Received: 3 June 2021 | Revised: 23 November 2021 | Accepted: 4 December 2021

DOI: 10.1111/jvp.13036

JOURNAL OF Veterinary Pharmacology and Therapeutics WILEY

ORIGINAL ARTICLE

Phenylbutazone pharmacokinetics in southern white rhinoceros (*Ceratotherium simum simum*) after oral administration

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Funding information
This study was financially supported by the International Rhino Foundation Student Grant R-2019–10 and the American Association of Zoo Veterinarian's Wild Animal Health Fund.

Abstract

Southern white rhinoceros (*Ceratotherium simum simum*) frequently develop painful conditions, such as traumatic injuries or osteoarthritis, necessitating the administration of pain-relieving medications. One of the preferred treatments is the nonsteroidal anti-inflammatory drug phenylbutazone because of the availability of oral formulations and the familiarity of its use in horses. For the main study, a single oral dose of phenylbutazone at 2 mg/kg was administered to healthy adult rhinoceros (n = 33) housed at six North American zoological institutions. Each rhinoceros had up to four blood samples collected under voluntary behavioural restraint at up to four predetermined time points (0, 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 30 and 48 h). Drug analysis was performed by high-performance liquid chromatography. The population pharmacokinetic parameters were calculated with nonlinear mixed-effects modelling, and analysis showed a peak concentration (C_{MAX}) of 3.8 μg/ml at 1.8 h and an elimination half-life of 9 h. The concentrations achieved were similar to what has been reported for horses and were within the half maximal effective concentration for horses for at least 10 h. A multi-dose trial in five rhinoceros receiving 2 mg/kg orally once daily for five days found mild accumulation at a predicted factor of 1.2. This study represents the first pharmacokinetic data of phenylbutazone in any rhinoceros species.

KEYWORDS
analgesia, *Ceratotherium simum*, pharmacokinetics, phenylbutazone, rhinoceros

1 | INTRODUCTION

Approximately, 18,000 southern white rhinoceros (*Ceratotherium simum simum*, SWR) inhabit wild and semi-wild environments (Emslie et al., 2019) as an important apex consumer in the savannas of southern Africa (Cromsigt & te Beest, 2014; Emslie et al., 2019). These wild populations have been declining since 2012 due to increased poaching (Emslie et al., 2019), and each year approximately 200 to 300 SWR are found alive after poaching attacks, often with gunshot wounds or wounds to their frontal and nasal bones (Leiberich et al., 2018). Appropriate medical management of these injuries following systemic stabilization includes wound lavage and effective analgesia (Meyer et al., 2015).

Analgesia is also important for at least 700 SWR residing in accredited zoological gardens (Species360 Zoological Information Management System, 2020a) who suffer from acute injuries (Hamerton, 1939; Singh et al., 2014), surgery (Esson et al., 2006; Harrison et al., 2011; Nance, 1998; Smit et al., 2016), and neoplasia (Greunz et al., 2016; Wack et al., 2010). In addition, axial (Rothschild et al., 2001) and appendicular (Galateanu et al., 2013; Regnault et al., 2013; Stilson et al., 2016) osteoarthritis is very common in Rhinoceritidae. SWR in captive settings are particularly at risk of pain associated with osteoarthritis, a chronic, age-related condition (Loeser, 2017), because 10.5% of the captive SWR population currently exceeds the median life expectancy of 31.4 years (AZA Population Management Center, 2020; Species360 Zoological Information Management System, 2020a).

The most commonly used pain-relieving medication in North American captive rhinoceros is phenylbutazone (4-butyl-1,2-diphenylpyrazolidine-3,5-dione), a nonsteroidal anti-inflammatory drug (NSAID; Kottwitz et al., 2016). Despite its frequent use to treat painful conditions in rhinoceros, there is no peer-reviewed, experimental evidence for an appropriate dosing regimen. Based on prescription reports of oral phenylbutazone use in SWR from a zoological information database (Species360 Zoological Information Management System, 2020b), clinically used multi-dose regimens include 1.1–5.7 mg/kg (median 2.4 mg/kg) once daily, 0.5–5.2 mg/kg (median 1.5 mg/kg) twice daily and 1.6–3.3 mg/kg (median 2.7 mg/kg) every other day (Species360 Zoological Information Management System, 2020b). These dosages are similar to those used in horses (*Equus caballus*; Papich, 2016), African elephants (*Loxodonta africana*; Bechert et al., 2008) and Asian elephants (*Elephas maximus*; Bechert et al., 2008). In horses, the closest domestic model of SWR digestion, the recommended phenylbutazone dosage is 2.2 to 4.4 mg/kg orally once daily based on pharmacologic studies (Papich, 2016). In elephants, a pharmacokinetic study of oral phenylbutazone showed that African elephants require 2 mg/kg once daily dosing, whereas Asian elephants require 3 mg/kg every other day (Bechert et al., 2008).

Only two NSAIDs, carprofen (Leiberich, Krebber, et al., 2018) and flunixin meglumine (East et al., 2019), have been investigated for use in rhinoceros. A single 1 mg/kg intramuscular injection in SWR of carprofen reduced thromboxane B_2 activity for 48 h and had an elimination half-life ($T_{1/2}$) of 106 h, which is the longest reported $T_{½}$ for that drug in any mammalian species (Leiberich, Krebber, et al., 2018). The $T_{1/2}$ of a single oral dose of flunixin meglumine in SWR was determined to be approximately 8 h, which is over 5 times the $T_{½}$ of the same dose reported in horses (East et al., 2019). These examples show that SWR have unique NSAID pharmacokinetics for some drugs; therefore, further species-specific studies with other medications and routes are necessary to ensure safe and effective pain relief.

The major objective of this study was to perform pharmacokinetic analyses of a single dose of phenylbutazone in SWR using a population pharmacokinetic design, which allowed for analysis in SWR in whom frequent, repeated sampling in a single individual is not feasible. Population pharmacokinetic designs with sparse sampling have been used in other veterinary studies and offer distinct advantages over traditional pharmacokinetic approaches, including few samples required from each patient and ability to screen for covariates (Bon et al., 2018; Martin-Jimenez & Riviere, 1998). This type of study design, however, does require more complex pharmacostatistical analyses and more intensive modelling (Charles, 2014).

To determine whether pharmacokinetic changes occur over time with multiple doses, we added time points from other individuals after multiple dosing and compared their concentrations with the rest of the single-dose population. We hypothesized that in healthy, adult SWR a single dose of phenylbutazone at 2 mg/kg and multiple doses of phenylbutazone at 2 mg/kg once daily will be adequately absorbed after oral administration to produce plasma drug concentrations in the range considered therapeutic in horses.

2 | MATERIALS AND METHODS

2.1 | Animals

All phases of this study were reviewed and approved by the North Carolina Zoo Animal Care and Use Committee. In addition, all participating institutions' animal care and research committees approved the applicable phases of the study. All participating zoological institutions are accredited by the American Zoological Association (AZA) and abide by the AZA's husbandry, medical care, enrichment and welfare guidelines. This study was also reviewed and endorsed by the Veterinary Advisor to the White Rhinoceros Species Survival Plan.

A total of 38 adult (2.3–51 years old; median 13.3 years old) SWR (26 females and 12 males) with no evidence of systemic illness based on clinical signs and routine veterinary evaluations and who had not received an NSAID within the past two weeks were enrolled in one or more phases of this study. All rhinoceros were housed in their AZA-zoological facility on a natural lighting cycle and fed their routine diet, which varied by institution but typically included grass hay, alfalfa and commercial pellets. Doses were based on body weights acquired via scale or estimation by caretakers (800–2486 kg; median 1800 kg). Phenylbutazone (Phenylbute Powder, 100 mg phenylbutazone/g powder, Phoenix Pharmaceuticals Inc., Burlingame, CA 94010, USA) was orally administered at the time of the routine morning meal by placing the powder on top of or mixed with a preferred food item, such as commercial pellets or grain-based feed, to facilitate voluntary consumption. All animals were monitored for adverse effects through general daily observations by caretakers.

2.2 | Study design

Because this study required voluntary participation of the study subjects for venipuncture and to improve reliability of the samples collected, randomization of time points was not performed and samples were not collected at all time points from a single individual. Instead, we used a sparse sampling protocol and population pharmacokinetic modelling.

A pilot study to determine optimum time points for sampling and validation of the analytical assay was first conducted in five SWR (3 females and 2 males) at the North Carolina Zoo who were administered a single dose of phenylbutazone and then had samples collected at one to three predetermined time points of 1, 2, 4, 6, 8, 12, 24 and 48 h following drug administration, which provided one sample per time point.

Based on the pilot study data from these five SWR (data not shown), a single-dose study was performed by administering

2 mg/kg of phenylbutazone once to 33 (23 females and 10 males) SWR from six AZA-accredited institutions ranging from 2.3 to 39.8 years old (median 10.4 years old) and weighing 800 to 2300 kg (median 1791 kg). All rhinos were sampled at one to three of the predetermined time points of 1, 1.5, 2, 3, 4, 6, 8, 10, 24, 30 and 48 h following drug administration, which provided six to 13 samples per time point.

Following analysis of the single-dose study, a multi-dose study was initiated, which consisted of 2 mg/kg oral doses given every 24 h for five doses to four SWRs (2 females and 2 males) from two AZA-accredited institutions ranging from 14.6 to 29.6 years old (median 21.5 years old) and weighing 1689 to 2486 kg (median 2054.5 kg). All rhinoceros in the multi-dose study were also subjects in the pilot or the single-dose study and had a minimum 11-month washout period. Rhinos in the multi-dose study were sampled at three to four of the predetermined time points of 96, 97.5, 102, 120, 126 and 144 h after the first dose administration, and the final dose of phenylbutazone was administered at 96 h immediately after the first sampling time point. This provided one to four samples per time point.

2.3 | Sample collection

Under voluntary, behavioural restraint with positive reinforcement, blood samples were collected from the auricular, radial or cephalic vein. Specific blood collection supplies varied by institution and rhinoceros to replicate the blood collection process that each venipuncturist and rhinoceros were comfortable with. We included a large population of 38 rhinoceros using a sparse sampling protocol because it is difficult to collect frequent, repeated blood samples from a single individual. All blood samples were placed in lithium heparin spray-coated tubes (BD Vacutainer Lithium Heparin 56 USP Units Blood Collection Tubes, Becton, Dickinson and Company, Franklin Lakes, NJ 07417, USA) and inverted five to 10 times to thoroughly mix anticoagulant. Each institution processed the samples on-site by centrifuging the samples for 10 min and transferring the plasma into a screw-top cryovial. Plasma was frozen at −80°C or 0°C until shipment. Samples were shipped on ice overnight to the Clinical Pharmacology Laboratory at North Carolina College of Veterinary Medicine where they were stored at −80°C until analysis.

2.4 | Phenylbutazone analysis

The samples were processed by thawing at room temperature prior to extraction of phenylbutazone from the plasma. A liquid phase extraction method was used in which the sample was mixed with acetonitrile and vortexed. The supernatant was evaporated to dryness and reconstituted with the mobile phase. Samples were analysed via high-performance liquid chromatography (HPLC) for phenylbutazone levels using a previously validated method in the Clinical Pharmacology Laboratory at North Carolina College of Veterinary Medicine. This laboratory uses the ICH validation guidelines for analytical methods (http://www.ich.org/products/guidelines/quality/quality-single/article/validation-of-analytical-procedures-text-and-methodology.html) and the guidelines published in Chapter <1225> of the United States Pharmacopeia (www.USP.org).

The samples were injected into an HPLC system that consisted of a quaternary solvent delivery system (flow rate, 1 ml/min), an autosampler (Agilent 1200 Series solvent delivery system, Agilent Technologies, Wilmington, Delaware 19808, USA) and an ultraviolet detector (Agilent 1200 Series Variable Wavelength Detector, Agilent Technologies, Wilmington, Delaware 19808, USA) set at a wavelength of 254 nm. Chromatograms were integrated with a computer program (Agilent OpenLAB software, Agilent Technologies, Wilmington, Delaware 19808, USA). The column was a reverse-phase, 4.6 mm × 15 cm C8 column (Zorbax Rx-C1, MAC-MOD Analytical, Inc., Chadds Ford, PA, USA) kept at a constant temperature of 40°C. The mobile phase for HPLC analysis consisted of acetonitrile and ammonium acetate buffer. Fresh mobile phase was prepared, filtered (0.45 μm) and degassed for each day's run.

The incurred samples were quantitated using a calibration curve that consisted of fortified, blank rhinoceros plasma with seven calibration standards ranging from 0.05 μg/ml to 10 μg/ml and included a blank (0 μg/ml). The limit of quantitation for the assay was 0.05 μg/ml, based on the signal/noise ratio and the lowest point determined with acceptable accuracy on a linear calibration curve. Fresh calibration standards and quality control samples were prepared daily for each day's run. The value of R^2 averaged >0.999 for all the calibration curves. The accuracy of the assay was within 108%, 93% and 98%, for high, medium and low concentrations, respectively. The precision of the assay was (expressed as CV%) 6.0%, 5.8% and 3.3% for high, medium and low concentrations, respectively.

2.5 | Pharmacokinetic analysis

Because of the sparse sampling design, it is not possible to apply a pharmacokinetic model to the samples from each rhinoceros using a conventional standard two-stage (STS) approach. The pharmacokinetic parameters were calculated using population pharmacokinetic methods and nonlinear mixed-effects (NLME) modelling using Phoenix® software (Phoenix®, NLME™, version 8.4, Certara Inc., St. Louis, Missouri 63105, USA), which produces the typical value for the population parameters (fixed effects) as well as estimates for the between-subject variability (random effects) and identify the sources of variation. A naïve averaged pooled analysis using a one-compartment model was used to obtain initial estimates (data not shown). From these initial estimates, the NLME model was fitted to these. Compartmental analysis of the data from phenylbutazone administration to the rhinoceros was

calculated using a one-compartment model according to the following formula:

$$C(T) = \frac{DK01}{V(K01 - K10)} \times [e^{(-K10 \times T)} - e^{(-K01 \times T)}]$$

where C is the phenylbutazone concentration, D is the dose, V is the apparent volume of distribution, $K10$ is the elimination rate constant, K01 is the absorption rate constant, and T is time. Secondary parameters calculated include $T_{1/2}$, area under the curve (AUC), peak concentration (C_{MAX}), time to peak concentration (T_{MAX}) and systemic clearance (CL).

Various models were tested with different error structures to determine the best-fit base model. Final model selection was based on goodness of fit plots, diagnostic plots of residuals, scatter plots of predicted vs. observed values and statistical significance between models using the minimum value of the objective function (MOF).

Samples from the multiple-dose study were compared with the single-dose study samples at the same time points. To compare these time points, calculation of an accumulation factor (AF) was used. The AF is a pharmacokinetic parameter that predicts accumulation of drugs in plasma after multiple dosing using the following formula:

$$AF = \frac{1}{(1 - e^{(-K\tau)})}$$

where AF is the accumulation factor, K is the elimination rate, and τ (tau) is the dose interval (24 h).

3 | RESULTS

This study confirmed that phenylbutazone administered to SWR at a dosage of 2 mg/kg orally was absorbed and resulted in measurable plasma levels. Administration compliance was good with all SWR in this study, and no adverse effects were noted.

Pharmacokinetic parameters for the single-dose study of 33 rhinoceros are shown in Table 1. The plots of the average concentrations are shown in Figure 1, and an obvious, secondary peak occurring at approximately 6 h for SWR sampled during that time interval. Mean concentrations from the multi-dose study are overlaid in Figure 1 as solid triangles for comparison; the four rhinoceros of the multi-dose study received five doses of oral phenylbutazone to steady-state before these samples were collected.

Figure 2 shows the plots from NLME after fitting the data to the best-fit pharmacokinetic model. The left panel of Figure 2 shows the spaghetti plots of 33 individual rhinoceros after the model was fit to these data. The right panel of Figure 2 shows the predicted plot for the typical model after accounting for inter-individual (between-subject) variability.

The mean concentrations after multiple doses are shown in Table 2, compared with the mean concentration from the single-dose administration using an accumulation factor. The AF is included in Table 2, which was calculated to predict accumulation of phenylbutazone in plasma after multiple dosing.

TABLE 1 Pharmacokinetic estimates for the population of 33 rhinoceros administered an oral dose of 2 mg/kg phenylbutazone using nonlinear mixed-effects modelling

Parameter	Estimate	Units	Shrinkage	CV%
θKa	1.83	1/h	1.00	0.03
θVD	0.46	L/kg	0.37	19.92
θKe	0.077	1/h	0.43	10.72
T_{MAX}	1.80	h		
AUC	56.92	μg•h/ml		
C_{MAX}	3.80	μg/ml		
CL	0.035	L/kg/hr		
Absorption T½	0.38	h		
Elimination T½	9.04	h		

Note: h, hour; T½, half-life; θVD (theta VD), volume of distribution; θKe, elimination rate; θKa, absorption rate; AUC, area under the curve; C_{MAX}, peak plasma concentration; T_{MAX}, time to peak concentration; CL, clearance; (theta notation, θ, is used to show that this is the 'typical value' for the population). The values of VD and CL are implied to mean VD/F and CL/F (per fraction absorbed) because this was not an intravenous dose. CV%, coefficient of variation expressed as a per cent; shrinkage is computed as 1—standard deviation for the residual values.

4 | DISCUSSION

In the study subjects, the $T_{1/2}$ for a single oral dose of phenylbutazone was approximately 9 h, which is longer than the $T_{1/2}$ in horses (4–6 h) and shorter than the $T_{1/2}$ in cattle (42–64 h) (Lees et al., 2004). Among mammals, the $T_{1/2}$ of phenylbutazone ranges widely from 1 to 2 h in donkeys to 96 h in humans (Lees et al., 2004). Without further study, we cannot speculate on the cause of these differences from other species. It may be caused by differences in protein binding or hepatic clearance, difference in diet consumed or some other unidentified factor (Lees et al., 2004). For example, horses and SWR have very different elimination curves for carprofen with a $T_{1/2}$ of 21.9 and 106 h, respectively (Leiberich, Krebber, et al., 2018). Despite a similar gastrointestinal tract and having the same cytochrome P450 family genomes present, horses possess a greater number of sequences for iso-enzymes than SWR, which may cause differences in drug elimination between these two species (Leiberich et al., 2018).

Based on the results of this study, a single oral dose of phenylbutazone at 2 mg/kg produces concentrations within the half maximal effective concentration (EC50) for horses of 1.5–4.3 μg/ml for at least 10 hours (Lees et al., 2004; Toutain et al., 1994). However, plasma concentration of phenylbutazone may not directly represent the extent and duration of their anti-inflammatory effects. Because phenylbutazone is highly protein bound, it is sequestered in the protein of exudates and persists longer in exudates than in transudates and plasma (Lees et al., 2004). For example, ponies administered 4.4 mg/kg IV had phenylbutazone concentrations higher in exudate than in

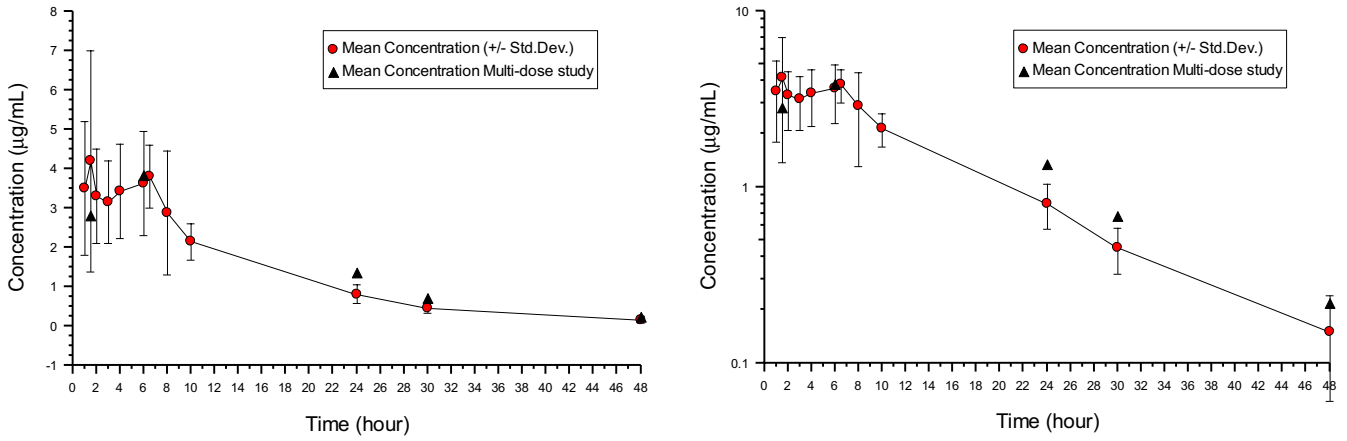

FIGURE 1 Phenylbutazone plasma concentrations in 33 southern white rhinoceros (*Ceratotherium simum simum*) after a dose of 2 mg/kg oral. Each round point represents the mean of 6–13 samples per time point (+/- std. deviation). The solid triangles represent the average concentration of samples from additional four rhinoceros after multiple dosing. The left panel is represented on a linear axis; the right panel is represented on a semi-logarithmic axis

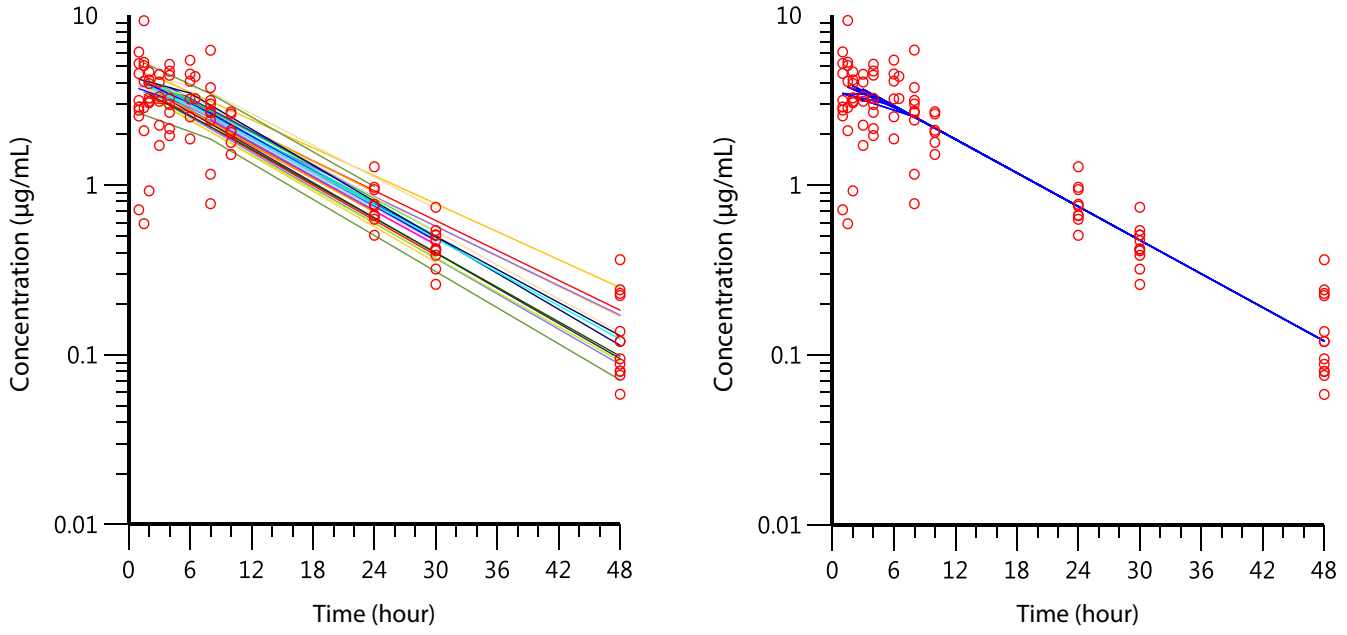

FIGURE 2 Phenylbutazone plasma concentrations in 33 southern white rhinoceros (*Ceratotherium simum simum*) after a dose of 2 mg/kg oral. Each open point represents a single sample, with 6–13 samples per time point. The solid line represents the fitted line according to the pharmacokinetic model. The left panel is the spaghetti plot of each individual rhinoceros fitted to the model. The right panel represents the fitted curve to the model after accounting for random variation (between-subject variability) in the model. Notice the improved population model fit on the right after adjusting for between-subject variability

plasma at 12 and 24 h following administration and had significant reductions in prostaglandin E2 activity in the exudate for at least 24 h, suggesting that the effect of phenylbutazone in inflammatory exudate likely extends beyond the plasma concentrations (Higgins et al., 1984).

We observed a distinct 'double peak' at approximately 6 h after oral administration for rhinoceros sampled at those time points (Figure 1). This phenomenon also was observed in elephants and horses (Baggot, 1992; Bechert et al., 2008). According to this review (Baggot, 1992), double peaks after oral administration to horses are common. The second peak in ponies occurred at approximately 6 h, which is similar to our results in rhinoceros. The explanation for this phenomenon in horses is that while some of the administered dose may be absorbed in the small intestine, some may become adsorbed on to the feed and subsequently released by fermentative digestion for absorption in the colon or caecum. Without additional undue study, we cannot speculate on the cause of the secondary peaks in our study.

The multi-dose study administered phenylbutazone for five consecutive days to steady-state to an additional four rhinoceros. The mean plasma concentrations for five time points are shown in Figure 1, and the concentrations are listed in Table 2. There was some accumulation at the later time points that ranged from a factor of 1.05 to 1.67 (Table 2). According to the formula for calculating the accumulation

TABLE 2 Comparison of mean concentrations at 5 time points after the single-dose administration (2 mg/kg) and after multiple doses of 2 mg/kg for 5 days to steady-state. The accumulation factor represents the ratio of multi-dose concentration to single-dose concentration. Accumulation factor $=1/(1\text{-}e^{-K\tau})$

	Concentration (μg/ml)		
Time (hour)	Multi-dose	Single dose	Accumulation factor
1.5	2.78	4.18	0.67
6	3.81	3.61	1.05
24	1.33	0.80	1.67
30	0.68	0.45	1.50
48	0.22	0.15	1.49

factor, the predicted accumulation is approximately 1.2. Thus, at the later time points the observed accumulation was slightly higher than predicted. Lower concentrations would have indicated some metabolic enzyme induction and increased clearance, which did not appear to occur. The slightly higher accumulation than predicted may reflect increased absorption, slower clearance or changes in the volume of distribution (such as protein binding) with multiple doses. Without further undue study and an intravenous dose to measure absolute values for bioavailability, clearance and volume of distribution, the cause for the accumulation we observed is undetermined.

One of the strengths of our study is that we showed that pharmacokinetic analysis using a sparse sampling design is possible, provides robust pharmacokinetic parameters to produce typical pharmacokinetic values for the population and allows an examination of the extent of variability. As seen in Table 1, the variability for the modelled primary pharmacokinetic parameters of elimination rate and volume of distribution was very low (10.7% and 19.9% CV, respectively). The overall model prediction (Figure 2) was improved by accounting for inter-individual (between-subject) variability. A sparse sampling strategy is an important tool in zoo and exotic species because repeated sampling of a single animal that may induce stress from handling is unnecessary. Sparse sampling pharmacokinetics combined with NLME can provide useful population pharmacokinetic data in animals where sampling is restricted (Ette et al., 2004).

The limitations of this study include non-standardized food intake with medications, which may have affected the variability of our data. There was a wide range of study subject ages and an unequal number of males and females in the study. We do not know the influence of age or sex on the disposition of phenylbutazone in rhinoceros. Although concentrations in this study reached targeted concentrations for efficacy in horses (Toutain et al., 1994), we cannot speculate on the efficacy of phenylbutazone in SWR, and it is unknown whether phenylbutazone at these dosages will provide analgesia or anti-inflammatory effects. In addition, no rhinoceros in this study experienced overt adverse effects. Clinicians using phenylbutazone in SWR should monitor for the adverse effects that have been seen in domestic horses, such as gastrointestinal ulceration, right dorsal colitis with hypoproteinemia and renal injury (Papich, 2016).

5 | CONCLUSION

This study determined that phenylbutazone administered at 2 mg/kg orally as a single dose and once daily for five doses produced plasma concentrations within the EC50 of horses without evidence of excessive accumulation and without clinically apparent adverse effects.

DATA AVAILABILITY STATEMENT

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

ANIMAL WELFARE AND ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have adhered to either US standards for the protection of animals used for scientific purposes.

ACKNOWLEDGEMENTS

The authors thank the keepers, managers, research coordinators and veterinary teams of the following zoological institutions: Busch Gardens Tampa Bay, Fossil Rim Wildlife Center, Houston Zoo, Lion Country Safari, North Carolina Zoo, The Wilds, and White Oak Conservation. We thank Delta R. Dise of the Clinical Pharmacology Laboratory for providing sample analysis.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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How to cite this article: Houck, E. L., Papich, M. G., & Delk, K. W. (2021). Phenylbutazone pharmacokinetics in southern white rhinoceros (*Ceratotherium simum simum*) after oral administration. *Journal of Veterinary Pharmacology and Therapeutics*, 00, 1–7. https://doi.org/10.1111/jvp.13036