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Pharmacokinetics of Two Formulations of Altrenogest Administered to Mares

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ABSTRACT

Altrenogest is a synthetic progestin that suppresses reproductive behaviours and assists pregnancy maintenance in female horses. Two formulations are available, a 'weekly' intramuscular injection and a daily oral formulation. Altrenogest administration has returned positive swabs for steroids; consequently, using injectable altrenogest in racing mares is prohibited. Oral administration may be permitted in race mares if there is one clear day between dosing and racing. The only pharmacokinetic data available were generated from geldings. Therefore, to assist veterinarians and analysts in determining accurate dosing and detection intervals, pharmacokinetic analysis using mares is required. Blood samples were taken from 10 mares pretreatment to obtain baseline concentrations. Mares were administered altrenogest, either oral (PO; 0.044 mg/kg; daily for 15 days) or intramuscular (IM; 0.3 mg/kg; twice; Days 0 and 7). On the first and last treatment day, blood samples were taken at designated times post dosing. After a 3-week washout, mares received the alternative treatment with sampling repeated. At the initial dose, for IM administration mean (± SD) plasma altrenogest C_{max} was 18.0 ± 6.6 ng/mL at 7.9 ± 3.9 h compared with PO dosing 13.2 ± 5.8 ng/mL at 0.8 ± 0.8 h. Plasma C_{max} on the final day was significantly higher ($p = 0.002$ [IM]; $p = 0.006$ [PO]). At 24 h post final oral treatment, mean (± SD) plasma altrenogest was 1.0 ± 0.8 ng/mL and at 48 h were 0.65 ± 0.5 ng/mL. Plasma concentrations well exceeding this may indicate that the one clear day rule or dosage recommendations have not been adhered to.

1 | Introduction

Altrenogest (allyltrenbolone, 17α-allyl-17β-hydroxy-estra-4,9,11-tren-3-one) is a synthetic progestogen approved for use in female horses as a breeding management tool during seasonal transition, for control and suppression of oestrus and its associated behaviours, and for maintenance of pregnancy [1–4]. Owners and trainers of race and competition mares often want to suppress oestrus and oestrus-related behaviours in mares as this can make them unpredictable and detract from competition performance with both female and entire males competing in close proximity. Oral altrenogest, requiring daily administration, has been used since the 1980s as it was the only product clinically demonstrated to have the necessary progestational effects in horses. Not only is daily administration labour intensive and logistically difficult in many situations (e.g., if the animal is fractious or needs to be treated for an extended period of time), but altrenogest also poses health risks to the administrator as it is an oil-based preparation that is readily absorbed through the skin [5]. Reported human risks from cutaneous exposure include disruption of menstrual cycles, prolongation of pregnancy, decreased libido in men, headaches, fever, abdominal pain, nausea, diarrhoea and rashes [6]. As a result, there is a need for a more convenient and safer formulation of altrenogest. In 2016, a 'weekly' long-acting injectable product was introduced, reducing the labour requirement and potential risks to personnel as it does not require daily handling or administration.

However, in 2018, Australian racehorses tested positive to traces of trenbolone and trendione, banned anabolic steroids. This was proposed to be due to impurities from the manufacture of altrenogest with injectable products containing higher amounts of these substances compared with oral products [7]. As a result, the use of altrenogest in racing or competition horses became an issue of debate. Across Australia the advice to trainers from racing authorities is to not use products containing altrenogest as there is the potential of a positive swabs to steroids and trainers risk the consequences associated with the detection of prohibited substances [8]. Equestrian Australia and Racing NSW continue to advise participants not to administer injectable products containing altrenogest. Oral altrenogest is permitted in both equestrian sports and Racing NSW, but racehorse trainers should avoid using oral altrenogest products within one clear day of racing [9]. Despite being permissible in competition horses, the oral administration must comply with the recommended dosage as changes to dosage may result in adverse analytic findings.

For effective suppression of oestrus, it is recommended that oral altrenogest is administered daily. This suppression is temporary, and when treatment is removed, the inhibition of reproductive hormones is removed, which may result in a rapid return to oestrus and subsequent display of oestrus-related behaviours [10]. In racehorses, altrenogest treatment is to cease one clear day before racing; as such, there is concern amongst trainers that it may result in mares returning to oestrus within that window. Pharmacokinetic analysis may help determine the likelihood of a rapid return to behavioural oestrus.

Existing pharmacokinetic research was completed in geldings (for which altrenogest use is contraindicated) and may not provide an accurate determination of plasma altrenogest concentrations (PAC) in mares. This becomes important if quantification is required to determine if the one clear day rule or dosing recommendations have been abided. The aim of this study was to determine the pharmacokinetic parameters for mares administered both oral and intramuscular altrenogest.

2 | Material and Methods

In accordance with CSU ACEC Protocol No. A19050, 10 healthy mares (mean age: 9.3 ± 3.2 years; mean bodyweight: 512 ± 57 kg) were administered altrenogest in a 2-period, randomised crossover study. Mares were randomly assigned to receive either oral altrenogest or intramuscular injectable altrenogest for 2 weeks before undergoing a 3-week washout period. Mares then received the alternative treatment (Table 1). Due to the health risks associated with handling altrenogest, a single person administered all treatments, in all mares, wearing long cuff nonporous gloves and protective overalls.

Pre-treatment blood samples were collected to ensure no traces of altrenogest were present in any mare. Blood samples were collected daily from 8.00 am for 21 days regardless of administration method.

Oral altrenogest (PO; 0.044 mg/kg) was administered via 10-mL syringe over the tongue at the back of the mouth, once daily, for 15 days. All treatments were successful with no spilling or expelling of any of the dose. Blood sampling occurred daily, prior to treatment and occurred at the same time every day. Following the first and last dose of altrenogest (Day 0 [first dose] and Day 14 [final dose; dose 15]), blood samples were collected at frequent intervals over a 12-h period.

TABLE 1 | Experimental design for the crossover study of altrenogest administration by the oral and intramuscular route.

Group	Period 1	Period 2
Mares 1, 3, 5, 7 and 9	Oral, daily 0.044 mg/kg, q 24 h for 15 days	Intramuscular, 0.3 mg/kg, q 7 days (twice)
Mares 2, 4, 6, 8 and 10	Intramuscular, 0.3 mg/kg, q 7 days (twice)	Oral, daily 0.044 mg/kg, q 24 h for 15 days

Abbreviation: q = frequency of treatment administration.

Injectable altrenogest (IM; 0.3 mg/kg) was administered by syringe and 18-G 1.5 in. needle into the neck muscle on Day 0 and Day 7 (once weekly; q 7). Blood sampling occurred daily and frequent blood samples over a 12-h period were taken on the treatment days (Day 0 [first dose] and Day 7 [final dose; second dose]).

On frequent sampling days, mares were removed from the paddock at 6 am and restrained with a halter and lead. A jugular catheter was inserted aseptically into the left or right jugular vein, and pre-treatment samples were taken. Treatments occurred at the set time for each mare and frequent blood sampling commenced after treatment. Blood was collected at 0.08, 0.17, 0.33, 0.5, 0.67, 0.83, 1, 1.25, 1.5, 2, 2.5, 3, 4, 6, 8 and 12 h. At each timepoint, 5 mL of blood was removed from the catheter and discarded prior to the sample being taken. Once the sample was taken, the catheter was flushed with sterile heparinised saline. After the 2-h sample mares were put into yards in pairs with free access to food and water for the remainder of the day. Catheter were removed after the 12 h sample, and horses were returned to their shared paddock. Outside of the frequent sample days, horses were maintained on pasture for the duration of the study. Blood sampling for elimination profiles occurred once daily for 7 days after the final PO treatment. Mares underwent a 14-day wash out period (21 days from final treatment) prior to receiving the alternative treatment with sampling repeated. Plasma samples were stored at −20°C until analysis.

Plasma was shipped to the Australian Racing Forensic Laboratory (Sydney, NSW, Australia) for analysis. The method was adapted from one routinely used by the Australian Racing Forensic Laboratory for the analysis of neutral compounds in equine plasma. Quantification was based on an internal standardisation using 50 ng/mL altrenogest-d_5. Samples were thawed and equilibrated to room temperature. Each sample was mixed by gentle inversion for 1 min, and duplicate 1 mL plasma aliquots were transferred into 15-mL culture tubes labelled with the corresponding sample number. The internal standard (20 μL) was added to each aliquot of 1 mL of plasma for a final concentration of 10 ng/mL and the sample was mixed well. The samples

underwent protein precipitation using trichloroacetic acid (10% *v/v*, 125 μL) and pH adjustments to pH 3–3.5 were made using H_2O and dilute hydrochloric acid (3% *v/v*) prior to centrifugation at 3000 rpm for 10 min.

Solid-phase extraction was completed using Xtract cartridges (United Chemical Technologies, Bristol, PA). Cartridges were conditioned using methanol and subsequently washed with distilled water. The supernatant of the sample was loaded into the conditioned cartridge, washed with acetic acid solution, dried and eluted with 3 mL ethyl acetate:hexane (3:2 *v/v*, 3 mL; Merck, Kilsyth, Australia). The eluted sample was evaporated to dryness under nitrogen flow at 60°C. The residue was reconstituted in 50 μL of 0.1% formic acid in methanol and 50 μL of 0.1% formic acid in water (Merck, Kilsyth, Australia). The samples were then transferred into autosampler vials for analysis.

Altrenogest concentrations for all plasma samples were determined using a Shimadzu (Kyoto, Japan) 8060 high-performance liquid chromatograph triple quadrupole mass spectrometer (HPLC-MS/MS) equipped with a Shim-pack ODS III (2 × 75 mm; 1.6 μM) column maintained at 60°C. Mobile phase A consisted of ammonium formate (5 mM, pH 3.0), and mobile phase B consisted of 0.1% formic acid in acetonitrile. The run time for each sample was 12 min, following the gradient: 0–0.25 (30% B), 0.25–8 min (52% B), 8–10 min (95% B). The mobile phase returned to initial condition for the final 2 min. The flow rate was 0.3 mL/min, and an injection volume of 5 μL was used. MS conditions were interface temperature 400°C, desolvation temperature 650°C, heating block temperature 400°C, nebulising gas flow 2.5 L/min, heating gas flow 12.0 L/min and drying gas flow 8.0 L/min. Detection and quantification were conducted using multiple reaction monitoring (MRM; Table 2) with a lower limit of quantification (LLOQ) of 0.05 ng/mL and a limit of detection (LOD) of 0.02 ng/mL. Quantification was achieved using matrix-matched calibration standards with a minimum of $r^2 > 0.99$, prepared using the area ratio of a range of altrenogest concentrations (0.05, 1, 2, 5, 10, 20 and 50 ng/mL) and the internal standard (10 ng/mL). Calibration standards and quality control samples were prepared fresh for each assay. Samples were prepared in duplicates to account for any variance between the day of sampling and spiking techniques. Incurred sample reanalysis was undertaken on 150 samples (> 10%) in duplicates to demonstrate reproducibility. Accuracy and precision were within acceptable conditions.

Sample data was exported from Shimadzu LabSolutions to Microsoft Excel. Plasma data were analysed using Phoenix WinNonLin software (Certara, USA, Inc, Princeton, NJ). A standard non-compartmental analysis was performed on concentrations above the LLOQ. Plasma data was plotted using SigmaPlot Version 13.0. Paired *t*-tests to determine differences in the pharmacokinetic parameters between the first and last dose (PO Day 0, Day 14; IM Day 0, Day 7) of each treatment were performed using Microsoft Excel Version 2302. A p-value < 0.05 was considered statistically significant. Mean drug accumulation ratio (R_{acc}) was calculated using the equation $R_{acc} = C^{SS}{}_{max} / C^{1}{}_{max}$, where $C^{1}{}_{max}$ is the peak concentration during the first dose interval (Day 0) and $C^{SS}{}_{max}$ is the peak level at steady state (in this study, after the final dose at Day 7 for IM administration and Day 14 for PO administration).

3 | Results and Discussion

An overview of pharmacokinetic parameters is included in Tables 3 and 4. It should be noted that the maximum plasma concentration (C_{max}) for oral and intramuscular administration were similar on their respective days. However, as oral altrenogest is administered daily, C_{max} and area under the curve (AUC) would be experienced by each mare daily.

As calculation of half-life and AUC are impacted by re-dosing prior to complete elimination of the drug, assessment of half-life and complete AUC is accurately determined following the final dose (Table 4).

A comparison of the PAC in mares administered their first dose of oral altrenogest compared with the same mares administered their first dose of injectable altrenogest with a 21-day washout period in between is shown in Figure 1.

Altrenogest was rapidly absorbed into the blood stream with all mares having detectable PAC within 5 min regardless of treatment as shown in the Figure 1 insert plot. Mean plasma concentrations for IM administrations are shown in Figure 2. Mean C_{max} values associated with IM administration to mares was 18.0 ng/mL (range 11.1 to 26.7 ng/mL) after the first injection. Following a single IM dose of the same altrenogest product at the same dose rate in geldings, mean C_{max} of 33.52 ng/mL (range 19.07–87.33 ng/mL) at 13.0 ± 9.4 h was reported [11]. In the present study, mares experienced lower maximal concentrations with peak PAC occurring in a shorter period of time. Time of maximum concentration (T_{max}) was reached within 2 h in two horses, 6 h in three horses, 8 h in one horse and 12 h in four horses. Mean T_{max} for the mares was also lower than previously reported for geldings (7.9 ± 3.9 h

TABLE 2 | Multiple reaction monitoring acquisition parameters for altrenogest and the internal standard.

Compound (ESI polarity)	Precursor ion (m/z)	Product ions (m/z)	Collision energy (eV)
Altrenogest (+)	311.25	227.15	−24
		251.20	−20
		269.10	−15
		159.20	−25
		199.10	−34
d_5-Altrenogest (+)	316.35	227.30	−24
		269.15	−16
		251.30	−20

TABLE 3 | Non-compartmental pharmacokinetic parameters for 10 mares administered oral (PO) and injectable (IM) for their respective dosing intervals (PO 24 h; IM 168 h).

Pharmacokinetic parameter	PO first dose (0–24 h)	PO final dose (336–360 h)	IM first dose (0–168 h)	IM final dose(168–336 h)
C_{max} (ng/mL)	13.2 ± 5.8	21.3 ± 7.1*	18.0 ± 6.6	24.6 ± 6.7*
T_{max} (h)	0.8 ± 0.8	1.6 ± 1.0*	7.9 ± 3.9	8.4 ± 2.6
AUC_{0-t} (h*ng/mL)	60.1 ± 29.6	104.6 ± 39.7*	593.9 ± 194	688.1 ± 111.6*
$AUC_{0-\infty}$	65.7 ± 34.3	115.1 ± 46.6	624.1 ± 184.8	713 ± 118.2
R_{acc}	—	1.8 ± 0.65	—	1.45 ± 0.42

Note: Data are presented as mean ± SD.
*indicates statistically significant difference between the first and last dose of altrenogest ($p < 0.05$).

TABLE 4 | Non-compartmental pharmacokinetic parameters for 10 mares administered oral (PO) and injectable (IM) altrenogest from the final dose (PO Day 14; IM Day 7) for the remainder of the sampling period.

Pharmacokinetic parameter	PO final dose (336–504 h)	IM final dose(168–504 h)
Half-life (h)	11.1 ± 6.23	35.0 ± 12.9
$AUC_{0-\infty}$ (h*ng/mL)	126.5 ± 59.2	715.9 ± 118.5
Hours to LLOQ following final dose (h)	74.4 ± 27.3	245.3 ± 50.6[a]

Note: Data are presented as mean ± SD.
[a]One mare remained above LLOQ for the entire the sampling period (14 days following final treatment) and was not included. The mean baseline was determined from nine horses; as a result, the SD would be higher if the final horse had been included.

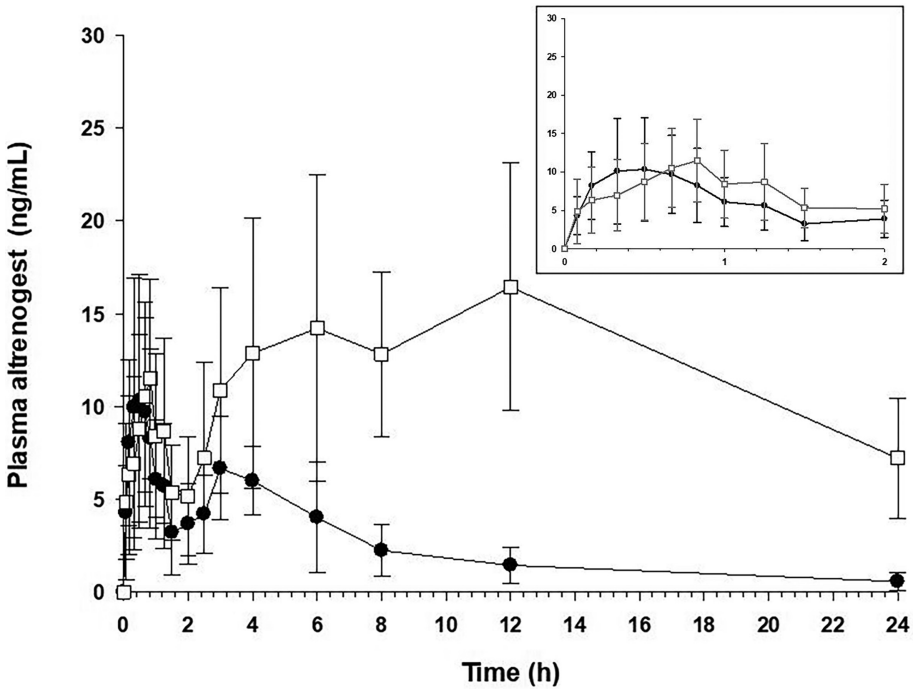

FIGURE 1 | Comparison of plasma altrenogest concentrations over a 24-h period of 10 mares administered 0.044 mg/kg altrenogest orally [●] on Day 0 and the same 10 mares administered 0.3 mg/kg intramuscularly [□] using a 5 × 5 crossover design. Insert: Concentration over time diagram is shown in detail for the first 2 h post-treatment.

compared to 13.0 ± 9.4 h). However, the half-life for the first IM dose (32.5 ± 11.4 h) was longer than that previously reported in geldings (15.71 ± 7.40 h) [11]. Total exposure to the IM drug did not differ greatly between genders with geldings in previous studies recording a numerically larger AUC (873 ± 166 h*ng/mL) when compared to the mares final dose in this study (715.9 ± 118.5 h*ng/mL). Unfortunately, direct comparisons are complicated by the use of different sexes. There are hormonal differences between males

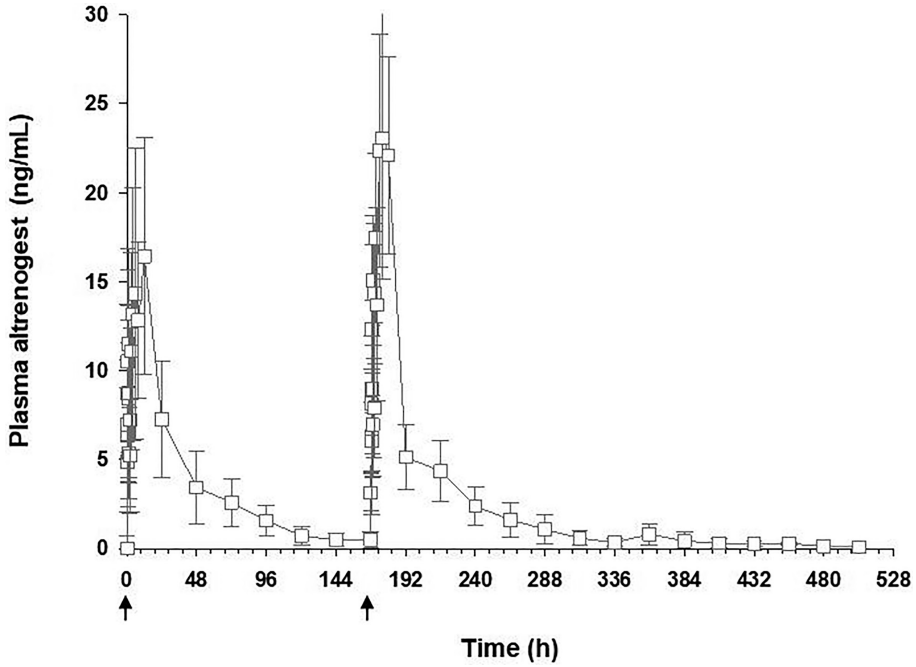

FIGURE 2 | Mean plasma altrenogest concentrations (±SD) from 10 mares administered 0.3 mg/kg altrenogest IM at Time = 0 h (Day 0) and Time = 168 h (Day 7) with elimination sampling to 504 h (Day 21). ↑ indicates treatment administration.

and females that have been associated with changes to metabolism, absorption and elimination of drugs in humans and other species [12, 13]. As geldings do not have gonads or uterine tissue, it is not unexpected that their pharmacokinetics differ to that of mares. Regardless of the reason for this variation, this drug is only permitted for use in female horses, and as such, pharmacokinetic profiles reported from the present study should be of particular interest to veterinarians and racing authorities when establishing appropriate dosage or withholding periods.

After initial dosing, maximum concentrations associated with PO administration were reached between 10 and 50 min in nine horses. The last horse reached concentrations near peak at 30 min but did not reach peak concentrations until 3 h post-administration. Compared with a study conducted in geldings [14], maximum concentrations reached in the present study were lower than previously reported (mean 13.2 ng/mL (Day 0) to 21.3 ng/mL (Day 14) compared with 35 ng/mL (Day 0) and 31 ng/mL (Day 5)). The mean AUC for the treatment interval (24 h) after the first dose was 60.1 ± 29.6 h*ng/mL for the oral dose (0.044 mg/kg) which is half of that reported for double the registered dose (0.088 mg/kg) in mares (mean AUC 128.1 ± 28.11 h*ng/mL) that would be expected if linear pharmacokinetics are assumed [15]. Mean plasma concentrations associated with oral altrenogest treatment are shown in Figure 3. Frequent sampling occurred on Day 0 and Day 14 with these peaks in plasma concentrations indicated in Figure 3. These peaks were expected to occur daily with oral altrenogest treatment, however sampling was only taken at the nadir each day (with the exception of Day 0 and Day 14) and have been presented in this figure.

To the authors knowledge this is the first study characterising PAC at the registered treatment interval (15 days) of oral altrenogest for oestrus suppression in normal cycling mares [16]. To date published pharmacokinetic studies are limited to treatment over 5 days at either the labelled dose (0.044 mg/kg) in geldings or twice the labelled dose (0.088 mg/kg) in mares. Dosing interval can have an impact on withholding estimates as the metabolism of the dose and subsequent elimination is altered with increasing concentrations and dosage. As the drug is not completely metabolised within the treatment period (PO 24 h; IM 7 days), the mean AUC of the last dose (PO Day 14; IM Day 7) were significantly higher than the first dose (Day 0; $p = 0.01$ [PO]; $p = 0.01$ [IM]; paired t-test, $n = 10$). Similarly, C_{max} was significantly different between the first and last dose of both altrenogest formulations ($p = 0.006$ [PO]; $p = 0.002$ [IM]) as the administration of multiple doses were associated with accumulation of drug and an increase in C_{max} in most mares (R_{acc} PO = 1.8 ± 0.65; IM = 1.45 ± 0.42). As accumulation can occur, mares undergoing chronic altrenogest treatment may have increased detection times. Competition or racing mares receiving oral altrenogest are likely on long term treatment to ensure continued suppression of oestrus and oestrus-related behaviours. As such, the plasma profile following withdrawal of altrenogest after multiple doses can be useful in determining withdrawal and withhold times as well as expected concentrations associated with one clear day of treatment as drug concentrations decline from their steady state. Mean minimum PAC (±SD) 24 h following the withdrawal of oral altrenogest treatment (360 h) was 1.0 ± 0.8 ng/mL (range 0.42 to 2.8 ng/mL; $n = 10$). At 48 h post-withdrawal (one clear day of administration, 384 h), mean PAC was 0.65 ± 0.5 ng/mL (range 0.29–1.38 ng/mL; $n = 6$ [4 mares below LLOQ]). Plasma concentrations well exceeding this range may indicate that the one clear day rule has not been adhered to.

Altrenogest was detected for at least 24 h after the first (24 h) and final (360 h) PO dose. As a result, the concern that race mares may return to oestrus after abrupt withdrawal of altrenogest

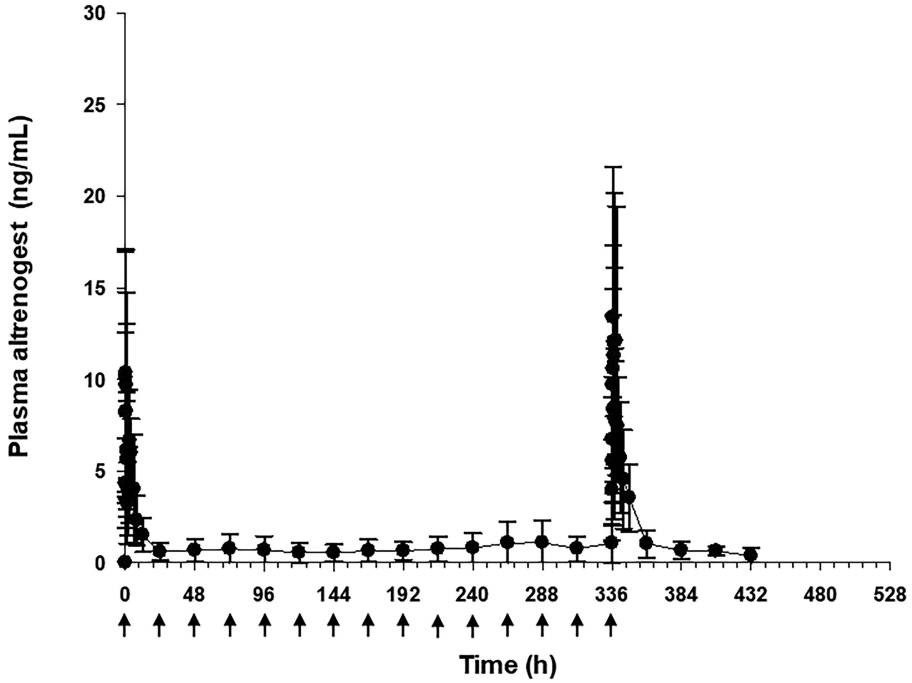

FIGURE 3 | Mean plasma altrenogest concentrations (±SD) from 10 mares orally administered 0.044 mg/kg altrenogest daily from Time = 0 h (Day 0) to Time = 336 h (Day 14) with elimination sampling to 504 h (Day 21). ↑ indicates treatment administration. With the exception of Day 0 and Day 14, sampling occurred at the nadir so only minimum concentrations are presented.

to adhere to the one clear day rule is likely unfounded. Concentrations returned to baseline (below LLOQ) within 48 h of the final dose for four mares (384 h), by 72 h in three mares (408 h), by 96 h in one mare (432 h), and the final two mares returned to baseline by 120 h (456 h). After the final IM administration, PAC returned to baseline 8 days (360 h) following treatment in three mares, one mare returned to baseline each day for the next 4 days (9 days [384 h], 10 days [408 h], 11 days [432 h] and 12 days [456 h]), two mares returned to baseline by 13 days (480 h), and the final mare did not return to baseline by end of sampling period 14 days following the final treatment (504 h). Importantly, this final mare would have retuned a positive race-day swab for altrenogest, 14 days following their last IM treatment, when all other mares ($n = 9$) would not have.

No treatment site reactions or adverse effects were observed from IM drug administration at the recommended dose. Handling and administration of intramuscular altrenogest did not result in visibly detectable oil residues on equipment or personnel. However, despite cautious handling, oily residue was observed on the bottle and gloves when using the oral formulation and therefore the risk of transfer or contamination was more obvious. Owners and trainers should be cautious and wear appropriate protective equipment when handling altrenogest to reduce the risk to personnel and potential contamination between treated and untreated animals. Importantly, this study is reporting pharmacokinetic data in mares for two formulations of altrenogest and does not determine the suitability of using injectable altrenogest as a substitute for daily oral altrenogest, despite these safety observations. Further pharmacokinetic assessment is required to compare the two formulations to determine bioequivalence and therefore efficacy.

4 | Conclusion

Administration of altrenogest resulted in rapid absorption in the mare. Altrenogest was detected in all blood samples within 5 min of treatment regardless of the formulation. Maximum plasma concentrations for oral and intramuscular administration were similar on their respective days. However, IM treatment was associated with a longer time between final dosing and plasma concentrations falling below LLOQ. There were increases in plasma pharmacokinetic parameters between the first dose and the final dose for both oral and IM administration. This is an important consideration for determining withhold or detection times as duration of dosing may influence PAC.

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Conflicts of Interest

The authors declare no conflicts of interest.

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