, nandrolone, and boldenone is complicated by their endogenous nature.[7–8] The concentrations of these three steroids vary between individuals and between the sexes, and may be elevated in individual animals due to endocrine disruptions such as pregnancy, residual testicular activity in a castrated male, or animals exhibiting both male and female sexual characteristics.[10–12] In a recent publication[13] it has also been suggested that nandrolone concentrations in cattle may be raised following trauma. Such considerations have led to control being established through the use of thresholds, either absolute or by ratio to other endogenous steroids, or by the ratio of one metabolite to another.

The naturally occurring endogenous AAS testosterone, nandrolone, and boldenone are usually administered to horses as their synthetically produced esters by intra-muscular injection. In this form and by this route of administration AAS achieve greater bioavailability and exert longer lasting effects.[14] A large number of esterified steroid preparations are available to buy, many via uncontrolled suppliers through the Internet. Not all of these preparations are licensed for use in the equine and therefore their safe administration is questionable. Regardless, in most rac-

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ing authorities around the world their use is either banned or controlled.

Once injected into muscle tissue the AAS are slowly released into the bloodstream, whereupon they are rapidly hydrolyzed by esterase enzymes into the free steroid, enabling interaction with a receptor. Generally, the longer the ester side chain the lower the water solubility of the compound and the longer it will take to diffuse into the bloodstream. Thus esters can be used to provide a constant and sustained dose of parent steroid, without the need for daily injections or oral administrations. To prolong and maximize this steady release, preparations are available as a mixture or esters of different chain length and/or structure.

From a regulatory point of view, the detection of an intact steroid ester would provide unequivocal evidence of an illegal administration of an endogenous steroid. However, the detection of intact steroid esters presents a number of analytical challenges which need to be overcome before the approach can be successfully applied in doping control. The main challenge is presented by the very low concentration of intact steroid ester found, following enzyme hydrolysis in the bloodstream. Therefore the techniques employed must be selective and exceptionally sensitive. Furthermore, the esters are also easily hydrolyzed by basic or acidic solutions and therefore extraction procedures have to be carefully designed to ensure that they remain intact prior to instrumental analysis.

Scientific literature contains a large body of work on the analysis of steroid esters in hair samples, but to date, there are few reports on the analysis of plasma samples. In 1986 Tang *et al*.[15] reported detection of three different testosterone esters by gas chromatography – mass spectrometry (GC-MS) following the intra-muscular injection of a solution of mixed esters to three geldings. However, this method required 10 mL of plasma and the detection period achieved (10 days) was less than that seen for testosterone in urine samples collected during the study, even at the International Federation of Horseracing Authorities (IFHA) and Fédération Equestre Internationale (FEI) threshold of 20 ng/mL. Shackleton *et al*.[16] described a method for detection of esters of testosterone in plasma following administration to human volunteers. These workers used the then relatively new technique of electrospray ionization (ESI) coupled to mass spectrometry to detect the esters. To achieve the high sensitivity required it was necessary to make use of a derivative (Girard P hydrazone), adding a complicated step to the extraction procedure. In 2000, Kim *et al*.[17] reported the measurement of intact esters of nandrolone in equine plasma, with limits of quantification ranging from 0.1 to 5 ng/mL. The reported method was used to analyze plasma samples generated following the administration of nandrolone decanoate to a Thoroughbred mare. Nandrolone was detected in plasma samples for 23 days post administration but the intact nandrolone decanoate could not be detected. Such observations are consistent with extensive hydrolysis and consequent low concentrations of intact ester. Recently, McKinney[7] referenced work presented by another group at an international conference reporting the detection of the enanthate ester of testosterone in plasma for 40 days post administration. This work shows that the analysis of equine plasma for the presence of intact esters of exogenously administered endogenous steroids is a promising route and one which requires further investigation.

Experimental

Materials

Methoxyamine hydrochloride was obtained from Sigma-Aldrich (Poole, UK), sodium hydroxide, hexane and ethyl acetate were from Fisher Scientific Ltd (Loughborough, UK), testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, testosterone decanoate, boldenone undecylenate and metenolone enanthate were from Steraloids Inc. (Newport, RI, USA). Equine plasma samples were obtained from stocks held at HFL Sport Science and previously shown to be free of steroid esters.

Animal administration and collection of samples

Plasma samples were obtained following the administration of Durateston®. A 500 mg dose of Durateston® (60 mg testosterone propionate, 120 mg testosterone phenylpropionate, 120 mg testosterone isocaproate and 200 mg testosterone decanoate) was administered by intra-muscular injection into the hindquarter muscle mass of a Thoroughbred filly. Post-administration blood samples were collected twice daily for the first 7 days after administration, then daily for 7 days, every other day for 16 days and finally every third day for 9 days. Total collection time for the study was 41 days post administration. Pre-administration blood samples were collected daily for five days prior to administration. Blood was collected into tubes containing sodium fluoride and potassium oxalate (Greiner Bio-One Ltd, Stonehouse, UK) in an effort to minimize esterase activity and to prevent clotting. The blood samples were centrifuged within 30 min of collection and the separated plasma stored at −20 °C until analyzed. The administration study was performed using ethically approved protocols.

Sample extraction and derivatization

To a 2 mL aliquot of equine plasma in an 8 mL screw-top glass tube 50 µL of a 20 ng/mL methanolic solution of metenolone enanthate (500 pg/mL) was added. The plasma was vortex mixed and 1.2 mL of 0.1M NaOH solution added and mixed again. After adding 4 mL of hexane : ethyl acetate (7 : 3, *v/v*) the tube was capped and rotary mixed for 60 min. The tube was centrifuged at 3000 rpm for 10 min after which the solvent layer was transferred to a clean glass tube (13 × 100 mm). The solvent was evaporated to dryness at 40 °C under a gentle stream of nitrogen and the dried residue was redissolved in 150 µL of hexane : ethyl acetate (7 : 3, v/v). The tube was vortex mixed and the hexane : ethyl acetate was transferred to a glass injection vial and evaporated to dryness at 40 °C under a gentle stream of nitrogen. 100 µL of 100 mM methoxyamine hydrochloride solution in 80% methanol (aqueous) was added to the vial and the vial capped and heated at 80 °C for 60 min to complete the methyloxime derivatization. A 20 µL aliquot was injected into the ultra-performance liquid chromatography (UPLC) system.

UPLC-MSMS

All samples were analyzed using a Waters Acquity UPLC coupled to a Waters Quattro Premier triple-quadrupole mass spectrometer (Waters, Milford, MA, USA). The source was operated in positive electrospray ionization mode, with the probe at 4.5 kV and a cone voltage of 50 V. The source temperature was 120 °C with a desolvation temperature of 450 °C. Nitrogen desolvation gas was applied at 800 L/hour and cone gas flow of 20 L/hour. The SRM

analysis was carried out using argon collision gas at a flow of 0.35 mL/min. SRM transitions were established by the infusion of 1 μg/mL solutions of the derivatized steroid esters at 10 μL/min into a mobile phase flow of 0.4 mL/min.

Chromatographic separation was achieved on a Waters Acquity BEH C18 reverse phase UPLC column (100 × 2.1 mm, 1.8 μm). A gradient elution program using 0.1% formic acid in methanol and 0.1% formic acid (aqueous) was employed, with the methanol concentration starting at 80% and being increased in a linear gradient to 100% after 4 min. A 1.5-min hold period at 100% methanol was followed by a rapid return to initial conditions and a 1.4-minute re-equilibration period. Total run time was 8.0 min. Flow rate was 0.4 mL/min and the column oven was maintained at 60 °C.

UPLC-MSMS quantification of steroid esters

Mixed calibration standards containing testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, testosterone decanoate, and boldenone undecylenate were prepared at concentrations of 100, 200, 400, 1000, 2000, 5000, 10 000, and 20 000 pg/mL in methanol. A calibration line was prepared in duplicate at concentrations of 5, 10, 20, 50, 100, 250, 500, and 1000 pg/mL by adding 100 μL of the appropriate spiking solution to 2 mL of equine plasma. An extracted standard calibration curve was incorporated before and after the analyses of each batch of samples.

Validation of the UPLC-MSMS method

The method was validated in terms of recovery, linearity, accuracy, precision, and sensitivity.

Extraction recoveries were investigated by spiking six different equine plasma samples (2 mL) with testosterone propionate, testosterone phenylpropionate, testosterone isocaproate, testosterone decanoate, and boldenone undecylenate at 1000 pg/mL. Separate aliquots were spiked before and after liquid/liquid extraction (LLE). Metenolone enanthate was added to the hexane : ethyl acetate organic phase after extraction at a concentration of 500 pg/mL. All extracts were derivatized with methoxyamine hydrochloride prior to analysis. The relative response for each steroid ester against metenolone enanthate was calculated and the pre- and post-extraction samples compared in order to calculate extraction efficiency.

Linearity was determined by the analysis of a spiked calibration curve, prepared in duplicate, at 5, 10, 20, 50, 100, 250, 500, and 1000 pg/mL. Two validation batches were prepared, extracted, derivatized, and analyzed on two separate days.

The lower limit of quantification (LLOQ) was determined by the analysis of six different equine plasma samples spiked with esters of testosterone and boldenone. Spiked samples were extracted and derivatized as described. The LLOQ was defined as the lowest concentration which returned a precision of <20% (% CV).

To assess the robustness of the method a mixed equine plasma sample was spiked with testosterone and boldenone esters at concentrations of 40 and 400 pg/mL. Six replicates at each concentration were extracted, derivatized and analyzed alongside the duplicate calibration curve to act as quality control (QC) samples. The QC samples were analyzed on two separate occasions to provide intra- and inter-day precision and accuracy calculations.

Analysis of post-administration plasma samples

Post-administration plasma samples obtained following the administration of Durateston® to a Thoroughbred filly were extracted and analyzed as described above. Extracts were quantified against an eight point calibration curve (in duplicate) and alongside QC samples (n=3) at concentrations of 40 and 400 pg/mL.

Results and Discussion

UPLC-MSMS

Derivatization of the 3-keto function of the steroid esters investigated during the study produced the methyloxime derivatives, shown in Figure 1. Infusion of the derivatized steroid esters produced prominent $[M+H]^+$ ions. Product ion scans of the protonated molecular ions produced limited fragmentation, which gave rise to a small number of candidate ions for use in SRM transitions. The product ion spectra obtained are shown in Figure 2. The SRM transitions selected for inclusion in the method are shown in Table 1 along with the collision energies employed. Derivatization efficiency was investigated by the analysis of a derivatized steroid ester standard (testosterone propionate, isocaproate, phenylpropionate, decanoate, and boldenone undecylenate) using both SRM transitions relating to the methyloxime derivatives and the non-derivatized steroid esters. The derivatized standard produced no significant peaks in the SRM transitions for non-derivatized esters, thereby indicating that the derivatization process was highly efficient.

The derivatized testosterone esters display prominent ions at *m/z* 126 and 138 and a minor ion at *m/z* 152, as shown in Figure 2. The pathways associated with the formation of these prominent A and B ring fragments, as shown in Figure 3, are fully discussed by Liu *et al*.,[18] and are consistent with the results obtained during the course of this study. Testosterone phenylpropionate also displays a prominent fragment ion at *m/z* 105, which is consistent with the presence of the phenylic group, as reported by Shackleton *et al*.[16] Boldenone undecylenate exhibits similar fragmentation through the A and B rings to produce major ions at *m/z* 120 and 150. The primary SRM transitions chosen for screening purposes were the

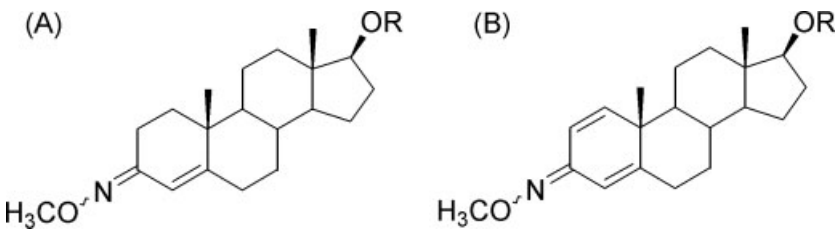

Figure 1. Structures of the methyloxime derivatives of testosterone (A) and boldenone (B); *E* and *Z* configurations are not specified. 'R' = ester side chain.

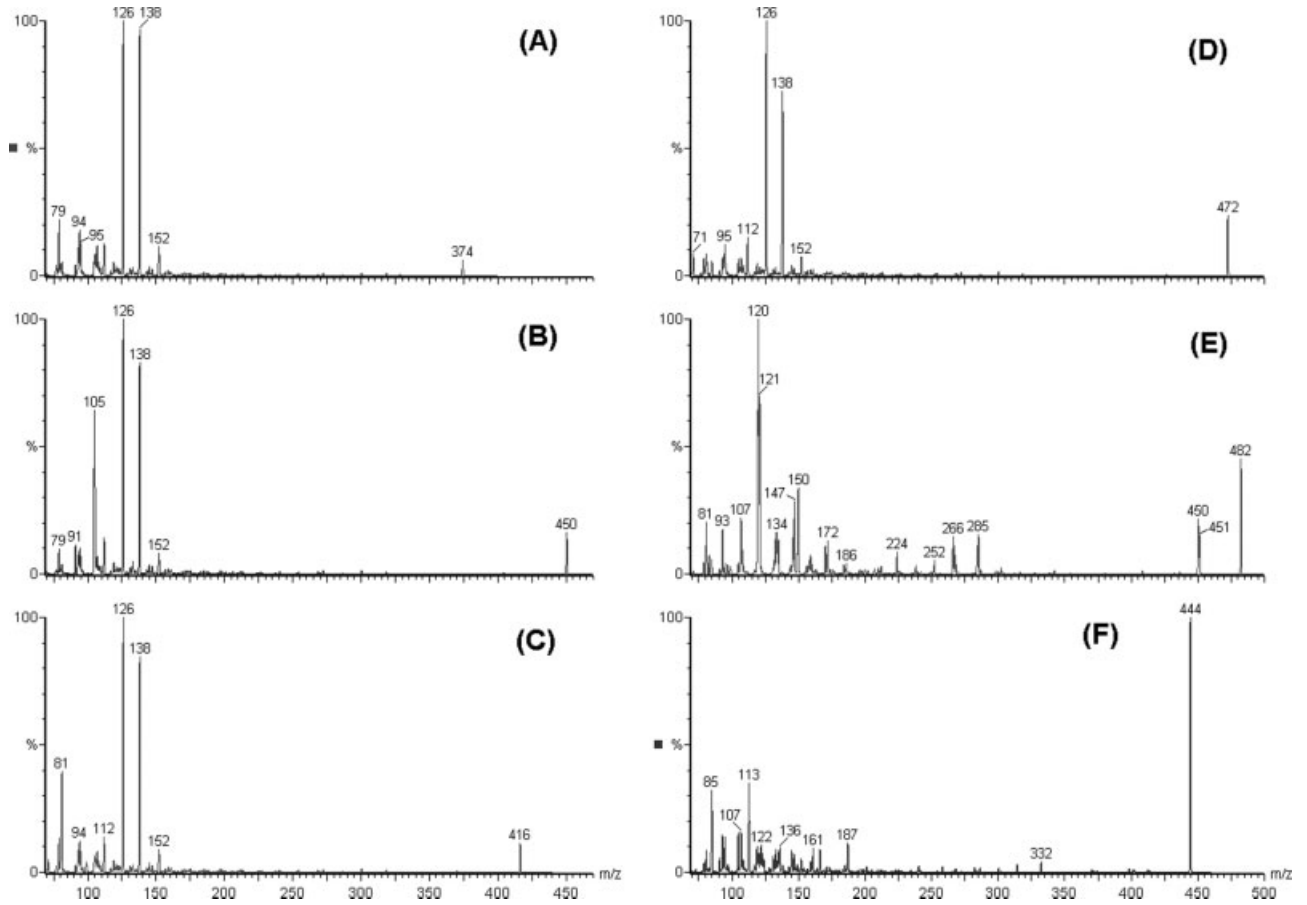

Figure 2. Product ion spectra of protonated molecular ions for the methyloxime derivatives of testosterone propionate (A), testosterone phenylpropionate (B), testosterone isocaproate (C), testosterone decanoate (D), boldenone undecylenate (E) and metenolone enanthate (F).

Table 1. SRM transitions and collision energies for methyloxime derivatives of intact steroid esters

	SRM transition for screening	2nd SRM for verification	Collision energy (eV)
Testosterone propionate	374 → 126	374 → 138	30
Testosterone phenylpropionate	450 → 126	450 → 105	35
Testosterone isocaproate	416 → 126	416 → 138	35
Testosterone decanoate	472 → 126	472 → 138	35
Boldenone undecylenate	482 → 120	482 → 150	25
Metenolone enanthate (IS)	444 → 113	–	30

$[M+H]^+$ to *m/z* 126 ion for testosterone esters and the $[M+H]^+$ to *m/z* 120 ion for boldenone undecylenate.

Separation of the methyloxime derivatives of the propionate, phenylpropionate, isocaproate, and decanoate esters of testosterone and the undecylenate ester of boldenone was achieved using a gradient elution and reversed phase C18 column. Derivatization with methoxyamine hydrochloride produced two baseline resolved chromatographic peaks for the esters of testosterone and metenolone enanthate, relating to the *E* and *Z* geometries. A single chromatographic peak was observed for the methyloxime derivative of boldenone undecylenate. It is not clear if this was due to the *E* and *Z* isomers not being resolved using the current UPLC conditions or as a result of the flatter A-ring conformation of boldenone causing one isomer to be thermodynamically favoured over the other. The SRM acquisition method was split into three time windows in order to maximize sensitivity; representative chromatograms of the SRM transitions used for screening are shown in Figure 4 for a 10 pg/mL spike and a blank plasma sample.

The formation of oxime derivatives of the 3-keto function of steroids offers potential improvements in sensitivity and specificity when using electrospray ionization (ESI), as reported by other groups.[16–20] The use of methyloxime derivatization during this study produced an approximate 2- to 4-fold increase in peak response (area) over the underivatized steroid esters investigated (B. Gray, unpublished work). Signal to noise ratios were increased for all steroid esters; however, the gains were not large (30–100%), as a result of the increased response being split over two chromatographic peaks. Background interference was not found to increase significantly as a result of the derivatization process. The increase in method sensitivity, whilst small, was considered to

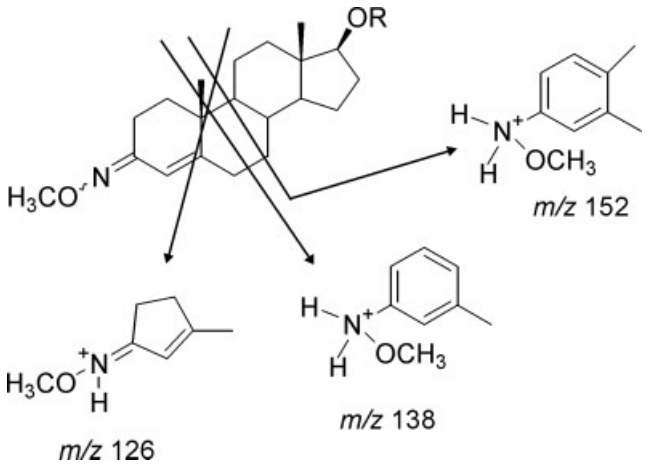

Figure 3. Fragmentation pathway of the methyloxime derivative of testosterone esters. 'R' = ester side chain.

be a worthwhile improvement and also offered the added benefit of increasing confidence in any positive finding as both the *E* and *Z* isomers could be observed.

Validation of the UPLC-MSMS method

The developed method was validated for linearity, sensitivity, precision, accuracy, and extraction efficiency. The validation data is summarized in Table 2. Calibration curves covering the range 5–1000 pg/mL were prepared in duplicate in pooled equine plasma and extracted as described. Calibration curves were obtained with correlation coefficients between 0.990 and 0.998 over two validation batches, which indicated good linear regression. The lower limit of quantitation was determined by the repeat analysis of six different equine plasma samples spiked with testosterone and boldenone esters. The LLOQ was defined as the lowest concentration which produced a % CV of less than 20%. For all of the esters investigated the LLOQ was 5 pg/mL, with % CVs of between 8.0 and 17.3% at this concentration. The limit of detection (LOD) was not determined directly, but analysis of the LLOQ samples and post-administration samples indicated that the LOD for all of the steroid esters investigated would be in the range of 1–3 pg/mL, at a S/N ratio of greater than 3 : 1.

The results of intra- and inter-day precision and accuracy are shown in Table 3. Six quality control samples at 40 and 400 pg/mL were extracted and analyzed on two separate occasions to provide intra- and inter-day results. The intra-day precision ranged from 4.5 to 9.2% for all analytes at both concentrations while the accuracy ranged from −5.0 to 5.3%. Inter-day precision ranged from 4.9 to 8.9% and the accuracy from −4.7 to 5.5%. These results show that the assay is accurate and reproducible over the concentration range validated. Metenolone enanthate was used as an internal standard and can be regarded as having intermediate chain length in this assay, i.e. seven carbons. It operates adequately as an internal

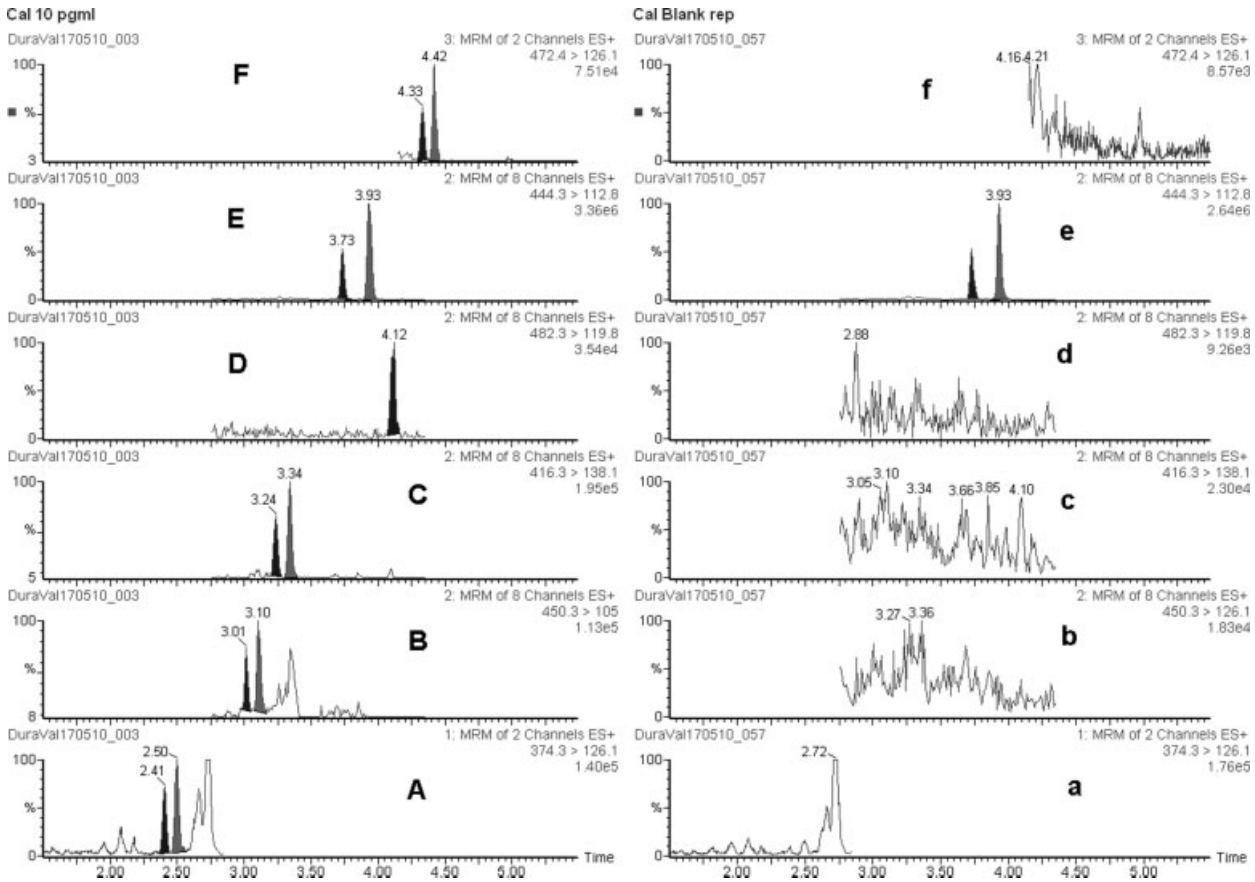

Figure 4. SRM chromatogram of equine plasma sample spiked at 10 pg/mL with testosterone propionate (A), testosterone phenylpropionate (B), testosterone isocaproate (C), testosterone decanoate (F), boldenone undecylenate (D) and metenolone enanthate (E). Blank equine plasma sample (spiked with metenolone enanthate) also shown (a,b,c,d,e,f).

Table 2. Validation data for the analysis of testosterone and boldenone esters in equine plasma

	Concentration range (pg/mL)	Linearity (R^2)	LLOQ (pg/mL)	% CV of LLOQ	Extraction Efficiency (%)
Testosterone propionate	5–1000	0.994	5	9.8	91.1
Testosterone phenylpropionate	5–1000	0.995	5	8.0	97.5
Testosterone isocaproate	5–1000	0.994	5	9.8	88.5
Testosterone decanoate	5–1000	0.997	5	11.4	62.2
Boldenone undecylenate	5–1000	0.993	5	17.3	85.2

Table 3. Precision[a] and accuracy[b] for the determination of esters of testosterone and boldenone

	Nominal Concentration (pg/mL)	Intra-day		Inter-day	
		Precision % CV	Accuracy % RE	Precision % CV	Accuracy % RE
Testosterone propionate	40	9.2	4.0	5.7	−0.3
	400	6.4	−1.6	4.9	−4.7
Testosterone phenylpropionate	40	7.3	3.3	8.9	4.2
	400	6.1	−1.4	8.6	−1.2
Testosterone isocaproate	40	5.7	5.3	7.3	3.5
	400	5.9	−1.3	7.4	−1.2
Testosterone decanoate	40	5.9	2.5	5.7	5.5
	400	6.0	−1.5	7.0	−1.1
Boldenone undecylenate	40	5.1	−0.4	5.6	2.6
	400	4.5	−5.0	6.3	−1.5

[a] Expressed as coefficient of variance of the peak area ratios of analyte/internal standard.
[b] Calculated as [(mean calculated concentration − nominal concentration)/nominal concentration] ×100.

standard for all of the esters investigated during the course of this study producing % CV's of under 20%.

Extraction efficiency was calculated by spiking six different equine plasma samples with testosterone and boldenone esters at a concentration of 1000 pg/mL. Once extracted, these were analyzed and compared with the same six equine plasma samples spiked post extraction at 1000 pg/mL. The internal standard, metenolone enanthate was added to all samples post extraction. Extraction efficiency for testosterone propionate, phenylpropionate, isocaproate and boldenone undecylenate was between 85 and 97%, with testosterone decanoate being extracted at 62% efficiency.

Analysis of post-administration plasma samples

The analytical procedure was applied to the analysis of plasma samples generated following the intra-muscular administration of 500 mg of Durateston® to a Thoroughbred filly. Blood samples were collected into tubes containing NaF as a potential esterase inhibitor. Previous investigations performed at our laboratory (B. Gray, unpublished work) had shown that the use of tubes containing NaF reduced the enzyme hydrolysis of steroid esters in equine blood stored at 4 °C for 72 hours by approximately 50% when compared to standard lithium heparin tubes. Blood samples were extracted and analyzed as described. The pre-administration blood samples collected prior to injection did not reveal any interference in the SRM transitions used for screening purposes.

All four of the testosterone esters contained in Durateston® were detected for over 200 hours post administration. As the smallest ester, testosterone propionate was quickly released into the bloodstream, with maximum plasma concentrations reached within 6 hours. The maximum concentration observed for testosterone propionate was 1000 pg/mL, considerably higher than that seen for the other three esters. The two mid-sized esters in the Durateston® mix, testosterone phenylpropionate and testosterone isocaproate, were detected for significantly longer than the propionate ester, although maximum concentrations were considerably lower.

Having the largest ester side chain, testosterone decanoate was expected to have the longest detection period following intra-muscular administration. The increased hydrophobicity of this compound results in a very slow release into the bloodstream, which in turn results in long detection periods but also very low concentrations of circulating drug. Testosterone decanoate is present in Durateston® at 200 mg per 500 mg and is therefore the major constituent. However, the maximum concentration observed in post-administration samples was around 100 pg/mL, significantly lower than the other three esters present. Using the method presented it was possible to detect testosterone decanoate in all of the samples analyzed during the course of this study.

Verification of the presence of testosterone decanoate was achieved by the use of two SRM transitions along with a retention time matched to a reference standard of the detected analyte. Figure 5 shows data obtained for testosterone decanoate in the 500 hours post-dose sample, along with the matching SRM traces for a testosterone decanoate reference standard and a pre-administration blank plasma sample from the same horse.

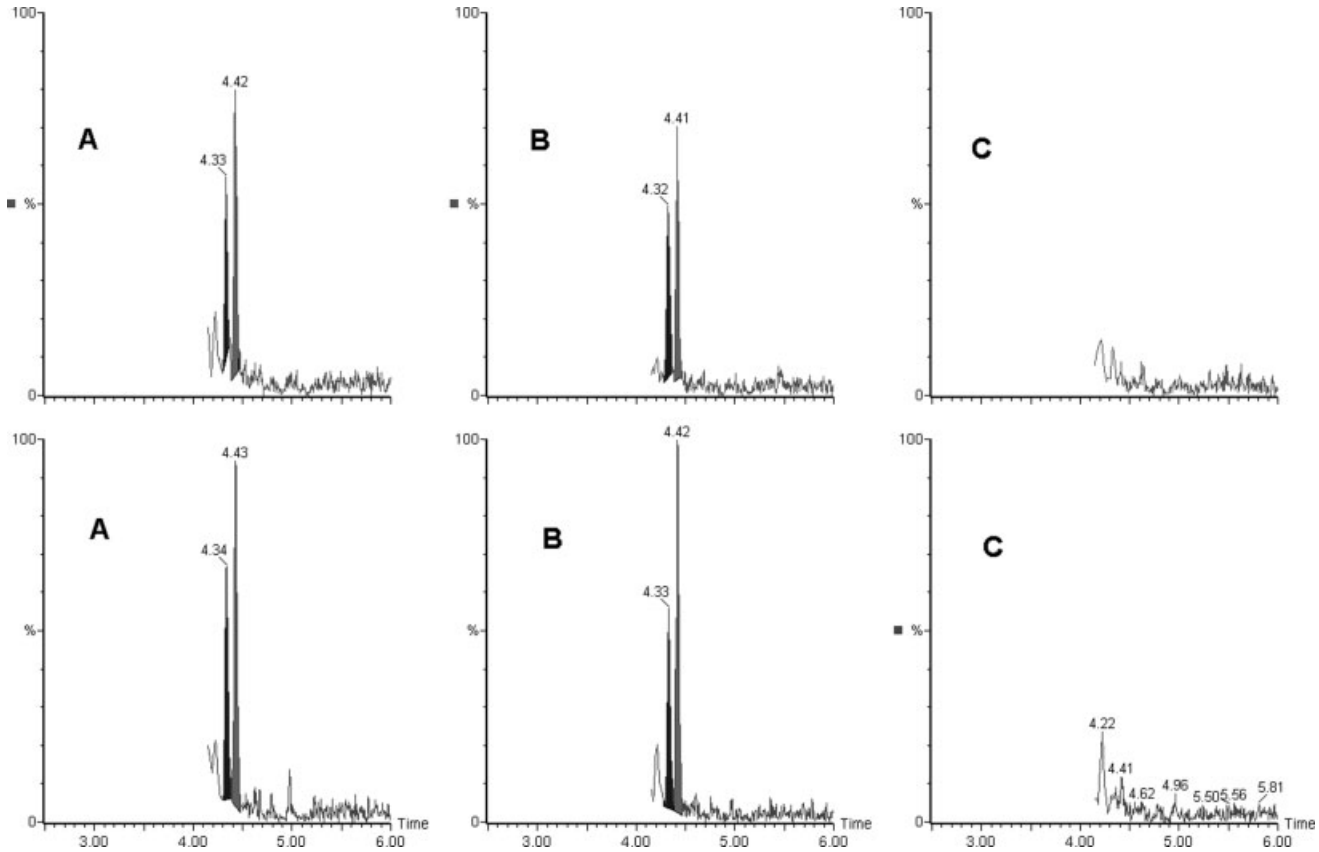

Figure 5. Verification data, showing two SRM transitions for testosterone decanoate. Reference standard (A), 500 hours post dose sample (B), pre-administration blank sample (C).

Other applications

The developed extraction and analysis method can be used for the analysis of a wide range of steroid esters in equine plasma and quickly expanded to include additional steroid esters if required. In our laboratory, the method has been used for the detection of various esters of testosterone, boldenone, and nandrolone in equine plasma samples. Additional SRM transitions were added to the method and used to successfully analyze post-administration plasma samples following the administration of boldenone undecylenate, nandrolone laurate, and nandrolone phenylpropionate.

Conclusions

A method for the extraction, derivatization, and analysis of intact esters of testosterone and boldenone in equine plasma has been successfully validated. The validated method was used to analyze post-administration blood samples generated following the intramuscular injection of Durateston® to a Thoroughbred filly. All four of the testosterone esters present in Durateston® were detected in plasma for a minimum of 200 hours post administration. Testosterone decanoate was the longest lasting ester, being detected in plasma for the full duration of the study. This method offers routine screening of equine plasma samples for the presence of the intact esters of anabolic steroids known to be endogenous to the horse, namely testosterone, nandrolone, and boldenone. Detection of intact steroid esters provides unequivocal proof of illegal administration and further development of this method in our laboratory is ongoing.

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