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Metformin in the Horse: Pharmacokinetic Modeling, Detection Methods, and Environmental
Exposure

By

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THESIS

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Metformin in the horse: pharmacokinetic modeling, detection methods, and environmental exposure

Abstract

The horseracing industry has strict regulations regarding the presence of chemical substances in equine athletes at the time of competition. Metformin, a commonly prescribed diabetes medication in human medicine, is considered a prohibited substance in horseracing, as there is no accepted therapeutic use for this compound in young, fit horses. Recent metformin detections in post-race blood and urine samples resulting in adverse analytical findings and severe disciplinary action against the trainers of the horses involved necessitates further study of this drug in horses. This thesis aims to characterize the pharmacokinetic behavior of metformin in the horse and explore environmental contamination as a means of exposure to inform regulation of metformin in horseracing.

In the first study, a pharmacokinetic (PK) study of metformin was performed utilizing twelve horses. Metformin was administered orally and intravenously in a balanced, randomized, two-way crossover design. Serum drug concentrations were determined using a highly sensitive liquid chromatography-tandem mass spectrometry (LC-MS/MS) assay and data was analyzed via noncompartmental analysis (NCA) for calculation of select pharmacokinetic parameters. Subsequently, concentrations were analyzed using a non-linear mixed effects (NLME) population model. The final model was then used to perform Monte Carlo simulations utilizing the estimated fixed effects and interindividual variability of the study horses to generate serum

concentrations in a simulated population of 1000 Thoroughbred horses. These simulations allowed for determination of a detection time using the current screening limit and estimation of withdrawal times for metformin corresponding to several additional proposed screening limits. The current serum screening limit of 0.5 ng/mL necessitated a prolonged detection time of 475 hours (~20 days).

To better understand the prolonged detection time of metformin in horses, a second PK study was performed wherein six horses were administered metformin orally (15 grams) for evaluation of sequestration into erythrocytes, a phenomenon that has been reported in humans. Metformin concentrations were determined in blood matrices, including plasma, serum, hemolyzed plasma, hemolyzed serum, serum from a serum separator tube (SST), red blood cells (RBCs), and whole blood, collected post administration. The ratios of blood to plasma average metformin concentrations were found to be >10 at later time points. The average concentrations in serum and plasma were also compared and found to be $>15\%$ different at several time points. The results of this study suggest RBCs act as a reservoir for metformin, contributing to extended detection time in blood samples.

Another study was performed to examine environmental exposure as a source for metformin detection in blood and urine of horses. Metformin is widely prescribed in human medicine and is excreted in urine as unchanged metformin. To simulate proposed metformin exposure at racetracks, six horses were exposed to metformin-contaminated urine. Blood and urine samples were collected for determination of metformin concentration following exposure. The blood matrices analyzed included plasma, serum, serum collected in serum separator tubes, whole blood, and RBCs. Over the course of the study all horses had detectable amounts of metformin in at least one matrix for at least one time point; urine concentrations were much

greater than blood. The results of this study indicate that environmental contamination may be a possible route of metformin exposure for horses housed at racetracks.

Chapter 1. Introduction

Metformin

Metformin (1,1-dimethylbiguanide) is a medication that is prescribed for the treatment of type II diabetes mellitus (T2DM) (Bailey & Turner, 1996). It is one of the most widely prescribed medications in human medicine, presumably because of its good oral bioavailability (50 – 60%; Pentikäinen et al., 1979), effectiveness, and wide safety margin (Rena et al., 2017). Metformin is a hydrophilic molecule, relying on the organic cation transporter 1 (OCT1) for absorption and distribution; polymorphisms in these transporters are thought to be responsible for the few instances of metformin intolerance in humans (Wang et al., 2002; Dujic et al., 2014). Metformin distributes to highly perfused tissues, including the liver, kidney, and intestinal wall. It does not undergo metabolism and is eliminated in urine as unchanged metformin with an elimination half-life of 7.83 – 8.99 hours (Pentikäinen et al., 1979).

Metformin lowers blood glucose levels through multiple mechanisms. It acts in the liver to inhibit glucose output by interacting with multiple molecular pathways, including blocking mitochondrial respiration and activating AMP-activated protein kinase (Owen et al., 2000; Zhou et al., 2001). This leads to a reduction in gluconeogenesis and an increase in glucose metabolism. Metformin also decreases glucose uptake in the digestive tract (Ikeda et al., 2000). These effects on blood glucose levels, favorable pharmacokinetics, and wide safety margin make metformin an optimal first-line treatment for T2DM.

Metformin in Veterinary Medicine

Metformin is not as commonly used in veterinary medicine as it is in human medicine. In horses, while it is sometimes prescribed, its use is rare, due to its apparent lack of efficacy. Tinworth et al. (2012) demonstrated that metformin is not effective at improving insulin

resistance in insulin-resistant ponies, indicating it would not be useful in treating metabolic diseases in equines. Furthermore, metformin has been shown to have a very low oral bioavailability (~6%) in horses, which could be responsible for the limited efficacy of the drug (Hustace et al., 2009). Hustace and colleagues found other pharmacokinetic parameters to be comparable between horses and humans, particularly its short elimination half-life and urinary excretion of unchanged metformin. Still, there is much that is unknown about its pharmacokinetic properties and behavior in the horse.

Drug Testing in Horseracing and Metformin Regulation

In the United States, horseracing safety and medication use in Thoroughbred horses is regulated by the Horseracing Integrity and Safety Authority (HISA). In 2023, HISA implemented the Anti-Doping and Medication Control (ADMC) Program, administered by the Horseracing Integrity and Welfare Unit (HIWU) for the purpose of centralized drug testing, results management, and penalty applications within the Thoroughbred racehorse industry (HISA, 2023). HIWU manages and enforces the directive set by the ADMC program, educates stakeholders, and accredits laboratories for investigatory sample testing. The regulations and standards enforced by HIWU as part of the ADMC program are informed by the Racing Medication and Testing Consortium (RMTC). The RMTC works with scientists, veterinarians, and analysts to develop drug testing policies in the best interest of the health and welfare of the equine athletes, as well as preserve the integrity of horseracing (RMTC, 2025).

HISA/HIWU classifies metformin as a banned substance in horseracing, as it does not have a recognized therapeutic benefit in young, fit racehorses. This means that detection of metformin in any post-race blood or urine sample will result in an adverse analytical finding (AAF), with trainers facing disciplinary action. Penalties for an AAF include forfeiture of

winnings, fines, or suspension. Multiple detections of metformin in post-race samples following adoption of the Horseracing Integrity and Safety Act highlighted the need for a better understanding of the pharmacokinetic behavior of metformin in horses. HISA in partnership with HIWU tasked the RTMC with reviewing the currently available science related to metformin. The scarcity of information led the RTMC to recommend additional research be conducted.

Drug Testing Matrices

For drug testing and regulation, urine is a commonly used matrix for regulation of therapeutic and banned substances. It is easy to collect and provides a longer detection window than blood. However, urine concentrations of a drug tend to fluctuate more than blood concentrations due to biological variables (Saugy et al., 2009). This can be problematic for doping control, since factors such as urine pH, hydration status, and urine flow can affect the measured concentration of a drug (Masereeuw & Russel, 2002). For many drugs, urine is considered suitable for screening purposes, but blood may be required for confirmation of the presence of the substance in excess of published screening limits. In the case of metformin, the ADMC utilizes both urine and serum samples for regulatory purposes, however there is no published research describing the relationship or correlation between the two matrices. To that end, more information is needed to determine if urine can be used as a reliable matrix for metformin testing and regulation in horseracing.

Blood is frequently used for regulation of both therapeutic and prohibited substances in horse racing. When compared to urine, blood is less susceptible than urine to fluctuations in concentration due to biological variation (Saugy et al., 2009). Blood provides a “real time” concentration, but the drug detection window is generally shorter for most drugs, compared to urine. Depending on the racing jurisdiction, plasma or serum may be utilized. For most drugs,

the difference in plasma and serum concentrations is negligible, however, in high stakes situations, such as drug testing, it is crucial to consider any factors (i.e. physiochemical properties of the drug, handling of the sample) that could impact the concentration of a regulated substance. For example, while most drugs are found in the plasma or serum fraction, some drugs can partition into red blood cells (RBCs). In this scenario, differences in sample handling may result in different concentrations in serum compared to plasma.

Sequestration of Metformin

In humans, there is evidence that metformin distributes into and sequesters in RBCs following administration. The drug slowly redistributes from RBCs to plasma leading to a prolonged terminal half-life and detection time for metformin (Robert et al., 2003). This is an important consideration for performance horses, whereby a long residence time may necessitate a prolonged withdrawal time should this drug be administered for therapeutic reasons to equine athletes. The potential for metformin sequestration in equine RBCs and the effects on detection time of the drug along with the implications of RBC sequestration on metformin concentrations in plasma versus serum are addressed in Chapter 3.

Monte Carlo Simulations and Regulatory Recommendations

Monte Carlo simulations have a history of use in human medicine for informing the clinical use of therapeutics. The use of Monte Carlo simulations in veterinary medicine, including for regulating drugs in performance horses is gaining in popularity (Toutain, 2024). This approach begins with modeling of plasma concentrations using a population pharmacokinetic model. Serum or plasma concentrations are then simulated for a larger virtual population of horses using the estimated fixed effects and interindividual variability from

modeling of the data from the experimental animals. This method, provides a statistically robust prediction of concentration-time curves of a drug for a much larger population than is realistically possible to sample, which can be used to inform therapeutic dosage guidelines, dosing intervals, or, in the case of drug regulations, withdrawal times. These simulations can account for physiological variations between horses in the simulated population for the most accurate detection and withdrawal time estimate as described by Toutain (2010).

Environmental Contamination of Metformin

Metformin is eliminated as the parent compound and appears to be resistant to degradation in the environment. Based on this and the widespread use of the medication in human medicine, it is perhaps no surprise that metformin has been detected in rivers and other waterways (Scheurer et al., 2012). Furthermore, the degradation resistant nature of metformin and the possibility of direct exposure to contaminated water or urine from humans taking the medication has raised the question of whether some of the detections of low levels of metformin in blood and urine samples were a result of exposure. This possibility is addressed in Chapter 4 of this thesis.

Summary

Metformin regulation in horseracing must be based on scientific data to ensure the safety and welfare of the horse as well as ensure fairness within the industry. In addition to understanding the behavior of the drug following intentional administration, it is imperative to understand the potential for exposure through environmental contamination. This thesis aims to fully describe the pharmacokinetics of metformin to inform regulatory standards. The studies included here aim to describe (1) the pharmacokinetic behavior of metformin in the horse

through population pharmacokinetic modeling and Monte Carlo simulations, (2) the concentrations of metformin in different blood matrices, and (3) the potential for metformin exposure through environmental contamination.

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**Chapter 2. Metformin in the Horse: Pharmacokinetics and Detection Times Using
Monte Carlo Simulations**

Metformin in the Horse: Pharmacokinetics and Detection Times Using Monte Carlo Simulations

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Abstract

The racehorse industry has strict regulations regarding the detection of prohibited substances in horses. Metformin, a diabetes medication, is a prohibited substance that has been reported in post-race blood and urine samples collected from racehorses. For further characterization of the disposition of metformin, 12 Thoroughbred horses were administered metformin orally and intravenously in a randomized, balanced, two-way crossover design. Serum and urine samples were collected and drug concentrations determined via liquid chromatography-tandem mass spectrometry. The serum data was analyzed using both noncompartmental analysis and a population pharmacokinetic model. Metformin concentrations were below the LOQ (0.25 ng/mL) in 6 of 12 horses at by day 11 post administration, and below the LOQ for all horses on day 25. The maximum serum concentration of metformin (mean $\pm$ SD) was 941.0 ± 467.8 ng/mL, and the mean terminal $t_{1/2}$ was 85.5 ± 11.6 h. Based on Monte Carlo simulations the time that serum metformin concentrations fell below the proposed HISA Anti-Doping and Medication Control (ADMC) minimum reporting level (MRL; 0.5 ng/mL) in a simulated population of 1000 Thoroughbred horses, was 13 days. Metformin concentrations in urine fluctuated significantly between and within individual horses and there was not a consistent relationship between blood and urine samples across time points. Results of the present study demonstrate a prolonged detection time, thus a prolonged withdrawal time is needed to prevent a positive finding following exposure to metformin. Additionally, these results suggest that blood is the preferred matrix for regulatory purposes due to the inconsistent elimination in urine.

Introduction

Metformin (1,1-dimethylbiguanide) is a medication used commonly for the treatment of type 2 diabetes mellitus and is one of the most prescribed drugs for humans.¹ It lowers hepatic glucose production and increases peripheral tissue sensitivity to insulin, lowering blood glucose concentrations.² In humans, metformin has a wide safety margin, moderate oral bioavailability, and minimal side effects, making it a good choice as a first line treatment for type 2 diabetes. Reported adverse effects associated with metformin are usually related to gastrointestinal intolerance, which is thought to be due to variations in organic cation transporter 1 (OCT1) function in the gastrointestinal tract.³

The use of metformin is much less common in veterinary medicine. Its use in cats and dogs is limited, due to minimal efficacy. Similarly in horses, its effectiveness is questionable. In one study, investigators noted that it did not improve insulin sensitivity in insulin-resistant ponies following oral administration.⁴ There is limited information regarding the pharmacokinetics of metformin in horses, but unlike humans, the oral bioavailability of metformin in horses is reportedly poor (~6%).⁵ Metformin is characterized by a short plasma half-life and is eliminated primarily as the parent compound in horses.⁵

In recent years, the detection of metformin in blood and urine samples collected from racehorses has raised concern amongst regulators. Metformin is considered a prohibited substance by the Horse Racing Integrity and Safety Authority (HISA) due to its lack of recognized therapeutic benefit in young, fit Thoroughbred racehorses, and its potential to impact performance. Although the potential impact of metformin on athletic performance in horses is unknown, there is some evidence that the drug improves heart rate and respiratory exchange ratio during exercise in humans.⁶

The increase in detection of this prohibited substance in regulatory samples, coupled with its widespread use in human medicine and potential for exposure through contamination, necessitates a better understanding of the behavior of this drug in horses. To that end, the first objective of the current study was to describe blood and urine concentrations and the pharmacokinetics of metformin in horses, using a highly sensitive analytical assay. A second objective was to use Monte Carlo simulations to provide estimated withdrawal times based on measured concentrations from experimental horses.

Methods

Animals

Twelve healthy, university-owned, exercised Thoroughbred horses (5 mares and 7 geldings, aged 2-7 years, weighing 471-660 kg) were studied. Horses were exercised via Equineciser (Centaur Horse Walkers Inc, Mira Loma, CA, USA) and a high-speed treadmill (Mustang 2200, Graber AG, Switzerland) prior to and for the duration of the study, with the exception of the day of drug administration, following standard protocols established by our laboratory.⁷ Before beginning the study, horses were subject to a physical examination, complete blood count, and a serum biochemistry panel to determine health status. Blood sample analyses were performed by the Clinical Pathology Laboratory of the William R. Pritchard Veterinary Medical Teaching Hospital of the University of California, Davis. An appropriate washout period was observed for any drugs administered to horses prior to enrollment to ensure the animals had no other medications in their system prior to beginning the study. All animals were housed in 12 x 12-foot stalls for

the duration of the study. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis.

Instrumentation and Drug Administration

This study was conducted in two phases, using a balanced, randomized, 2-way crossover design. In Phase I, 6 horses received metformin orally (15 grams; 22.7-31.8 mg/kg) and 6 received the drug intravenously (IV; 5 mg/kg). In phase II, the horses received the opposite treatment. Phase II commenced when metformin concentrations were no longer detected in serum and urine from the Phase I administration (based on analysis of samples collected prior to administration of drug in Phase II). The order in which each horse received each administration type was determined with a computerized random number generator. The oral dose was selected based on evaluation of medication reports filed by owners and trainers for horses competing in other performance horse disciplines. The IV dose was selected based on a previous report.⁵ Food was withheld for oral administration for 12 hours prior and 2 hours post administration; Horses had free access to food for IV administration. All horses had access to water ad libitum for the duration of the study. For oral administration, commercially available metformin tablets (Aurobindo Pharma, Hyderabad, Telangana, India) were crushed and suspended in water and administered within 1 hour of dosing. The suspension was administered within preparation directly into the oral cavity via a dosing syringe. As there is no commercially available injectable metformin formulation, commercially available metformin powder (>95% purity) was suspended in 0.9% NaCl using sterile technique. The formulation was filter sterilized and administered within 1 hour of preparation. The formulation was tested via liquid chromatography-tandem mass spectrometry (LC-MS/MS), as described below, to ensure the concentration was within 10% of the nominal concentration. Immediately prior to dosing, a 14-gauge IV catheter was placed aseptically in one

jugular vein for blood sample collection and a second in the contralateral vein for IV drug administration. Dosing catheters were removed 1 hour after dosing; sampling catheters were removed 18 hours after dosing. All subsequent blood samples were collected via direct venipuncture.

Sample Collection

Blood samples were collected into tubes devoid of anticoagulant (Kendall/Tyco Healthcare, Mansfield, MA) for drug concentration determination at time 0 (prior to drug administration) and at 5, 10, 15, 30, 45 minutes and 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 18, 24, 30, 36, 48, 72, 96, 120, 144, and 168-hours post administration for both treatment groups. At 168 hours (7 days), samples were analyzed using LC-MS/MS for determination of metformin concentrations. As drug was still detected, additional samples were collected at 24-hour intervals until 14 days post administration, then 3 times per week until 28 days post administration. Samples were kept at room temperature for approximately 20 minutes until centrifugation at 3000 x g for 10 minutes. Serum was separated, transferred to storage vials and stored at -20°C until drug concentration analysis.

Horses utilized in this study are trained to urinate upon whistle command and therefore urine samples were collected via free-catch. Samples were collected at 4, 24, 48, 72, 96, 120, 144, and 168-hours post dosing. Urine samples collected at 168-hours were analyzed for metformin using LC-MS/MS (in Phase I). As metformin was still detected in the 168-hour sample, additional samples were collected at 24-hour intervals until 14 days post administration, then 3 times per week until 25 days post administration. The day 25 urine samples were analyzed for determination of metformin concentrations sprior to commencement of Phase II. Since drug was detected in the last urine samples collected during Phase I (day 25), the collection duration

was extended in Phase II. In Phase II, additional samples were collected 3-4 times per week until the drug concentration was below the limit of quantitation (LOQ) for all horses (80 days post administration). Following collection, urine samples were kept on ice, then stored at -20°C until drug concentration determination.

Determination of Metformin Concentrations

Serum Sample Analysis: Metformin working standard solutions were prepared by dilution of the metformin stock solutions (Cerilliant, Round Rock, TX) with methanol to concentrations of 0.01, 0.1, 1, 10 and 100 ng/μL. Serum calibrators were prepared by dilution of the working standard solutions with drug free equine serum to concentrations ranging from 0.25 to 25,000 ng/mL. Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (drug free equine serum fortified with analyte at three concentrations within the standard curve) were included as an additional check of accuracy.

Prior to analysis, 0.5 mL of the serum samples was diluted with 0.5 mL of acetonitrile (ACN):1M acetic acid (9:1, v:v) containing 20 ng/mL of d6-metformin (Cayman Chemical Company, Ann Arbor, MI), to precipitate proteins. The samples were vortexed for 1.5 minutes to mix, refrigerated for 20 minutes, vortexed for an additional 1 minute, centrifuged (4300 rpm/3102 g) for 10 minutes at 4 °C and 500 μL of the organic layer transferred to a tube and dried under nitrogen at 55 °C. Samples were dissolved in 140 μL of ACN:1M acetic acid (9:1, v:v). All samples were then transferred to 2 mL Eppendorf tubes, centrifuged at 14,000 rpm (12,753 g) for 5 minutes, and 20 μL was injected into LC-MS/MS system.

The concentration of metformin was measured in serum by LC-MS/MS using positive heated electrospray ionization (HESI(+)). Quantitative analysis was performed on a TSQ Altis triple quadrupole mass spectrometer coupled with a Vanquish liquid chromatography system

(Thermo Scientific, San Jose, CA). The spray voltage was 3500V, the vaporizer temperature was 350 °C, and the sheath and auxiliary gas were 50 and 10 respectively (arbitrary units). Product masses and collision energies of analyte and stable isotopically labeled internal standard were optimized by infusing the standards into the TSQ Altis. Chromatography employed a EZ:faast 4u AAA:MS 250x2mm column (Phenomenex, Torrance, CA) and a linear gradient of ACN in water with a constant 0.2% formic acid at a flow rate of 0.3 mL/ min. The initial ACN concentration was held at 1% for 0.6 minutes, ramped to 25% over 2.4 minutes, ramped to 95% over 0.5 minutes, held at that concentration for 0.1 minutes, before re-equilibrating for 3.3 minutes at initial conditions.

Detection and quantification were conducted using selective reaction monitoring (SRM) of initial precursor ion for metformin (mass to charge ratio (m/z) 130.1) and the internal standard d6-metformin (m/z 136.1). The response for the product ion for metformin (m/z 60.0) and the internal standard (m/z 59.9), were plotted, and peaks at the proper retention time integrated, using Quanbrowser software (Thermo Scientific). Quanbrowser software was used to generate calibration curves and quantitate analytes in all samples by linear regression analysis. A weighting factor of $1/X$ was used for all calibration curves.

Urine Sample Analysis: Urine calibrators were prepared by dilution of the working standard solutions with drug free equine urine to concentrations ranging from 0.5 to 25,000 ng/mL. Prior to analysis, 0.5 mL urine was diluted with 2.0 mL 0.1M pH 7 phosphate buffer and 0.1 mL water containing the internal standard (d6-metformin; 0.05 ng/mL). All samples were vortexed gently to mix and subjected to solid phase extraction using Strata-X-CW columns 200 mg/3mL (Phenomenex). In brief, the columns were conditioned with 2 mL of methanol and 2.5 mL of water. The samples were loaded onto the column and a minimum of 2 minutes was allowed for

samples to pass through the column. The columns were rinsed consecutively with 2.8 mL of water and 1 mL methanol prior to eluting with 2.5 mL methanol:ACN (1:1, v:v) containing 2% formic acid. Samples were dried under nitrogen and dissolved in 150 μ L of 10% ACN in water, both with 0.2% formic acid. The injection volume was 10 μ L into the LC-MS/MS system.

Detection and quantification were the same as serum.

The instrument response was linear and gave correlation coefficients of 0.99 or better. For both serum and urine, the precision and accuracy of the assay was determined by assaying quality control samples in replicates (n=6). Quality control samples met accuracy and precision acceptance criteria as described in the FDA's Guidance for Bioanalytical Method Development and Validation⁸ (Table 2.1). The limit of quantitation (LOQ) was the lowest calibrator that could be measured with acceptable precision (back calculated concentration not exceeding 20% of the coefficient of variation (CV)) and accuracy (within 20% of the nominal concentration) and the limit of detection (LOD) was established based on the lowest calibrator with a 3:1 signal to noise ratio. The technique was optimized to provide an LOQ of 0.25 ng/mL in serum and 0.5 ng/mL in urine. The LOD was approximately 0.1 ng/mL and 0.25 ng/mL for metformin in serum and urine, respectively.

Metformin Concentration and Pharmacokinetic Analysis

To evaluate the relationship between blood and urine concentrations, the urine to blood ratio was calculated for each horse at each timepoint, using the formula:

$$\text{Urine concentration/blood concentration}$$

The maximal concentration (C_{max}) and time of maximal concentration (T_{max}) were determined directly from the concentration data. As an aid in determining initial estimates for model fitting, non-compartmental analysis (NCA) was performed on metformin serum

concentrations using commercially available software (Phoenix WinNonlin v8.3; Certara, Princeton, NJ).

Following NCA, concentration data for all horses from both routes of administration (IV and oral) were modeled simultaneously with a population model using a nonlinear mixed effect (NLME) modeling approach with the Phoenix software. Different compartmental models and different residual error models (additive, multiplicative, Poisson, and mixed additive/multiplicative residual) were assessed. Goodness of fit and the appropriate error term were selected through visual analysis of observed compared with predicted concentration graphs and residual plots, as well as Akaike Information Criterion, and % CV of parameter estimates.

Monte Carlo Simulations

Monte Carlo simulations were performed in WinNonlin using the final population PK model. Serum concentrations for a virtual population of 1,000 horses were simulated using the estimated fixed effects and interindividual variability from the 12 experimental horses. Each simulated subject received a standard dose matching the experimental protocol. Using this meta-population, the withdrawal time corresponding to the proposed MRL of 0.5 ng/mL and screening limits of 1, 2 and 4 ng/mL were estimated. The concentration data generated for the simulated population were then analyzed with the WinNonlin statistical tool in order to compute the quantiles of interest (50 and 99th).

Results

Following oral metformin administration five horses showed mild signs of abdominal discomfort, such as pawing and rolling 4 hours after the drug was administered; these signs resolved on their own within 2 hours.

Individual and mean metformin serum concentrations with respect to time following intravenous and oral administration are depicted in Figure 2.1. Metformin concentrations were below the LOQ (0.25 ng/mL) in all 12 horses following both oral and intravenous administration at 504 hours (day 21). The mean $\pm$ SD and median and range of select pharmacokinetic parameters following noncompartmental analysis for both routes of administration are listed in Table 2.2. The $C_{\max}$ (mean $\pm$ SD) was 941.0 ± 467.8 ng/mL at 0.86 ± 0.66 hours following oral administration. The terminal half-life 85.5 ± 11.6 and 85.8 ± 15.1 hours following IV and oral administration, respectively. Using a compartmental modeling approach, based on evaluation of goodness of fit criteria, a 3-compartment multiplicative error model, parameterized with respect to clearance, gave the best fit to the data. Pharmacokinetic parameters generated from compartmental analysis are listed in Table 2.3. The absorption rate constant was 0.362 1/h and the bioavailability was 11.6% following oral administration.

Individual and mean metformin urine concentrations with respect to time for both routes of administration are depicted in Figure 2.2. Metformin was not detected in urine samples prior to drug administration in Phase II. For both IV and oral administration, urine concentrations fluctuated unpredictably with some horses showing decreases followed by increases at later time points. Metformin concentrations were below the LOQ (LOQ=0.5 ng/mL) in 4 of 12 horses at 25 days post administration (the last time point collected in Phase I). At 80 days post administration

(the last time point collected in Phase II), metformin concentrations in urine were below the LOQ in 3 of 12 horses (2 IV and 1 oral).

Corresponding metformin urine and blood concentrations as well as the urine to blood ratio from 24 hours to 14 days are listed in Tables 2.4 and 2.5 for IV and oral administration, respectively. The urine-to-blood concentration ratios varied widely between horses at each time point for both routes of administration.

Based on the Monte Carlo simulations, the time at which 50% and 99% of the simulated population would fall below the proposed MRL of 0.5 ng/mL was approximately 245 and 475 hours, respectively (Figure 2.3). Monte Carlo simulations showing the time at which metformin concentrations would fall below screening limits 1, 2, and 4 ng/mL are shown in Figure 2.4.

Discussion

Recent reports of the detection of metformin in urine and blood samples collected from racehorses post- race is a concern for regulators. The current study describes blood and urine concentrations and the pharmacokinetics of metformin following IV and oral administration to horses, utilizing a NLME population modeling approach.

The maximum concentration ($C_{\max}$) in the presently reported study (941.0 ± 467.8 ng/mL) was approximately 2.5 times higher than a previous report describing the pharmacokinetics of metformin in horses (400 ± 100 ng/mL),⁵ consistent with the higher dose administered here (15 grams versus 6 grams PO). The time of maximum concentration was comparable between the two studies (0.86 ± 0.66 versus 0.9 ± 0.1 hours).⁵

Serum concentration versus time data were fit to an NLME population PK (Pop PK) model. This modeling approach is used commonly in human medicine and is slowly being utilized more in veterinary medicine.⁹ Pop PK models may also be useful in the regulation of drugs in horseracing and other equestrian disciplines.¹⁰ The reader is referred to a recent review for more details regarding the utility of this modeling approach in this area.¹⁰ Serum concentration data in the current study was best fit by a 3-compartment model. The use of both 2- and 3- compartment models have been reported in metformin pharmacokinetic studies in humans.^{11,12} Metformin absorption was less rapid in the current study ($K_a=0.399$ 1/h) compared to reports in humans ($K_a=1.19$ 1/h).¹¹ In humans, metformin is absorbed in the small intestine primarily by active transporters;³ and is saturable.¹³ In the current study, a linear absorption model provided the best fit indicating saturation was not apparent. Since the current study only evaluated a single oral dose, additional study is needed to ascertain whether absorption is saturable in horses at higher doses.

The oral bioavailability of metformin was low ($7.86 \pm 1.65\%$ (NCA), 11.6% (Pop PK)). This is comparable to previous reports in horses ($7.1 \pm 1.5\%$),⁵ but much lower than that reported for humans (50-60%).^{12,13} In the presently reported study the terminal half-life (mean $\pm$ SD) of metformin in horses was 85.5 ± 11.6 h (IV) and 85.8 ± 15.1 h (oral), which is significantly longer than previously reported in horses (24.9 ± 0.4 h).⁵ This is likely due to differences in sampling protocols, with sample collection being more prolonged in the current study. Additionally, the LC-MS/MS assay used in the current study was more sensitive, compared to the previous study in horses (0.25 ng/mL vs 0.50 ng/mL). Both the longer sampling time and the lower LOQ in the present study allowed for more complete characterization of the terminal portion of the concentration time curve, explaining the longer terminal half-life. This is

especially important from a regulatory perspective, when extremely low concentrations of metformin can result in regulatory action against the trainer. The reason for the prolonged residence time in horses, and hence the long terminal half-life is not clear. In humans, there is evidence that metformin partitions into erythrocytes and may be sequestered for extended periods of time.¹⁴ Although further study is necessary, it is possible that this is the case in horses as well, and may explain the long terminal half-life of metformin in this species.

Urine is a common biological matrix for drug testing of equine athletes. In the current study, urinary metformin concentrations varied greatly between horses resulting in a large range of concentrations at each time point. To understand the relationship between urine and blood, the metformin urine to blood ratio was calculated for each horse at each timepoint where urine was collected and blood concentrations remained above the LOQ. At all timepoints, for both routes of administration, there was a large degree of variability between and within horses with respect to the urine to blood ratio, resulting in a wide range. Additionally, when comparing the ratios between timepoints the urine to blood ratio was inconsistent. It is also important to note that for some horses, unpredictable increases in metformin urine concentrations were noted at later timepoints. With any drug exposure, there are multiple factors that can influence urine concentrations. This includes things such as hydration status and urinary pH,¹⁵ both of which may be different after high intensity exercise compared to a horse at rest. Another potential explanation for the “spikes” in urine concentrations, is sequestration in tissues and/or cells and subsequent release. As discussed above, in humans, metformin sequesters in various sites within the body (i.e. erythrocytes). If this proves to be the case in horses, this may explain the fluctuating urine concentrations. Drug may be slowly and/or inconsistently released into the serum and subsequently eliminated in the urine. These fluctuations in urine metformin

concentrations, along with the inconsistent relationship between blood and urine, calls into question the reliability of urine as a matrix for regulating metformin. Aside from the unpredictable behavior of metformin in urine, the prolonged detection time, would necessitate excessive restriction of this drug.

Monte Carlo simulations provide a powerful and statistically rigorous approach to estimate withdrawal times for medications by generating a large theoretical population of horses and determining the time at which concentrations would be expected to fall below an established screening limit for this population. The theoretical population is generated from data gathered from an experimental group with varying individual pharmacokinetic parameters; thus, it reflects the natural biological variability within the investigated horse population.^{16,17} Monte Carlo simulations were used in the current study to determine the metformin withdrawal time in serum, for a virtual population of 1000 horses using the current proposed HISA MRL for serum (0.5 ng/mL). Based on these simulations, a very prolonged withdrawal time (~20 days) is necessary for 99% of the virtual 1000 horse population to avoid an adverse analytical finding with the current HISA MRL. Although notably, concentrations of metformin in blood following unintentional exposure resulting from environmental contamination, are unknown, based on the widespread use of metformin in human medicine and the reported prevalence of this compound in the environment, Monte Carlo simulations were used to make predictions. For the purposes of this exercise, withdrawal times for hypothetical MRLs of 1, 2 and 4 ng/mL were chosen in the current study. Based on these simulations, for 99% of the virtual horse population withdrawal times of 306 hours (~13 days), 168 hours (7 days), and 120 hours (5 days) would be appropriate for 1, 2, and 4 ng/mL, respectively.

The results of the current study show that metformin is detectable in the serum and urine of horses for an extended period of time after administration by both IV and oral routes. Urine concentrations can be unpredictable, making blood a more effective matrix for confirming the presence of metformin in regulatory samples in relation to the application of reporting limits. In the case of an oral administration, the current proposed HISA MRL would correspond to a minimum detection time of 20 days. It is also important to note that with how common metformin is as a prescription medication in humans and reports of environmental contamination,¹⁸ accidental exposure may pose a risk of horses testing positive for the drug with the proposed HISA MRL. Results of the present study supports revisiting the way in which metformin is regulated in racehorses. Furthermore, based on the widespread use of this drug and concern over administration and exposure in horseracing, further investigation into the behavior of this drug in horses is warranted.

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Conflicts of Interest:

The authors have no conflicts of interest to declare.

Data Availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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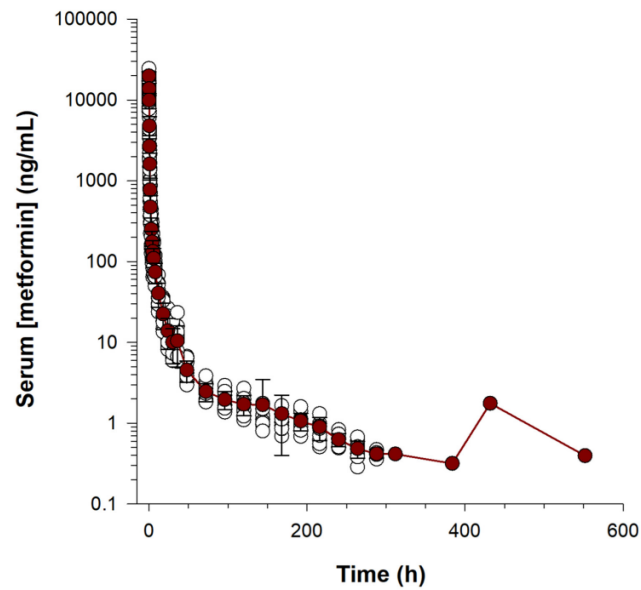
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Figures and Tables

A.)



B.)

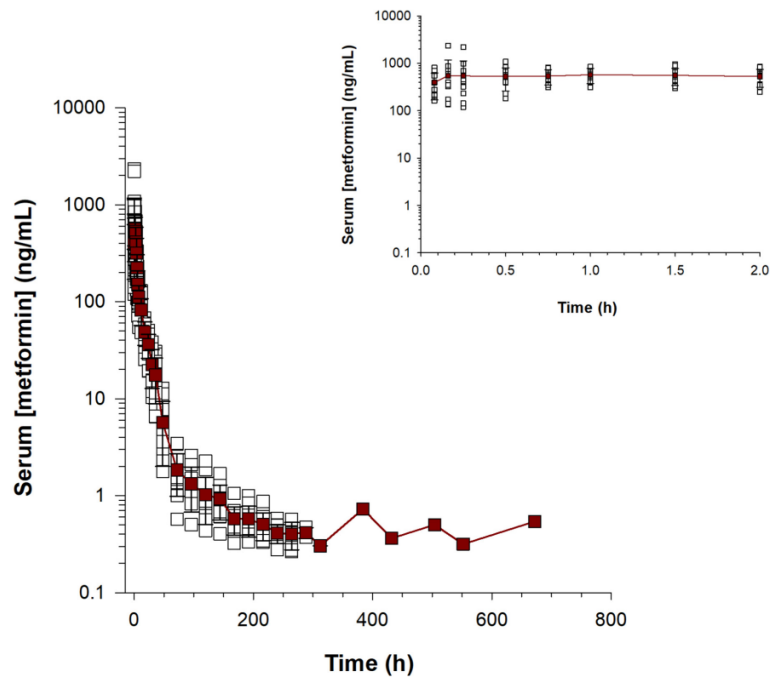


Figure 2.1. Serum metformin concentrations following a single (A) IV administration of 5 mg/kg or (B) oral administration of 15 grams to 12 horses. Mean concentrations are shown with solid points ($\pm$ SD), and individual points are shown with open circles. The inset for B represents the first 2 hours post drug administration.

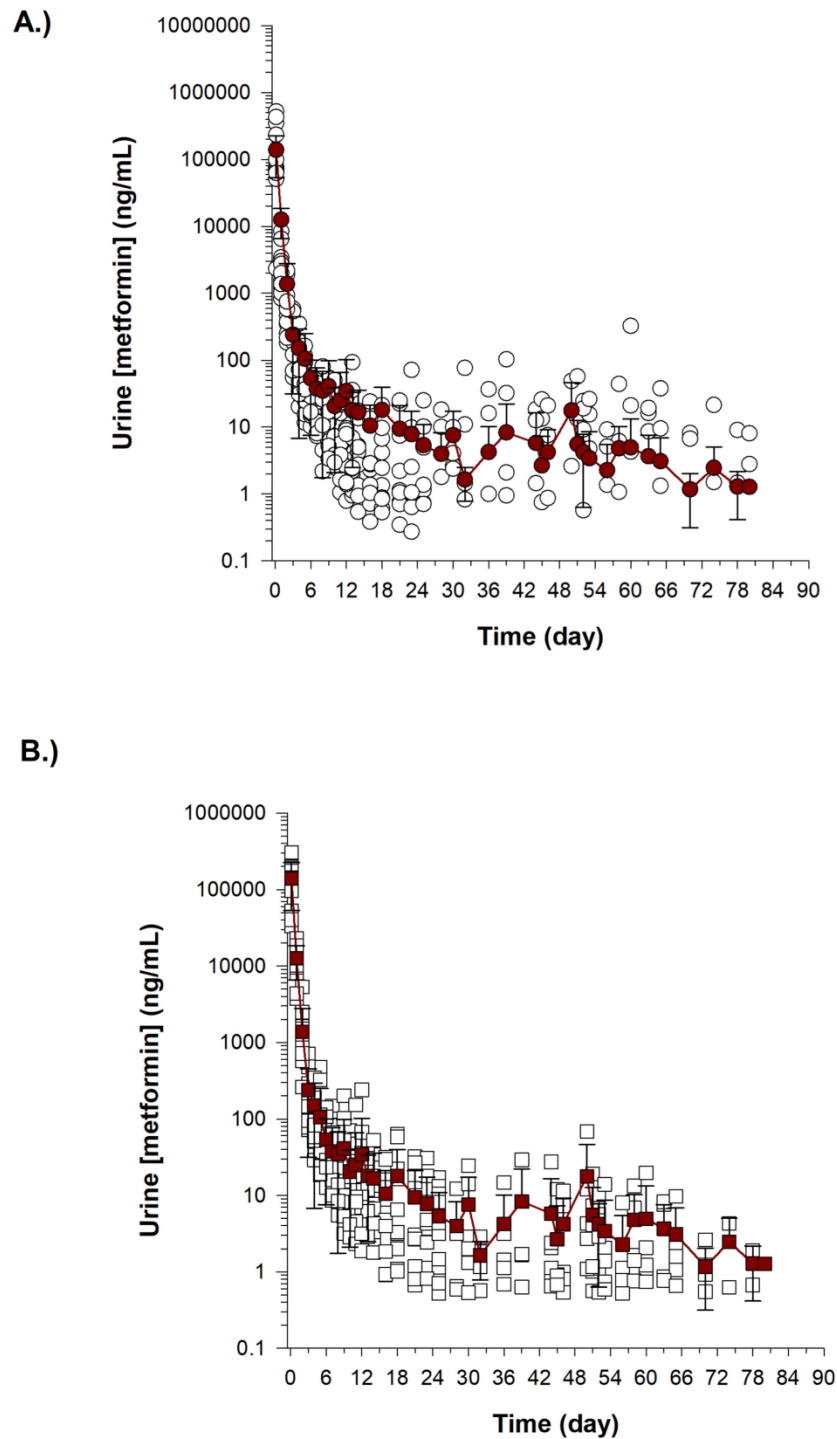


Figure 2.2. Urine metformin concentrations following a single (A) IV administration of 5 mg/kg or (B) oral administration of 15 grams to 12 horses. Mean concentrations are shown with solid points ($\pm$ SD), and individual points are shown with open circles. Samples after day 25 were only collected from 6 horses.

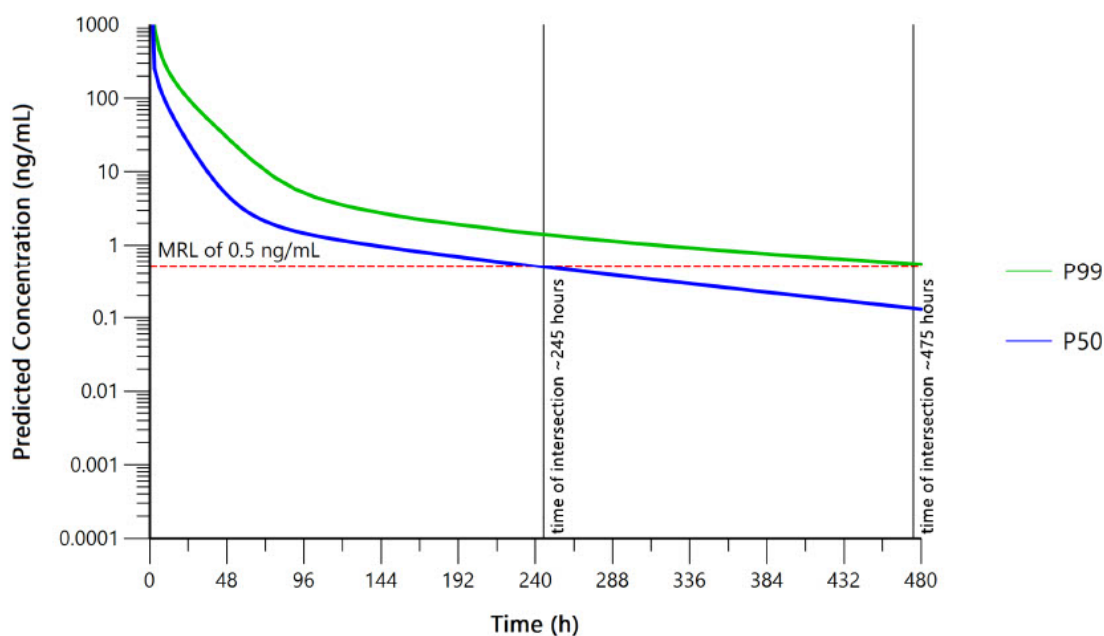
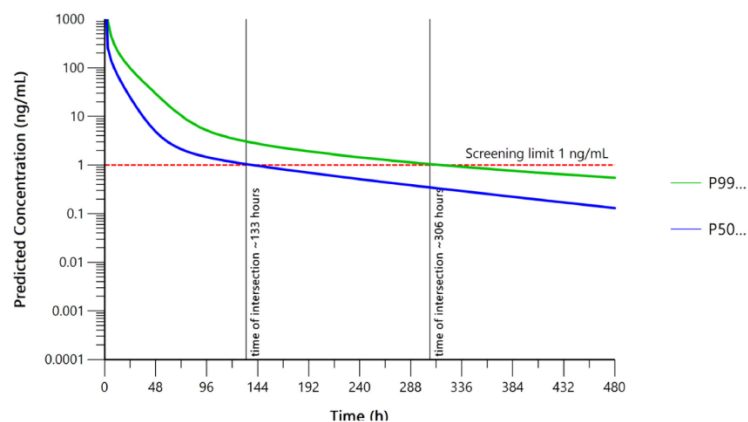
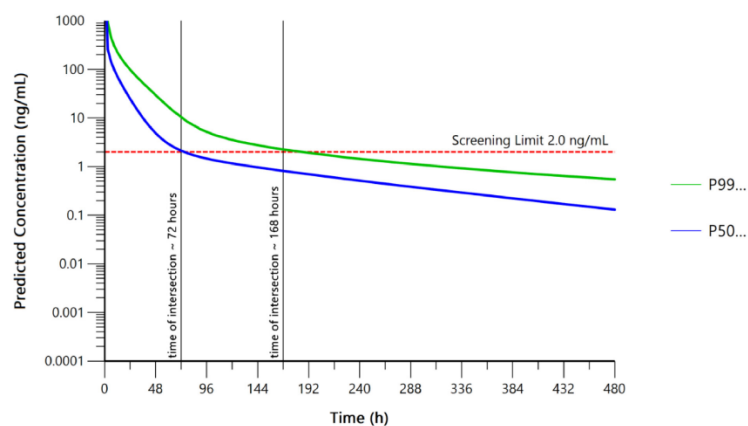


Figure 2.3. Plot of the 50% and 99% quantiles for a hypothetical horse population (1000 Thoroughbred horses) obtained via Monte Carlo simulation for the proposed Horseracing and Integrity Safety Authority (HISA) minimum reporting limit (MRL) of 0.5 ng/mL for metformin. The times of intersection of the predicted concentration curves with the screening limit line represent the detection time for 50% and 99% of the hypothetical population.

A.)



B.)



C.)

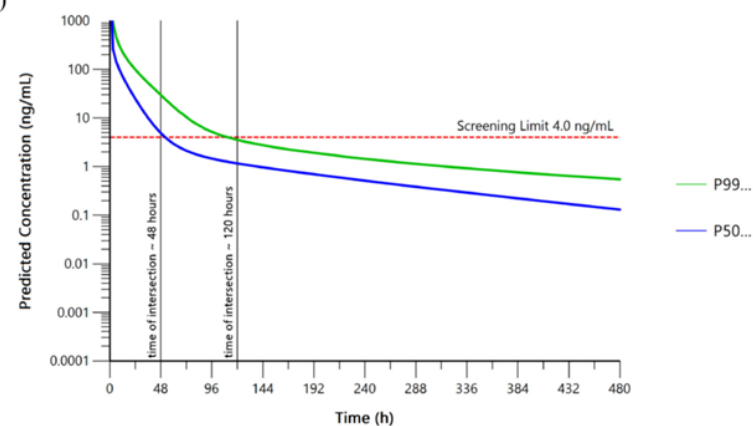


Figure 2.4. Plot of the 50% and 99% quantiles for a hypothetical horse population (1000 Thoroughbred horses) obtained via Monte Carlo simulation for theoretical screening limits of (A) 1.0 ng/mL, (B) 2.0 ng/mL, and (C) 4.0 ng/mL for metformin. The times of intersection of the predicted concentration curves with the screening limit line represent the detection time for 50% and 99% of the hypothetical population.

Table 2.1. Accuracy and precision values (average of 6 replicates) for LC-MS/MS analysis of metformin in equine serum and urine.

Matrix	Concentration (ng/mL)	Intra-day accuracy (% nominal concentration)	Intra-day precision (% relative SD)	Inter-day accuracy (% nominal concentration)	Inter-day precision (% relative SD)
Serum					
	0.75	88.0	5.0	87.0	6.0
	40.0	96.0	3.0	98.0	5.0
	5000	92.0	2.0	94.0	3.0
Urine					
	0.75	85.0	4.0	85.0	7.0
	100	87.0	6.0	92.0	4.0
	5000	94.0	2.0	99.0	4.0

Table 2.2. Pharmacokinetic parameters for metformin following a single IV (5 mg/kg) or oral (15 grams) administration to 12 horses.

Parameters generated using non-compartmental analysis.

Parameter	Intravenous		Oral	
	Mean $\pm$ SD	Median (range)	Mean $\pm$ SD	Median (range)
C(0) (ng/mL)	25,553 $\pm$ 3850.7	28,243.7 (20,340.9 - 34,370.8)	---	---
C _{max} (ng/mL)	---	---	941.0 $\pm$ 467.8	832.6 (542.8 – 2346.0)
T _{max} (h)	---	---	0.86 $\pm$ 0.66	0.88 (0.08-1.5)
AUC _{inf} (h*ng/mL)	10,489.8 $\pm$ 2,364.7	9,987.0 (6,972.6 - 14,107.3)	4,324.7 $\pm$ 1,072.3	4,586.4 (2,785.5 – 6,263.2)
AUC % extrap	0.55 $\pm$ 0.16	0.50 (0.37-0.83)	1.09 $\pm$ 0.45	0.96 (0.60 – 2.34)
Bioavailability (%)	---	---	7.86 $\pm$ 1.65	8.02 (5.25 – 10.9)
Clearance (mL/h/kg)	499.8 $\pm$ 114.1	500.8 (354.4 – 717.1)	---	---
V _{dss} (L/kg)	4.37 $\pm$ 1.62	4.15 (2.48 – 8.19)	---	---
Terminal t _{1/2} (h)	85.5 $\pm$ 11.6	86.7 (64.1 – 105.6)	85.8 $\pm$ 15.1	87.8 (56.5 – 111.0)

C(0), calculated initial plasma concentration following IV administration; C_{max}, maximum serum drug concentration; T_{max}, time of maximum serum drug concentration; AUC_{inf}, area under the curve extrapolated to infinity; AUC % extrap, percent of area under the curve extrapolated; V_{dss}, volume of distribution at steady state; HL lambda z, terminal half-life.

Table 2.3. Model typical values (tv) for metformin following a single IV (5 mg/kg) and oral (15 grams) administration to 12 horses as determined using a 3-compartment multiplicative error population model and modeling IV and oral administration simultaneously.

Parameters		Random Effects: Between subject variability			
		Estimate	CV(%)	Estimate	CV(%)
Fitted Model	Parameters				
	tvKa (1/h)	0.362	25.8	0.799	110.6
	tvV (L/kg)	0.313	5.99	0.013	11.1
	tvV2 (L/kg)	5.11	17.2	0.249	53.2
	tvV3 (L/kg)	1.57	11.6	0.088	30.3
	tvCl (mL/min/kg)	9.22	5.11	0.023	15.3
	tvCl2 (mL/min/kg)	0.537	8.24	0.048	22.1
	tvCl3 (mL/min/kg)	3.16	7.52	0.001	3.05
	tvFbio (%)	11.6	5.47	0.023	15.1
Derived	Parameters				
	K10 HL (h)	0.393	6.40		
	Effective HL (h)	8.77	13.4		
	Vd _{ss} (L/kg)	7.00	13.3		
	stdev0	0.410	3.37		

tvKa, rate of absorption; tvV, the volume of the central compartment;

tvV2, denotes the volume of the peripheral compartment; tvV3, denotes the volume of the deep peripheral compartment; tvCl, the clearance of drug from plasma; tvCL2, the clearance of drug from the peripheral compartment; tvCL3, the clearance of drug from the deep peripheral compartment; tvFbio, oral bioavailability; K10 HL, K10 half-life; effective HL, half-life that accounts for **drug accumulation and redistribution**; Vd_{ss}, volume of distribution at steady state; stdev0 = the estimated residual standard deviation for plasma data. CV(%), coefficient of variation.

Table 2.4. Metformin blood (serum) and urine concentrations as well as the urine to blood ratio following a single IV administration of 5 mg/kg to 12 horses.

Time	[Blood] (ng/mL)	[Urine] (ng/mL) Range	Urine: Blood
24 h	8.14 – 42.7	835.3 – 19,600.9	64.7 – 387.2
48 h	2.97 – 12.8	182.1 – 2,484.4	65.0 – 375.4
72 h	1.58 – 3.84	55.3 – 534.7	29.8 – 183.7
96 h	0.85 – 2.93	20.2 – 350.7	13.2 – 141.9
120 h	0.87 – 2.71	11.6 – 163.0	10.6 – 80.8
144 h	0.61 – 7.27	14.7 – 52.1	3.70 – 54.6
168 h	0.54 – 3.94	7.33 – 54.6	4.03 – 53.3
192 h	0.55 – 1.60	2.17 – 80.3	1.91 – 50.1
216 h	0.47- 1.49	3.16 – 28.92	5.02 – 59.2
Day 10	0.34 – 0.83	2.49 – 39.1	6.01 – 69.0
Day 11	ND – 0.67	1.05– 23.2	ND – 45.3
Day 12	ND – 0.47	1.40 – 27.2	ND – 18.9
Day 13	ND – 0.42	0.94 – 93.0	ND – 10.4
Day 14	ND	1.34 – 17.5	---

*Urine to blood ratio = urine concentration/blood concentration

ND, not detected.

Table 5. Metformin blood (serum) and urine concentrations as well as the urine to blood ratio following a single oral administration of 15 grams to 12 horses.

Time	[Blood] (ng/mL)	[Urine] (ng/mL) Range	Urine: Blood
24 h	19.2 – 50.3	4,368.5 – 33,486.3	185 - 722
48 h	1.77 – 12.8	570.6 – 5,346.7	55 - 571
72 h	0.57 – 3.46	52.0 – 712.3	50 – 209
96 h	0.5 – 2.61	28.9 – 404.9	28 – 172
120 h	0.44 – 2.27	18.5 – 472.4	19 – 208
144 h	0.40 – 1.69	12.7 – 141.9	12 – 193
168 h	0.54 – 1.06	11.0 – 146.8	18 – 139
192 h	0.34 – 1.00	5.52 – 100.0	10 - 167
216 h	0.33-0.90	3.16 – 201.0	7 - 231
Day 10	<LOQ – 0.58	2.83 – 71.0	8 – 152
Day 11	ND – 0.57	2.30 – 152.0	17 – 276
Day 12	ND – 0.47	1.90 – 240.4	38 – 179
Day 13	ND – 0.30	2.19 – 50.6	31 – 172
Day 14	ND	7.70 – 51.8	---

*Urine to blood ratio = urine concentration/blood concentration

ND, not detected; LOQ, limit of quantitation

Chapter 3. Erythrocyte Sequestration of Metformin in Horses: Impact on Matrix-Specific Pharmacokinetics and Detection Windows

Erythrocyte Sequestration of Metformin in Horses: Impact on Matrix-Specific Pharmacokinetics
and Detection Windows

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Abstract

Metformin is used for the treatment of type 2 diabetes and is one of the most prescribed medications in human medicine. It is less commonly prescribed in equine medicine, and its use is tightly regulated in several performance horse disciplines. In horseracing, it is considered a banned substance. A previous pharmacokinetic study of metformin in horses demonstrated a prolonged, unpredictable elimination phase. In the current study it was hypothesized that this was due to sequestration of metformin in a “deep” compartment, specifically red blood cells. This could result in pharmacokinetic differences between blood matrices (i.e. blood and serum). The objective of the current study was to assess red blood cell partitioning and the concentrations of metformin in different blood matrices following oral administration to horses. To that end, six horses received a single 15 gram oral dose of metformin and plasma, serum, whole blood, red blood cells, and urine samples were collected starting at 5 minutes until 31 days post administration. Concentrations of metformin were determined using liquid chromatography-tandem mass spectrometry, and pharmacokinetic analysis performed. Red blood cells act as a reservoir for metformin in horses with the average blood to plasma ratio ranging from <1 to >10 at the later time points. The terminal half-life (mean $\pm$ SD) was 14.7 ± 7.25 , 75.4 ± 32.2 and 49.1 ± 7.01 in plasma, serum, and red blood cells, respectively. The difference between serum and plasma concentrations was $>15\%$ at several time points, especially at the later times. Concentrations in urine samples, fluctuated unpredictably over time. Red blood cells act as a reservoir for metformin leading to a prolonged detection time, necessitating an extended withdrawal time for oral administration prior to competition in performance horses to prevent inadvertent positive drug tests. Additionally, differences in metformin concentrations across

various biological matrices may preclude the extrapolation of data from one sample type to another.

Introduction

Metformin (1,1-dimethylbiguanide) is a medication commonly used in the treatment of type 2 diabetes mellitus in humans. It works by lowering hepatic glucose production and increasing peripheral tissue glucose sensitivity to lower overall blood glucose concentrations.¹ It is a small, hydrophilic molecule and primarily excreted in the urine as metformin.² As a result of its effectiveness and wide safety margin, metformin is one of the most commonly prescribed drugs in human, medicine.³

In contrast to its widespread use in human medicine, metformin is less commonly prescribed in veterinary medicine. While it is sometimes used for treating insulin dysregulation in horses, its efficacy is controversial. One study found that metformin did not improve insulin sensitivity in insulin-resistant ponies following oral administration.⁴ This is likely attributable, at least in part, to its low bioavailability in horses (~8%).^{5,6} In horses, similar to humans, metformin is excreted in the urine as the parent compound.⁶

Although metformin is not commonly administered to horses, recent reports of positive drug tests in performance horses, necessitates a thorough understanding of the behavior of this drug. The pharmacokinetics of metformin in horses have been reported.^{5,6} In a recent study, metformin pharmacokinetics were studied in 12 horses following a single oral administration of 15 grams and an IV dose of 5 mg/kg.⁶ Metformin concentrations remained above the limit of detection (LOD) of the analytical assay (0.1 ng/mL) for 21 days in blood and >80 days in urine.⁶ The terminal half-life ranged from 56.5 – 111.0 hours.⁶ In humans, there is evidence that metformin partitions into and sequesters in red blood cells (RBC).⁷ The drug is then slowly released back into the plasma, resulting in an extended residence time in the body.⁷ If this same phenomenon occurs in horses, this may explain the prolonged detection time and long terminal

half-life in this species. In addition, for drugs that exhibit significant sequestration in RBCs, concentrations can differ between blood matrices. For example, plasma and serum concentrations may not be comparable, primarily because of the way the samples are processed. Given that different performance horse disciplines regulate drugs in different matrices, this can have regulatory implications. Therefore, understanding how a drug behaves is imperative for appropriate regulation.

To that end, the primary objective of the current study was to determine if metformin partitions into RBCs by measuring metformin concentrations in different blood matrices and the effect of RBC hemolysis on serum and plasma concentrations. A secondary objective was to describe metformin concentrations and determine pharmacokinetic parameters in different blood matrices, to provide data to appropriately regulate administration of this drug to performance horses.

Methods

Animals

Six healthy, university-owned Thoroughbred horses (4 geldings, 2 mares; weighing 473-604 kg, aged 3-6 years) were used for this study. Health status was assessed by physical examination, complete blood count, and serum biochemistry panel prior to beginning the study. Horses did not receive any medications for 2 weeks prior to commencement of the study. Horses were housed in 12 x 24-foot pens and exercised 5 days a week using a Equineciser (Centaur Horse Walkers Inc, Mira Loma, CA, USA) and a high-speed treadmill (Mustang 2200, Graber AG, Switzerland) following standard protocols established by our laboratory.⁸ Horses were exercised prior to and for the duration of the study, with the exception of the day of drug administration. Food and

water were available *ad libitum* for the entirety of the study, except for the 12 hours directly prior to and for 2 hours post drug administration, when food was withheld. This study was approved by the Institutional Animal Care and Use Committee of the University of California, Davis (Protocol # 23732).

Instrumentation and Drug Administration

A 14-gauge catheter was placed aseptically in the external jugular vein of all horses for blood sample collection. Horses received a single oral administration of 15 grams (25-32 mg/kg) of metformin. Metformin tablets (Aurobindo Pharma, Hyderabad, Telangana, India) were crushed and suspended in water in a dosing syringe. Drug was administered directly into the oral cavity within 1 hour of formulation. Sampling catheters were removed 18 hours after dosing, and all following samples were collected via direct venipuncture.

Sample Collection and Processing

Blood samples were collected into Ethylenediaminetetraacetic acid (EDTA) blood tubes for determination of metformin concentrations in plasma, RBCs, and whole blood. For determination of metformin concentrations in serum, one sample was collected into a blood tube devoid of anticoagulant and one sample was collected into a tube containing a serum separator (SST). Since metformin partitions into RBCs in other species, one additional plasma sample (EDTA) and one additional serum sample (without serum separator) were collected to assess whether hemolysis of red blood cells could lead to the release of metformin into the serum or plasma fraction of the blood samples. Samples were collected at times 0 (prior to drug administration), 5, 10, 15, 30, 45 minutes, and 1, 1.5, 2, 3, 4, 5, 6, 8, 12, 18, 24, 30, 36, 48, 72, 96, 120, 144, 168 hours, and 9, 11, 14, 16, 20, 22, 24, 27, 29, and 31 days post drug

administration. Immediately following sample collection, the blood samples from one EDTA tube and one tube devoid of anticoagulant were repeatedly drawn up and reinserted back into the tubes with a clean syringe to induce hemolysis (Figure 3.1). Following collection, all samples in EDTA tubes were kept on ice. The tubes devoid of anticoagulant and the SST were kept at room temperature. All samples were processed within 1 hour of collection.

An aliquot of whole blood was taken from one EDTA tube, while the other 2 EDTA tubes were centrifuged at 3000 g at 4°C for 10 minutes. One aliquot of plasma (one hemolyzed, one non-hemolyzed) was taken from each tube, as well as an aliquot of RBCs from the non-hemolyzed tube. The 2 tubes devoid of anticoagulant and SST were incubated for 30 minutes to 1 hour at 37°C, then centrifuged at 3000 g for 10 minutes. An aliquot of serum was taken from each tube (1 hemolyzed, 2 non-hemolyzed). All samples were stored at -20°C until drug concentration analysis.

As horses are trained to urinate on command, urine was collected via free catch. Samples were collected at 24, 48, 72, 96, 120, 144, 168 hours, and 9, 11, 14, 16, 20, 22, 24, 27, 29, 31, 34, 36, and 41 days post drug administration. Samples were kept on ice and subsequently stored at -20°C until drug concentration analysis.

Drug Concentration Determination:

For both blood and urine samples, calibration curves, quality control samples, and negative control samples were prepared fresh for each assay. Metformin stock solutions (Cerilliant, Round Rock, TX) were generated by diluting working standard solutions with methanol. Fifteen calibrators for each blood sample type were prepared by dilution of the working standard solutions with drug free matrix to concentrations ranging from 0.25 to 5,000 ng/mL. Similarly, urine calibrators (13 calibrators between 0.5 and 25,000 ng/mL) were prepared by dilution of the

working standard solutions with drug free equine urine. Quality control samples for blood were prepared at concentrations of 0.75, 40, and 5000 ng/mL by fortifying drug free matrices with analyte. For urine, quality control samples were prepared by fortifying drug free urine with analyte at 0.75, 100, and 5000 ng/mL.

Serum samples were processed and extracted as described previously.⁶ For RBC lysis in RBC and whole blood samples, 0.5 mL of sample was combined with 0.5 mL water, mixed for 1 minute, incubated for 10 minutes at room temperature, and mixed for an additional 1 minute prior to freezing at -80 °C. Samples were thawed at room temperature and 0.5 mL was aliquoted to a new vial. Whole blood, RBCs, and plasma samples underwent protein precipitation as described previously for serum samples.⁶ Urine samples were extracted as previously described.⁶

For all matrices, 20 µL was injected into the liquid chromatography tandem mass spectrometry (LC-MS/MS) system and quantitative analysis was conducted as previously described.⁶

The limit of quantitation (LOQ; the lowest calibrator that could be measured with acceptable precision) was 0.25 ng/mL for plasma and serum, and 0.3 ng/mL for RBCs and whole blood. The LOD (lowest calibrator with a 3:1 signal to noise ratio) was 0.1 ng/mL for plasma and serum and 0.2 ng/mL for RBCs and whole blood. For urine the LOQ and LOD were 0.5 ng/mL and 0.25 ng/mL, respectively.

Quality control samples met accuracy and precision acceptance criteria as described in the Food and Drug Administration's (FDA) Guidance for Bioanalytical Method Development and Validation.⁹ (Table 3.1).

In vitro Assessment of Matrix comparability (Plasma vs Serum):

An *in vitro* study was conducted to compare metformin concentrations in plasma and serum. Blood samples collected from six horses not administered metformin were compared *in vitro*. For each horse, three tubes were collected: (1) EDTA blood tube, (2) tube devoid of anti-coagulant, and (3) tube devoid of anticoagulant but with a serum separator. Three aliquots were taken from each tube type for each horse. Each aliquot was fortified with metformin at the low and high end of the calibration curve. Samples were processed and analyzed for determination of metformin concentrations as described for administration samples.

Comparability between plasma and serum was assessed by calculating the % relative difference with plasma concentration as the reference matrix, as plasma is the standard matrix for pharmacokinetic characterization of metformin. The percent relative difference was calculated for each paired sample at each time point using the formula:

$$\%Relative\ Difference = \frac{C_{serum} - C_{plasma}}{C_{plasma}} \times 100$$

To calculate the % relative difference between serum samples collected in tubes without a separator compared to serum collected in SSTs, serum samples from tubes without a separator were used as the reference matrix with the formula:

$$\%Relative\ Difference = \frac{C_{serum} - C_{SST}}{C_{serum}} \times 100$$

Where C_{serum} is serum collected in tubes without a separator and C_{SST} is serum collected in SSTs. A mean bias $\leq \pm 15\%$ was considered an indication of comparable concentrations between the matrices in alignment with international regulatory guidelines for bioanalytical cross-validation and analytical accuracy.¹⁰

Concentration and Pharmacokinetic Analysis:

Metformin concentrations at each timepoint post administration were compared between plasma and serum samples and between serum samples collected in SST tubes and tubes without a separator as described above for “Assessment of Matrix Comparability.” The blood to plasma (B/P) ratio was calculated using the formula:

$$B/P = [metformin]_{whole\ blood} / [metformin]_{plasma}$$

Non-compartmental analysis (NCA) was performed using a commercially available software program (Phoenix WinNonlin V8.3, Certara, Princeton, NJ). For determination of pharmacokinetic parameters in all matrices, concentration data was truncated due to a prolonged terminal phase with extremely low concentrations (see Supplementary Figure 3.1). For plasma data, concentrations up to 96 hours were utilized. For serum, red blood cells, and whole blood, concentrations up to 9 days were utilized. Plasma concentrations were uniformly weighted for pharmacokinetic analysis. The area under the curve extrapolated to infinity (AUC_{inf}) was calculated using the linear up log down method with the extrapolated portion calculated using the formula:

$$= \text{Concentration}_{last} / \lambda_z$$

Where λ_z represents the slope of the terminal portion of the curve and includes a minimum of 3 data points.

The percent of the AUC extrapolated (AUC extrap) was calculated using the formula:

$$= 100 * \left[\frac{AUC_{inf} - AUC_{last}}{AUC_{inf}} \right]$$

The half-life λ_z was calculated using the formula:

$$= \ln(2) / \lambda_z$$

The maximal concentration ($C_{\max}$) and time of maximal concentration ($T_{\max}$) were determined directly from the concentration curves.

Results

In vitro Assessment of Matrix comparability (Plasma vs Serum):

The % relative difference in metformin concentrations between plasma and serum and serum collected in tubes with and without a separator was <15% (Table 3.2).

Metformin Pharmacokinetic Study:

At approximately 4 hours post drug administration, two horses showed mild signs of abdominal discomfort (pawing and rolling); these signs resolved on their own within 2 hours. On day 3 (72 hours) after beginning the study, one horse was administered oral sulfamethoxazole and trimethoprim for treatment of a condition unrelated to the nature of the present study. Treatment lasted for 14 days. Samples collected from this horse were analyzed for determination of metformin concentrations and were included in the pharmacokinetic analysis.

Serum concentrations (mean $\pm$ SD) of metformin are depicted in Figure 3.2. Samples were collected into tubes with and without a serum separator for comparison purposes. Metformin concentrations were > 15% different between the 2 tube types at 15 minutes (16.6%), and 18 (15.8%), 30 (17.0%), 120 (23.9%), 144 (113.9%), and 168 (35.7%) hours (Supplementary Table 3.1). At all times, where the concentration difference was >15%, the concentrations in the SST were higher. Metformin serum concentrations were below the LOQ in all horses at 24 and 27 days but were again detectable in one horse at 29 days (Figure 3.2).

Plasma metformin concentrations over time are depicted in Figure 3.3. Metformin was not detected in any of the plasma samples collected between 9 and 14 days. Drug was detected in plasma samples from one horse on day 16 (1.53 ng/mL) and then in a different horse again on day 29 (1.80 ng/mL) and day 31 (0.42 ng/mL). The percent difference between plasma and serum metformin concentrations are reported in Supplementary Table 3.1. Concentrations were higher in plasma compared to serum (25.6% higher at 10 minutes; Supplementary Table 3.1). Serum concentrations were >15% higher, compared to plasma at 3 (25.1%), 4 (31.4%), and from 48-168 hours (16.1-150.5%) post drug administration (Supplementary Table 3.1). Metformin was detected on days 9 and 11 in serum but not plasma (Supplementary Table 3.1). Metformin concentrations in plasma were higher than RBCs until 24 hours post administration. After 24 hours, concentrations remained higher in RBCs compared to plasma (Figure 3.4). A comparison of metformin concentrations in plasma, RBCs and whole blood at each sample collection time post administration are depicted in Figure 3.5. In whole blood samples, metformin concentrations fell below the LOQ in all horses between 14 and 16 days and were detectable again at 20 days (0.32 ng/mL) in one horse. Between 20 and 24 days, concentrations were non-detectable in all horses, returning to detectable concentrations on day 27 (15.5 ng/mL) and day 29 (0.77 ng/mL) in one horse (same horse that had detectable concentrations at 20 days). The B/P ratio for each time point is listed in Table 3.3. The B/P ratio was less than 1 until the 24-hour timepoint. The B/P ratio at the 30-hour time point was >1 with the highest value observed at 96 hours (10.6 ± 4.63).

Metformin concentrations in hemolyzed serum and plasma samples are compared to non-hemolyzed samples in Figure 3.6. Metformin concentrations were > 15% higher in the 72-hour hemolyzed plasma sample compared to non-hemolyzed plasma samples. In 2 horses, metformin

was detected at 16 and 24 days post administration in hemolyzed plasma samples, whereas drug was not detected in the corresponding non-hemolyzed samples (Figure 3.6A). Metformin concentrations were >15% higher in hemolyzed serum samples compared to non-hemolyzed serum samples at 5 minutes (480.2 ± 203.6 ng/mL vs 380.7 ± 171.6 ng/mL (mean $\pm$ SD) and 10 minutes (338.5 ± 258.5 ng/mL vs 250.6 ± 99.4 ng/mL) and 18 hours (61.8 ± 26.1 ng/mL vs 43.4 ± 3.59 ng/mL) and 144 hours (0.77 ± 0.37 ng/mL vs 0.61 ± 0.26 ng/mL) post drug administration. In one horse, metformin was detected at 9 and 11 days post administration in hemolyzed serum samples, whereas drug was not detected in the corresponding non-hemolyzed samples (Figure 3.6B)

The mean $\pm$ SD, median, and range of select pharmacokinetic parameters following noncompartmental analysis for serum, plasma, RBCs, and whole blood are listed in Table 3.4. The $C_{\max}$ (mean $\pm$ SD) of metformin was highest in serum (788.0 ± 289.2 ng/mL) and plasma (800.0 ± 352.2 ng/mL) (Table 3.4). The $C_{\max}$ was 148.7 ± 54 ng/mL and 478.0 ± 209.8 ng/mL for RBCs and whole blood, respectively. The $T_{\max}$ was similar between matrices (Table 3.4). The number of data points used in the calculation of lambda z was 7.3 ± 5.9 , 8.0 ± 5.7 , 7.2 ± 2.6 , and 5.3 ± 1.6 for serum, plasma, RBCs, and whole blood, respectively. The terminal half-life of metformin (mean $\pm$ SD) was shortest in plasma at 14.7 ± 7.25 hours (Table 3.4). The terminal half-life was 49.1 ± 7.01 , 50.6 ± 15.6 , and 75.2 ± 32.2 hours for RBCs, whole blood, and serum (Table 3.4). The terminal half-lives for the horse that received sulfamethoxazole and trimethoprim was within the range reported for other horses (serum: 55.8 hours; plasma: 10.0 hours; RBCs: 51.3 hours).

Metformin urine concentrations (mean $\pm$ SD) with respect to time are depicted in Figure 3.7. Metformin concentrations fluctuated over time for each horse. At day 41 post administration

(the last time point collected), urine metformin concentrations in 5/6 horses were above the LOQ.

Discussion

A previous study conducted by our laboratory demonstrated a prolonged terminal half-life for metformin following administration to horses.⁶ Studies in humans have described a “deep” compartment that contributes to this prolonged elimination.^{2,7,11} This deep compartment includes RBCs^{2,7,11} and sequestration in these cells creates a depot for the drug, contributing to a prolonged terminal elimination phase.⁷ One of the objectives of the current study was to assess whether a similar “deep” compartment exists in horses, perhaps explaining the prolonged terminal half-life reported previously.⁶

Serum pharmacokinetic parameters, including $C_{\max}$ and $T_{\max}$ were consistent with the previous report describing the pharmacokinetics of metformin in horses following oral administration of the same dose.⁶ The prolonged terminal serum half-life in the current study (75.8 ± 32.2 hours) was also in agreement with the previous report (85.8 ± 15.1 hours).⁶

Maximum metformin concentrations in RBCs were lower compared to serum and plasma, similar to what has been reported in humans.⁷ In contrast to human studies, whereby maximum concentrations occurred at 4.7 hours post drug administration,⁷ $T_{\max}$ in the current study occurred quickly following drug administration (0.83 hours). This is not unexpected as the plasma $T_{\max}$ in the current study is also much earlier (0.90 hours) compared to the previous study in humans (3.0 hours).⁷ Distribution of metformin from plasma to RBCs is a result of passive diffusion and therefore the most rapid rate of diffusion into the RBCs would be expected to correlate with the time at which plasma concentrations are highest.^{7,12} In the current study,

equilibration of metformin concentrations between plasma and RBCs occurred between 18 and 24 hours post administration, with concentrations in RBCs remaining higher than plasma for the remainder of the sample collection period. The longer detection time resulted in a longer terminal half-life in RBCs (49.1 ± 7.01 hours) compared to plasma (14.7 ± 7.25 hours). This longer detection time in RBCs and subsequently, longer RBC half-life (23.4 ± 1.9 hours) compared to plasma (2.7 ± 0.2 hours) has also been reported for humans.⁷ Notably, the terminal half-life in both plasma and RBCs was longer in the current study compared to the previous report by Robert and colleagues. While this may be related to species differences, it is also important to be cognizant of differences in sampling protocols and the greater sensitivity of the analytical instrumentation utilized in the current study (plasma LOQ: 0.25 ng/mL; RBC LOQ: 0.3 ng/mL) compared to the previous study (plasma LOQ: 20 ng/mL and RBC LOQ: 30 ng/mL).⁷

The blood to plasma (B/P) ratio for a drug describes distribution between RBCs and plasma.¹³ In an *in vitro* study, the B/P ratio for metformin was reported to be 1.2 and 1.4 in humans and rats, respectively.¹⁴ *In vivo* clinical studies, report B/P ratios ranging from 1 to >10, depending on the time post administration.^{2,11} Tucker and colleagues reported that the metformin B/P ratio was <1 between 0 and 4 hours post drug administration and >10 at 24 hours.¹¹ A similar trend was observed in the current study, whereby the B/P was <1 until 18 hours post administration, increasing to a B/P of 8 at 72 hours post administration and reaching 10.6 at 96 hours. This high B/P ratio is also a likely explanation for the long terminal half-life observed in the current study. It is interesting to note that in one horse, the metformin concentration was 15.1 ng/mL at 648 hours. Metformin concentration in the two previous samples collected from this horse were non-detectable. There was nothing notably different about this horse or sample and

re-extraction and analysis resulted in a similar concentration. Based on this, the most likely explanation for this finding is sequestration in RBCs.

Another notable finding in the current study were differences in metformin concentrations between serum and plasma. This is especially important in equine drug testing, whereby some disciplines or even different horseracing jurisdictions may use serum, and others may utilize plasma for regulatory purposes. Differences between matrices were considered negligible if the % relative difference remained within $\pm 15\%$, ensuring that any observed variation is within the limits of acceptable analytical error in accordance with standard bioanalytical method validation acceptance criteria.¹⁰ While the magnitude and significance of the concentration difference varied, depending on the time of sample collection, there were notable differences between plasma and serum. For drugs that sequester in RBCs, such as metformin, concentrations in serum may be artificially elevated due to liberation of the RBC bound fraction into the serum during the clotting process. Since plasma samples are immediately placed on ice and centrifuged, concentrations represent the drug circulating in the aqueous phase and leakage of drug from RBCs into plasma is less of a concern. In the current study, concentrations of metformin were initially higher in plasma compared to serum ($>15\%$ difference). This is not unexpected as concentrations in plasma at these early timepoints represent levels prior to significant distribution into RBCs and tissue. In the case of serum, the time necessary for the clot to form provides a period of time in which the drug in the liquid phase can continue to interact with the RBCs, if distribution equilibrium has yet to be reached, as is likely at these early timepoints, metformin continues to move into the cells, thereby decreasing serum concentrations. Although at most time points post distribution the difference was $<15\%$, metformin concentrations tended to be higher in serum compared to plasma, most likely due to

“cellular leakage.” The difference between the two matrices was more pronounced (>15%) at the later times (48 hours onwards) when more drug was present in the RBCs. The presence of higher concentrations of metformin in RBCs is supported by the much higher B/P ratio at the later time points. Based on these results, it is important to be cognizant of differences in the matrices utilized in experimental studies versus what is used to regulate metformin in equine performance events. If they are different (i.e. plasma versus serum), comparisons may not be appropriate. An additional consideration related to collection of blood samples for drug testing purposes, especially if serum is the preferred matrix, is that the longer the samples sit prior to centrifugation, the greater the likelihood of “cellular leakage” from RBCs into serum due to cellular lysis.¹⁵

Hemolysis, or the rupture of RBCs and subsequent release of cellular contents can occur during sample collection and handling. In the case of drugs like metformin, that are sequestered in RBCs, hemolysis can lead to artificially elevated concentrations of the drug in serum or plasma. In the case of plasma, hemolysis is most likely during sample collection and in the case of serum, this can occur during collection and/or during the clotting time leading to “cellular leakage”, as discussed above. In the current study, overall hemolysis had a minimal impact on blood metformin concentrations. It is important to note that while all attempts were made to induce maximum hemolysis, that the degree of experimentally induced hemolysis may not represent a real-life scenario.

Similar to the report by Jacobs et al, urine metformin concentrations varied greatly between horses.⁶ Also as described previously, decreases in urine concentrations would be followed by subsequent increases in individual horses.⁶ Jacobs and colleagues speculated that in addition to well-known factors that can affect urine concentrations (i.e. hydration, status, urinary

pH, etc), that these “spikes” may also be due to slow and/or inconsistent release of drug from sequestration sites, such as RBCs.⁶ Results of the current study, demonstrating that metformin does sequester in RBCs in horses, supports this theory.

Results of the current study demonstrate that, as described for humans, metformin sequesters in RBCs, resulting in a prolonged detection time and long terminal half-life. This sequestration led to differences in metformin concentrations between the different blood matrices (i.e. plasma vs serum). These findings are important as the distribution of drugs to “deep” compartments can lead to a prolonged terminal half-life, ultimately affecting efficacy, toxicity, and in the case of regulation of drugs, the detection time.

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Conflicts of Interest:

The authors have no conflicts of interest to declare.

Data Availability:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures and Tables

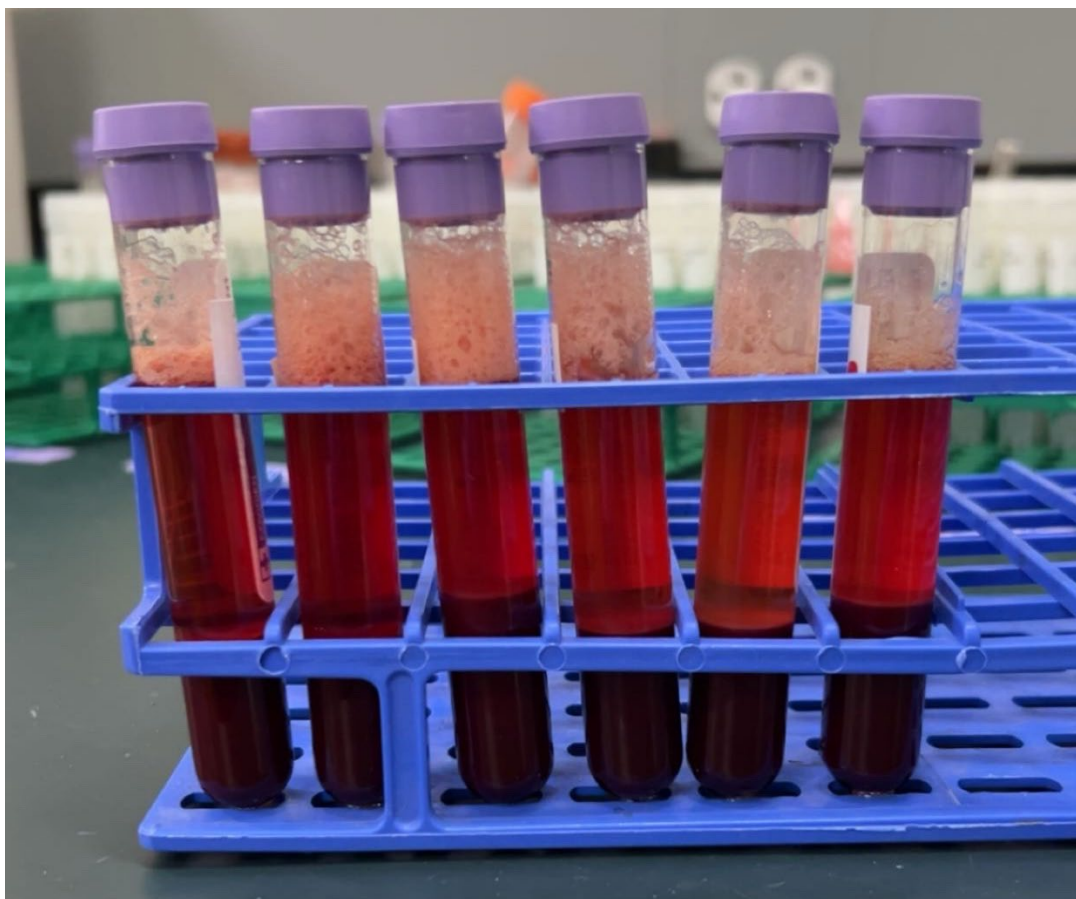


Figure 3.1. Example of hemolyzed plasma samples collected post metformin administration.

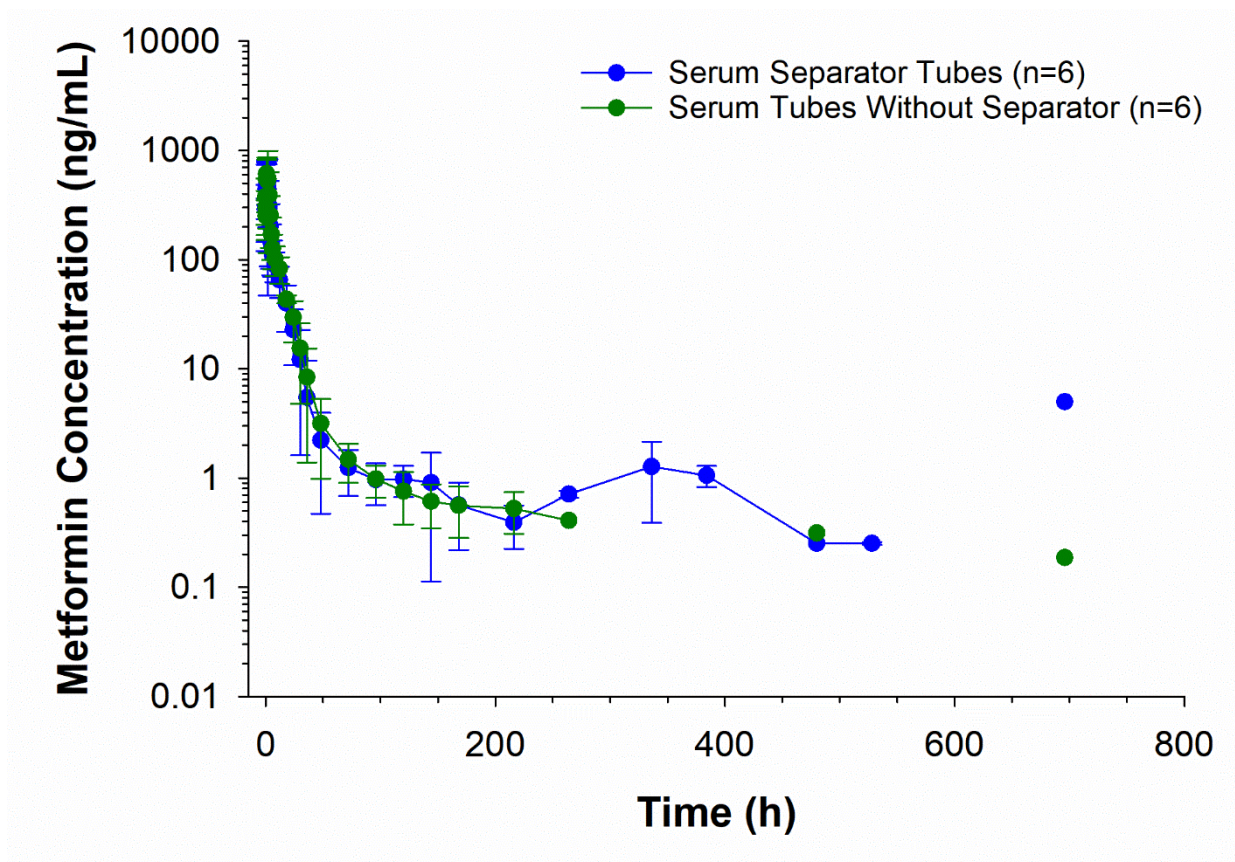


Figure 3.2. Serum metformin concentrations (mean $\pm$ SD) following a single oral administration of 15 grams to 6 horses.

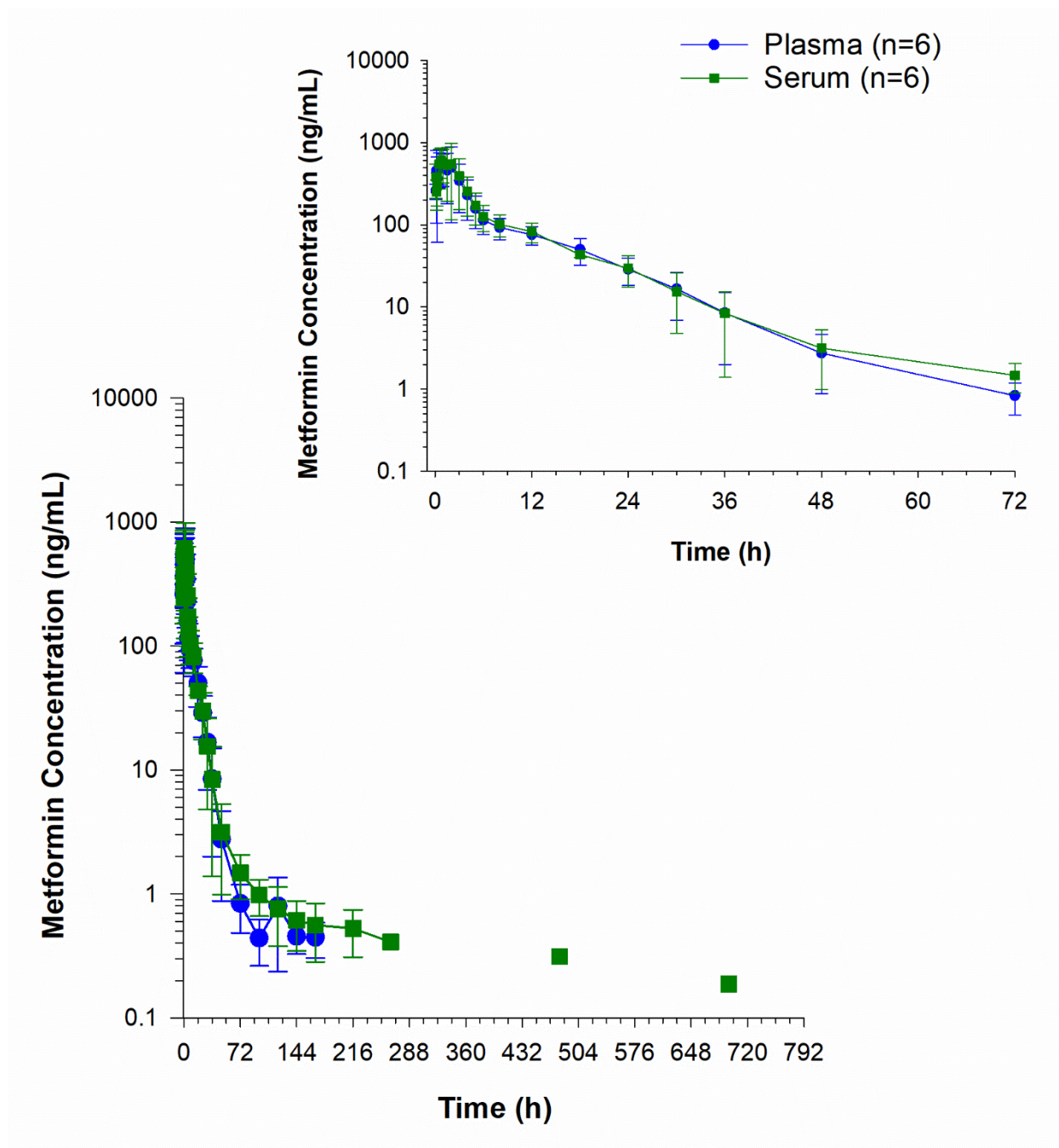


Figure 3.3. Serum and plasma metformin concentrations (mean $\pm$ SD) following a single oral administration of 15 grams to 6 horses. Inset represents the first 72 hours post drug administration.

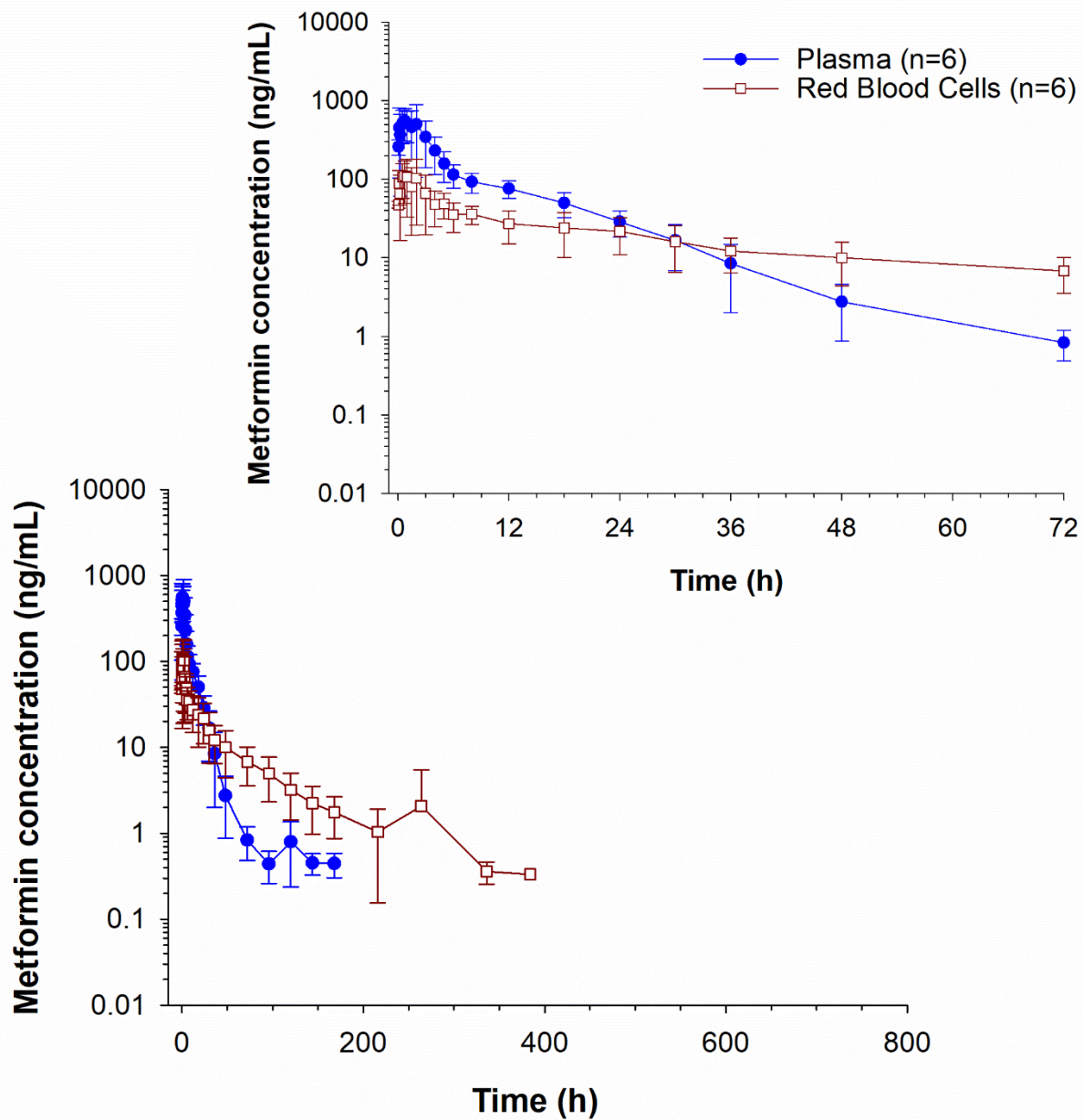


Figure 3.4. Metformin concentrations (mean $\pm$ SD) in plasma and red blood cells (RBC) following a single oral administration of 15 grams to 6 horses. Inset represents the first 72 hours post drug administration.

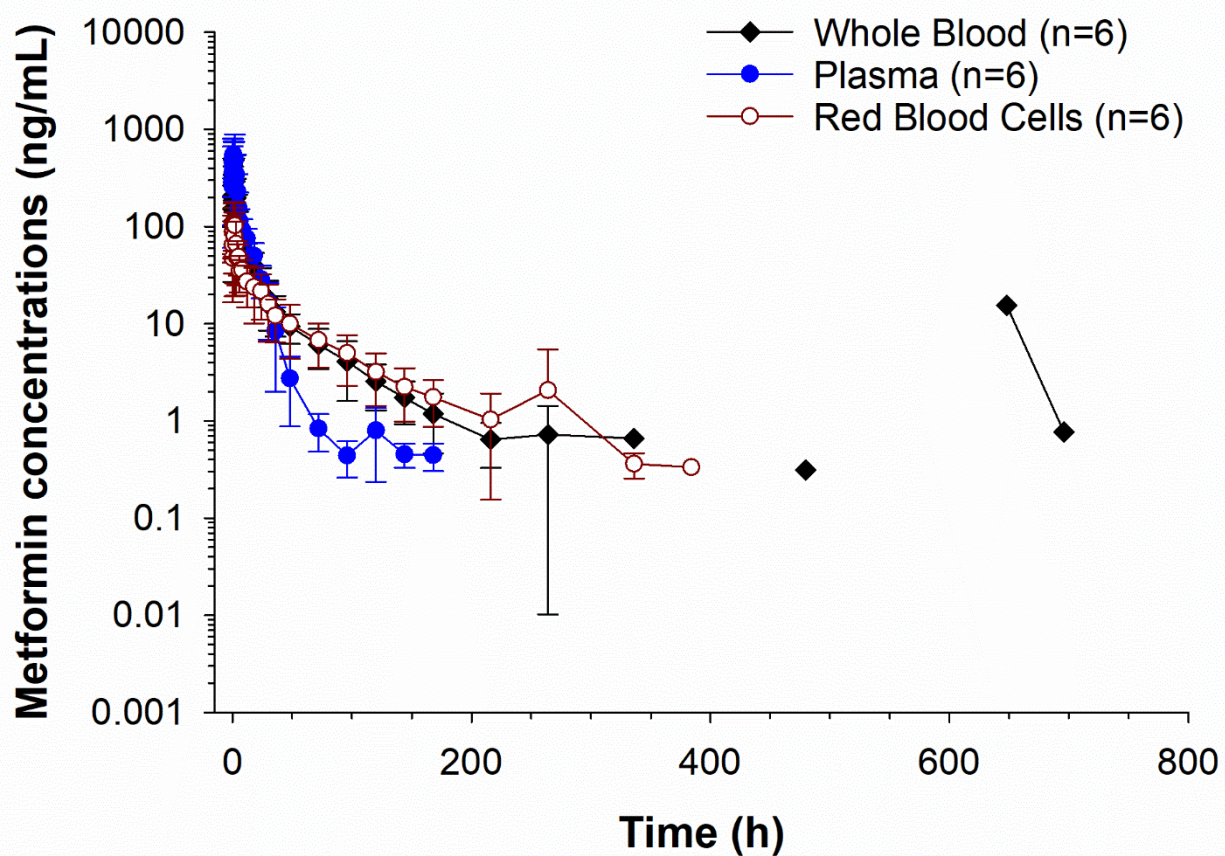


Figure 3.5. Metformin concentrations (mean $\pm$ SD) in whole blood, plasma, and red blood cells following a single oral administration of 15 grams to 6 horses.

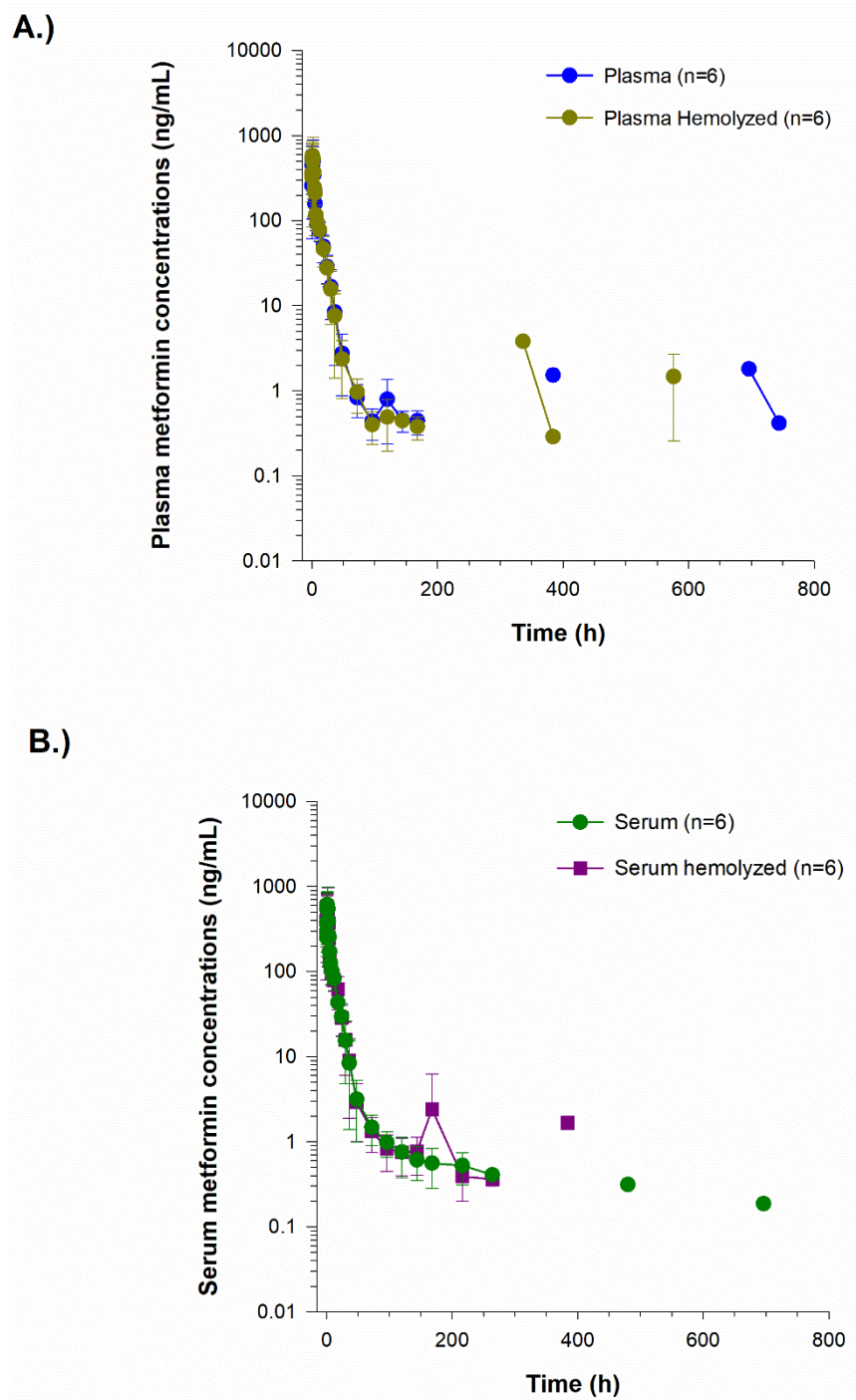


Figure 3.6. Metformin concentrations (mean $\pm$ SD) in hemolyzed and non-hemolyzed (A) plasma and (B) serum samples following a single oral administration of 15 grams to 6 horses.

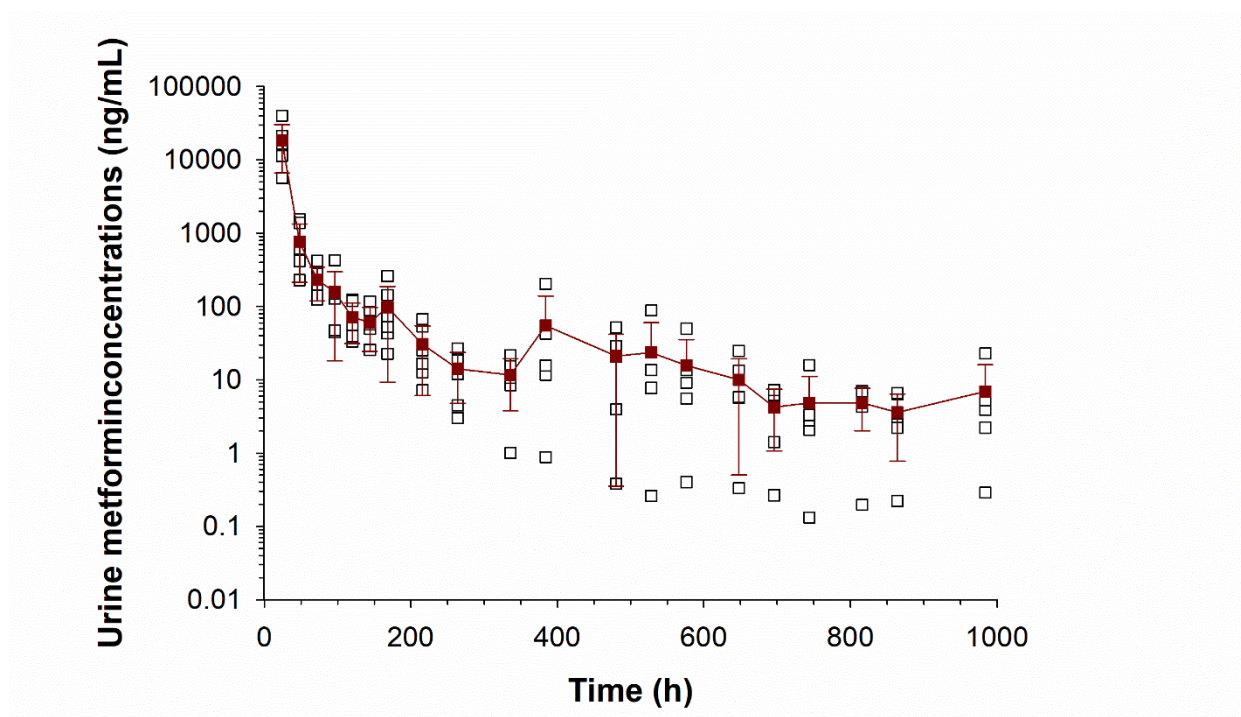


Figure 3.7. Urine metformin concentrations following a single oral administration of 15 grams to 6 horses. Mean concentrations are shown with solid points ($\pm$ SD), and individual points are shown with open squares.

Table 3.1. Accuracy and precision values for LC-MS/MS analysis of metformin in equine serum, plasma, RBCs, whole blood, and urine.

Matrix	Concentration (ng/mL)	Accuracy (% nominal concentration)	Precision (% relative SD)
Serum	0.75	91.0	12.0
	40.0	101	1.0
	5000	100	2.0
Plasma	0.75	106	4.0
	40.0	106	1.0
	5000	107	3.0
RBCs	0.75	98.0	13.0
	40.0	108	1.0
	5000	101	2.0
Whole Blood	0.75	100	9.0
	40.0	104	2.0
	5000	99.0	4.0
Urine	0.75	92.0	6.0
	100	105	6.0
	5000	95.0	3.0

Table 3.2. Differences in metformin concentrations between plasma and serum (A) and serum collected with and without a serum separator (B) as determined *in vitro*.

A.)

	Plasma %CV	Serum %CV	% relative difference *
QC1 (40 ng/mL)	1.9	1.9	0.54
QC2 (5000 ng/mL)	4.3	4.6	2.63

*Plasma used as the reference.

B.)

	Serum %CV	SST CV	% relative difference *
QC1 (40 ng/mL)	1.9	1.8	1.93
QC2 (5000 ng/mL)	4.6	3.1	3.23

*Serum used as the reference.

Table 3.3. Metformin concentrations in whole blood and plasma and the blood to plasma (B/P)

ratio following a single oral administration of 15 grams to six horses. Values are mean $\pm$ SD.

Time (h)	[Metformin] (ng/ml)		B/P ratio*
	Whole Blood	Plasma	
0	ND	ND	---
0.08	206.6 $\pm$ 107.5	258.8 $\pm$ 55.7	0.77 $\pm$ 0.23
0.16	264.2 $\pm$ 237.2	453.3 $\pm$ 349.4	0.61 $\pm$ 0.33
0.25	152.8 $\pm$ 48.6	366.4 $\pm$ 305.4	0.53 $\pm$ 0.18
0.5	311.2 $\pm$ 105.3	516.4 $\pm$ 233.4	0.66 $\pm$ 0.27
0.75	342.0 $\pm$ 156.4	553.7 $\pm$ 245.3	0.62 $\pm$ 0.05
1	335.4 $\pm$ 147.3	512.4 $\pm$ 220.2	0.65 $\pm$ 0.06
1.5	309.2 $\pm$ 193.8	463.1 $\pm$ 281.9	0.66 $\pm$ 0.03
2	292.7 $\pm$ 245.2	499.5 $\pm$ 393.4	0.58 $\pm$ 0.09
3	198.3 $\pm$ 113.5	344.5 $\pm$ 203.7	0.58 $\pm$ 0.04
4	146.1 $\pm$ 66.9	231.2 $\pm$ 117.0	0.64 $\pm$ 0.05
5	100.8 $\pm$ 41.7	158.3 $\pm$ 67.3	0.64 $\pm$ 0.06
6	68.4 $\pm$ 23.6	114.2 $\pm$ 37.4	0.60 $\pm$ 0.08
8	62.4 $\pm$ 14.7	92.8 $\pm$ 26.7	0.68 $\pm$ 0.06
12	50.8 $\pm$ 15.6	75.8 $\pm$ 19.0	0.67 $\pm$ 0.09
18	39.7 $\pm$ 14.2	50.0 $\pm$ 17.7	0.80 $\pm$ 0.12
24	26.1 $\pm$ 11.3	28.8 $\pm$ 10.6	0.90 $\pm$ 0.08
30	18.1 $\pm$ 9.53	16.6 $\pm$ 9.76	1.16 $\pm$ 0.24
36	13.4 $\pm$ 5.97	8.45 $\pm$ 6.45	2.08 $\pm$ 1.09
48	9.41 $\pm$ 3.17	2.75 $\pm$ 1.87	4.42 $\pm$ 2.19
72	6.14 $\pm$ 2.70	0.83 $\pm$ 0.35	7.83 $\pm$ 3.39
96	4.12 $\pm$ 2.51	0.44 $\pm$ 0.18	10.6 $\pm$ 4.63
120	2.55 $\pm$ 1.26	0.80 $\pm$ 0.56	7.23 $\pm$ 6.98
144	1.73 $\pm$ 0.81	0.45 $\pm$ 0.13	4.73 $\pm$ 2.15
168	1.19 $\pm$ 0.73	0.44 $\pm$ 0.14	3.99 $\pm$ 1.15
Day 9	0.64 $\pm$ 0.31	<LOQ	---
Day 11	0.60 $\pm$ 0.71	<LOQ	---

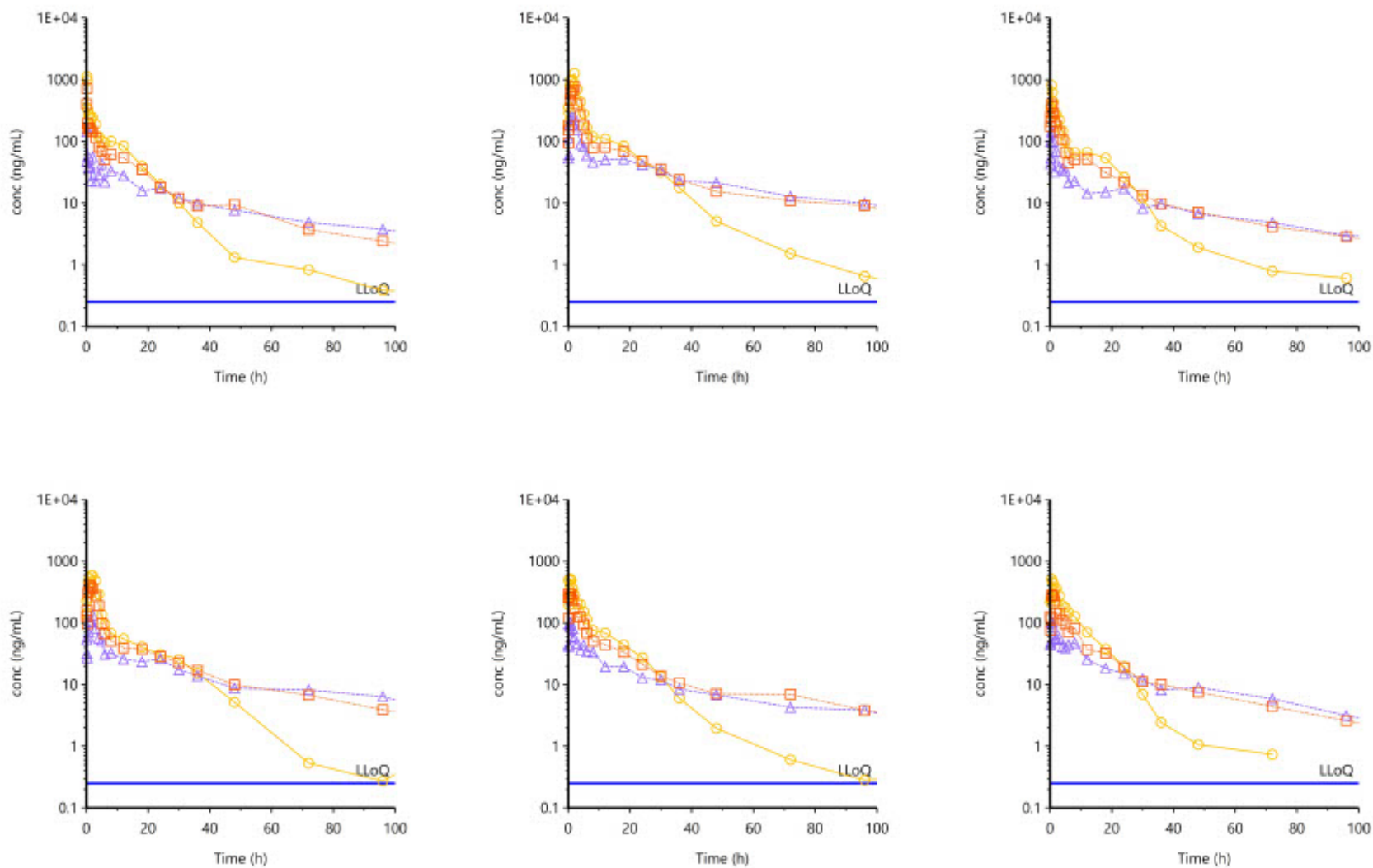
*B/P = whole blood concentration/plasma concentration

LOQ, limit of quantitation; ND, not detected; ---, not determined

Table 3.4. Pharmacokinetic parameters for metformin in various blood matrices following a single oral (15 grams) administration to 6 horses. Parameters generated using non-compartmental analysis.

	$C_{\max}$ (ng/mL)	$T_{\max}$ (h)	AUC_{inf} (h*ng/mL)	AUC extrap (%)	Lambda z (1/h)	Terminal $t_{1/2}$ (h)
Serum						
Mean $\pm$ SD	788.0 $\pm$ 289.2	1.0 $\pm$ 0.79	4012.3 $\pm$ 1344.1	4.72 $\pm$ 6.41	0.013 $\pm$ 0.010	75.4 $\pm$ 32.2
Median	732.7	0.63	3725.9	1.76	0.008	85.8
Range	527.3-1270.5	0.25-2.0	2892.9-6626.5	0.24-16.9	0.006-0.033	20.9-110.8
Plasma						
Mean $\pm$ SD	800.0 $\pm$ 325.2	0.90 $\pm$ 0.70	3592.6 $\pm$ 1358.7	0.45 $\pm$ 0.40	0.058 $\pm$ 0.028	14.7 $\pm$ 7.25
Median	695.2	0.63	2999.2	0.30	0.054	13.5
Range	512.6-1262.5	0.16-2.0	2590.3-6130.6	0.15-1.23	0.026-0.103	6.71-27.1
RBCs						
Mean $\pm$ SD	148.7 $\pm$ 54.1	0.83 $\pm$ 0.63	2031.7 $\pm$ 917.9	5.57 $\pm$ 9.85	0.014 $\pm$ 0.002	49.1 $\pm$ 7.01
Median	140.0	0.63	1762.5	1.55	0.014	51.2
Range	100.3-247.7	0.25-2.0	1442.6-3788.9	0.90-25.6	0.012-0.019	36.8-55.9
Whole Blood						
Mean $\pm$ SD	478.0 $\pm$ 209.8	0.76 $\pm$ 0.69	3417.2 $\pm$ 1456.8	4.59 $\pm$ 8.22	0.015 $\pm$ 0.003	50.6 $\pm$ 15.6
Median	402.2	0.63	2787.4	1.02	0.015	45.5
Range	285.0-764.6	0.16-2.0	2288.2-5841.7	0.60-21.3	0.008-0.017	40.4-81.6

$C_{\max}$, maximum drug concentration; $T_{\max}$, time of maximum drug concentration; AUC inf, area under the curve extrapolated to infinity; AUC % extrap, percent of area under the curve extrapolated; Lambda z, terminal slope of the concentration time curve; HL lambda z, terminal half-life.



Supplementary Figure 3.1. Concentration curves including all time points where drug was detected in individual horses. Plasma concentrations are represented by the yellow line, red blood cells by the purple line, and whole blood by the red line.

Supplementary Table 3.1. Percent differences between plasma and serum metformin concentrations following a single oral (15 grams) administration to 6 horses.

Plasma vs Serum

Time	Metformin Concentration Mean $\pm$ SD (ng/mL) Plasma	Metformin Concentration Mean $\pm$ SD (ng/mL) Serum	Relative mean difference (%) *
0 h	ND	ND	
0.16 h	258.7 $\pm$ 55.7	250.6 $\pm$ 99.4	25.6
0.25 h	453.3 $\pm$ 349.4	298.4 $\pm$ 129.8	0.4
0.5 h	366.4 $\pm$ 305.4	549.2 $\pm$ 277.2	4.6
0.75 h	516.4 $\pm$ 233.4	611.9 $\pm$ 246.6	12.0
1 h	553.7 $\pm$ 245.3	575.1 $\pm$ 251.4	12.0
1.5 h	512.4 $\pm$ 220.2	529.9 $\pm$ 337.1	13.2
2 h	463.1 $\pm$ 281.9	549.1 $\pm$ 434.3	9.8
3 h	499.6 $\pm$ 393.4	391.9 $\pm$ 239.4	25.1
4 h	344.5 $\pm$ 203.7	255.1 $\pm$ 127.2	31.4
5 h	231.2 $\pm$ 117.0	171.2 $\pm$ 71.6	8.8
6 h	158.3 $\pm$ 67.3	126.1 $\pm$ 44.0	9.8
8 h	114.2 $\pm$ 37.4	101.6 $\pm$ 30.7	9.3
12 h	92.8 $\pm$ 26.7	82.7 $\pm$ 22.5	8.7
18 h	75.8 $\pm$ 19.0	43.4 $\pm$ 3.59	7.2
24 h	50.0 $\pm$ 17.7	29.6 $\pm$ 12.1	2.3
30 h	28.8 $\pm$ 10.6	15.5 $\pm$ 10.7	11.6
36 h	16.7 $\pm$ 9.76	8.38 $\pm$ 6.99	3.3
48 h	8.45 $\pm$ 6.45	3.15 $\pm$ 2.16	16.1
72 h	2.75 $\pm$ 1.87	1.48 $\pm$ 0.57	87.4
96 h	0.83 $\pm$ 0.35	0.98 $\pm$ 0.32	150.5
120 h	0.44 $\pm$ 0.18	0.76 $\pm$ 0.38	96.1
144 h	0.80 $\pm$ 0.56	0.61 $\pm$ 0.26	70.8
168 h	0.45 $\pm$ 0.13	0.56 $\pm$ 0.28	85.6
Day 9	<LOQ	0.53 $\pm$ 0.22	
Day 11	<LOQ	0.41 $\pm$ 0.0	

*Plasma used as reference

No Serum vs Serum Separator (SST)

Time	Metformin Concentration Mean $\pm$ SD (ng/mL) No serum separator	Metformin Concentration Mean $\pm$ SD (ng/mL) SST	Relative mean difference (%) *
0 h	ND	ND	
0.16 h	250.6 $\pm$ 99.4	236.9 $\pm$ 64.2	8.6
0.25 h	298.4 $\pm$ 129.8	362.4 $\pm$ 299.5	16.6
0.5 h	549.2 $\pm$ 277.2	527.2 $\pm$ 229.9	0.4
0.75 h	611.9 $\pm$ 246.6	568.2 $\pm$ 259.6	8.9
1 h	575.1 $\pm$ 251.4	532.2 $\pm$ 236.3	7.7
1.5 h	529.9 $\pm$ 337.1	488.7 $\pm$ 303.9	7.4
2 h	549.1 $\pm$ 434.3	515.5 $\pm$ 394.6	5.6
3 h	391.9 $\pm$ 239.4	368.1 $\pm$ 218.7	4.3
4 h	255.1 $\pm$ 127.2	243.0 $\pm$ 120.3	8.1
5 h	171.2 $\pm$ 71.6	162.5 $\pm$ 69.2	5.5
6 h	126.1 $\pm$ 44.0	120.0 $\pm$ 39.8	4.3
8 h	101.6 $\pm$ 30.7	99.1 $\pm$ 27.2	1.7
12 h	82.7 $\pm$ 22.5	80.0 $\pm$ 20.7	3.1
18 h	43.4 $\pm$ 3.59	50.7 $\pm$ 18.2	15.8
24 h	29.6 $\pm$ 12.1	29.4 $\pm$ 12.1	1.3
30 h	15.5 $\pm$ 10.7	16.4 $\pm$ 10.6	17.0
36 h	8.38 $\pm$ 6.99	7.93 $\pm$ 6.4	3.4
48 h	3.15 $\pm$ 2.16	3.03 $\pm$ 1.75	1.6
72 h	1.48 $\pm$ 0.57	1.41 $\pm$ 0.56	1.6
96 h	0.98 $\pm$ 0.32	1.08 $\pm$ 0.40	9.8
120 h	0.76 $\pm$ 0.38	0.85 $\pm$ 0.31	23.9
144 h	0.61 $\pm$ 0.26	1.11 $\pm$ 0.79	113.9
168 h	0.56 $\pm$ 0.28	0.70 $\pm$ 0.35	35.7
Day 9	0.53 $\pm$ 0.22	0.43 $\pm$ 0.17	14.2
Day 11	0.41 $\pm$ 0.0	0.33 $\pm$ 0.05	3.3

*No serum separator tube used as reference

**Chapter 4. Investigation of Metformin Contamination in Horses: Implications for
Environmental Exposure and Doping Control**

Investigation of Metformin Contamination in Horses: Implications for Environmental Exposure and Doping Control

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Abstract

Metformin is a diabetes medication with minimal therapeutic efficacy in veterinary medicine. However, recently there have been several adverse analytical findings in regulatory samples collected from racehorses. Metformin is commonly prescribed to humans, is excreted unchanged in urine, and is resistant to environmental degradation, raising concerns with respect to environmental contamination. In this study 6 horses were exposed to shavings contaminated with metformin-positive urine at concentrations of 10, 135, and 270 µg/mL. Blood (plasma, serum, whole blood, and red blood cells) and urine samples were collected for 168 hours post exposure for determination of metformin concentrations. All horses had detectable metformin concentrations blood samples at least once during the sample collection period. Metformin concentrations in urine samples greatly exceeded those in blood and were detectable as early as 4 hours post-contamination. The presence of metformin in these samples raises the possibility of metformin exposure through contamination, especially given the housing conditions at racetracks. The potential for exposure through contamination, resulting in adverse analytical findings should be considered in the regulation of metformin in racehorses.

Introduction

Metformin (1,1-dimethylbiguanide) is a medication used for the treatment of type 2 diabetes mellitus. It is very widely prescribed in human medicine and prescriptions of metformin dispensed in the United States have increased significantly in recent years for both Food and Drug Administration (FDA)-approved and off-label uses.¹ Metformin is a water-soluble small molecule drug that is excreted in urine as unchanged metformin.² The degradation-resistant

nature of the drug, along with its widespread use, have led to detectable metformin levels in wastewater and surface waters near residential areas due to excretion in human urine.³

Metformin has recently been detected in post-race blood and urine samples from horses at racetracks throughout the United States. Unlike in human medicine, metformin is rarely prescribed to horses, largely due to its ineffectiveness in treating insulin-resistance in this species.⁴ Its lack of recognized therapeutic utility in young, fit horses has led to its classification as a prohibited substance by the Horse Racing Integrity and Safety Authority (HISA), making the adverse analytical findings (AAF) from horses at racetracks a cause for concern amongst horseracing regulators.

The prevalence of use in human medicine has led to speculation that some metformin AAFs are a result of exposure to human urine containing metformin. It has been suggested that stalls become contaminated when handlers urinate in the stalls. The potential for exposure to metformin contaminated urine resulting in AAF in horses warrants further study. To that end, the primary objective of this study was to explore environmental contamination as a source of metformin exposure by describing the blood and urine levels of metformin in horses that were in contact with metformin-positive urine.

Methods

Six university-owned exercised Thoroughbred horses (2 mares and 4 geldings, aged 3-5 years, weighing 452-570 kg) were used for this study. Prior to beginning the study, horses were subject to a physical examination, complete blood count, and serum biochemistry panel to determine health status. Horses were housed in 12 x 12-foot stalls bedded with approximately 18 cubic feet of premium Douglas wood shavings for the duration of the study. Stalls were stripped of all shavings and waste, power-washed with soap and water, and re-bedded with clean shavings

the day before contamination. Automatic waterers were also emptied, scrubbed, and allowed to refill prior to contamination of the stall.

To create the metformin-positive urine, equine urine that tested negative for metformin was spiked with commercially available metformin powder (>95% purity) to concentrations of 10, 135, and 270 µg/mL. As published data describing urine concentrations in humans was not found, concentrations were chosen based on unpublished data of concentrations in the urine of humans taking a 1000 mg oral dose of metformin. The prepared urine (800 mL/per stall) was spread over shavings in one corner of the stall (n=2 horses per concentration of prepared urine). Stalls were spot-cleaned every morning for the duration of the study, leaving most of the urine-soaked shavings in the stall but removing the solid waste.

Blood samples were collected via direct venipuncture into 2 EDTA blood tubes, one tube devoid of anticoagulant, and one serum separator tube (SST) for determination of drug concentration at times 0 (prior to contamination), 30 minutes, and 1, 2, 4, 6, 8, 12, 24, 30, 36, 48, 72, 96, 120, 144, and 168-hours post contamination. The EDTA tubes were kept on ice until processing, wherein one tube was centrifuged at 3000 g at 4°C for 10 minutes, plasma and red blood cells (RBCs) separated, and an aliquot of each stored at -20°C until drug concentration analysis. An aliquot of whole blood was taken from the second EDTA tube and stored at -20°C until drug concentration analysis. The serum tubes were kept at room temperature for approximately 30 minutes prior to centrifugation at 3000 g for 10 minutes. Serum was then separated and stored at -20°C until drug concentration analysis.

Horses utilized in this study are trained to urinate at the sound of a whistle, so that urine can be collected via free catch. Urine was collected for determination of drug concentration at

times 0 (prior to contamination), 4, 24, 48, 72, 96, 120, 144, and 168-hours post contamination.

Samples were kept on ice, then stored at -20°C until drug concentration analysis.

Metformin concentrations were determined in urine and blood by liquid chromatography-tandem mass spectrometry using a previously validated method.⁵ Calibration curves and negative control samples were prepared fresh for each quantitative assay. In addition, quality control samples (drug free equine blood matrices and urine) were fortified with analyte at three concentrations within the standard curve) as an additional check of accuracy.

Results

Quality control samples for all matrices met accuracy and precision acceptance criteria as described in the FDA's Guidance for Bioanalytical Method Development and Validation⁶ (Supplementary Table 4.1). The technique was optimized to provide a limit of quantitation (LOQ) of 0.25 ng/mL for serum and plasma and 0.3 ng/mL for whole blood and RBCs.⁵ The limit of detection (LOD) was approximately 0.10 ng/mL for serum and plasma and 0.2 ng/mL for whole blood and RBCs.⁵ For urine, the LOQ and LOD were 0.5 ng/mL and 0.25 ng/mL, respectively.⁵

Metformin concentrations following exposure to 10, 135, and 270 µg/mL of urine are listed in Tables 1,2, and 3, respectively. Metformin was detected at low levels in serum samples collected from all horses at some time during the sample collection period. Detection times differed between serum samples collected in tubes containing the silicon separator and those without. Although in some instances, concentrations were below the LOQ, metformin was detected in plasma samples from all horses (Tables 1-3). In whole blood samples, metformin was above the LOQ in one horse in the 10 µg/mL group and detectable but below the LOQ in 4 horses (Table 4.1). Red blood cell metformin was detectable in all horses and above the LOQ at

some time points in 4 horses (135 and 270 ug/mL groups; Tables 4.2 and 4.3). For all matrices, metformin concentrations did not follow a predictable pattern with concentrations fluctuating unpredictably for some horses.

Metformin urine concentrations for all horses are listed in Table 4.4. At 4 hours post contamination, 2 horses had metformin urine levels above the LOQ (0.25 ng/mL). At 24 hours post contamination, all 6 horses had metformin urine levels above the LOQ. Metformin levels remained above the LOQ for all time points for the duration of the study for all but 1 horse. The 10 µg/mL group had the lowest metformin urine levels throughout the study, while horses in the two higher concentration groups had similar levels. For each horse, metformin urine levels increased and decreased unpredictably between consecutive time points.

Discussion

Metformin detection in the blood and urine of horses housed at racetracks and claims of environmental exposure, is a concern for horseracing regulators. The current study describes blood and urine metformin concentrations in horses following exposure to bedding with metformin contaminated urine.

Reports in humans, demonstrate that metformin partitions into RBCs, accounting, at least in part for the prolonged residence time of this drug in the body.⁷ A recent study conducted by our laboratory, demonstrates that metformin behaves similarly in horses (manuscript currently under review). In the current study, concentrations of metformin in several different blood matrices (serum, plasma, RBCs, whole blood) were assessed. All horses exposed to metformin-positive urine in the presently reported study had detectable concentrations of metformin in their blood at some time during the study. Although metformin concentrations in different types of blood sample (serum, RBCs, etc) varied within and between horses, detection in all horses

supports the potential for low level environmental exposure. Interestingly, with respect to serum concentrations, metformin concentrations differed between the two serum tubes used in this study (serum separator versus no serum separator). Some drugs will bind to the silicon plug present in serum separator tubes, leading to differences in concentrations between the two types in the harvested serum. However, based on a previous study, this appears unlikely for metformin as it does not appear to bind to the silicon plug to an appreciable extent (manuscript under review). That same study demonstrated that metformin, as with other drugs that partition into RBCs, may “leak” from RBCs into serum as part of the liberation of the RBC bound fraction into the serum during the clotting process. As the serum separator reduces exposure of the serum to the RBCs, this may explain, at least in part, situations where concentrations are higher in tubes devoid of the separator. From a regulatory perspective this could be an important consideration in the type of tube used for drug testing purposes.

Notably, the horses had detectable concentrations of metformin in RBCs, suggesting that similar to humans, this drug partitions into RBCs in horses. This supports the previous study whereby investigators suggest that RBCs act as a compartment for metformin sequestration in horses; the drug would be allowed to slowly leach back into the blood plasma, leading to longer detection times in plasma or serum and thus a longer half-life. This may also explain the pulsatile like behavior of this drug, whereby metformin concentrations decline only to increase later. Recontamination offers another plausible explanation for this behavior. As horses continued to reside in the originally contaminated stalls throughout the sample collection period, they could be continuously exposed. It is also possible that horses re-contaminate themselves through their own metformin containing urine, a theory supported by the relatively high metformin concentrations detected in the urine of the horses in the current study. Urine metformin levels

were above the LOQ in all horses for the entire duration of the study (168 hours), beginning as early as 4 hours post contamination. One unexplained finding is the high concentration of metformin (34.6 ng/mL) detected in whole blood collected from horse 2 following exposure to 10 ug/mL of metformin. Metformin was not detected in serum and was at very low levels in plasma and RBCs. While the reason for this is not clear, a similar finding was reported by Jacobs and colleagues (manuscript under review). The findings reported here, both for blood and urine, should be considered when implementing screening methods for metformin, especially those using urine.

When horses are housed at racetracks, they are inevitably going to be exposed to the urine of other horses or humans. It should be noted in the current study that horse urine was used as the source of exposure and even though it is not anticipated that horses would react differently to human urine (i.e. avoid it), it is possible that concentrations detected in horses may be different depending on the source of the urine. While notably the sample size was small and the exact route of exposure (oral and/or transdermal) is unknown, low levels of metformin were detected in blood and urine samples collected from all horses (not previously administered metformin) housed in stalls that were intentionally contaminated with metformin containing urine. These findings suggest that exposure of horses to metformin contaminated stalls may result in detectable concentrations of drug in biological samples for at least 168 hours (last time point sampled). However, given the low blood and urine concentrations and the question surrounding how this drug is absorbed following low-level exposure, it may be prudent to study absorption of low levels of metformin via the oral and transdermal routes, as an additional means of validating this conclusion.

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Conflicts of Interest:

The authors have no conflicts of interest to declare.

Data Availability:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figures and Tables

Table 4.1. Metformin concentrations collected from horses following intentional contamination with urine (800 mL) containing 10 ug/mL of metformin. Values in italics are < LOQ (limit of quantitation) (0.25 ng/mL for serum and plasma and 0.3 ng/mL for whole blood and red blood cells (RBCs)) and those in bold are >LOQ.

Metformin Concentration (ng/mL)										
Time (h)	Serum (w/o separator)		Serum (w/separator)		Plasma		Whole Blood		RBCs	
	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2	Horse 1	Horse 2
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
1.0	ND	ND	ND	0.31	ND	ND	ND	ND	<i>0.07</i>	ND
2.0	<i>0.15</i>	ND	ND	ND	ND	ND	ND	ND	ND	ND
4.0	ND	ND	ND	ND	<i>0.10</i>	ND	ND	ND	ND	ND
6.0	ND	ND	ND	ND	ND	<i>0.20</i>	ND	ND	ND	ND
8.0	ND	ND	ND	ND	ND	ND	ND	<i>0.06</i>	ND	ND
12.0	ND	<i>0.23</i>	ND	ND	<i>0.09</i>	ND	ND	ND	ND	ND
24.0	ND	ND	ND	0.54	ND	ND	ND	ND	ND	ND
30.0	ND	ND	ND	ND	ND	0.29	ND	34.6	<i>0.10</i>	<i>0.14</i>
36.0	ND	ND	ND	ND	ND	<i>0.04</i>	ND	<i>0.07</i>	<i>0.06</i>	ND
48.0	ND	ND	ND	ND	ND	ND	ND	0.99	ND	ND
72.0	ND	ND	ND	ND	ND	ND	ND	0.38	ND	ND
96.0	ND	ND	ND	<i>0.11</i>	<i>0.09</i>	ND	ND	ND	ND	<i>0.07</i>
120	ND	ND	0.25	ND	ND	ND	ND	ND	ND	ND
144	ND	ND	ND	ND	ND	<i>0.09</i>	ND	ND	ND	ND
168	ND	ND	ND	ND	ND	ND	ND	ND	ND	<i>0.18</i>

ND, not detected.

Table 4.2. Metformin concentrations collected from horses following intentional contamination with urine (800 mL) containing 135 ug/mL of metformin. Values in italics are < LOQ (limit of quantitation) (0.25 ng/mL for serum and plasma and 0.3 ng/mL for whole blood and red blood cells (RBCs)) and those in bold are >LOQ.

Metformin Concentration (ng/mL)										
Time (h)	Serum (w/o separator)		Serum (w/separator)		Plasma		Whole Blood		RBCs	
	Horse 3	Horse 4	Horse 3	Horse 4	Horse 3	Horse 4	Horse 3	Horse 4	Horse 3	Horse 4
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.5	ND	ND	ND	ND	ND	<i>0.06</i>	ND	ND	ND	<i>0.05</i>
1.0	ND	ND	ND	ND	ND	<i>0.09</i>	<i>0.24</i>	ND	ND	ND
2.0	ND	ND	ND	ND	ND	<i>0.06</i>	ND	ND	ND	ND
4.0	ND	ND	ND	ND	ND	<i>0.08</i>	ND	ND	ND	<i>0.10</i>
6.0	ND	ND	0.65	ND	ND	<i>0.16</i>	ND	ND	ND	ND
8.0	ND	ND	0.80	ND	ND	ND	ND	ND	ND	ND
12.0	ND	ND	ND	ND	ND	<i>0.03</i>	ND	ND	ND	ND
24.0	ND	ND	ND	<i>0.18</i>	ND	<i>0.11</i>	ND	ND	ND	ND
30.0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
36.0	ND	ND	<i>0.13</i>	ND	0.51	ND	ND	ND	0.60	0.36
48.0	ND	ND	ND	ND	<i>0.04</i>	ND	ND	<i>0.22</i>	ND	ND
72.0	ND	ND	ND	ND	<i>0.05</i>	ND	ND	ND	ND	ND
96.0	ND	ND	ND	<i>0.15</i>	<i>0.18</i>	<i>0.08</i>	ND	<i>0.08</i>	ND	<i>0.07</i>
120	ND	ND	ND	<i>0.10</i>	ND	ND	<i>0.05</i>	<i>0.12</i>	ND	0.31
144	ND	ND	ND	ND	<i>0.08</i>	ND	ND	<i>0.04</i>	ND	<i>0.12</i>
168	ND	<i>0.24</i>	ND	ND	ND	ND	ND	ND	ND	<i>0.27</i>

ND, not detected.

Table 4.3. Metformin concentrations collected from horses following intentional contamination with urine (800 mL) containing 270 ug/mL of metformin. Values in italics are < LOQ (limit of quantitation) (0.25 ng/mL for serum and plasma and 0.3 ng/mL for whole blood and red blood cells (RBCs)) and those in bold are >LOQ.

Metformin Concentration (ng/mL)										
Time (h)	Serum (w/o separator)		Serum (w/separator)		Plasma		Whole Blood		RBCs	
	Horse 5	Horse 6	Horse 5	Horse 6	Horse 5	Horse 6	Horse 5	Horse 6	Horse 5	Horse 6
0	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.5	ND	<i>0.19</i>	ND	ND	ND	ND	<i>0.06</i>	ND	<i>0.05</i>	ND
1.0	ND	ND	<i>0.09</i>	ND	ND	ND	ND	<i>0.08</i>	<i>0.17</i>	ND
2.0	ND	0.11	ND	ND	<i>0.11</i>	ND	ND	<i>0.08</i>	<i>0.07</i>	<i>0.08</i>
4.0	0.37	ND	ND	ND	ND	<i>0.05</i>	ND	<i>0.08</i>	ND	ND
6.0	ND	<i>0.08</i>	ND	ND	ND	ND	ND	ND	ND	ND
8.0	ND	ND	ND	<i>0.12</i>	<i>0.07</i>	ND	<i>0.07</i>	ND	ND	<i>0.12</i>
12.0	ND	ND	ND	<i>0.07</i>	<i>0.05</i>	ND	<i>0.06</i>	<i>0.13</i>	<i>0.17</i>	ND
24.0	ND	ND	ND	ND	<i>0.10</i>	<i>0.06</i>	ND	ND	ND	<i>0.08</i>
30.0	ND	ND	<i>0.14</i>	ND	ND	ND	ND	ND	ND	ND
36.0	1.38	ND	<i>0.15</i>	ND	ND	ND	ND	ND	ND	<i>0.26</i>
48.0	ND	ND	ND	<i>0.19</i>	ND	ND	ND	<i>0.06</i>	<i>0.09</i>	ND
72.0	ND	ND	ND	ND	<i>0.06</i>	ND	ND	ND	ND	ND
96.0	ND	ND	ND	ND	<i>0.08</i>	ND	ND	ND	ND	ND
120	ND	ND	ND	ND	<i>0.14</i>	<i>0.06</i>	ND	ND	ND	ND
144	<i>0.16</i>	ND	ND	ND	<i>0.12</i>	ND	ND	ND	ND	<i>0.08</i>
168	ND	ND	ND	ND	ND	1.36	<i>0.06</i>	ND	0.51	ND

ND, not detected.

Table 4.4. Urine metformin concentrations collected from horses following intentional contamination with urine (800 mL) containing 10, 135, or 270 ug/mL of metformin. Values in italics are < LOQ (limit of quantitation) (0.5 ng/mL).

Dose Group	Metformin Concentration (ng/mL)					
	10 ug/mL		135 ug/mL		270 ug/mL	
Time (h)	Horse 1	Horse 2	Horse 3	Horse 4	Horse 5	Horse 6
0	ND	ND	ND	ND	ND	ND
4.0	<i>0.14</i>	<i>0.24</i>	<i>0.13</i>	0.45	0.29	<i>0.17</i>
24.0	0.60	0.69	0.63	20.7	13.2	2.66
48.0	<i>1.36</i>	0.60	7.47	2.80	4.47	1.25
72.0	<i>0.20</i>	0.41	2.66	3.84	9.33	5.48
96.0	0.42	0.66	4.01	5.31	6.91	3.93
120	<i>0.18</i>	0.64	4.16	2.56	3.12	4.46
144	0.36	0.26	3.78	3.03	2.58	3.26
168	0.35	0.85	1.99	1.61	1.63	4.36

ND, none detected.

Supplementary Table 4.1. Accuracy and precision values for LC-MS/MS analysis of metformin in equine serum and urine.

Matrix	Concentration (ng/mL)	Accuracy (% nominal concentration)	Precision (% relative SD)
Serum	0.75	91.0	12.0
	40.0	101	1.0
	5000	100	2.0
Plasma	0.75	106	4.0
	40.0	106	1.0
	5000	107	3.0
RBCs	0.75	98.0	13.0
	40.0	108	1.0
	5000	101	2.0
Whole Blood	0.75	100	9.0
	40.0	104	2.0
	5000	99.0	4.0
Urine	0.75	92.0	6.0
	100	105	6.0
	5000	95.0	3.0