# Title: Novel holistic pharmacokinetic model applied to plasma and urine concentrations of 2,5-dihydroxybenzene sulfonate following administrations of calcium dobesilate and etamsylate to exercised horses

Short Running Title: Pharmacokinetics of 2,5-dihydroxybenzene sulfonate

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## Abstract

Calcium dobesilate (CD) is a synthetic venoactive drug used in veterinary medicine to treat equine navicular disease. Etamsylate is a haemostatic agent used in horses for the treatment of exercise induced pulmonary haemorrhage. Both etamsylate and CD dissociate in the circulatory system to 2,5-HBSA as the active drug.

The aim of the research was to be able to provide Detection Time (DT) advice from pharmacokinetic (PK) studies in Thoroughbred horses to better inform trainers, and their veterinary surgeons, prescribing these substances for treatment of Thoroughbred racehorses. Two (pilot study) and six (final study) horses were given 28 and 9 repeated administrations of CD (3 mg/kg BID), respectively. Two horses were each given a single intravenous (IV)

administration of etamsylate (10 mg/kg). Plasma and urine 2,5-HBSA concentrations were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The CD pilot study revealed that steady-state could be reached with a few days and that 2,5-HBSA in plasma and urine shows instability during storage at -20°C but appears stable at -80°C.

A novel holistic non-linear mixed effects 3 compartmental PK model was developed that described both plasma and urine concentrations of 2,5-HBSA, from either CD or etamsylate administration. Typical values for 2,5-HBSA clearance and bioavailability were 2.0 ml/min/kg and 28%, respectively. Using the parameters obtained from this PK model, in conjunction with methodology developed by Toutain, afforded a possible screening limit (SL) that can regulate for a DT of 3 days in urine, however, a corresponding SL in plasma would be below current levels of detection. However, it is the responsibility of the individual racing authorities to apply their own risk management with regard to SLs and DTs.

## Introduction

Calcium dobesilate (calcium 2,5-dihydroxybenzene sulfonate; CD; Figure 1A) is a synthetic venoactive drug used in veterinary medicine to treat equine navicular disease (Janssen et al 2011). CD was investigated in a rabbit knee model of osteoarthritis (OA) where favourable effects on pressure dynamics in subchondral bone of osteoarthritic joints were found suggesting CD may be

useful for medical management of OA by reducing bone marrow oedema (Miles et al 2011). The biochemical functions of this drug include anti-platelet and fibrinolytic activities by inhibiting platelet activation factor and enhancing the release of tissue plasminogen activator, thereby improving the local blood flow to tissues, otherwise inhibited due to thrombosis (Tejerina and Ruiz, 1998).

A previous pharmacokinetic study has been reported in beagle dogs given CD via intravenous infusion and reported no measurable CD in plasma at 20 hours (Plessas et al, 1986).

Etamsylate (Figure 1B) is a haemostatic agent, which is believed to work by increasing the resistance in the endothelium of capillaries and promoting platelet adhesion. In horses it may be used as a blood coagulating treatment for exercise induced pulmonary haemorrhage (EIPH) (Segura et al 2007). After administration, both etamsylate and CD dissociate in the circulatory system to 2,5-dihydroxybenzene sulfonate (2,5-HBSA).

As legitimate therapeutic medications, permitted for use in training, but which must not have a pharmacological effect on race day, it is of interest to racing authorities to be able to provide Detection Time (DT) advice for these drugs to veterinary surgeons. To deal with the race-day scenario where a horse has received medication treatment in its training programme, a non-significant pharmacological effect in a population of horses has to be defined. One such definition can be a drug concentration in plasma that is less than the

concentration required for a significant therapeutic effect. Toutain & Lassourd (2002) proposed an approach based upon the above definition that also takes into account variation in both pharmacokinetic (PK) and pharmacodynamic (PD) parameters for a population of horses. An estimate of the irrelevant plasma concentration (IPC) is based upon a pharmacologically effective plasma drug concentration divided by an appropriate safety factor. Also, with knowledge of urine PK parameters, an irrelevant urine concentration (IUC) can also be estimated. The IPC and IUC can be used for the purpose of deriving possible drug screening limits (SL), which, in turn, can be used in conjunction with drug clearance profiles to obtain appropriate DT for the matrices concerned. The DT is the time post last therapeutic administration when the drug concentration in plasma or urine drops below the IPC/IUC for six observed horses. By using a DT to form the basis of a Withdrawal Time (WT), veterinary surgeons working with racehorses can help avoid an Adverse Analytical Finding ('positive') on race day following legitimate therapeutic treatment in training.

There are no published pharmacokinetic studies for the administration of CD or etamsylate in the horse. As both drugs dissociate to 2,5-HBSA, when monitoring for either CD or etamsylate administration, the same detection methodology can be used.

The aim of the herein paper is to develop a novel holistic PK model that describes both plasma and urine concentrations of 2,5-HBSA in thoroughbred

horses for either CD or etamsylate administration. Furthermore, the PK model will be used to assess possible screening limits and a DT for CD and etamsylate administration.

## Materials and Methods

### Horses

Two healthy Thoroughbred gelding horses aged 5 years and 6 years and weighing 542 kg and 570 kg respectively were used for the pilot CD administration study and an additional six healthy Thoroughbred horses (5 geldings and 1 mare) aged 4-6 years (mean bodyweight 559±55 kg) were used for the final CD administration study. Two healthy Thoroughbred horses (2 geldings) aged 4 and 6 years (bodyweight 680 and 481 kg) were used for the etamsylate administration study. They were exercised in a manner consistent with that used in British training yards, fed solely a normal racehorse diet, and housed at the British Horseracing Authority's Centre for racehorse studies (Newmarket, UK). All horses were at least 1 month without medications prior to the studies. Ethical approval was obtained for the studies and all horses and personnel involved were licensed under the UK Animals (Scientific Procedures) Act.

### Drug administration and sampling

For both the CD and etamsylate studies, urine was collected as free catch samples in order to mimic a competition sample. Control urine samples were collected from each horse over 3 days preceding dosing at 08:00 and 16:00 and control blood samples were taken by direct venepuncture on each of the 3 days preceding dosing at 09:00. Further blood samples collected during the studies were via an intravenous 14 G catheter (Milocath®) placed in the left jugular vein of each horse on the first day of dosing.

A pilot study was conducted for CD prior to the final CD and etamsylate studies in which doses of 3 mg/kg/BID per os (orally) powdered calcium dobesilate monohydrate (FSP Galena, Poland) were given twice daily in a small amount of feed (observed) for 14 consecutive days, totalling 28 doses, to two Thoroughbred horses. Blood and urine samples were collected and stored at -20°C. The information obtained was used to design the final CD study including administration protocol and storage conditions. Hay was fed ab libitum and horses had free access to fresh water during the study. For the final CD study, a dose of 3 mg/kg/BID per os (orally) powdered calcium dobesilate monohydrate (FSP Galena, Poland) was given in a small amount of feed (observed), twice daily at 09:00 and 16:00 for 4.5 consecutive days, totalling 9 doses. For the etamsylate, a dose of 10 mg/kg Hemoced (Grovet Equine Health Company, The Netherlands) was given IV (intravenously) via the catheter as a single 10 mg/kg

dose at 09:00. Hay was fed ab libitum and horses had free access to fresh water during the studies.

For the CD study, including the pilot study, all naturally voided urine was collected as free catch samples following the first CD dose at 09:00 on day 1 and on day 5, then twice daily pre first and second CD doses at 09:00 and 16:00 on day 2-4 and day 6-12. On day 1 and day 5 blood samples were collected immediately before each dose was administered and at 15, 30, 45, 60, 90, 120, 150, 180 minutes post dose then hourly for 5 collections, then at 2-hour intervals for 5 collections, then twice per day day 2-4 and day 6-12.

For the etamsylate study, all naturally voided urine was collected as free catch samples following the 09:00 dose on day 1 until 05:00 on day 2 and then twice daily at 08:00 and 16:00 on days 2-5. Blood samples were collected immediately before the dose was administered and at 5, 15, 30, 45, 60, 90, 120, 150 minutes post dose then continued hourly for 5 collections and, then at 2-hour intervals for 4 sample collections, then twice per day at 08:00 and 16:00 on days 2–5.

For both studies, blood was collected into lithium heparin tubes, centrifuged immediately to separate the plasma after collection. Urine and plasma samples were stored at -80°C prior to analysis.

**Chemicals and Reagents**

Reagent grade water (RG $H_2O$) was purified by a Triple Red ultrapure water system (Triple Red Ltd., Buckinghamshire, UK). Acetonitrile (ACN; ≥99.9 %), methanol (MeOH; ≥99.9 %), formic acid (≥98 %), acetic acid (≥99 %) and ammonia solution (35 %) were purchased from Fisher Scientific UK Ltd. (Loughborough, UK). Trimethyl orthoacetate (TMOA; 99 %) was purchased from Sigma-Aldrich (Dorset, UK).

Calcium dobesilate and etamsylate were purchased from Santa Cruz Biotechnology (Dallas, USA) and $D_3$-calcium dobesilate was purchased from TLC Pharmaceutical Standards (Ontario, Canada). All reference standards were prepared to give 1 mg/mL of the constituent 2,5-HBSA in MeOH prior to storage at -20°C.

**Sample Preparation**

All pre- and post-administration plasma and urine samples were quantitatively analysed for 2,5-HBSA. Plasma calibration samples were prepared between 50-500 ng/mL with quality (QC) samples spiked to give final concentrations of 90, 250 and 400 ng/mL. Urinary calibration samples were prepared between 50-1000 ng/mL with QC samples spiked to give final concentrations of 250, 400 and 800 ng/mL. Aliquots of 0.5 mL for each calibration, QC and administration sample were prepared in fresh glass tubes and spiked with 25 µL of $D_3$ -2,5-HBSA solution (4 µg/mL) to act as an internal standard. All samples were then diluted with 0.45 mL of 1 % aqueous formic acid, followed by solid-phase

extraction, performed using Supelco Discovery® DSA-6S cartridges (1 mL, 50 mg) preconditioned with 1 mL of MeOH and 1 % aqueous formic acid. Samples were loaded onto the cartridge and allowed to pass through under positive pressure. The cartridges were washed with 1 mL of 1 % aqueous formic acid followed by 1 mL of MeOH. Samples were eluted with two 0.5 mL aliquots of 5 % ammonia solution in 50:50 RG $H_2O$:MeOH (*v:v*) into fresh glass tubes. Each eluate was then immediately transferred to a maximum recovery LC vial and evaporated to dryness at 40°C. Samples were subsequently reconstituted in 50 µL of glacial acetic acid and 400 µL of TMOA, capped and incubated at 80°C for 60 minutes. Following incubation, the samples were decapped and evaporated to dryness at 40°C prior to reconstitution in 100 µL of 0.1 % formic acid in 95:5 RG H2O:ACN (*v:v*), mixing and centrifugation.

**Calibration Method**

Following validation, the analytical methods were deemed suitable for application to the study samples. Specifically, inter- and intra-batch precision (% coefficient of variation) and accuracy (% relative error) were within 20 % (25% at the lower limit of quantification (LLOQ)), linearity of dilution (% relative error and % coefficient of variation) was within 20 % and no significant matrix suppression or interferences were observed.

**Analytical Methodology**

Ultra-performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) analysis was performed with a Waters Acquity UPLC system coupled to an Applied Biosystems Sciex API 5500 QTrap mass spectrometer using electrospray ionisation in negative mode. Chromatographic separation was achieved using a Restek Pinnacle DB IBD column (100 x 2.1 mm, 1.9 μm) with the column temperature set to 60℃ and applying 0.1 % formic acid in ACN and 0.1 % aqueous formic acid mobile phases at a flow rate of 0.4 mL/min. The gradient started at 5 % organic and was held for 1 minute before increasing to 20 % at 1.5 minutes, to 40 % at 5.5 minutes and to 95 % at 6 minutes. This was held for 0.5 minutes before resuming the initial conditions and re-equilibrating. An injection volume of 10 μL was used.

Mass spectrometric analysis was performed using the selected transition monitoring acquisition mode. The quantitative transitions for 2,5-HBSA and $D_3$ -2,5-HBSA consisted of 203.0 > 188.0 (collision energy (CE) -24 V, collision exit potential (CXP) -17 V) and 206.0 > 191.0 (CE -22 V, CXP -18 V) respectively. The LLOQ was 50 ng/mL for 2,5-HBSA in both urine and plasma.

**Stability assessment of 2,5-HBSA in matrix**

Stability samples in equine plasma and urine were prepared by spiking blank matrix to give final 2,5-HBSA concentrations of 200 and 1000 ng/mL in plasma and 200, 2,000 and 20,000 ng/mL in urine. These samples were then subjected to long-term storage at -20°C and -80°C for 0, 1, 2, 4, 8 and 17 weeks. Each

stability sample analysis batch were prepared, extracted and analysed as previously described alongside duplicate matrix blanks and freshly spiked samples at each analyte concentration investigated. Degradation was measured as a percentage relative to the freshly prepared spiked samples at the equivalent concentration.

## Pharmacokinetic Analysis

### Novel approach for simultaneously modelling the PK of plasma and urine drug concentrations

The clinical effects of most drugs are driven by the plasma concentration, however, the most common matrix sampled in equine sports is urine. Therefore, for medication control in equine sports an understanding of the urine to plasma drug concentration relationship at a given time is paramount.

The theoretical rate of change of amount of drug with time in the urine of the nephrons can be expressed as follows:

$$\frac{dA_{UN}}{dt} = k_{ren} x\ A_P(t) - \ k_N x\ A_{UN}(t) \qquad Equation 1$$

Where A represents amount of drug, P and UN represent plasma and urine in nephrons, $k_{ren}$ and $k_N$ represent micro rate constants for renal excretion from plasma and urine movement from nephrons into the bladder, respectively. As urine is being made in and emptied from the nephrons, the volume of urine in

the nephrons can be considered as a fixed parameter. Therefore, the rate of drug removal from the nephrons is:

$$-\frac{dA_{UN}}{dt} = k_N x\, A_{UN}(t) = UP\ x\ C_{UN}(t) \qquad Equation\ 2$$

Where UP is the urine production rate and $C_{UN}$ (t) is the concentration of the drug in the urine of the nephrons at time (t).

The bladder acts as a reservoir for the urine being emptied from the nephrons. However, it is not possible to determine the instantaneous rate of drug elimination from the nephrons unless a catheter is inserted into the bladder to continuously collect urine. Therefore, it is necessary to make use of the average rate for a time interval (Δt), where Δt represents the time between filling and empting of the bladder. Martin, 1967 showed that as long as Δt is less than the half-life during the interval then the amount of drug voided in urine ($\Delta A_{UV}$) divided by Δt approximates to $k_{ren}$ x $A_p$ at the mid-point of Δt.

In a typical medication control PK study, urine is collected at specific time points post administration and measured for drug concentration, however, Δt is not recorded. Assuming in a healthy animal that urine production rate (UP) is constant then:

$$-\frac{\Delta A_{UV}}{\Delta t} = \frac{C_{UV}\ x\ \Delta V_{UV}}{\Delta t} = \frac{C_{UV}\ x\ \Delta t\ x\ UP}{\Delta t} = UP\ x\ C_{UV} \qquad Equation\ 3$$

Where $\Delta V_{UV}$ and $C_{UV}$ are the volume and drug concentration of the voided urine. While $C_{UV}$ represents an average concentration for $\Delta t$ it is assigned to time (t) post administration for comparison to plasma concentration. The deviation ($\lambda$) for the departure of Equation 3 from Equation 2 can be expressed as follows:

$$-\frac{\Delta A_{UV}}{\Delta t} = -\lambda \frac{dA_{UN}}{dt} \; with \; \lambda = \frac{C_{UV}}{C_{UN}(t)} \qquad Equation\ 4$$

Alternatively, the deviation can be expressed by comparing the amount of drug in voided urine over interval $\Delta t$ with Equation 2.

Using Laplace transformation, Equation 3 can be expressed as follows:

$$\frac{\Delta A_{UV}}{\Delta t} = \frac{D_0 e^{-tk_{ren}}}{\Delta t}\left(\frac{k_N}{k_{ren} - k_N}\right)\left(e^{\Delta t k_{ren}} - 1\right) - \frac{D_0\, e^{-tk_N}}{\Delta t}\left(\frac{k_{ren}}{k_{ren} - k_N}\right)\left(e^{\Delta t k_N} - 1\right) \qquad Equation\ 5$$

Where $D_0$ is the initial dose and $e^{\Delta t}$ is the exponential change over $\Delta t$.

Equation 2 can be expressed as follows:

$$\frac{dA_{UN}}{dt} = -k_N x\, A_{UN}(t) = k_N\, x \left(D_0 e^{-tk_{ren}}\left(\frac{k_{ren}}{k_{ren} - k_N}\right) - D_0 e^{-tk_N}\left(\frac{k_{ren}}{k_{ren} - k_N}\right)\right) \qquad Equation\ 6$$

Deviation can now be expressed by comparing Equations 5 and 6:

$$\lambda_1 = \left(\frac{\left(e^{\Delta t k_{ren}} - 1\right)}{\Delta t k_{ren}}\right) and\ \lambda_2 = \left(\frac{\left(e^{\Delta t k_N} - 1\right)}{\Delta t k_N}\right) \qquad Equation\ 7$$

Where $\lambda_1$ and $\lambda_2$ represent the deviations in equation components for drug renal excretion from the blood and movement from urine in nephrons to the bladder,

respectively. Equation 7 indicates that the deviation between the theoretical rate of change of amount of drug at time (t) in the urine of the nephrons and the corresponding measured change of amount of drug in voided urine per $\Delta t$ will be dependent only on $k_{ren}$, $k_N$ and $\Delta t$ and therefore constant for a specific value of $\Delta t$. Assuming a constant rate of urine production (UP) and that $\Delta t$ is less than the half-life then Equation 1 can be used to model free catch urine drug concentration measurements within a PK model.

While the LLOQ for urine was 50 ng/ml, one horse had its last 2 urine concentrations just above this LLOQ but with little change in concentration. For the purposes of modelling any urine concentrations below 100 ng/ml were considered censored data. The compartmental model used to describe 2,5-HBSA concentrations in plasma and urine from either IV or oral administrations of etamsylate or CD, respectively, is shown in Figure 2 where: Ka and F are the oral absorption rate constant and bioavailability of 2,5-HBSA for oral administration of CD. $CL_{ren}$ and CLD are the clearances for 2,5-HBSA renal excretion from plasma and distribution between the central compartment (1) and peripheral compartment (2), respectively. V1, and V2 are the volumes of the central and peripheral compartments, respectively. $V_{UN}$ and $k_{UN}$ are the volume and drug elimination rate constant for urine of nephrons, respectively. $C_P$ and $C_{UN}$ are the plasma and urine concentrations of 2,5-HBSA, respectively.

Pharmacokinetic analyses were conducted using non-linear mixed effects methods (NLME) with Phoenix WinNonlin 8.3 (Certara, Princeton, NJ, USA). Compartmental NLME PK models were applied simultaneously to the plasma and urine concentration data for 2,5-HBSA which included censored data below 50 and 100 ng/ml for plasma and urine, respectively. The Laplace engine within Phoenix was used as inclusion of censored data required a non-gaussian approach. Doses were inputted as the dose of 2,5-HBSA per kg bodyweight resulting from either etamsylate or calcium dobesilate monohydrate administration. Residual error was modelled on a proportional error model. An exponential random effect model was chosen to describe inter-individual variability e.g. parameter = typical parameter * $\exp^{(eta)}$. A categorical covariate for either etamsylate or CD administration was implemented on the model parameters in a multiplicative exponential way. The model analysis started from the basic compartmental models without the covariate and random effects were added stepwise to parameters. Next, any contribution of the covariate to the fixed parameters and correlation on the random effects were assessed by a reduction in the objective function (OBF) using stepwise forward inclusion. The OBF was optimised while maintaining shrinkage below 30%. Selection of the best model was based on the lowest value of the Akaike and Bayesian Information Criteria (AIC and BIC), chi-square p-value based on the likelihood ratio test, visual inspection of the population predicted concentration versus the observed concentrations and the resulting conditional weighted residual errors.

Finally, the best model was checked for robustness using a bootstrap resampling method. The effective plasma concentration (EPC) and irrelevant plasma and urine concentrations (IPC and IUC) were estimated using the Toutain and Lassourd, 2002 methodology which were used for the basis of possible SLs. Briefly, the EPC for therapeutic CD administration was estimated from the total daily oral dose of 2,5-HBSA divided by $CL_{ren}/F$ over a 24-hour period. The IPC was determined by dividing the EPC by a factor of 500 and the IUC determined by multiplying the IPC by the steady-state ratio of urine to plasma concentration (Rss). The Rss value for 2,5-HBSA was determined by dividing $CL_{ren}$ by UP, with the latter determined from $V_{UN} \times k_{UN}$ . The DTs were determined by the time post dose where all horses had concentrations below the possible plasma and urine SLs.

## Results

### Pilot administration study assessment

A pilot study was conducted to optimise the final CD study protocol. No bioaccumulation was observed during the 14-day dosing period; therefore, it was deemed appropriate to reduce the number of administrations given to 9 doses over 4.5 days for the final study. This adjustment was beneficial in terms of horse welfare whilst still achieving steady state during the dosing period. Furthermore, from this study, a potential instability of 2,5-HBSA was identified dependent on storage condition; thus, prompting further assessment.

## Stability assessment of 2,5-HBSA in matrix

Plasma and urine samples were spiked with 2,5-HBSA and subjected to extended stability analysis at -20 °C and -80 °C up to 17 weeks. Stability profiles of samples spiked at multiple concentration levels for plasma and urine are presented in Figure 3 (A and B).

Initially, there appeared to be only minor variations between the analyte responses of samples stored at the two storage temperatures over the first 2 weeks of the stability study. However, as the duration of the stability analysis continued, the differences became more pronounced with significant deterioration of the samples stored at -20°C compared to those stored at -80°C. For the latter condition, no significant deterioration was observed after 17 weeks. This contrasts with samples stored at -20°C in both matrices which, on average, were 18.5 % of their original response following the same time period. Therefore, it was concluded that analyte stability would be greatly improved by storing samples at -80°C. This resulted in the adoption of this storage conditions for administration samples as soon as was practical following collection during the final studies for CD and etamsylate.

## 2,5-HBSA pharmacokinetics for IV administration of etamsylate

The plasma and urine PK profiles for 2,5-HBSA resulting from IV administration of etamsylate (2 horses) are displayed in Figure 4 (A and B).

Plasma decay curves appear to have two main phases of decline entering into the second phase at approximately 12 hours post administration. As expected, the urine PK lag behind plasma with an initial absorption phase followed by two phases of decay with the later phase at approximately 12-24 hours post administration.

## 2,5-HBSA pharmacokinetics for BID oral administration of calcium dobesilate

The plasma and urine PK profiles for 2,5-HBSA resulting from BID oral administration of CD (6 horses) are displayed in Figure 5 (A and B). 2,5-HBSA concentrations appear to reach steady-state after 2 doses (1 day) following CD administration. After the final CD dose, 2,5-HBSA plasma and urine curves initially decline in a similar manner, with urine showing a longer terminal phase comparable to that observed for etamsylate urine.

The most parsimonious compartmental NLME model contained central, peripheral and urine compartments with random effects included on $CL_{ren}$, V1, V2, F and $k_{UN}$ with partial correlation ($CL_{ren}$, F and $k_{UN}$). The categorical covariate for either etamsylate or CD administration did not reduce the OBF for any of the parameters. The addition of a metabolism pathway to the model, in addition to the renal elimination route, did not reduce OBF. Shrinkage for all eta values was less than 30% and residual error standard deviation for $C_P$ and $C_{UN}$ were 0.45 and 0.57, respectively. Table 1 shows the outputted typical values

(TV) for the parameters resulting from the final model which were encompassed by the 2.5 and 97.5% confidence intervals of the bootstrap resampling analysis. CV% for theta based on the bootstrap analysis were less than 30% with the exception of V2 and CLD (32 and 40% respectively). Table 2 shows the variance, co-variance, correlation and shrinkage for the final model with high correlation between $CL_{ren}$, F and $k_{UN}$ and shrinkage less than 30% for all parameters. Figures 4 and 5 also show the individual horse concentration predictions using the EBE parameters as green lines superimposed onto the measured concentrations (black symbols) versus time graph.

## Calcium dobesilate EPC, IPC, Rss and IUC

The estimated TVs for 2,5-HBSA plasma clearance ($CL_{ren}$) and bioavailability (F) are 2.0 mL/min/kg and 28%, respectively. The estimated EPC, IPC, Rss and IUC values for 2,5-HBSA from a CD oral administration (3 mg/kg BID) are 583 ng/mL, 1.17 ng/mL, 140 and 164 ng/mL, respectively, based on the TVs for $CL_{ren}$, F, $k_{UN}$ and $V_{UN}$.

## Discussion

The analytical methods applied in the current study are more sensitive for detecting 2,5-HBSA, with an LLOQ of 50 ng/mL in both plasma and urine, compared to those previously reported in equine plasma, with a limit of detection of approximately 200 ng/mL identified (Russell *et al*, 2006). It is

possible that the comparatively lower LLOQ could be a result of enhanced mass spectrometric detection and chromatographic separation afforded by the derivatisation step with TMOA, which was not included by Russell *et al* (2006). Furthermore, no storage conditions or considerations regarding analyte stability are described by Russell *et al* (2006); therefore, it is feasible that the disparity between reported LLOQs is due to analyte instability during sample storage, preparation and/or analysis. Thermal degradation of 2,5-HBSA was identified as a significant concern during the pilot study described herein, with sample storage at -80ºC subsequently adopted as an effective preventative measure. To the best of the authors' knowledge, there are no existing published methodology which measures the concentration of 2,5-HBSA in urine.

Due to the improved detection capabilities provided by the analytical methods described herein, an enhanced window of detection is permitted. This was anticipated to result in a more accurate assessment of the PK disposition of the drug at the later time points. Furthermore, the concurrent measurement of plasma and urine concentrations allows for the calculation of urine to plasma drug ratios, which enables urinary drug concentrations to be used as surrogates for plasma concentrations when regulating the use of these medications.

Traditional clinical PK studies investigating drug renal excretion typically collect urine and quantify the amount of drug excreted in a time interval from the product of the measured concentration and the volume of urine voided. The

rate of change of amount of drug in urine at the mid-point of the time intervals are then analysed to determine PK parameters. However, this historical approach assumes that movement of urine from the nephrons into the bladder is much faster than the renal excretion rate from plasma (Martin, 1967).

Moreover, this approach is not appropriate for medication control as it is the relationship between urine and plasma concentration that is important. Kuroda et al., 2022 have proposed an approach that allows the simultaneous PK modelling of plasma and selected drug urine concentrations at apparent pseudo steady-state by using a proportionality parameter between plasma and urine. However, this approach relies on the subjective determination of pseudo steady-state which becomes complex for multi-phasic decay PK profiles. Furthermore, this approach is empirical, assumes that urine production is constant and that the urine interval ($\Delta t$) is less than drug half-life.

The resulting NMLE model here affords a quantitative description of both plasma and urine concentrations of 2,5-HBSA, either from IV administration of etamsylate or oral administration of CD. There is evidence in other species that 2,5-HBSA is almost exclusively eliminated via renal excretion and the NMLE models supports this in horse as a single elimination route via renal excretion gave the most parsimonious model. Moreover, comparing the dose normalised average 2,5-HBSA urine AUCs between the IV and oral routes gave a ratio of 0.84 further supporting renal excretion as the major route of clearance. The

CV% from the boot strap analysis suggested that the majority of the TV parameter estimates are robust. The bioavailability determined (TV) for oral CD BID administration was 28% which while on the low side produces significant concentrations of 2,5-HBSA. The estimated value for $k_{UN}$ is greater than $k_{ren}$ indicating that (pseudo) steady-state is reached quickly and rate of decline is driven by the rate of renal excretion from plasma. However, for a drug with a higher rate of renal excretion from plasma, that is greater than the rate of drug movement from the urine in the nephrons to the bladder, the rate of decline may be driven by movement into the bladder from nephrons. The CV% for residual error on CP and CU was 45 to 57% suggesting concentrations in urine are slightly more variable than plasma as expected. Figure 5B indicates that for some horses the measured urine concentrations are lower than predicted from the post-hoc EBEs at approximately 24 hour post last administration. In addition, there is evidence of a flat phase for a couple of horses at 72 hours post administration although these levels are close to the LLOQ. These observations may suggest that there is a further phase of decline, however, this was not observed in plasma. The latter may be due to this potential plasma phase being well below the plasma limit of detection. An NMLE model was constructed with 2 peripheral compartments that visually gave a better fit to the terminal urine profiles, however, this model was inferior to the model presented here based on the CV% of typical values from bootstrap analysis.

The Toutain approach for determining the EPC should technically be applied to multiple dose administrations leading to steady-state. This is because the EPC is determined from the average steady-state concentration for a therapeutic dosing regimen. The Toutain approach is more problematic for single IV dosing, as is the case for IV etamsylate administration, as it is not apparent what constitutes an effective plasma concentration. Given that CD is administered as a multi dose regimen to steady-state, and is more commonly used by equine veterinarians compared to etamsylate, the EPC, IPC and IUC were only determined for CD administration (3 mg/kg BID). The estimated IPC and IUC values of 1.17 and 164 ng/mL, for CD oral administration (3 mg/kg BID), are either lower (plasma) or higher (urine) than the LLOQ for this study (50 ng/mL). IPC and IUC values form the basis of possible screening limits (SL) and it is down to the individual racing authorities to apply their own risk management. A plasma SL based on the estimated IPC of 1.17 ng/mL is not practical based on current instrument sensitivity and therefore no plasma detection time can be determined. However, a urine SL based on the IUC could afford a possible value of 200 ng/mL and would correspond to a DT of 72 hours after final administration.

While the Toutain approach may not be appropriate for the IV etamsylate study, both horses in this study have 2,5-HBSA urine concentrations below a possible CD urine SL of 200 ng/ml at a DT of 72 hours. While only 2 horses were

investigated for IV etamsylate a single urine SL and DT may be applied to control for both oral CD and IV etamsylate at the therapeutic doses described.

A holistic model has been described for the simultaneous modelling of plasma and urine drug concentration data and has been applied to 2,5-HBSA resulting from IV and oral administration of etamsylate and calcium dobesilate, respectively. From this model estimated values for IPC, Rss and IUC have been determined giving a DT of 72 hours. However, it is the responsibility of the individual racing authorities to apply their own risk management with regard to SLs and DTs. Given the limited number of horses used in this study extra time must be added to the DT, by the treating veterinarian, in order to form the basis of a withdrawal time.

## Animal welfare and Ethics

The study was approved by the British Horseracing Authority's (BHA) Centre for Racehorse Studies (CRS) Animal Welfare and Ethics Review Board and the BHA ethics board, with the horses and personnel involved licensed under the UK's Animals (Scientific Procedures) Act.

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## Conflict of interest

The authors have no commercial conflict of interests; however, the authors are either employed by, working on behalf of or consulting to a regulatory agency.

## Data availability statement

The data that support the findings of this study are available from the British Horse Racing Authority (BHA). Restrictions apply to the availability of these data, which were used under license for this study. Data are available from the authors with the permission of the BHA.

## Authors' contribution

CW, JZ, MV and CH contributed to the analytical development method, validation and sample analysis. JHB led the administration study, PH performed project management, JHB and PH performed manuscript editing. SP co-ordinated the data and performed pharmacokinetic analysis. All authors contributed to the writing of the manuscript and have read and approved the final manuscript.

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Table 1: NLME typical values (TV) and bootstrap analysis for final model

| Parameter | Typical Value (TV) | Mean Value* | CV%* | 95% Confidence Interval* | |
|---|---|---|---|---|---|
| | | | | lower | upper |
| V1 (ml/kg) | 359 | 380 | 29 | 216 | 618 |
| Ka ($hr^{-1}$) | 0.179 | 0.183 | 9 | 0.161 | 0.210 |
| V2 (ml/kg) | 17.20 | 17.8 | 32 | 7.64 | 25.4 |
| CLD2 (ml/hr/kg) | 1.52 | 1.40 | 40 | 0.27 | 2.11 |
| $V_{UN}$ (ml/kg) | 0.643 | 0.667 | 26 | 0.372 | 1.03 |
| $CL_{ren}$ (ml/hr/kg) | 120 | 125 | 11 | 106 | 153 |
| $k_{UN}$ ($hr^{-1}$) | 1.33 | 1.36 | 26 | 0.861 | 2.10 |
| F (%) | 28 | 29 | 13 | 24 | 37 |

*obtained from bootstrap analysis

Table 2: Variance, co-variance, correlation and shrinkage for final model

| | $nCl_{ren}$ | nF | $nk_{UN}$ | nV1 | nV2 |
|---|---|---|---|---|---|
| **$\omega^2$** | | | | | |
| $nCl_{ren}$ | 0.0842 | | | | |
| nF | 0.0766 | 0.125 | | | |
| $nk_{UN}$ | -0.0481 | -0.0493 | 0.0280 | | |
| nV1 | 0 | 0 | 0 | 0.273 | |
| nV2 | 0 | 0 | 0 | 0 | 0.183 |
| | | | | | |
| **Correlation** | | | | | |
| $nCl_{ren}$ | 1 | | | | |
| nF | 0.745 | 1 | | | |
| $nk_{UN}$ | -0.990 | -0.832 | 1 | | |
| nV1 | 0 | 0 | 0 | 1 | |
| nV2 | 0 | 0 | 0 | 0 | 1 |
| | | | | | |
| **Shrinkage** | 0.0267 | 0.0732 | 0.0276 | 0.0289 | 0.214 |

## Figure Legends

Figure 1: Chemical structures of (A) CD and (B) etamsylate

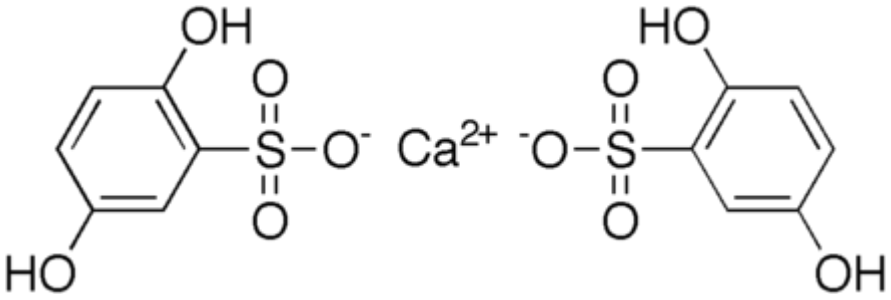
Figure 2: Compartmental model describing plasma and urine concentrations of 2,5-HBSA from either IV administration of etamsylate or oral administration of CD.

Figure 3: 2,5-HBSA stability profiles in (A) plasma and (B) urine over a 17-week period for a range of concentrations (black symbols; circles represent 200 ng/mL in both matrices, triangles represent 1,000 ng/mL in plasma and 2,000 ng/mL in urine, and squares represent 20,000 ng/mL in urine). The solid and dotted lines represent storage conditions of -80ºC and -20ºC.

Figure 4: Pharmacokinetic profiles (black symbols) for 2,5-HBSA and corresponding post-hoc EBE model fits (green lines) for (A) plasma and (B) urine concentrations resulting from the IV administration of etamsylate (10 mg/kg). LLOQ = 50 ng/ml (red dashed line).

Figure 5: Pharmacokinetic profiles (black symbols) for 2,5-HBSA and corresponding post-hoc EBE model fits (green lines) for (A) plasma and (B) urine concentrations resulting from the oral administration of calcium dobesilate (3 mg/kg BID). LLOQ = 50 ng/ml (red dashed line).

A




B

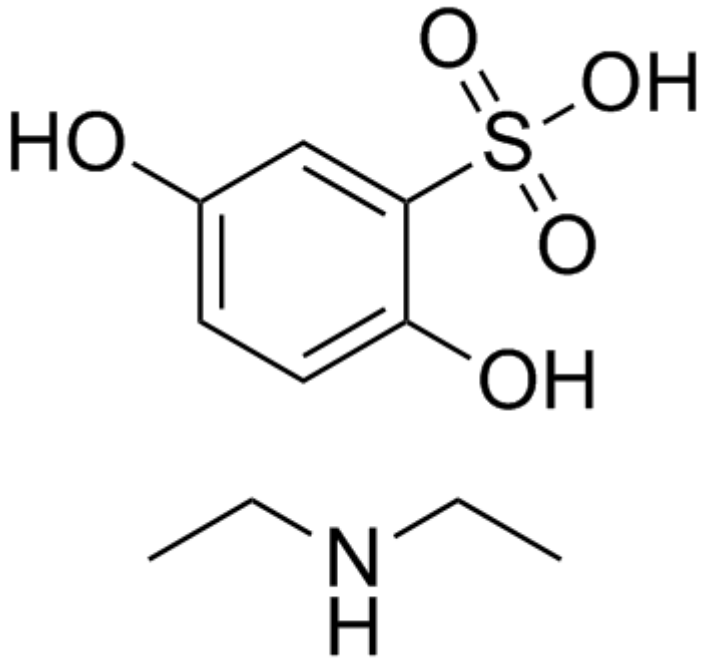



Figure 1 A and B

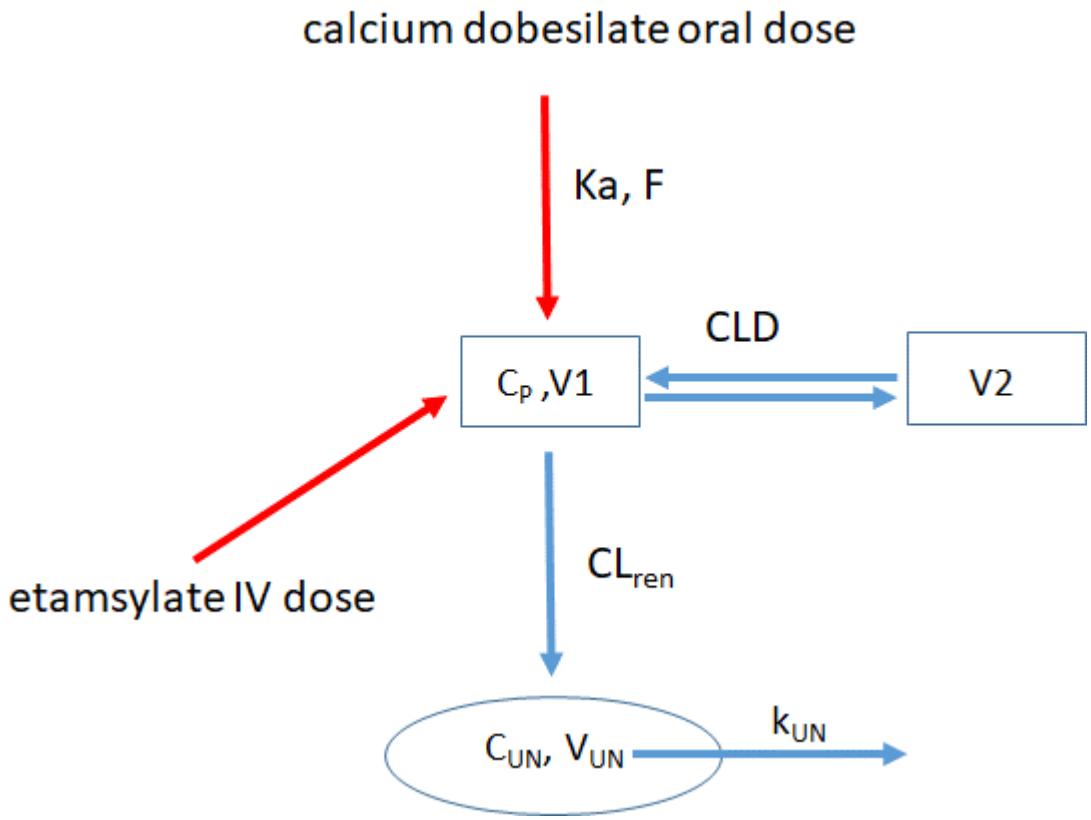
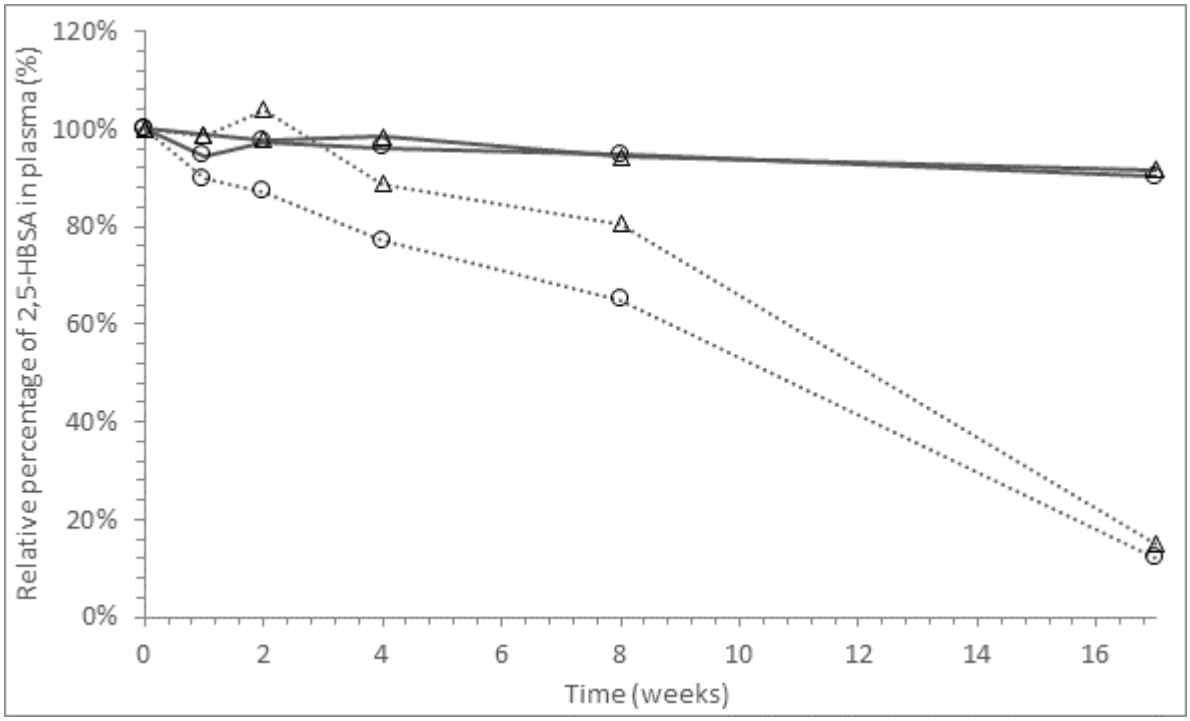

calcium dobesilate oral dose
Ka, F
CLD
Cp ,V1
V2
etamsylate IV dose
CLren
CUN, VUN
kUN

Figure 2



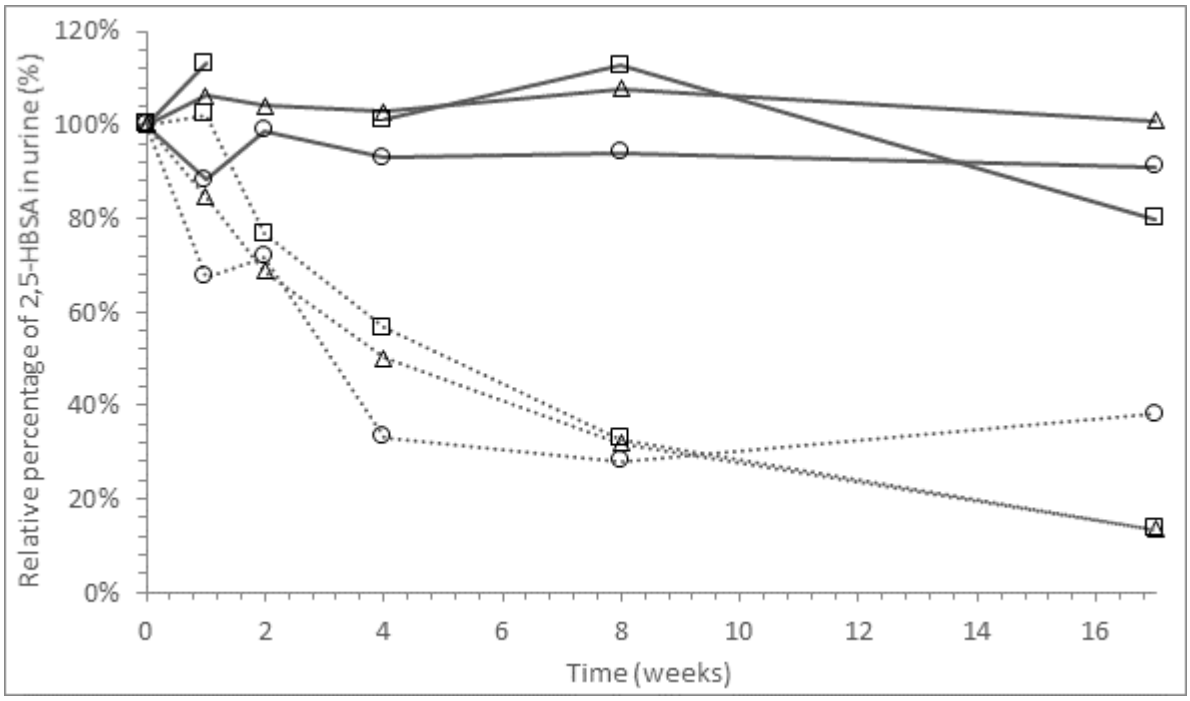
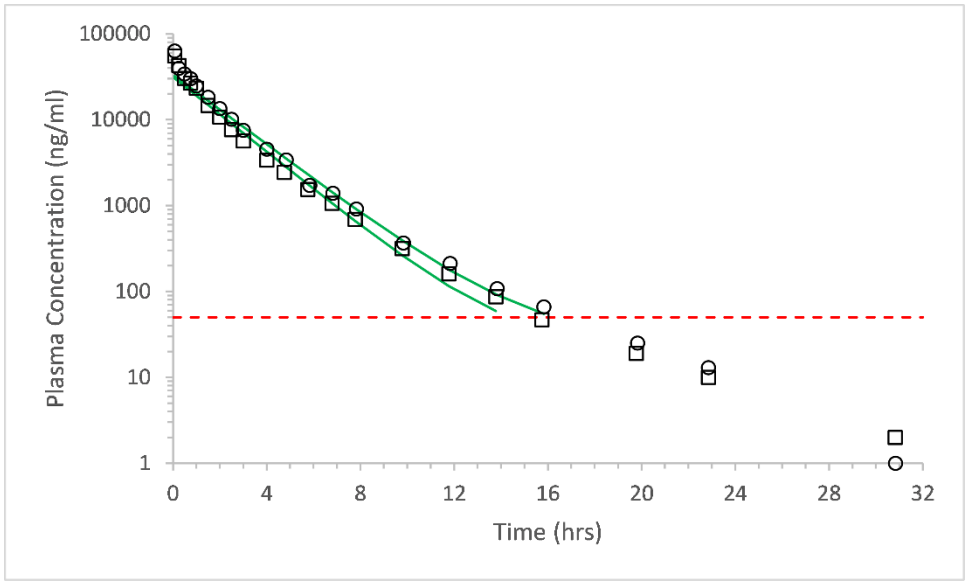




Figure 3 A and B



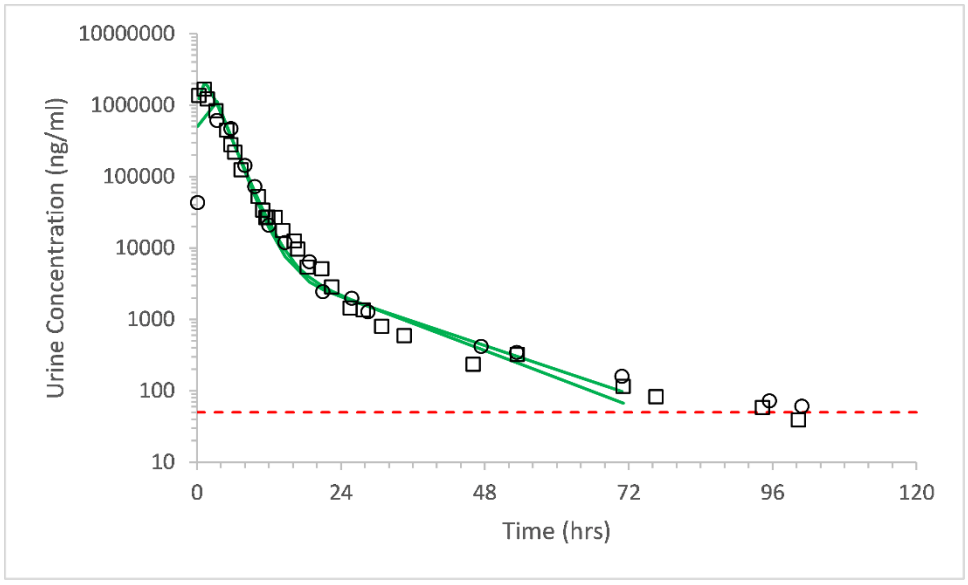
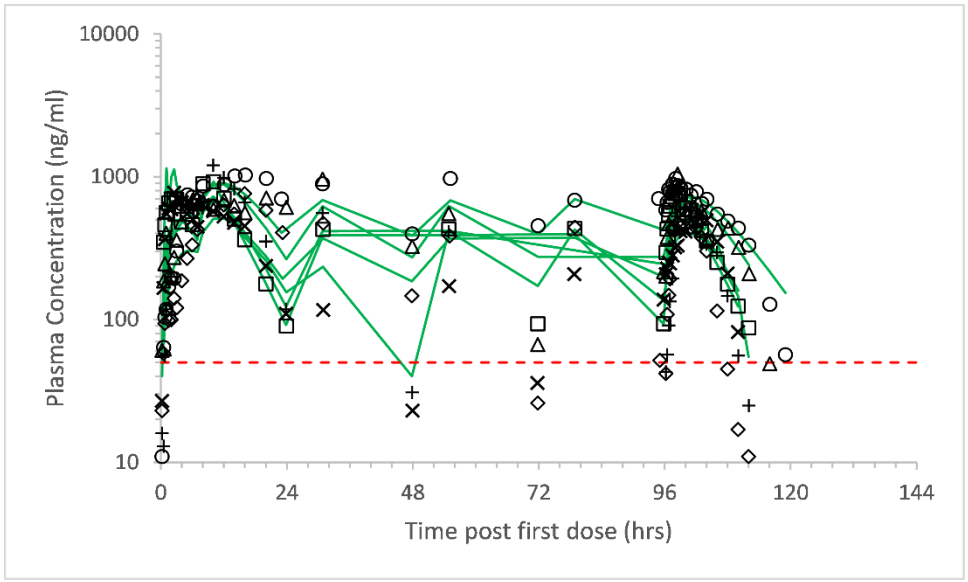




Figure 4 A and B



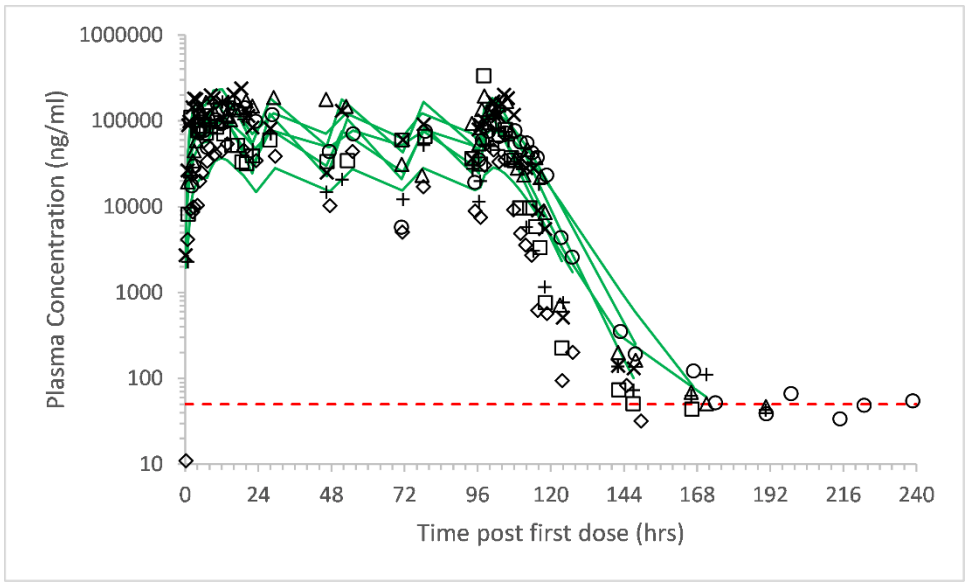




Figure 5 A and B