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An interlaboratory study of the pharmacokinetics of testosterone following intramuscular administration to Thoroughbred horses

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Testosterone is an anabolic androgenic steroid (AAS) that is endogenously produced by both male and female horses that also has the potential for abuse when administered exogenously to race horses. To recommend appropriate withdrawal guidelines so that veterinarians can discontinue therapeutic use prior to competition, the pharmacokinetics and elimination of testosterone were investigated. An aqueous testosterone suspension was administered intramuscularly in the neck of Thoroughbred horses (n = 20). The disposition of testosterone from this formulation was characterized by an initial, rapid absorption phase followed by a much more variable secondary absorption phase. The median terminal half-life was 39 h. A second focus of this study was to compare the testosterone concentrations determined by two different laboratories using a percentage similarity model with a coefficient of variation of 16.5% showing good agreement between the two laboratories results. Based on the results of this study, a withdrawal period of 30 days for aqueous testosterone administered IM is recommended.

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INTRODUCTION

Testosterone is an anabolic androgenic steroid (AAS) used in the horse and is classified as a Class 3 drug by the Association Racing Commissioners International (ARCI). It is a potent sex steroid hormone that is produced endogenously in varying degrees by both intact male and female horses (Silberzahn *et al.*, 1983; Inoue *et al.*, 1993; Bonnaire *et al.*, 1995; Roser, 2008). Testosterone is available in several different pharmaceutical formulations and is recommended for use in veterinary medicine to treat chronic wasting conditions and improve appetite and physical appearance. In addition, veterinary compounding pharmacies offer for sale numerous testosterone ester preparations in oil and testosterone suspension in aqueous vehicle. Recent published reviews describing the effects of testosterone and other AAS have found little evidence for their continued use. This is simply because of a the lack of studies showing efficacy or enhancement of performance along with the strong negative correlation on breeding careers of horses (Berndtson *et al.*, 1979; Squires *et al.*, 1982; Maher *et al.*, 1983; Pitts & Davis, 2007; Fajt & McCook, 2008).

Several studies characterizing the pharmacokinetics of testosterone in the horse have been undertaken (Houghton & Dumasia, 1979; Thompson *et al.*, 1980; Dumasia & Houghton, 1981; Bonnaire *et al.*, 1995). However, many of these studies focused on either the determination of testosterone administration or the pharmacodynamic effects of the drug. To the best of the authors' knowledge, there is only one previous report describing the pharmacokinetics of testosterone describing the administration of testosterone enanthate to eight horses (Martinez *et al.*, 1991).

With the advent of more sensitive analytical instrumentation used in testosterone analysis, specificity has increased and limits of detection (LOD) and limits of quantitation (LOQ) have improved when compared to previously used radioimmunoassay (RIA) (Thompson *et al.*, 1980; Martinez *et al.*, 1991) and gas chromatography–mass spectrometry (Houghton & Dumasia, 1979; Bonnaire *et al.*, 1995) methods. The improvements from these methodologies may ultimately allow for longer detection periods following testosterone administration.

Because of the lack of information detailing the pharmacokinetics of testosterone, a study was undertaken to determine the pharmacokinetics of aqueous testosterone following IM administration. In the United States, there are several regulatory laboratories responsible for testing samples collected from performance horses. Each laboratory may utilize different

technologies and techniques and have differing capabilities. Therefore, a secondary goal of this study was to compare results obtained from concurrent analysis of plasma testosterone concentrations by two analytical laboratories. Information obtained from these studies will be used to develop withdrawal guidelines.

MATERIALS AND METHODS

Animals

Twenty healthy adult Thoroughbred horses (13 geldings and seven mares with a mean ± SD weight of 534.1 ± 43.1 kg and an age of 7.0 ± 1.8 years) were selected for the study. All horses were considered fit, as assessed by the ability to run one mile in 2 min without undue stress. Food and water were available *ad libitum* throughout the study. Horses did not receive any medications for at least 2 weeks prior to commencement of this study. Administrations for this study were conducted at the University of Florida (UF), and the study was approved by the Institutional Animal Care and Use Committee.

Drug administration

Horses received a single IM injection in the neck of 0.15 mg/kg of an aqueous compounded testosterone suspension (75 mg/mL; Franck's Pharmacy, Ocala, FL, USA). Each horse was weighed prior to drug administration.

Sample collection

Blood samples were collected immediately before (predose control) and at 1, 2, 4, 6, 8, and 24 h, and at 2, 3, 4, 5, 7, 9, 11, 14, 17, 21, 25, 28, and 34 days postdrug administration. Blood samples were collected from each horse until there were at least two consecutive samples with no detectable testosterone. Blood samples were collected into lithium heparin blood tubes (15 USP units; Becton Dickinson, Franklin Lakes, NJ, USA) and stored on ice until centrifugation at ~1300 ***g*** at 4 °C for 15 min. Plasma was immediately transferred into cryovials (Phenix Research Products, Chandler, NC, USA) and stored at −70 °C until analysis. Plasma samples were split with one set shipped frozen by overnight courier to the University of California – Davis (UCD) for analysis and the remaining set being analyzed at UF.

Chemicals and reagents

For samples analyzed at UF, testosterone was purchased from US Pharmacopeia (Rockville, MD, USA) and d3-testosterone for use as the internal standard (IS) was purchased from Cerilliant (Round Rock, TX, USA). All working standard solutions were quantitatively prepared in methanol. Separate working standard solutions of testosterone were used for the generation of calibrators and quality control samples (QCs). Standard solutions were validated by LC-MS–MS to ensure minimum variability in the concentration from different preparations. HPLC grade solvents including methanol and methyl-*tert*-butyl ether (MTBE) were purchased from Fisher Scientific (Fairlawn, NJ, USA). Deionized water used for dilutions was generated in house using a Millipore water purification system (Millipore, Billerica, MA, USA).

For samples analyzed at UCD, separate working solutions used for the generation of calibrators and QCs were used. Testosterone and d3-testosterone (IS) were purchased as powders from Steraloids (Newport, RI, USA), and a 1.0 mg/mL solution of testosterone was purchased from Cerilliant. Testosterone from Steraloids was used in the generation of calibrators, and testosterone from Cerilliant was used in the generation of QCs. Acetonitrile, MTBE, methanol, and water were HPLC grade and purchased from Burdick and Jackson (Muskegon, MI, USA). Acetone, isopropanol, and ammonium hydroxide were Optima grade and purchased from ThermoFisher (St Louis, MO, USA). ACS grade formic acid was purchased from EMD (Gibbstown, NJ, USA).

Sample analysis

Plasma samples were analyzed in racing chemistry laboratories located at the UF and the UCD. Samples were analyzed in both laboratories' using liquid–liquid extraction (LLE) and quantified by high-performance liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a triple quadrupole mass spectrometer with electrospray ionization operating in the positive mode. Both laboratories independently developed and validated analytical methods to quantify testosterone. Both laboratories methods assessed inter-/intra-assay precision and accuracy, analyte recovery, ion suppression, assay linearity, and analyte stability (freeze/thaw and long term) during the validation of the methods and the results met guidelines outlined by the Food and Drug Administration (FDA) guidelines for bioanalysis (FDA, 2001). Both laboratories used Thermo triple stage quadrupole mass spectrometers that were controlled using Xcalibur 2.0 or 2.07 software (Thermo Scientific, San Jose, CA, USA). The UF used a method targeting only testosterone while the UCD laboratory used a multiple analyte method to quantify testosterone along with three additional AAS.

UCD employed a LC-MS/MS system consisting of a TSQ Vantage triple quad mass spectrometer (Thermo, San Jose, CA, USA) equipped with a TLX2 turbulent flow chromatography HPLC system (ThermoFisher Scientific Inc., Franklin, MA, USA). This method was developed and validated with the details of this method reported elsewhere (Moeller *et al.*, 2010). Briefly, 1 mL of plasma fortified with an IS of d3-testosterone was extracted by LLE using MTBE. Extracts were dried and dissolved in 100 μL of 90/10 water/methanol and 30 μL injected onto the LC-MS/MS system. Linear calibration curves were generated from matrix-matched calibrators from 25 to 10 000 pg/mL by weighted ($1/X$) linear regression analysis using the ratio of analyte peak area to IS peak area by the equation $y = m(x) + b$. The LOD for testosterone was 10 pg/mL and the LOQ was 25 pg/mL. QCs ($n = 6$ per concentration) were used at three concentrations (75, 750, 3000 pg/mL) to asses inter-/intra-assay accuracy and

precision and were analyzed with samples during analysis. Testosterone was eluted at a retention time of 1.55 min and was quantified by highly selective reaction monitoring (HSRM) of the 289.2 *m/z* precursor ion and the 97.1 *m/z* product ion. Qualitative determination was confirmed by monitoring four additional product ions (77.1, 79.1, 81.1, and 109.1 *m/z*). Samples from testosterone administrations were analyzed once (repeat analysis was conducted on outliners) and results were used in pharmacokinetic analysis.

For samples analyzed at UF, 1 mL of plasma was supplemented with d3-testosterone in 0.5 mL of 0.9% saline solution and subjected to LLE using 5 mL of MTBE. Extracts were dissolved in 60 μL of 50% methanol in water with 0.1% formic acid and 20 μL injected onto an LC-MS/MS system consisting of a TSQ Quantum Ultra triple quad mass spectrometer (Thermo, San Jose, CA, USA) equipped with a Accela HPLC system (ThermoFisher Scientific Inc.) and a CTC PAL auto-sampler (Leap Technologies, Carrboro, NC, USA). Testosterone was separated over a 6-min method using a linear reverse-phase gradient consisting of water with 0.1% formic acid (solvent A) and methanol with 0.1% formic acid (solvent B) on a T3 UPLC column (1.8 μm, 2.1 × 50 mm; Waters Corp., Milford, MA, USA) with a guard column (Waters Corp.) held at 25 °C. The gradient consisted of the following steps 50% solvent A from the start to 10% solvent A at 4 min, isocratic at 10% solvent A from 4.0 to 4.55 min, and return to 50% solvent A at 4.55 min. Testosterone and d3-testosterone were detected by selected reaction monitoring (SRM) using the 97 *m/z* product ion for both testosterone and the IS with a LOD of 10 pg/mL and LOQ of 25 pg/mL. QCs (n = 2) used at three concentrations (40, 500, 2500 pg/mL) were run with each batch. Linear calibration curves were generated from matrix-matched calibrators from 25 to 10 000 pg/mL by weighted ($1/X^2$) linear regression analysis using the ratio of analyte peak area to IS peak area by the equation $y = m(x) + b$. Study samples were run in duplicate, and the average of the two determinations was used in pharmacokinetic analysis.

Pharmacokinetic calculations

Plasma concentration vs. time data were analyzed by noncompartmental analysis (NCA) by the linear trapezoidal rule using commercially available software (WinNonlin version 5.2; Pharsight, Cary, NC, USA). Data points included in the analysis were plasma concentrations until drug was no longer detectable. Pharmacokinetic parameters are reported as median and range from each testing laboratory.

Statistical analysis

Plasma concentrations were determined at both laboratories and the percent similarity model was used to assess the agreement between the laboratories (Graph-Pad Prism version 5; GraphPad Software, La Jolla, CA, USA) (Scott *et al.*, 2003). Pharmacokinetic parameters generated by NCA from each laboratory's results were compared using the Mann–Whitney rank sum test (SigmaPlot version 11; Systat Software, San Jose, CA, USA).

RESULTS

Median plasma concentrations at each time point from both laboratories following IM administration of aqueous testosterone suspension to 20 horses are shown in Fig. 1. Pharmacokinetic parameters including λ_z, $T_{1/2}$ λ_z, $AUC_{0-T\text{last}}$, $AUMC_{0-T\text{last}}$, plasma clearance per fraction of the dose absorbed (Cl/F), apparent volume of distribution per fraction of the dose absorbed (Vz/F), $T_{\max}$, T_{last}, and $C_{\max}$ were determined for each horse by NCA. The median pharmacokinetic parameters along with the minimum and maximum are shown in Table 1. Pharmacokinetic parameters were determined from data generated at both laboratories.

The percentage similarity between the results from the two laboratories was 92.5%, with a mean percentage difference of 7.45%, and a standard deviation of 15.3% (Table 2). A percent similarity histogram comparing the two laboratories results is shown in Fig. 2. A comparison of authentic testosterone reference standards used for calibration and QCs between the two laboratories was undertaken by injecting equivalent amounts of standards from both labs onto the LC-MS/MS system. An 8% difference in the concentration of testosterone between the calibration solutions used at the UF and UCD laboratories was observed (data not shown).

DISCUSSION

Testosterone is a naturally produced AAS with a high potential for abuse in horseracing and as such is classified as a Class 3 drug by the ARCI. The primary goal of this study was to determine the plasma pharmacokinetics following IM administration of aqueous testosterone in geldings and mares to recommend a withdrawal time prior to racing. The secondary goal was to provide a comparison of the quantitation of testosterone following administration by two separate laboratories using similar but different methodologies.

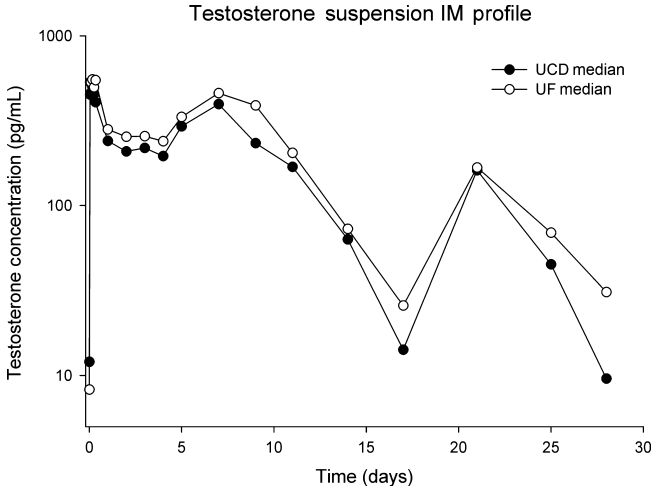

Fig. 1. Concentration vs. time profiles. Data are shown as the median for each laboratory following aqueous testosterone IM administration.

Table 1. Select pharmacokinetic parameters – pharmacokinetic parameters determined by noncompartmental analysis for each horse (n = 20) were determined and the median, minimum, maximum are shown. Results from data analyzed at University of California – Davis are shown in (a), while results from data analyzed at University of Florida are shown in (b)

Horse	λ_z (1/h)	$T_{1/2}$ λ_z (h)	$AUC_{0-Tlast}$ (h·pg/mL)	$AUMC_{0-Tlast}$ (h·h·pg/mL)	Vz/F (L/kg)	Cl/F (L/h/kg)	T_{max} (h)	C_{max} (pg/mL)	T_{last} (h)
(a)									
Median	0.021	33.0	9.13×10^4	1.28×10^7	73.6	1.63	6.00	646	336
Min	0.012	16.3	6.69×10^4	5.61×10^6	42.9	1.33	1.00	302	216
Max	0.042	56.8	1.10×10^5	2.82×10^7	137	2.18	408	1308	672
(b)									
Median	0.018	39.0	1.09×10^5	1.61×10^7	69.0	1.35	6.00	759	336
Min	0.007	15.9	8.63×10^4	7.46×10^6	37.9	1.12	1.00	361	216
Max	0.044	104	1.34×10^5	4.15×10^7	173	1.74	408	1270	672

Table 2. Percent similarity model – plasma testosterone concentrations were compared between the two laboratories using the percentage similarity model and the mean, standard deviation (SD), mean percentage difference (MPD) and co-efficient of variation (CV) are shown

Mean similarity	SD similarity	MPD ± SD	CV
92.5%	15.3%	7.45 ± 15.3%	16.5%

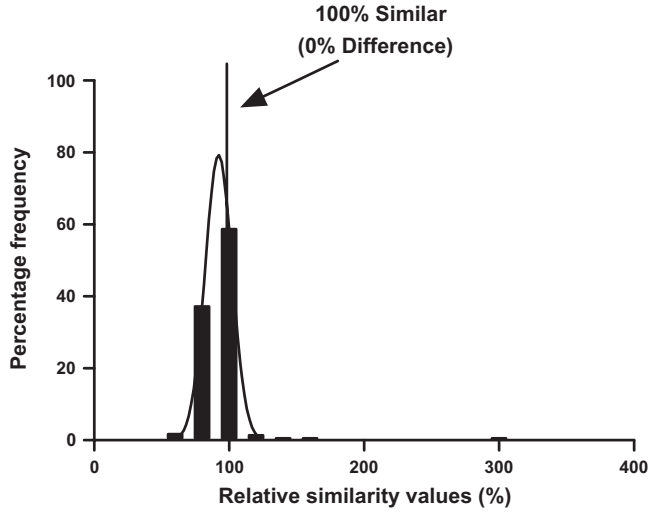

Fig. 2. A percentage similarity histogram comparing testosterone concentrations determined at University of California – Davis and University of Florida.

In the study reported here, the disposition of aqueous testosterone following IM administration was highly variable. This is especially evident in the comparison of C_{max} values between horses. Horses administered IM aqueous testosterone suspension had what appeared to be a very rapid absorption phase with T_{max} for most horses appearing between 1 and 2 h following drug administration (Fig. 1). This was followed by a slow decrease in plasma testosterone concentrations and a second peak in plasma concentration between 4 and 17 days after administration. Several horses had two or more peaks which lead to the high variability seen in the T_{max} from horses investigated (Table 1). The large variability in T_{max}, C_{max} and the presence of multiple local maxima has been seen in horses following IM testosterone enanthate adminstration (Martinez *et al.*, 1991). The early peak concentration of testosterone is attributed to absorption of testosterone in solution from the formulation after IM administration. The second and subsequent peaks are attributed to dissolution of testosterone from the solid material in the suspension with similar results being reported in humans (Misra *et al.*, 1997).

Although IV administration has not been investigated in the horse, several authors have monitored the reduction or disappearance of testosterone following castration of intact males, with testosterone no longer being detectable or reaching a minimum basal level in <12 h (Ganjam & Kenney, 1975; Thompson *et al.*, 1980; Martinez *et al.*, 1991). A previous study determined two half-lives with distribution and elimination half-lives of 48 and 173 min, respectively (Thompson *et al.*, 1980). These studies were not designed as pharmacokinetic studies and relied on less selective RIA in the determination of testosterone concentrations although they do provide information that the elimination half-life of testosterone following castration is <3 h in recent castrates. This is in contrast to the studies detailed in this paper with terminal half-lives ranging from 15.9 to 104 h. The large difference between the elimination half-lives following castration and IM administration clearly shows that the clearance of free testosterone is not the rate limiting step but rather that the release or absorption of testosterone from the injection site into the central compartment controls the terminal slope of the concentration vs. time profile. This large difference in half-lives suggests that flip-flop kinetics are occurring after both IM and SC administration of aqueous testosterone and testosterone cypionate as has been observed in humans following nandrolone administration (Van der Vies, 1985). However, pharmacokinetic studies after IV administration are necessary for a definitive conclusion.

Testosterone was no longer detected in any horse dosed with aqueous testosterone IM after 28 days (672 h) and the last collection (T_{last}) with a detectable concentration of testosterone was highly variable. The earliest T_{last} was observed at 9 days (216 h), and the median was 336 h postadministration. A recommended withdrawal guideline for aqueous testosterone is 30 days following IM administration. The administration of

aqueous testosterone at a dose higher than 0.15 mg/kg or via an alternate route or site of administration may result in altered pharmacokinetics. Therefore, the 30 day withdrawal guideline only applies to horses dosed identically as those in the study. The use of a compounded testosterone formulation may also result in altered pharmacokinetics as the strength, purity, and other formulation factors of these products are not assured as in FDA approved formulations. Testosterone, like many other endogenous steroids, may produce pharmacodynamic effects at concentrations less than its limit of quantification in plasma. Measurements of pharmacodynamic responses were not included in this study.

Testosterone is rapidly cleared as demonstrated by the fast decrease in testosterone concentrations following castration. The administration of ^{14}C-testosterone to a gelding resulted in a T_{max} of 3 h with labeled testosterone no longer being detectable in plasma after 24 h and the majority cleared from urine by 200 h (Houghton & Dumasia, 1979). Testosterone is extensively metabolized by a large number of drug metabolizing enzymes, and its *in vivo* metabolism has been studied by several groups following IM administration with <5% of testosterone excreted in the urine unchanged (Houghton & Dumasia, 1979; Dumasia & Houghton, 1981; Bonnaire *et al.*, 1995). Testosterone undergoes both phase I and II metabolism in the horse with the majority of urinary metabolites being 17-hydroxylated compounds that are conjugated with sulfate (~66%) and the remainder with glucuronic acid moieties (Dumasia & Houghton, 1981). The most commonly used markers of testosterone administration in either mares or geldings following cleavage of phase II metabolites in urine are elevated concentrations of 5α-androstane-3β,17α-diol in the glucuronide fraction and 5α-androstane-3β,17α-diol and testosterone in the sulfate fraction (Dumasia & Houghton, 1981; Bonnaire *et al.*, 1995). Plasma testosterone sulfate and glucuronide concentrations following administration to two mares were markedly increased over basal values with testosterone glucuronide being found to be a good marker of testosterone administration (Bonnaire *et al.*, 1995). Some investigators have measured testosterone in plasma to detect the administration of testosterone in the horse with a threshold value of 2000 pg/mL for stallions and 200 pg/ml for geldings and mares (Soma *et al.*, 2008).

A secondary goal of the study reported here was to compare the reproducibility of measurements from two different laboratories and their ability to provide similar withdrawal time estimates. A comparison of plasma testosterone concentrations determined in the two laboratories was undertaken using a percent similarity model (Scott *et al.*, 2003). Differences in values were seen for several time points although there was good agreement in concentrations between the two laboratories for the majority of samples with a coefficient of variation of 16.5%. These data suggest that laboratories operating under similar, although not identical conditions, can achieve reproducible results for the quantitative determination of testosterone in plasma. The bias observed between values reported by the laboratories can partially be explained by the 8% difference in concentration between the working standard solutions used to prepare the calibrators at each laboratory. Possible explanations for this difference are evaporation of the solvent over time leading to higher concentration remaining in the vial, variability in weighing or diluting, and the differences in purity, stability, and storage of the analytical standards used to prepare the working standard solutions.

In addition to the percent similarity analysis of the plasma concentrations, a statistical comparison of pharmacokinetic parameters determined by the individual laboratories was undertaken using the Mann–Whitney rank sum test. Differences between the two data sets were seen for *AUC* ($P < 0.001$) and Cl/F ($P < 0.002$). Both of the differences in the *AUC* and Cl/F can be explained by the different plasma concentrations determined at either laboratory as seen by the percent similarity analysis (Table 2). No other differences in the selected pharmacokinetic parameters were observed.

CONCLUSION

In conclusion, there are large inter-individual differences in pharmacokinetics following IM administration of aqueous testosterone in the Thoroughbred horse. A withdrawal guideline of 30 days following IM administration of a single dose of 0.15 mg/kg is recommended. Furthermore, as demonstrated by the study reported here, different regulatory laboratories using similar methods can produce similar results for the quantitative analysis of testosterone in equine plasma.

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