Assessing the value of enrofloxacin and carprofen combination treatment in Southern white rhinoceros (*Ceratotherium simum*) through the use of pharmacokinetic modelling

Marion Sarah Leiberich

2018

Department of Companion Animal Clinical Studies
Faculty of Veterinary Science
University of Pretoria
South Africa

Assessing the value of enrofloxacin and carprofen combination treatment in southern white rhinoceros (*Ceratotherium simum*) through the use of pharmacokinetic modelling

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

by

Marion Sarah Leiberich

Department of Companion Animal Clinical Studies

Faculty of Veterinary Science

University of Pretoria

Promotor: Prof Vinny Naidoo

2018

Declaration

I, Marion Sarah Leiberich, hereby declare that the dissertation, "*Assessing the value of enrofloxacin and carprofen combination treatment in Southern white rhinoceros (Ceratotherium simum) through the use of pharmacokinetic modelling"* is submitted in accordance with the requirements for the Doctor of Philosophy degree at the University of Pretoria, is my own original work and has not previously been submitted to any other institution of higher learning. All sources cited or quoted in this research paper are indicated and acknowledged with a comprehensive list of references.

Marion Sarah Leiberich
20th March 2018

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Prof. Vinny Naidoo for providing me with the opportunity to complete a PhD at the University of Pretoria, for your ongoing support, your invaluable guidance and patience. Your motivating words, your criticism and your ideas have helped me greatly, while your wealth of knowledge and enthusiasm have inspired me.

I would also like to thank my co-supervisor Dr. Johan Marais for the initiation of this project and for your help and support finding the suitable study animals and for introducing me to the owner and the staff of the Rhino Orphanage.

A special thanks also goes to Dr. Michael Hewetson for the help at the Rhino Orphanage during the fieldwork. Thank you for the time invested, the early mornings and late nights, trying to get all the required blood samples from the rhinos.

A big thank you also goes to Dr. Pierre Bester for the help with the immobilisation of the study animals. Thank you for your commitment and for dedicating many hours to the project, collecting the blood samples and overcoming issues with the auricular catheters.

This thesis also owes its existence to the help of 'The Rhino Orphanage'. Thank you very much, Arrie van Deventer, owner of the orphanage for your support and trust and for letting us perform the study at 'The Rhino Orphanage' with the rhinoceros in your care.
A very big thank you also goes to the staff at the Rhino Orphanage, in particular to Laura Ellison, Jamie Traynor and to Gabriela Benavides for the great support during the study. For planning the rhino movements and groupings of the study animals, for calming down the rhinos when needed and for the countless extra hours you put in to help with the study and the overall goal to be able to offer better treatment to poaching victims. Without your dedication and your relationship to the orphans we would have never been able to get all our blood samples.

A huge thank you also goes to my faithful friend Dr. Robin Gielding, the best field assistant I could have hoped for during the first phase of the trial. Thank you so much for your hard work, doing the job of several people all at once. I highly value your support and all the energy you put into making the field work a success. Furthermore, I would also like to thank Aminisha Somers and Klaudia Pruczkowska, my team of field assistants during the second trial. Your support was essential to the success of the trial and I am grateful for the very good moments and also the very tiring moments we got to share. Without you girls, it would have not been possible!

I thank Arina Ferreira and Annette Venter for all the help in the pharmacology laboratory. Arina, you taught me techniques and tricks and cheered me up on long lab days. Annette, thank you for your invaluable technical support with the computer, machines and the software in the lab.

Also, Dr. Emmanuel Adawaren deserves a special thanks, your introduction into phylogenetics has helped me greatly.

I would also like to express my gratitude to the bacteriology laboratory of the Department of Tropical Diseases of the University of Pretoria for letting me use your database on all records of bacterial infections in rhino from 2008 to 2015.

Thank you Dr. Matthias Pohlmeier from Bayer Animal Health for your efforts in resolving the CITES permit issues and Dr. Ralph Krebber and the team for performing the sample analysis for us. Your help was invaluable.

My sincere thanks also goes to the German Academic Exchange Service (DAAD) and to the Univeristy of Pretoria for the financial support granted and further to Bayer Animal Health, Zoetis and Wildlife Pharmaceuticals for donating the required drugs for our study.

And finally, a very big thank you goes to my parents and my sister for being only a phone call away from me when I need you. For always being understanding, supportive and loving, and for being my first place to go to for general advice. Even if you are far, I know you stand right behind me and I know I can count on you.

And I would like to thank my partner Christoff van der Walt. I am grateful for your ongoing support and your unconditional love.

Publications and conferences output

Published

Leiberich, M., Krebber, R., Hewetson, M., Marais, J., Naidoo, V. (2018). A Study of the Pharmacokinetics and Thromboxane Inhibitory Activity of a Single Intramuscular Dose of Carprofen as a Means to Establish its Potential Use as an Analgesic Drug in White Rhinoceros. *Veterinary Pharmacology and Therapeutics*. *41*(4), 605-613. 10.1111/jvp.12508

Publication submitted for review

Leiberich, M., Krebber, R., Hewetson, M., Marais, J., Naidoo, V.: Is the White Rhinoceros a Large Horse? The Use of Allometry and Pharmacokinetic Modelling to Evaluate the Importance of Interspecies Differences for One of Africa's Iconic Species. Submitted for consideration to PLOS ONE.

Leiberich, M., Marais, J., Naidoo, V.: Phylogenetic analysis of the cytochrome P450 (CYP450) nucleotide sequence of the horse and predicted CYP450 of the white rhinoceros (Ceratotherium simum) and other mammalian species. Submitted for consideration to PeerJ.

Conference presentations

February 2017, Veterinary Management of African Wildlife Conference, Pretoria, South Africa:
Pharmacokinetics and pharmacodynamics of intravenous and oral enrofloxacin and ciprofloxacin in white rhinoceros – a possible treatment option for injured poaching victims?

March 2018, Wildlife Group of the SAVA congress, Johannesburg, South Africa: Pharmacokinetics and Pharmacodynamics of Intramuscular Carprofen in White Rhinoceros – Exploring its Potential for Critically Injured White Rhinoceros.

Abstract

Over the past decade, the poaching of white rhinoceros (*Ceratotherium simum*) has increased dramatically. More than 7000 animals have been killed and approximately 200 animals annually survive the poaching attempts with life threatening injuries, which require immediate medical attention. Despite their need for veterinary care, knowledge on the treatment of white rhinoceros is scarce. Approved drugs are non-existent and dosages need to be extrapolated arbitrarily from other species. In order to successfully treat injured poaching victims and to increase their chance of survival, potentially effective drugs and corresponding dosages need to be assessed. For this study, we evaluated the pharmacokinetic and pharmacodynamic properties of enrofloxacin in combination with carprofen, administered to immobilised rhinoceros.

From the pharmacokinetic evaluation, intravenous enrofloxacin (12.5 mg/kg) was characterised by an AUC, Cl and $t_{1/2}$ (Gmean ± SD) of 64.5 ± 14.44 µg*h/mL, 0.19 ± 0.04 L/h*kg and 12.41 ± 2.62 hours, respectively. Of these, the unexpected finding was the extremely long half-life of elimination, which was significantly longer than that of the horse and of any other mammalian species. Subsequent pharmacodynamics modelling showed that daily intravenous application could be useful and could be achieved by means of a light butorphanol sedation, followed by the enrofloxacin administration through an indwelling catheter. To further evaluate if the intravenous priming dose could be maintained by subsequent oral dosing, animals in phase two of the study were treated with a second dose of enrofloxacin in the feed following immobilisation. The oral treatment was deemed inappropriate, as the oral bioavailability of 33.30 ± 38.33% was surprisingly low. Intramuscular carprofen (1 mg/kg), was characterised by an AUC, Cl and $t_{1/2}$ of 904.61 ± 110.78 µg*h/ml, 0.0011 ± 0.0001 L/h*kg and 105.71 ± 15.67 hours, respectively. As for enrofloxacin, the major finding was the considerably long half-life, which was the longest reported in any species this far. Based on the changes in plasma thromboxane concentration, we surmise that the drug would have an anti-inflammatory effect for a minimum of 48 hours.

To gain a better understanding of the prolonged half-life of elimination, two *in silico* studies were undertaken. For the first study, allometric pharmacokinetic modelling was conducted with enrofloxacin to ascertain if the slower metabolism evident in the rhino could be attributed to the size of the species. Despite the goodness of fit of different models, none were predictive of the actual pharmacokinetic parameters. This leads to the conclusion that the slower metabolism resulted from metabolic constraints as opposed to size-related slower metabolism.

To provide insight into the metabolic constrains in the rhino and to ascertain if the rhino genome coded for the enzymes described in the horse, a species closest related to the rhinoceros, the published rhino genome was evaluated using BLAST algorithms. The white rhino nucleotide sequences were 90.74% identical to the equine sequences, which represents the highest degree of similarity amongst all evaluated species sequences. Even though all CYP450 families present in the horse coud be identified in the white rhino, the horse genome contained additional gene sequences for a larger number of iso-enzymes, which were not present in the rhino. Thus, the difference in the metabolic capacity cannot be directly attributed to an absolute deficiency of a CYP enzyme family. However, with the difference possibly being the absence of specific isoenzymes in the rhino, this could suggest that the horse is a more efficient metabolizer.

Based on the results of this study, we believe that carprofen shows good promise as an analgesic for use in rhinoceros. While enrofloxacin should be effective, its use is rather limited due to the frequently required intravenous administration. The subsequent *in silico* modelling suggested that the rhino may exhibit metabolic constraints, which may result from their lower number of isoenzymes. The latter assumption does however require further evaluation.

Key terms: white rhinoceros, pharmacokinetics, pharmacodynamics, poaching, antimicrobial, enrofloxacin, non-steroidal anti-inflammatory drug, carprofen.

Table of contents

List of Figures

List of Tables

List of supplementary information

Abbreviations

ABC transporter	ATP-binding cassette transporter
AUC_{last}	Area under curve to the last quantifiable time point
AUC_{tot}	Total area under curve extrapolated to infinity
AUMC	Area under the moment curve
B/B0	Sample bound/maximal bound
BCRP	Breast cancer resistant protein
C	Concentration
CINOD	COX-inhibiting nitric oxide donator
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora
CK	Creatinine kinase
Cmax	Maximum concentration
Cl	Clearance
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CLSI	Clinical & Laboratory Standards Institute
CYP	Cytochrome P450
E	Effect
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
Gmean	Geometric mean
IASP	International Association for the Study of Pain
IM	Intramuscular
IV	Intravenous
HPLC	High pressure liquid chromatography
HR	Heart rate
HPLC-MS/MS	High pressure liquid chromatography-tandem mass spectrometry
λ	Terminal elimination rate constant
Ln	Natural logarithm
LOD	Limit of detection
LOQ	Limit of quantification
MAT	Mean absorption time
MEGA	Molecular Evolutionary Genetics Analysis
MIC	Minimum inhibitory concentration
ML	Maximum Likelyhood Method
MRT	Mean residence time
MUSCLE	Multiple Sequence Comparison by Log Expectation
NaOH	Sodium hydroxide
NO	Nitric oxide
NSAID	Non steroidal anti-inflammatory drug
online-SPE-MS/MS	Online-solid phase extraction-tandem mass spectrometry
PGE2	Prostaglandin E_2
PGEM	Prostaglandin E_2 Metabolites
PO	Per os
RR	Respiratory rate
SC	Sub cutaneous

SD	Standard deviation
SE	Standard error
SEM	Standard error of the mean
T	Time
t1/2	Half-life
Tmax	Time at maximum concentration
TXA2	Thromboxane A2
TXB2	Thromboxane B2
Vd	Volume of distribution
Vss	Volume of distribution at steady state
Vz	Volume of distribution during terminal phase
W	Weight

1. Chapter 1: Introduction

1.1 The poaching crisis

'Even though rhinos are well built for survival, they were not prepared to face an organized gang of humans with an insatiable taste for greed.' (Fargnoli)

In 2016, a staggering three rhinoceros were poached a day bringing the total number killed mercilessly in the past decade to more than 7000 individuals (Poaching statistics, 2018). This injudicious slaughter of the animals has been caused by the high demand and value placed on the horn by individuals in Asian countries, making rhino horn more valuable than gold, diamonds and even cocaine (Biggs et al., 2013). After the near extinction of the white rhinoceros in the 1980s' and the successful reintroduction of the iconic species in the early 20th century, the current poaching crisis results once again in the decline of the rhino populations, dashing the conservation efforts and achievements of the last century to an extent that the white rhino is once again threatened and in danger of extinction (Emslie et al., 2016). It may be argued that the population can be rescued once again, however in addition to the great loss in population numbers, the species is facing a great losses in genetic variability, which may make such future rebreeding efforts difficult (Ripple et al., 2015). As a result, numerous efforts have been put in place by private owners, local and international conservation organisations and the South African government, including demand reduction campaigns and educational programs in Asia, involvement of local communities, anti-poaching and law enforcements measures. However, besides all measures aimed at reducing the poaching of white rhinoceros, less effort has been directed towards the protections of the surviving victims. About 20% of the targeted rhinoceros initially survive the poaching attack with variable degrees of life-threatening injuries (J. Marais, personal communication, 2016). Veterinary interventions are indispensable for reasons of animal welfare, to increase their chance of survival and to promote the survival of the species. However, the knowledge on appropriate treatment of wildlife species, including information on antimicrobial and analgesic support, is generally scarce and results in largely empirical treatment, based on the experience of the treating veterinarian. More so, thus far, the horse (*Equus caballus*) has often been used as a model species for dosage extrapolations for the rhinoceros. However, interspecies differences in metabolic capacity and drug metabolising enzymes could be resulting in rhino being treated at non-optimal doses.

Since effective and adequate antibiotic and analgesic treatment is of prime importance to prevent the infection of wounds; to fight an existing infection; and to reduce pain and inflammation, the following study was aimed at looking into optimising one antimicrobial and

one analgesic drug combination for the use in rhino. More specifically, this study will look into the potential efficacy of enrofloxacin and carprofen in combination for the treatment of the Southern white rhinoceros (*Ceratotherium simum simum*). For this study, we rely on pharmacokinetics, allometric scaling and pharmacodynamics modelling to evaluate the potential of the mentioned combination to be effective. The study will also use these two drugs to evaluate if further drug dose predictions can be based on the horse, or whether more species-specific work needs be undertaken for further drug dose prediction.

1.2 Hypotheses

1. Enrofloxacin and carprofen present a promising combination for the management of pain, inflammation and secondary infections in the Southern white rhinoceros.
2. The rhino is metabolically distinct as a species to the domestic horse.

1.3 Objectives

1. To evaluate the pharmacokinetic and predict the pharmacodynamic properties of enrofloxacin in white rhinoceros.
2. To compare the pharmacokinetics of enrofloxacin after intravenous administration and after concurrent intravenous and oral administration.
3. To evaluate the pharmacokinetics of carprofen in white rhinoceros.
4. To determine the degree and duration of thromboxane B2 inhibition as an indicator for COX-1 inhibition following a single dose of carprofen.
5. To investigate the value of allometric scaling for the extrapolation of pharmacokinetic parameters from common domestic species to the rhinoceros.
6. To assess the phylogenetic relationship of the cytochrome P450 enzymes of the horse, the rhino and other selected species.

1.4 Aims

1. To evaluate the potential benefit of enrofloxacin in the management of rhino infections, when administered by the intravenous and oral route.
2. To evaluate the potential benefit of carprofen for the treatment of white rhinoceros against inflammation.
3. To evaluate the relatedness of Southern white rhinoceros to the domestic horse using a combination of allometric pharmacokinetic scaling and phylogenetic comparison of predicted populations sequences of relevant cytochrome P450 enzyme.

2. Chapter 2: Literature review

2.1 The rhinoceros

Despite growing human populations and the re-tasking of land towards agricultural and industrial developments in the place of natural habitats, South Africa is still characterised by its abundant and unique species of wildlife and counts as one of the countries with the highest biodiversity in the world (South African National Biodiversity Institute, 2015). While the most popular species associated with Africa are the "Big Five" including elephant (*Loxadanta Africana*), leopard (*Panthera pardus*), buffalo (*Syncerus caffer*), lion (*Panthera leo*) and rhinoceros (*Rhinocerotidae*), Africa is home to many more species of various sizes and habitats. The rhinoceros, along with the elephant is one of the biggest living terrestrial species. Often described as the charismatic mega herbivore, it is attracting many tourists yearly (Lindsey et al., 2007). Alarmingly, this diverse range of species is at different risks of extinction. Five remaining rhinoceros species exist in the world (Figure 2-1) and are all listed in the IUCN list of endangered species (Emslie et al., 2016). The white rhinoceros is listed near threatened; the greater one-horned rhinoceros; is listed as vulnerable and the black rhinoceros, the Sumatran rhinoceros and the Javan rhinoceros are listed as critically endangered (Emslie et al., 2016).

Figure 2-1: The five remaining rhinoceros species (Rhino Resource Center, n.d.)

The Southern white rhinoceros (*Ceratotherium simum simum*) represents the white rhinoceros subspecies occurring in South Africa and Namibia, Botswana, Zimbabwe, Swaziland and Mozambique. It has also been introduced to Kenya, Uganda and Zambia (Dinerstein, 2011). However, South Africa with 93% of the total number of the southern subspecies, holds the majority of the overall population (Milliken & Shaw, 2012). Weighing up to 2700 kg, it is the biggest of all rhino species. The white rhinoceros is a grazer and an important umbrella species in the ecosystem. As one of the last living mega-herbivores species, Cromsigt and te Beest (2014) suggested that it plays an important role in the maintenance of

the African grassland, in enhancing plant diversity and in creating grazing lawns. The increase in short grass areas may lead to the creation of habitats for several other species such as birds, insects, small mammals and ungulates. The white rhino is thus speculated to represent a keystone species which increases grassland heterogeneity within the ecosystem (Cromsigt & te Beest, 2014).

Once abundant, the white rhinoceros was close to extinction due to malicious poaching of the animals for their horns at the end of the 19th century. With less than 100 animals left in KwaZulu-Natal, South Africa committed to what is known today as one of the biggest conservation achievements in the world. From a single population of 20 to 50 rhinoceros in KwaZulu-Natal, South Africa, the numbers of rhinoceros grew back to more than 20,000 animals in the 21st century (Emslie et al., 2016)

2.2 The new poaching crisis

Unfortunately, the poaching crisis has rekindled, with thousands of rhinos killed in the last ten years. The illegal trade in rhino horn and therewith the killing of rhinoceros has been increasing uncontrollably and led to the white rhino population being once again threatened. South Africa and Vietnam, the main driver in the market for rhino horn (Emslie et al., 2016), represent the major countries of focus. Organised crime syndicates are illegally exporting rhino horn in large quantities mainly to Vietnam where the rhino horn is used in traditional Chinese medicine against fevers, headaches, delirium, epilepsy, strokes, measles and for detoxification and further for ceremonial purposes and as a status symbol (Challender & MacMillan, 2014). However, the main use of rhino horn is unrelated to traditional Chinese medicine and the belief in curative properties, but is based on contemporary, recent developments (Emslie et al., 2016). Rhino horn has become a status symbol and is used as expensive, high value gifts and as luxury goods, demonstrating prosperity amongst the growing wealthy middle class. Further, it is used as a cure for hangovers after excessive alcohol consumption, in social business elite events at functions and so called ‘rhino wine associations’. The increase in demand and value of rhino horn has taken dramatic extents and is driving the rhinoceros towards extinction. The numbers of poached rhino victims from 2007 until 2017 are highlighted in Figure 2-2.

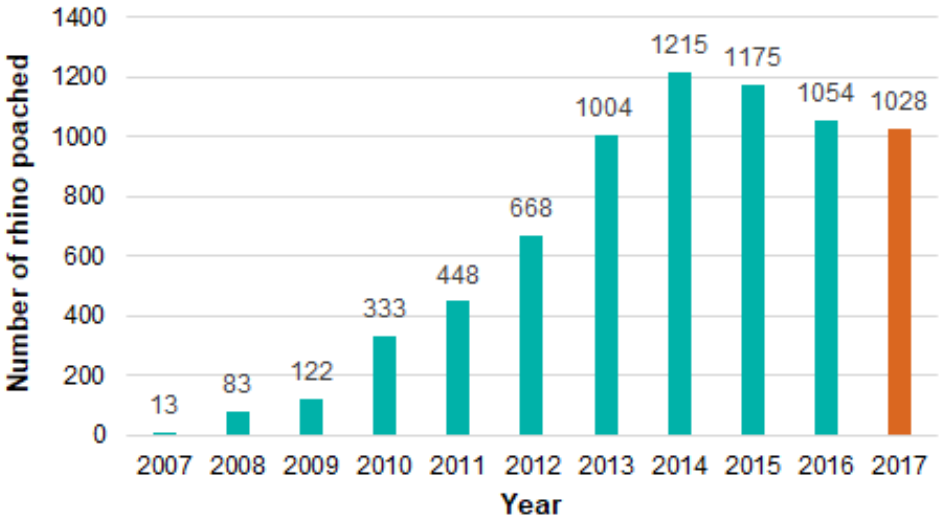


Figure 2-2: Recorded numbers of poached rhinoceros in South Africa from 2007 until 2017 (Save the Rhino, 2018)

Rhino poaching has not surprisingly become a tremendously lucrative business that consists of a highly organised network of well trained and equipped poachers, middlemen, dealers and masterminds. So far, the efforts to reduce the ongoing killing of the rhinoceros, in terms of government strategies and on the private sector have not lead to any significant success (Emslie et al., 2016). Strict law enforcement, highly trained and dedicated anti-poaching units, the lucrative involvement of the local communities in the protection of the natural heritage, dehorning and awareness campaigns in Asia are all valuable approaches, which need to be established, continued and improved. However, while working on a long term strategy to combat poaching, immediate help for the affected victims, survivors of brutal poaching attacks is currently needed (Figure 2-3).

Figure 2-3: Poached rhino victim (Photo courtesy of Dr. Marais, Saving the Survivors)

Amongst all killed rhinoceros for their horns, between 200 and 300 rhinos per year are found alive after poaching attacks (J. Marais, personal communication, 2016). Victims of poaching incidents often suffer from gunshot wounds or from wounds situated on the frontal and nasal bone after the removal of the horn and require veterinary treatment against both infections and pain. Despite the need to treat these rhinoceros for their injuries, very little is known about their veterinary management. Several reports and case studies have been published on some bacterial, viral and parasitic diseases in different rhino species (Bauwens et al., 1996; Bryant, et al., 2012; Flach et al. 2003; Jones & Thomsett, 1972; Miller & Buss, 2014; von Houwald et al., 2001). However, despite an extensive literature review, little information is available regarding the management of infection or pain with drugs. To our knowledge, no scientifically evaluated drug dosage recommendations, nor pharmacokinetic and pharmacodynamic studies have been published. As a result, current treatment in rhino is based on equine doses, which may not be accurate since the horse has undergone years of domestication. As a result, a different manner of dose selection is needed. One such method described in literature makes use of pharmacokinetic modelling. Three different methods, namely linear extrapolation, metabolic scaling and allometric scaling have been described and attempted for the calculation of the appropriate dose and the dose interval of drugs for new species.

2.2.1 Linear extrapolation

Linear extrapolation is the simplest method and assumes that weight and dosage are directly proportional. This means that with the increase in body weight, the amount of drug needed increases linearly, even between different species (Hunter & Isaza, 2008). However, it has been found that the basal metabolic rate of an animal is not proportional to its body weight but to the body surface area (Kleiber, 1947). Thus, small animals with a relatively higher body surface feature a higher basal metabolic rate, which renders the method inaccurate for extrapolation. Another point of criticism is that linear extrapolation does not consider species-specific differences in pharmacokinetics and pharmacodynamics nor the weight range. Pharmacologic differences between species are not rated clinically relevant. This method risks to overdose large animal species and to under-dose small species. If the extrapolated drug has a narrow margin of safety, the linear extrapolation can have serious consequences in terms of toxicity (Hunter & Isaza, 2008).

2.2.2 Metabolic scaling

By comparison, metabolic scaling incorporates the metabolism and body surface area in the calculations. It is based on the conversion of body weight into metabolic size. The idea is to scale physiological processes across species of very different sizes. Metabolic scaling includes information about physiologic functions or anatomic features such as basal metabolic rate and body surface area in order to extrapolate a dose from an approved species. It results in a relatively higher dosage recommendation for small species and in a relatively lower dose recommendation for large species. It is presumed that because most physiologic functions can be described with allometric equations relative to body weight, similar allometric relationships apply to the pharmacologic parameters (Hunter & Isaza, 2008). As a result, the basal metabolic rate or the body surface can be used as a scaling factor. Assuming that there is a direct relationship between size and metabolic rate within certain species, every animal is put in one of five groups: passerine birds, non-passerine birds, placental mammals, marsupial mammals or reptiles (Sedgwick, 1993). Depending on the group, a predetermined K value is chosen in order to calculate the metabolic rate of the species. The constant K, for placental mammals is 70. For the estimation of the right dosage, the specific minimum energy cost (SMEC) is calculated for each species with the formula:

$SMEC = K(W_{kg}^{0.75}/W_{kg}) = K(W_{kg}^{-0.25})$ with W_{kg} being the body weight in kg.

In order to calculate the SMEC dose of a drug for a new species, the dose rate in mg/kg for the control species is divided by the control species' SMEC. To further calculate the new species' treatment dose, the SMEC dose has to be multiplied by the new species' SMEC (Sedgwick, 1993). The advantage of this method is that a metabolic rate can be calculated for a species and can then be used for the extrapolation of any drug. The method developed by Sedgwick (1993) has been used in zoo medicine and a commercial computer programme is available to calculate dosages. However, the method has not been scientifically validated and inaccurate dose estimations compared to results of pharmacokinetic studies have been reported (Mortenson, 2001). Like the linear scaling method, the metabolic scaling method does not consider species variations in pharmacology as clinically relevant. It assumes that the metabolic rate can be used for any drug in order to estimate the right dose for target species. However, the relation between metabolic rate and the pharmacokinetics is more complex than this method presumes (Hunter & Isaza, 2008). Metabolic scaling has been described as inappropriate for the calculation of dosages following several pharmacokinetic studies in elephants (Lodwick et al., 1994; Bechert & Christensen, 2007).

2.2.3 Allometric scaling

Allometric scaling is based on the idea that the pharmacokinetic properties of a drug are not linear to the weight of the animal. The aim is to allometrically scale the pharmacokinetic parameter instead of the dose. Pharmacokinetic parameters such a half-life ($t_{1/2}$), clearance (Cl) and volume of distribution (V_d) are collected for different well studied species. They are plotted against the weight using the equation $Y=aW^b$ in order to calculate the pharmacokinetic parameter for the unknown species (Y = the pharmacokinetic parameter of interest, a = coefficient of allometry, b = exponent of the allometry and W = body weight) (Cox et al., 2004; Hunter & Isaza, 2008). It is the method of choice for interspecies extrapolation and is used for the dose selection in humans (Hunter & Isaza, 2008). While the method has not been critically evaluated for zoo medicine it may provide a more accurate method of dose estimation in wild animal species. The accuracy and success of the method of allometric scaling also depends on the drug type. Riviere (2011) states that drugs with blood-flow dependant clearance and limited hepatic metabolism seem to be ideal for allometric scaling. Drugs with a hepatic clearance markedly lower than the hepatic blood flow and hepatic metabolism through phase II pathways (glucuronidation, acetylation or sulfation) are not suitable for allometric scaling. Cox et al. (2004) concluded that allometric analysis of enrofloxacin and ciprofloxacin could predict Cl and V_d proportional to the body weight whereas $t_{1/2}$ could not be predicted. Riviere et al. (1997) was able to demonstrate that 75% of the 44 drugs he evaluated in the study were not allometrically scalable. However, in case of some drugs, the apparent lack of scalability could be due to the differing data sources leading to differing results or due to non-existent quality data. Nevertheless, Hunter and Isaza (2008) suggest that, if no species specific pharmacokinetics data is available, the allometric scaling method is the most appropriate to use to determine a therapeutic dose in non-approved species.

2.2.4 Pharmacokinetic scaling for rhino: Is the rhino a big horse?

It has been put forward that the metabolic capacity of the rhino should be similar to the horse since they are both hindgut fermenters. However, this topic is still controversially discussed. In a survey by Mortenson (2001), 27 zoos in North America showed that the dosage of antibiotics used by clinicians in zoo medicine were mostly based on equine dosage recommendations or pharmacokinetic research results. Mortenson (2001), subsequently compared dosages from published studies to doses calculated using metabolic scaling and doses derived from the recommendations for horses. This study was able to demonstrate that for both, the African and Asian elephant, the equine dose could lead to overdosing in mg/kg and a overly

high frequency of dosing. Furthermore, the dosages based on metabolic scaling compared to dosage recommendations derived from pharmacokinetic studies did not correspond well. Mortenson (2001) suggested that this could be due to the differences in biological functions such as biotransformation of the drug, cardiac output, tissue receptor sites, plasma protein binding, enzyme systems, hepatic and renal clearance and drug distribution in the different species. Even though this study focused on the elephant, the findings are believed to be applicable for the rhinoceros who represents another very large herbivore and indicate that the treatment of megaherbivores according to drug properties in horses is not appropriate.

2.3 Principles in the treatment of infected wounds

Besides the estimation of the correct dose, it is of prime importance to select a drug, which meets the requirements for the field of application. The surviving poaching victims are often suffering from life-threatening injuries such as gunshot and big facial wounds. Apart from reducing pain and inflammation, infections need to be prevented and existing infections require adequate antimicrobial treatment. The aim of selecting an appropriate antimicrobial drug is to support the host's immune system so that the invading pathogen can be contained and killed. Therefore, it is important that the therapeutic drug concentration is rapidly reached at the site of infection and is kept high for an adequate period of time in order to reduce or inhibit the ability of the microorganisms to replicate and to further impede the formation of toxic products by the host and the microorganism (Giguère, 2013).

2.3.1 Pharmacokinetic features

Current understanding of the antimicrobial drugs indicates that the pharmacokinetic properties play a major role in the choice of appropriate treatment. Successful therapy of bacterial infections depends on an effective drug concentration, which rapidly reaches the site of infection and remains on a therapeutic level for a sufficient period of time. Four major processes; absorption, distribution, metabolism and elimination determine the time course of the drug in the body and can be quantified by the pharmacokinetic parameters (Riviere & Papich, 2009).

Absorption has been defined as “the movement of the drug from the site of administration into the blood” (Riviere & Papich, 2009) and includes the gastrointestinal, dermal, respiratory, intravenous, intramuscular, subcutaneous, intraperitoneal, topical, intra-articular, intramammary and subconjunctival absorption. The most important site for drug absorption after oral administration, particularly in monogastric species, is the small intestines

due to a more alkaline pH and a highly absorptive intestinal epithelium. Additionally, the blood flow to the small intestines is higher than that to the stomach. A considerable part of the intestinal microbial flora is able to metabolise specific drugs which, along with the presystemic drug metabolism occurring mainly in the liver, leads to the so called "first pass effect". The application of the drug with food can, depending on the properties of the drug and the species, also reduce the absorption considerably (Riviere & Papich, 2009).

The distribution of the drug plays an important role in the antimicrobial reaching the site of action at a sufficiently high concentration. The four major factors characterising the distribution are:

1. the physicochemical properties of the drug such the pKa value, the lipid solubility and the molecular weight
2. the difference in drug concentration between the blood and the tissue
3. the ratio of blood flow to tissue mass
4. the affinity of the drug for tissue components

Other factors influencing distribution are the route of administration, the rate of metabolism and the rate of excretion. Additionally, high plasma protein binding, mostly to albumin, leads to reduced distribution. Furthermore, the distribution to certain tissues is inhibited by specific barriers such as the blood brain barrier or the placental barrier (Riviere & Papich, 2009).

The metabolism of a drug can alter pharmacokinetic and pharmacodynamic parameters. It can result in pharmacological activation or deactivation, in alteration of disposition kinetics of drug absorption, distribution and excretion. Metabolism can be divided into phase I and II reactions with phase I primarily responsible for drug inactivation.

Lastly, the two main routes for elimination are through renal excretion (most ß-lactam antibiotics and aminoglycosides) and hepatic metabolism followed by renal and biliary excretion (Riviere & Papich, 2009).

The pivotal pharmacokinetic parameter include the half-life ($t_{1/2}$), clearance (Cl), maximum concentration (C_{max}), time to maximum concentration (T_{max}), the volume of distribution (V_d) and the area under the curve (AUC). The total serum concentration (and thus extent of exposure) is determined through the total area under the curve through the application of the linear trapezoidal rule. The maximum concentration is determined from the plasma concentration-time curve, while the time to maximum concentration is the time to reach the

maximal serum concentration. The clearance and the volume of distribution characterise the drug disposition. The clearance is defined by the volume of blood cleared of the drug per unit of time and indicates the ability of the body to eliminate the drug. The systemic body clearance is a sum of the clearance of the different organs, which contributes to the elimination of the drug. The volume of distribution expresses the relation between the administered drug dose and the plasma concentration of the drug, thereby indicating the extent of distribution of a drug in the body. The half-life is defined as the time for the drug concentration to decrease by 50%. Furthermore, the bioavailability is an important pharmacokinetic parameter, which describes the rate and the degree of the drug reaching the systemic circulation unaltered e.g. after oral or non-vascular parenteral administration. It is characterised by the absorption, the drug formulation, the dosage and the route of administration. Only intravascular administration leads to total systemic availability (Riviere & Papich, 2009).

2.3.2 Pharmacodynamic features and the minimum inhibitory concentration (MIC)

Pharmacodynamics are defined as "the science of drug action on the body or on microorganisms and other parasites within or on the body" (Lees et al., 2004a). The evaluation of the pharmacodynamics of a drug is important in order to be able to compare and classify drugs on the basis of their affinity, efficacy, potency and sensitivity (Lees et al., 2004a).

The most commonly used parameter to determine and compare the efficacy and potency of antimicrobials against pathogens is the minimum inhibitory concentration (MIC), which is defined as the minimum concentration of the antimicrobial drug needed to visibly inhibit the growth of microorganisms after an overnight incubation. It is assessed under standard culture conditions and represents the 'gold standard' for the assessment of the susceptibility of pathogens to antimicrobial drugs (Andrews, 2001). It is routinely used by diagnostic laboratories when testing for resistance and is also widely applied in research for the evaluation of the in vitro activity of new antimicrobial drugs and for the establishment of breakpoint values (Andrews, 2001).

However, it is important to recognise that the MIC values on their own represent an inadequate indicator of the efficacy of the drug in vivo. Immunocompetence, primary diseases, the location of the infection as well as pharmacokinetics, pharmacodynamics and the consideration of potential adverse effects play an important role in order to determine the success of the antibacterial treatment and to select the right drug (Estes, 1998).

2.4 Bacterial profile of wounds in rhinos

Very limited published information is available on the infectious agents present in rhino following poaching incidents. However, since the gun shot sites and horn sites are external, we would expect the infectious agents of these wounds to mimic other skin wounds and bone infections. Information from a local laboratory indicate that *Streptococcus agalactiae* (a beta-hemolytic Group B streptococcus) was the most commonly isolated wound contaminant (M. Henton, personal communication, 2015). This is similar to a study by Flach et al. (2003), who identified a streptococcus, albeit *S. equisimilis* in a greater one-horned rhinoceros suffering from osteomyelitis of the middle digit of the forefoot. Furthermore, a bacterial survey of black rhinoceros carried out by Clausen and Ashford (1980) isolated most often ß-haemolytic *Streptococcus* group L. It was found in skin lesions and wounds, even leading to septicaemia and death of two animals. Three rhinoceros were diagnosed with *Staphylococcus aureus* infection resulting in the death of one of the rhinos, while 16 other bacteria species were identified, including *Klebsiella* spp, *Salmonella* spp, *E. coli, Bacillus* spp, different streptococci and *Pseudomonas*.

Group L *Streptococcus*, isolated from healthy-appearing rhinos after chemical immobilisation, appeared to be normal residents. However, the fact that they were often found in wounds caused by filaria nematodes and other injuries demonstrates that they can exhibit pathogenic properties leading to septicaemia and death in several cases when the skin is damaged and is providing little resistance (Clausen & Ashford, 1980). A survey in Hluhluwe National Park assessed the skin lesions of black rhinoceros and reported the primary cause of the damage to be due to *Stephanofilaria dinniki*. Secondary infections of those wounds were a result of haemolytic and non-haemolytic streptococci, staphylococci and small Gram-negative bacilli (Hitchins & Keep, 1970).

In order to obtain further information on the type of infections in rhinos, the database of the bacteriology laboratory at the Department of Tropical Diseases, University of Pretoria was mined (unpublished data). From 2008 until 2014 a total of 33 cases (excluding faecal sample analysis) were submitted for culture at the laboratory. Of these, 15 samples were also evaluated by antimicrobial sensitivity testing. The samples were very diverse and were obtained either from patients of (private) veterinarians or from the pathology section of the Faculty of Veterinary Science, University of Pretoria following post mortem evaluations. Bacterial isolates for the antimicrobial susceptibility testing were cultured from synovial fluid (2), exudate (2), tissue/organ samples (4), wound swabs (2), a bone fragment and a left and right

ventral swab (Table 2-1, Table 2-2). The bacteria isolated and cultured at the laboratory without evaluation by antimicrobial sensitivity testing are listed in Table 2-3.

Table 2-1: The culture and sensitivity results from 15 samples obtained from rhinos through routine diagnostic work by the bacteriology laboratory of the Department of Tropical Diseases, University of Pretoria

Organism	Susceptible	Intermediate	Resistant
Staphylo-coccus aureus	amikacin, ceftiofur, doxycycline/oxy-tetrazycline, enrofloxacin, gentamicin, sulfamethoxazole/ trimethoprim, chloramphenicol	amoxicillin/ampicillin, penicillin G	X
Staphylo-coccus aureus **isolate 2**	amoxicillin/ ampicillin, ceftiofur, florfenicol, kanamycin, doxycycline/oxy-tetrazycline, sulfamethoxazole/ trimethoprim, tilmicosin	enrofloxacin	penicillin G
Staphylo-coccus **spp**	amikacin, ampicillin, ceftiofur, chloramphenicol, doxycycline, enrofloxacin, gentamicin, penicillin, sulfamethoxazole/ trimethoprim	X	X
Coryne-bacterium **spp isolate 1 (commensals)**	florfenicol, doxycycline/oxy-tetracycline, sulfamethoxazole/ trimethoprim	amoxycillin/ampicillin, kanamycin, penicillin G, tilmicosin	ceftiofur, enrofloxacin
Coryne-bacterium **spp isolate 2 (commensals)**	enrofloxacin, florfenicol, doxycycline/oxy-tetracycline	ceftiofur, kanamycin, penicillin G, tilmicosin	amoxycillin/ampicillin, sulfamethoxazole/ trimethoprim

Organism	Susceptible	Intermediate	Resistant
Salmonella enteridis	amoxicillin, ceftiofur, enrofloxacin, florfenicol, kanamycin, oxytetracycline/ chlortetracycline, sulfamethoxazole/ trimethoprim	penicillin G	tilmicosin
E.coli	enrofloxacin, polymixin B, tulathromycin, imipenem	X	amikacin, amoxicillin/ampicillin, ceftiofur, doxycycline/oxy-tetracycline, gentamycin, penicillin G, sulfamethoxazole/ trimethoprim, cephalexin, kanamycin, lincomycin, lincospectin, orbifloxacin, amoxycillin/clavulanate, tylosin, florfenicol, tilmicosin, metronidazole, carbenicillin, ceftazidime, piperacillin, tobramycin, vancomycin
Proteus vulgaris	amikacin, ceftiofur, synulox	X	amoxicillin/ampicillin, doxycycline/oxy-tetracycline, enrofloxacin, gentamycin, penicillin G, sulfamethoxazole/ trimethoprim, cephalexin, kanamycin, lincomycin, lincospectin, orbifloxacin, tylosin,

Organism	**Susceptible**	**Intermediate**	**Resistant**
			polymixin B, florfenicol, tilmicosin, tulathromycin, metronidazole, carbenicillin, ceftazidime, piperacillin, tobramycin, vancomycin
Enterobacter cloacae	ceftiofur, enrofloxacin, florofenicol, kanamycin, oxytetracycline/ chlortetracycline	amoxicillin	penicillin G, sulfamethoxazole/ trimethoprim, tilmicosin
Streptococcus canis	ceftiofur, doxycycline/oxy-tetracycline, enrofloxacin, sulpha/ trimethoprim, cephalexin/ cephalothin, lincospectin, amoxycillin/ clavulanate, tylosin, florfenicol, chloramphenicol, tilmicosin	amoxicillin/ampicillin, penicillin G, lincomycin, orbifloxacin	amikacin, gentamycin, kanamycin, tulathromycin, polymixin B, metronidazole
Streptococcus pyogenes	ampicillin, ceftiofur, chloramphenicol, doxycycline, enrofloxacin, penicillin, sulfamethoxazole/tr imethoprim	gentamycin	amikacin, tulathromycin
***Moraxella* spp**	amikacin, ampicillin, ceftiofur, chloramphenicol, doxycycline,	penicillin G	X

Organism	**Susceptible**	**Intermediate**	**Resistant**
	enrofloxacin, gentamicin, sulfamethoxazole/ trimethoprim, tulathromycin		
Mixed culture of pyogenic *Streptococcus* Group G	amoxycillin/ampicillin, florfenicol, sulfamethoxazole/ trimethoprim, tilmicosin	ceftiofur, enrofloxacin, doxycycline/oxy-tetracycline, penicillin G	kanamycin
Pseudomonas aeruginosa	polymixin B	imipenem	amikacin, ceftiofur, amoxycillin/ampicillin, doxycycline/oxy-tetracycline, enrofloxacin, gentamicin, penicillin G, sulfamethoxazole/ trimethoprim, cephalexin, kanamycin, lincomycin, orbifloxacin, amoxycillin/clavulanate, florfenicol, tilmicosin, tulathromycin, metronidazole, carbenicillin, ceftazidime, tylosin, piperacillin, tobramycin, vancomycin
Arcanobacterium pyogenes	ampicillin, ceftiofur, gentamicin, penicillin, sulfamethoxazole/ trimethoprim	doxycycline	amikacin, enrofloxacin

Table 2-2: Bacterial isolates from rhino samples without availability of sensitivity results

Bacterial isolate	**Specimen**
***Enterococcus* spp (4)**	small intestine, fresh tissue (2), organ pool
***Citrobacter* spp**	colon
***Corynebacterium* spp (3)**	lung, swab/culturette, fresh tissue
***E.coli* (rough) (7)**	muscle, liver swab, peritoneal fluid (2), liver, spleen, duodenum, gastric and duodenal content, organ pool
***Bacillus* spp**	peritoneal fluid
***Staphylococcus* spp (3)**	lung (2), skin, skin swabs, liver abscess and swab
Staphyloccocus intermedius	fresh tissue
***Streptococcus bovis* (2)**	liver, fresh tissue
***Streptococcus pyogenes* (2)**	liver, spleen, kidney, heart blood, lung, skin, skin swabs
***Streptococcus canis* (4)**	liver abscess, liver swab, fresh tissue, lung, spinal cord, peritoneal fluid, tonsil
***Steptococcus* spp (5)**	liver, liver abscess, lung, skin biopsy, swab/culturette
Streptococcus agalactiae	peritoneal fluid
Streptococcus dysgalactiae	liver, spleen
Streptococcus equines	lung
***Lactobacillus* spp (3)**	mesenteric lymphnode, liver abscess and swab, organ pool
***Salmonella enteridis* (2)**	organs, duodenum
***Salmonella* spp**	intestines, lung, liver, spleen
***Clostridium perfringens* (3)**	duodenum, heart, liver, organ pool
***Clostridium septicum* (2)**	spleen (2), muscle, liver, peritoneal fluid
Clostridium sordelli	duodenum, intestines
Clostridium bifermetans	intestine
***Clostridium* spp**	spleen, muscle, organ pool

***Pasteurella* spp**	liver abscess and swab
***Micrococcus* spp (3)**	lung, skin biopsy,
***Acinetobacter* spp (2)**	skin biopsy, swab/culturette
Vibrio parahaemolyticus	liver, spleen
Morganella morgana	duodenum, gastric and duodenal content

2.4.1 Selecting an antimicrobial

The sensitivity results from the bacterial samples submitted to the university laboratory for several antibiotics were considered along with pharmacokinetic and pharmacodynamic properties of the drugs. As a result, two antimicrobials, namely ceftiofur and enrofloxacin were further considered as potential candidates. The culture results obtained from sensitivity tests for enrofloxacin and ceftiofur of all susceptibility tests performed are listed in Table 2-3 and are summarized in Table 2-4**.**

Table 2-3: A direct comparison of the culture and sensitivity results against enrofloxacin and ceftiofur from the same 15 samples listed in Table 2-1, obtained from rhinos through routine diagnostic work by the bacteriology laboratory of the Department of Tropical Diseases, University of Pretoria

Bacterial isolate	**Source**	**Sensitivity Results**	
		Enrofloxacin	**Ceftiofur**
Staphylococcus aureus	joint fluid	sensitive	sensitive
***Staphylococcus aureus* isolate 2**	joint fluid	intermediate	sensitive
***Staphylococcus* spp**	fresh tissue	sensitive	sensitive
***Corynebacterium* spp isolate 1 (commensals)**	nasal exudates	resistant	resistant
***Corynebacterium* spp isolate 2 (commensals)**	nasal exudates	sensitive	intermediate
Salmonella enteridis	liver, spleen, kidney, small intestine	sensitive	sensitive
E.coli	bone fragment and wound swab	sensitive	resistant

Proteus vulgaris	bone fragment and wound swab	resistant	sensitive
Enterobacter cloacae	tissue sample	sensitive	sensitive
Streptococcus canis	left and right ventral swab	sensitive	sensitive
Streptococcus pyogenes	fresh tissue	sensitive	sensitive
***Moraxella* spp**	fresh tissue	sensitive	sensitive
Mixed culture of pyogenic *Steptococcus* Group G	nasal exudates	intermediate	intermediate
Pseudomonas aeruginosa	bone fragment and wound swab	resistant	resistant
Arcanobacterium pyogenes	exsudate	resistant	sensitive

Table 2-4: Summarized sensitivity results for enrofloxacin and ceftiofur

Sensitivity	Enrofloxacin	Ceftiofur
Sensitive	60% (9 of 15)	67% (10 of 15)
Intermediate	13% (2 of 15)	13% (2 of 15)
Resistant	27% (4 of 15)	20% (3 of 15)

Of the two drugs evaluated, we selected enrofloxacin for further study. We decided against the use of ceftiofur as it is firstly a time-dependent drug (Riviere & Papich, 2009), i.e. the serum concentration must stay above the MIC for a certain period of time to be effective. This implicates that the drug needs to be administered frequently in order to achieve its therapeutic effect, which is not applicable in wild animal species. Another important feature of concern is the oral bioavailability. While no information is available for the rhino, cephalosporins such as cephalexin are only 5% absorbed in horses and have to be administered at high concentrations of 30 mg/kg every 8 hours in order to maintain a therapeutic level (Davis et al., 2005). In contrast, the concentration-dependent drug enrofloxacin in horses exhibits an oral bioavailability of 63% (Giguère et al., 1996) and therefore can be given orally or parenterally at a lower dose of 5 mg/kg once daily (Lees et al.,1994; McKellar et al., 1991; Papich et al., 2002).

2.5 Enrofloxacin

Enrofloxacin (1-Cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid) (Figure 2-4), a second generation fluoroquinolone was the first fluoroquinolone developed for veterinary purposes in the late 1980s. It is a bactericidal, concentration dependent broad spectrum antimicrobial, particularly effective against Gram-negative bacteria but also against Gram–positive bacteria and intracellular organisms. It reaches therapeutic drug concentrations in most tissues of the body and can be employed for the treatment of osteomyelitis, bone, sinus and many soft tissue infections (Lopez-Cadenas et al., 2013; Martinez et al., 2006), which makes it a potentially attractive drug for wound management in rhinoceros.

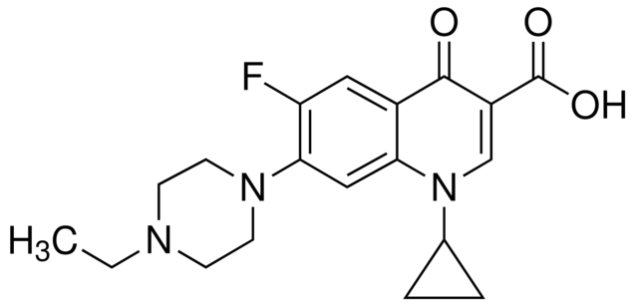


Figure 2-4: Molecular structure of enrofloxacin

2.5.1 Antibacterial spectrum

The antibacterial spectrum of enrofloxacin and fluoroquinolones in general covers most Gram-negative bacteria, especially *Enterobacteriaceae* such as *Escheria coli, Klebsiella* spp., *Proteus* spp, *Salmonella* spp and *Enterobacter* spp. Fluoroquinolones are generally effective against *Brucella* spp, *Chlamydia trachomatis*, *Staphylococcus* spp, *Mycoplasma* spp and *Mycobacterium* spp. However, staphylococci tend to have higher MIC levels than gram-negative bacteria. Fluoroquinolones may not be suitable for treating infections caused by obligate anaerobes or certain streptococcal and enterococcal infections and the susceptibility for *Pseudomonas aeruginosa* is variable (Brown, 1996; Lopez-Cadenas et al., 2013).

2.5.2 Mechanism of action

The bactericidal properties arise from the inhibition of the microbial DNA-gyrase and topoisomerase IV. By blocking the DNA-gyrase, the initiation of the DNA replication is disabled, the process of supercoiling is impaired and further DNA damage occurs. In conjunction, this results in the death of bacteria in both the growth and cell division phases, making the quinolones bactericidal. DNA-gyrase seems to be the target in Gram-negative

bacteria whereas in Gram-positive bacteria, enrofloxacin and other fluoroquinolones target the topoisomerase IV (Lopez-Cadenas et al., 2013).

Fluoroquinolones are concentration-dependent drugs and feature a post-antibiotic effect (PAE) due to the inhibition of protein or nucleic acid synthesis. *Saunders Comprehensive Veterinary Dictionary* (Blood et al., 2007) defines the PAE as the 'continued suppression of antibacterial growth after the administration of antibiotic has ceased and serum concentrations have fallen below the MIC'. The duration of the PAE depends on the drug–microorganism combination, the drug concentration, environmental factors and the duration of exposure of the microorganism and the drug (Craig, 1998; Spivey, 1992).

2.5.3 Resistance

The predominant mechanism of resistance is the mutation of genes encoding for the DNA-gyrase (topoisomerase II) and topoisomerase IV. Other mutations can target genes regulating the drug uptake into the bacterium or the efflux pumps leading to an increased excretion of the drug (Riviere & Papich, 2009).

Concerns have been expressed about causing resistance in veterinary medicine by the frequent use and misuse of fluoroquinolones in animals and thus, reducing the susceptibility of the bacteria and the efficacy of the fluoroquinolones for the treatment in human patients. While the decrease in susceptibility of bacteria did not have an instant clinical effect on many infections, the recent development of highly resistant strains requires attention and precautions. The use of fluoroquinolones needs to be restricted to sensible use with clear clinical indication (Piddock, 1998).

According to the Clinical and Laboratory Standards Institute (CLSI, 2015), the breakpoint for bacteria susceptible to enrofloxacin is ≤ 0.5 µg⁄mL. The intermediate breakpoint is 1–2 µg⁄mL. However, one must bear in mind that those breakpoints were established for small animals and are not necessarily applicable for all species. Bacteria with a MIC > 4 µg⁄mL are generally regarded as resistant to enrofloxacin (Boothe, 1994). Table 2-5 shows the current level of resistance to enrofloxacin with pseudomonas and enterococcus showing the highest degree of resistance.

Table 2-5: MIC of enrofloxacin for the different bacteria found in the literature

Bacteria spp	**MIC (µg/ml)**	**Source**
Streptococcus zooepidemicus	MIC_{90} = 1	Prescott & Yielding, 1990
Pseudomonas aeruginosa	MIC = 0.5–1 (0.031 - 0.063 mg/ml for its active metabolite ciprofloxacin)	Blondeau et al., 2012
Pseudomonas aeruginosa **(canine isolates)**	MIC_{90} = 8	Boothe et al., 2006
Pseudomonas aeruginosa	MIC_{90} = 1-8	Giguère & Dowling, 2013
Staphylococcus aureus **DSM 11823**	MIC_{90} = 0.125 – 0.25	Wetzstein, 2005
Enterococcus **spp**	MIC_{90} = 1-2	Giguère & Dowling, 2013
E.coli	MIC ≤ 0.016	Blondeau et al., 2012
E.coli **ATCC 8739**	MIC_{90} = 0.03 – 0.06	Wetzstein, 2005
E.coli **ATCC 2592**	MIC_{90} = 0.015 – 0.03	Wetzstein, 2005
E.coli **DSM 10650**	$MIC_{90} \leq 0.008$	Wetzstein, 2005
E.coli	MIC_{90} = 0.03 – 0.125	Giguère & Dowling, 2013
Streptococcus **spp**	MIC_{mean} = 0.2 MIC_{50} = 0.25 MIC_{90} = 1	Boothe et al., 2006

2.5.4 Pharmacokinetics of enrofloxacin

Despite the lack of pharmacokinetic studies in wildlife, particularly in rhino, enrofloxacin pharmacokinetic studies have been conducted in many domestic animal species such as cattle (Bregante et al., 1999), horses (Lees et al., 1994; McKellar et al., 1991; Papich et al., 2002), dogs (Küng et al., 1993; McKellar et al., 1990; 1994), cats (Seguin et al., 2004), chicken (Bugyei et al., 1999), sheep, rabbits, rats, mice (Bregante et al., 1999), pigs (Nielsen & GyrdHansen, 1997) and donkeys (Sekkin et al., 2012). Enrofloxacin can be administered orally, intramuscularly, subcutaneously and intravenously, depending on the species. Its general characteristics include a good absorption after oral as well as parenteral administration, low protein binding and a high volume of distribution indicating good tissue penetration abilities. The half-life is relatively long and varies between 2.8 hours in cattle, 2.4 hours in the dog, 10.96

hours in chicken and 6.7 hours in the horse after intravenous administration. The bioavailability of enrofloxacin after oral administration in the dog reaches close to 100% (Küng et al., 1993). In adult horses, enrofloxacin absorption after oral administration is about 63% (Giguère et al., 1996). Food has been reported to interfere with the rate of absorption, leading to a prolonged time to peak concentration without changing the overall absorption and the area under the curve (Giguère & Dowling, 2013). The excellent tissue penetration abilities lead to tissue concentrations which are at least as high as the plasma concentrations (Turnidge, 1999) and have led to detectable enrofloxacin concentrations in cortical bones of dogs (Duval & Budsberg, 1995), skin (DeManuelle et al., 1998) and the prostate (Dorfman et al., 1995) of dogs after parenteral administration.

Enrofloxacin is partially biotransformed into its active main metabolite ciprofloxacin through deethylation. The degree of transformation across species varies and exhibits large interspecies differences. In adult horses, ciprofloxacin serum concentration reaches about 20 to 35% of the enrofloxacin serum concentration (Lopez-Cadenas et al., 2013). In sheep (Otero et al., 2009), goats (Rao et al., 2002) and cattle (Kaartinen et al., 1995), ciprofloxacin serum concentration reaches 26%, 24% and 35% of the enrofloxacin concentration, respectively. In contrast, in pigs, ciprofloxacin formation reaches less than 10% of the parent drug concentration (Nielsen & GyrdHansen, 1997) and in elephants ciprofloxacin concentrations were measurable but very low and were not further quantified (Sanchez et al., 2005). In dogs, the portion of enrofloxacin metabolised to ciprofloxacin was about 40% (Cester & Toutain, 1997). The partial metabolism of enrofloxacin to ciprofloxacin leads to a simultaneous circulation of both antimicrobials and to an additive antimicrobial activity against certain bacteria such as *Pseudomonas aeruginosa* (Blondeau et al., 2012; Lautzenhiser et al., 2001). Finally, enrofloxacin is mainly eliminated though renal elimination while ciprofloxacin elimination involves hepatic and renal excretion (Martinez et al., 2006).

Table 2-6 summarizes the results from several pharmacokinetic studies carried out in the horse. Due to the phylogenic relationship between the horse and the rhino, the comparable anatomy and physiology of the gastrointestinal tract (monogastric) and the similar diet (herbivore, grazer) (Clauss et al., 2005; Kiefer, 2002; Oftedal et al., 1996; Price & Bininda-Emonds, 2009), the horse is regarded as the most suitable species to compare to the rhino and to derive drug related information from.

Table 2-6: Comparative pharmacokinetics results after intravenous, intramuscular and per os administration of enrofloxacin to horses. Results are presented as mean and standard deviation

Parameter	Kaartinen et al. (1997) 5mg/kg IV	Papich et al. (2002) 5mg/kg IV	Boeckh et al. (2001) 7.5 mg/kg IV	Kaartinen et al. (1997) 5mg/kg IM	Boeckh et al. (2001) 7.5 mg/kg PO
AUClast (µg*h/ml)	13.2 ± 3.1	25.28 ± 8.98	14.37± 3.58	25.2 ± 2.5	9.78 ± 4.03
$t_{1/2}$ (h)	4.4 ± 0.6	6.7 ± 2.9	5.92 ± 4.04	9.9 ± 0.5	10.7 ± 3.3
MRT (h)	5.1 ± 0.7	8.43 ±4.13	3.67± 0.96	15 ± 0.8	3.83 ± 1.38
V_{ss} (L/kg)	2.3 ± 0.5	1.66 ± 0.45			
V_z (L/kg)			4.2 ± 2.17		
Cl (L/h*kg)	0.51 ± 0.11	0.22 ± 0.084	0.56 ± 0.19		
C_{max} (µg/ml)				1.28 ± 0.07	2.22 ± 0.71
T_{max} (h)				2.67 ± 0.42	0.9 ± 0.31
MAT (h)				9.9 ± 1	

AUC_{last}, area under the curve until the last time point; $t_{1/2}$, half-life; MRT, mean residence time; Vss, apparent volume of distribution in steady state; Vz, apparent volume of distribution during the terminal phase; Cl, clearance; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; MAT, mean absorption time

2.5.5 Pharmacodynamics of enrofloxacin

Pharmacodynamics, which is defined as the study of drug action on the body, microorganism or parasite in or on the body (Lees et al., 2004a), depict the relation between plasma drug concentrations and the pharmacological and toxicological properties of the drug. With special regard to antimicrobials, the relation between plasma drug concentration and the antimicrobial drug effect is of major importance (Craig, 1998). The MIC represents the most important parameter for the quantification of the antimicrobial effect and for the assessment of the potency of a drug on the particular pathogen. However, in order to be able to evaluate the time-dependent course and the efficacy of an antimicrobial, the combined assessment of the MIC and pharmacokinetic parameters is of prime importance. Along with the MIC, the three most relevant pharmacokinetic parameters for determining pharmacokinetic/pharmacodynamic interrelationships are the AUC_{24}, C_{max} and the time above the MIC (Hyatt et al., 1995). Efficacy marker such as AUC_{24}:MIC and C_{max}: MIC and T > MIC have been identified for the evaluation of the treatment outcome. In concentration dependent drugs such as fluoroquinolones, the aim is to maximise the concentration of the antimicrobial as opposed to the duration above the MIC.

Consequently, the AUC_{24}: MIC and C_{max}: MIC ratios are best correlated with the success of an antimicrobial treatment (Hyatt et al., 1995). For the interpretation of the efficacy marker, three AUC_{24}: MIC breakpoint values have been determined. A value below 100 leads to a bacteriostatic effect and can only result in bacterial killing if the host immune system is capable of fighting the infection (Schentag et al., 2003; Thomas et al., 1998). Due to the risk of selecting for resistant bacteria, the use of fluoroquinolone doses resulting in a low ratio below 100 have been controversially discussed and should be avoided (Thomas et al., 1998). A breakpoint value of 100 – 250 leads to a bactericidal effect at a slow rate, after approximately seven days of treatment (Forrest et al., 1993; Schentag et al., 2003). It is recommended to reach a ratio of at least 125 in order to ensure clinical and microbiological cure as well as for a short time to microbial eradication and thus a successful treatment outcome (Forrest et al., 1993). At AUC_{24}: MIC ratios below 125, Forrest et al. (1993) reported a probability of clinical and microbiological cure of 42% and 26%, respectively. In contrast, ratios above a value of 125 led to a clinical and microbiological cure of 80 and 82%, respectively. Ratios above the third breakpoint of 250 are preferred and have shown to lead to a rapid bactericidal effect and to bacterial eradication within 24 hours (Schentag et al., 2003). With regard to the C_{max}: MIC ratio, a value greater than 8:1 and ideally a ratio greater than 12:1 is required in order to reduce the bacterial numbers by 99%, to inhibit the bacterial regrowth after 24 hours and to reduce the risk of resistance (Blaser et al., 1987).

2.5.6 Clinical use of enrofloxacin in horses, other rhino species and Asian elephants

Enrofloxacin dosage requirements have been established for a number of domestic species such as dogs, cats, cattle, small ruminants, horses and a number of exotic species (Riviere & Papich, 2009). With the rhinoceros believed to be similar to the horse, it has been assumed that the equine information may be the most applicable to the rhinoceros. From equine literature, enrofloxacin is not recommended for the treatment of horses due to concerns about causing quinolone induced arthropathy in young horses (Langston et al., 1996). However, enrofloxacin studies in adult horses have not revealed any arthrotoxicity and were considered safe at a parenteral dose of 5 mg/kg over a period of 3 weeks (Bertone et al., 2000) and at an oral dose of one and three times the recommended 4 mg/kg every 12 hours (Giguère et al., 1999). As a result, it is commonly used as an extra label medication. Several pharmacokinetic studies and clinical experience have since highlighted the benefits of the treatment in horses, viz. the various options of administration (*per os* (PO), intramuscular (IM) and intravenous (IV)), its broad spectrum of activity against susceptible microorganisms and the safety of the drug. In terms of side effects, dosing according to recommended guidelines does not cause any

gastro-intestinal disturbance as the spectrum of activity excludes anaerobic bacteria. The drug may not be suitable for IM administration, since a study conducted by Kaartinen et al. (1997) showed that IM injection of enrofloxacin in horses caused irritation and tissue damage. Clinical reactions were observed in two out of six horses and the serum creatine kinase (CK) values were ten times higher compared to the pre-injection values.

Considering the value of enrofloxacin in veterinary medicine, it is not surprising that it has been used in white rhinoceros on a case to case basis by veterinarians to treat pneumonia and other bacterial infections (M. Böer, personal communication 2014). In a case study, Flach et al. (2003) used, amongst others, enrofloxacin for the treatment of a greater one-horned rhinoceros (*Rhinoceros unicornis*) with osteomyelitis. Nonetheless, no supporting information is available for the optimal dosage, the enrofloxacin plasma concentrations or pharmacokinetic values in white rhinoceros.

While limited information on drugs is available for rhinoceros, several pharmacokinetic studies on different drugs have been published for the elephant (Adkesson et al., 2012; Bechert & Christensen, 2007; Bechert et al., 2008; Bush et al., 1996; Bush et al., 2000; Dumonceaux et al., 2005; Hunter et al., 2003; Lodwick et al., 1994; Page et al., 1991; Rosin et al., 1993; Sanchez et al,. 2005; Schmidt, 1978). As a large, wild, non-domesticated species, elephants provide similar challenges concerning the right dosage of drugs, the large volume of medication required and the problem of treatment frequency. Additionally, the elephant as well as rhinoceros and the horse, are hindgut fermenter with a similar digestive physiology (Clauss et al., 2005; Kiefer, 2002; Oftedal et al., 1996). Therefore, the studies conducted on elephants deliver valuable information on possible relevant drugs for the use in wild, large species such as the rhinoceros.

In a case of footrot in one Asian elephant, the animal was treated with a parenteral injection of 2.5 mg/kg enrofloxacin twice a day for 10 days in addition to topical preparations, daily debridement and foot soaks. The report indicates an improvement in walking and exercising from the 7th day (Shafiuzama et al., 2012). The successful treatment of a chronic abscess in an Asian elephant included the IM injection of 2.5 mg enrofloxacin/kg body weight for 15 days. The improvement was also noticeable after 7 days of treatment (Senthilkumar et al., 2014). However, while the animals' health improved, no studies on the efficacy and pharmacokinetics of enrofloxacin were performed.

Some pharmacokinetic information is however available as Sanchez et al. (2005) studied the pharmacokinetics of a single oral dose of 2.5 mg enrofloxacin/kg in six Asian

elephants. The aim was to assess the potential value of enrofloxacin for the oral administration at a 24-hour interval. The recorded half-life of 18.4 hours in the elephant was much longer than that in horses, despite a three times higher dose used for the horse. Sanchez et al. (2005) concluded that the oral treatment with enrofloxacin at a dose of 2.5 mg/kg once a day could be efficient for the treatment of bacterial infections against susceptible bacteria with a $MIC_{90} \leq$ 0.13µg/ml.

2.6 Principles of pain management

The International Association for the Study of Pain (IASP) defines pain as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage". Already in 1931, Dr. Albert Schweitzer stated "pain is a more terrible lord of mankind than even death itself" (Brabazon, 2000). Despite this knowledge, it is only in the past few decades that pain, suffering and pain treatment has been recognised in animals. Only several years ago, society and professionals still believed that animals did not feel pain in the same manner as people, due to their lower sentience. Nowadays it is generally accepted that pain and suffering in all animals has to be alleviated. It is a pivotal point in veterinary medicine, as pain causes physical and psychological harm and inhibits normal behaviour, delays healing, suppresses the immune system and increases blood viscosity (Gaynor & Muir, 2015).

Drugs for pain treatment can be categorised in five main classes, namely opioids, non-steroidal anti-inflammatory drugs (NSAIDs), α_2-agonists, local anaesthetics and adjunct/adjuvant analgesics (Gaynor & Muir, 2015):

- The opioids are a group of natural or synthetic drugs with morphine-like effects. They are seen as one of the most potent analgesic treatment option leading to species specific slight to moderate CNS effects such as sedation, respiratory and CNS depression. Despite their superior analgesic properties, the clinical use is limited and complicated as the opioids are strictly controlled drugs. Strict ordering, storage and record keeping regulations and the interdiction of the dispense of those drugs restricts their application (Gaynor & Muir, 2015). Other concerns relate to the ability of the highly potent analgesics to cause various degrees of sedation, which is generally not desirable when treating animals against pain. Specifically in wild rhino, when using drugs like etorphine for their immobilising effects, the subsequent reversal with naltrexone or similar also generally results in the reversal of the opioids analgesic effects.

- NSAIDs are producing analgesic, anti-inflammatory and antipyretic effects. They represent the most popular analgesic drugs for treatment of acute and chronic pain in several animal species. Due to synergistic effects with opioids, NSAIDs can also be applied to reduce the amount of opioids needed for analgesia, or may be used in addition to the effects of opioids. A major advantage of the NSAIDs is their availability for oral as well as parenteral treatment, and their relatively long duration of effect.
- α_2-agonists are characterised by moderate to extensive sedative effects, muscle relaxing and mild to moderate analgesic properties. Like the NSAIDs, they show synergistic effects when administered with opioids. However, their use as analgesics is limited to small medical or surgical procedures and pre-anaesthetic medication, often in combination with other analgesics. Administered in very small doses, they exhibit anxiolytic, sedative, opioid and anaesthetic sparing properties. As a result, the α_2-agonists on their own are not suitable as analgesics and more applicable during surgical procedures or intensive care management. Another major constraint in the use of the α_2-agonists is their short duration of action.
- Local anaesthetics block the initiation and transmission of electrical signals in sensory nerves resulting in a loss of sensation at the site of injection. Analgesia is produced locally by topical and local application to a particular site or by regional application onto nerves. However, there is no distinction between sensory nerves and motor nerves, which can lead to the loss of motor function and to motor paralysis.
- Adjunct and adjuvant analgesics are used to control acute and more often chronic pain. Adjunct drugs are generally employed to complement the impact of a primary drug. In contrast, adjuvant drugs alter the effect of the primary drug but often additionally possess other indications than pain (Gaynor & Muir, 2015).

2.6.1 Selection of a drug for the treatment of pain in rhinos

Amongst all analgesic drugs, NSAIDs appear to be the most suitable option for long-term in-field use in rhinoceros. They do not exhibit sedative effects and do not lead to behavioural changes; they are well recognised as potent analgesics, represent the most used drugs for pain treatment; and tend to have the longest duration of effect. In most cases, musculoskeletal and visceral pain can be treated equally well with either NSAIDs or opioids (Gaynor & Muir, 2015).

The NSAIDs commonly used in veterinary medicine include aspirin, ibuprofen, phenylbutazone, flunixin meglumine, ketoprofen, carprofen, etodolac, and meloxicam. The

classification of the NSAIDs can be done in three different ways. One method separates the drugs according to their chemical structure in two major groups – the carboxylic and the enolic acids. The first group can be further divided in the salicylic acids (aspirin, salicylate), acetic acids (etodolac, eltenac), propionic acids (ketoprofen, carprofen, naproxen) and fenamic acids (flunixin, meclofenamic acid). The enolic acids consists of the pyrazolones (phenylbutazone, dipyrone) and the oxicams (meloxicam, piroxicam). The second method classifies NSAIDs according to their mode of action in terms of cyclooxygenase (COX) inhibition. While drugs of the first class lead to simple, competitive and reversible COX enzyme inhibition, NSAIDs of the second class lead to a time-dependent, competitive and reversible inhibition. In contrast, class three NSAIDs result in time dependent, competitive but irreversible enzyme inhibition. Lastly, the NSAIDs can also be characterised according to their selectivity of COX-1 or COX-2 inhibition (Moses & Bertone, 2002).

2.6.2 Mode of action

Generally, NSAIDs are believed to function through the inhibition of COX-1 and 2, leading to a reduced production of eicosanoids such as prostaglandins, prostacyclins and thromboxanes (Moses & Bertone, 2002). The COX-1 is a constitutively expressed enzyme in the body tissues, which is also described as the housekeeping enzyme (Vane & Botting, 1995). It catalyses arachidonic acid into thromboxanes, prostacycline and prostaglandin. Even though its role in case of tissue injury and inflammation is less significant than that of COX-2, COX-1 expression increases by 2- to 3- fold after tissue injuries and plays a role in pain transmission (Brooks et al., 1999; Mathews, 2002).

COX-2 enzymes are inducible and upregulated during inflammatory processes and are produced by macrophages and other cells of the immune system in case of tissue damage and inflammation. Their concentrations can raise 20-fold above the baseline values (Brooks et al., 1999; Lee et al., 1992). The increase in enzyme concentrations leads to an increase in prostanoid synthesis and thus to the increase in nociception and the transmission in the peripheral as well as central nervous system. Particularly PGE2 and also PGI2 cause vasodilatation, increase the action of cytokines and inflammatory mediators and sensitise the nociceptors leading to inflammatory reactions, pain and hypersensitivity. However, besides their role in inflammatory processes, recent studies have shown that COX-2 enzymes also exhibit important constitutive functions such as the protection of the integrity of the gastro intestinal tract and the healing of gastric ulcer (Schmassmann et al., 1998). Furthermore, the inhibition of COX-2 has been shown to aggravate colitis in rats (Reuter et al., 1996), while constitutive functions of COX-2 have

been postulated in conjunction with uterine (St-Louis et al., 2010), ovarian, brain and nerve functions and bone metabolism (Mathews, 2002). Additionally, COX-2 enzymes play an important role in the development and function of the kidney maturation (Zhang et al., 1997).

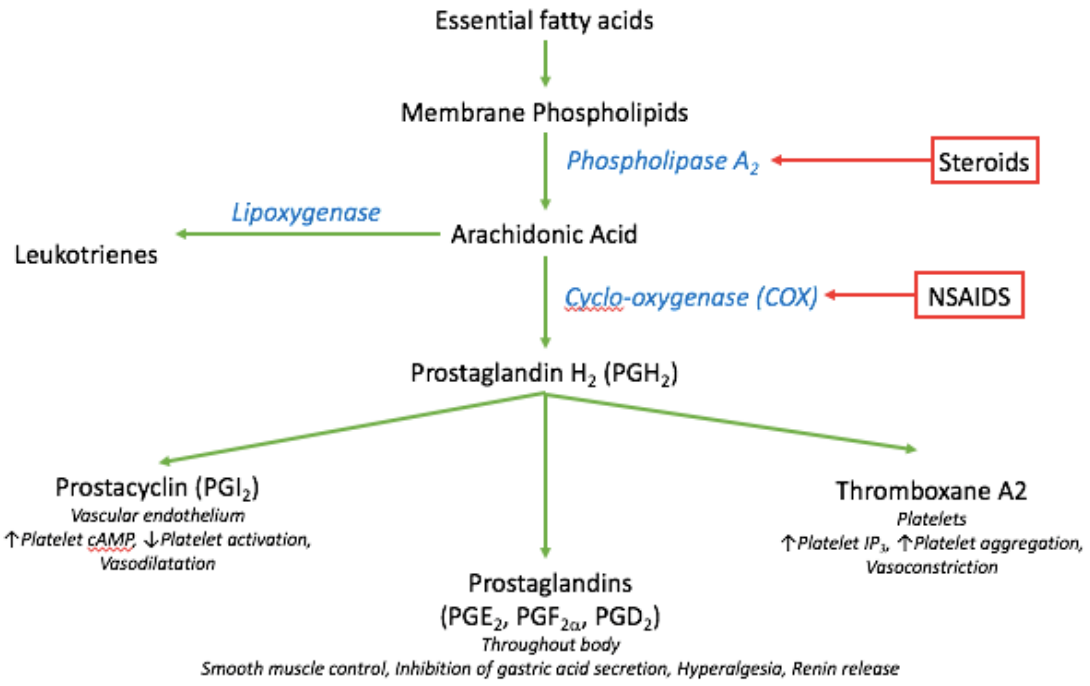


Figure 2-5: Prostaglandin synthesis (Hudson, 2017)

New research findings have unveiled another cyclooxygenase isoform, the COX-3, which has been mainly found in the brain of dogs (Chandrasekharan et al., 2002). It originates from the COX-1 gene but includes intron 1. Opinions on the clinical relevance of the COX-3 enzyme differ. While authors such as Botting and Ayoub (2005) postulate that the analgesic and hypothermic actions of paracetamol can be ascribed to the inhibition of COX-3 in the brain, Kis et al. (2005) states that the concentrations of COX-3 are too low to attribute clinical effects to its occurrence.

Although COX-inhibition plays a major role in the mode of action of NSAIDs, it cannot solely explain all effects. Additional, alternate, non-COX mediated effects have been described and include the inhibition of nitric-oxide, the inhibition of 5-lipoxygensase and also the inhibition of neutrophil aggregation.

1. Inhibition of nitric-oxide (NO)

Yoon et al. (2003) could show that NSAIDs block nitric oxide induced apoptosis and dedifferentiation of articular chondrocytes by altering the extracellular signal-regulated kinase (ERK), p38 kinase, protein kinase C alpha (PKCα) and protein kinase C zeta (PKCζ).

The effect of carprofen on the NO inhibition was also assessed by Armstrong et al. (1999), who demonstrated that R(-) carprofen inhibited NO production in chondrocytes to a small extent. In cartilage explants ex-vivo, rac-carprofen and the carprofen enantiomers inhibited NO synthesis significantly, however incompletely and not dependant on the drug concentration.

2. Inhibition of 5-lipoxygenase (5-LOX)

The 5-lipoxygenase is part of the lipid peroxidising enzymes and catalyses the first step of the transformation of arachidonic acid into leukotrienes. Leukotriene play a significant role in the inflammatory response as they can trigger the activation of leucocytes, function as chemoattractant and exhibit vasoconstrictive properties. Furthermore, they are able to alter the vascular permeability and enhance the contractibility of smooth muscle cells (Harizi et al., 2008; Holgate et al., 2003; Parameswaran et al., 2002; Parker, 1987). Along with the inhibition of the COX-1 and COX-2 enzymes, some NSAIDs are able to concurrently inhibit the lipoxygenase pathway and therewith improve overall anti-inflammatory properties and reduce undesired effects. The concurrent inhibition of both pathways prevents the increased leukotriene synthesis and its negative gastric effects, simultaneously with the inhibition of the gastro-protective prostaglandins caused by COX-1 and -2 inhibition (Leone et al., 2007). Lees et al. (2002) suggested that carprofen in the horse could be a weak 5-LOX inhibitor. However, the incomplete and dose independent inhibition leaves it clinical significance unconfirmed.

3. Inhibition of neutrophil aggregation

Another hypothesis has been constructed in order to explain some of the effects of NSAIDs, which cannot be fully ascribed to the COX induced inhibition of prostaglandins. Abramson and Weissmann (1984) postulated a direct effect of NSAIDs on neutrophils and posit that the NSAIDs can block the aggregation of neutrophils independently from the prostaglandin inhibition by integrating into the plasma membranes and by disrupting signal cascades and protein-protein interactions.

2.6.3 Pharmacologic effects

NSAIDs are used for their anti-inflammatory, analgesic and antipyretic properties in human medicine and, with the progressive awareness of the importance of pain management, in veterinary medicine. Besides their use for the alleviation of perioperative pain, NSAIDs are used against inflammatory conditions, fever, endotoxaemia and thrombosis (Blikslager &

Jones, 2005). The main fields of application in the horse are musculoskeletal disorders and abdominal pain. They are also used for musculoskeletal disorders in cattle and, in combination with antibiotics, in calves to treat acute respiratory diseases (Balmer et al., 1997; Moses & Bertone, 2002). In dogs, NSAIDs such as meloxicam and carprofen are routinely used for the symptomatic treatment of synovitis and osteoarthritis (Borer et al., 2003; Sessions et al., 2005). Due to their relatively long duration of effect and the lack of side effects on the central nervous system, they are often the drug of choice for analgesia alternatively to the use of opioids or for a prolonged pain relief after initial patient support with opioids.

Certain NSAIDs have also been found to exhibit anti-neoplastic properties in humans (Chan et al., 1998; Thun et al., 1991), laboratory animals (Reddy et al., 1992) and dogs (Knapp et al., 1994). Research in this field is ongoing, however, NSAIDs have shown promising antineoplastic characteristics for the application in human and veterinary medicine as a tumour prophylaxis, for the regression of existing tumours or as an accessory treatment in addition to the chemotherapy of colorectal tumours. The mechanisms of action have not yet fully been revealed, but strong evidence suggests that the anti-neoplastic effects could be the result of the inhibition of COX enzymes, leading to an accumulation of the substrate arachidonic acid. This in turn could lead to the activation of the enzyme sphingomyelinase which catalyses the synthesis of ceramide, the apoptosis inducer (Chan et al., 1998).

In case of fever, the raise in body temperature is caused predominantly by an increase in COX-synthesised prostaglandin E2, which results in an increase in the thermoregulatory set point for the body temperature in the preoptic anterior hypothalamus. The antipyretic effect of NSAIDs is mainly attributed to the inhibition of prostaglandin E2 synthesising COX-2 enzymes and therewith to the downregulation of the thermoregulatory set point to physiological, basal levels (Aronoff & Neilson 2001; Li et al., 1999; Saper & Breder, 1994).

Nevertheless, the use of NSAIDs is not limited to their anti-inflammatory, analgesic and antipyretic properties. Additionally, they can be employed for their antithrombotic effects and further against endotoxaemia. Endotoxin, a lipopolysaccharide of the outer membrane of Gram-negative bacteria, unleashes an inflammatory response by stimulating phospholipase A. The synthesis of pro-inflammatory cytokines and eicosanoids result in altered cardiovascular, pulmonary functions, fever and leukopenia. Furthermore, increased renal blood flow and intestinal permeability are common clinical signs associated with endotoxaemia (Andreasen et al., 2008; Moses & Bertone, 2002). The administration of NSAIDs leads to the inhibition of eicosanoid and thromboxane A2 production and thus to an anti-endotoxaemic effect.

Particularly flunixin and phenylbutazone have demonstrated very good properties against endotoxaemia in horses (King & Gerring, 1989; Semrad et al., 1987). The antithrombotic function of NSAIDs arises from the suppression of TXA2 formation through COX enzyme inhibition (Moses & Bertone, 2002). TXA2, which is produced in activated platelets, plays an important role in the stimulation and aggregation of platelets in haemostasis but also in pathological thrombosis, in case of strokes and myocardial infarction (Antithrombotic Trialists' Collaboration, 2002; Ogletree, 1987). Therefore, NSAIDs can further be applied in patients with pathologically increased coaguability for the prevention of thrombosis.

2.6.4 Pharmacokinetics

The NSAIDs are all lipid soluble, generally weak organic acids, which are well absorbed and are generally known for a good bioavailability after oral administration, IM and SC injection. They are able to penetrate the blood-brain barrier and the passage into inflamed exudate is enhanced due to the high degree of plasma protein binding of 95-99% (Riviere & Papich, 2009). The accumulation in exudate of inflamed areas is regarded as a possible explanation for the long duration of effect even after a significant decrease of the plasma concentrations (Lees et al., 2004b). However, the passage from the plasma to the interstitial and transcellular fluids is limited due to the high percentage of plasma protein binding (Riviere & Papich, 2009). The main organ for metabolism is the liver, and leads to oxidation, reduction, hydrolysis, conjugation. With the exception of the metabolism of phenylbutazone, it results in inactive metabolites. Excretion of the drug is mostly through renal tubular secretion. Due to the high level of protein binding, glomerular filtration is limited (Moses & Bertone, 2002).

2.6.5 Adverse effects

NSAIDs are widely used drugs alleviating pain, inflammation and fever. However, the inhibition of the COX enzymes and therewith the inhibition of prostaglandin synthesis does not only account for the therapeutic but also for the adverse effects. The use of NSAIDs has been associated with mild to serious, often dose-related side effects. Most commonly, these side effects include gastro-intestinal disorders such as vomiting, gastric ulceration and bleeding into the gastric or intestinal lumen (Kahn & Line, 2010; Lees et al., 1991; Lees et al,, 2002; Moses & Bertone, 2002; Riviere & Papich, 2009). The inhibition of gastro-protective prostaglandin synthesis leads to a reduced perfusion of the gastric mucosa, to the lack of protective buffer and mucous production and results in cell damage, gastritis and ulceration (Kahn & Line, 2010). Besides the reported hepatotoxicity (Lees & Higgins, 1985) and blood dyscrasias (Lees et al., 2002), the inhibition of platelet COX-1 enzymes and the inhibition of pro-aggregatory TXA2

synthesis can result in extended bleeding. Furthermore, the inhibition of renal prostaglandins, which regulate tubular function and haemodynamics of the kidneys leads to changes in electrolyte homeostasis and reduced renal perfusion and ultimately results in kidney damage (Epstein, 2002). Importantly, adverse drug effects can vary across different species and can be species specific. As an example, in cats the lack of the metabolising enzyme glucoronyl transferase leads to the accumulation of the reactive metabolite, to the damage of the hepatocytes and thus to a life threatening toxcity (Savides et al., 1984).

Initially, the adverse effects were solely attributed to the inhibition of the consecutively expressed COX-1 enzyme and efforts were made to increase the selectivity of the COX-inhibition towards suppressing COX-2 enzymes only (Moses & Bertone, 2002). However, even the efforts towards a COX-selective inhibition have not led to the complete elimination of side effects. The inhibited conversion of arachidonic acid to prostaglandins leads to an accumulation of arachidonic acid, which then activates the 5-LOX and leads to the increased synthesis of leukotrienes. Consequently, this results in gastro intestinal irritation and damage evoked by vasoconstriction, reduced blood flow through the gastric mucosa and leukocyte activation (Martel-Pelletier et al., 2003; Rainsford, 1987). For that reason, dual COX and 5-lipoxygenase (5-LOX) inhibitors such as tapoxalin and licofelone have been generated, as they exhibit anti-inflammatory properties but no gastrointestinal adverse effects (Argentieri et al., 1994; Knight et al., 1996; Laufer et al., 1994; Martel-Pelletier et al., 2003). The combined inhibition of the prostaglandin and leukotriene leads to synergistic effects and good anti-inflammatory properties. It prevents the excessive leukotriene synthesis by blocking both pathways, thus, prevents gastric vasoconstriction and leucocyte infiltration and ultimately leads to the protection of the gastric mucosa (Martel-Pelletier et al., 2003).

A fifth group, the COX inhibiting nitric oxide donors (CINODs), has recently been developed with the aim to provide a gastrointestinal sparing NSAID, which does not interfere with the healing of gastric ulcers. By adding a nitric-oxide (NO) releasing component, the blood flow through the gastrointestinal tract is maintained and the adherence and activation of leucocytes is impeded. The lesions in the gastrointestinal tract are significantly reduced while the suppression of the gastric and peripheral prostaglandin synthesis is not impaired and anti-inflammatory properties are unchanged or ameliorated. The gastrointestinal sparing effect of NO-NSAIDs is not yet fully understood. However, one possible explanation is a protective impact, which counteracts the detrimental effects of the COX inhibition. Another theory is that the release of nitric oxide, nitrate or nitrite exhibits vasodilatatory properties, which leads to its protective qualities (Wallace et al., 1994).

2.6.6 Carprofen

Carprofen (2-(6-Chloro-9H-carbazol-2-yl)propanoic acid) (Figure 2-6)), the propionic acid derivative represents one of the newer classes of NSAIDs. It is used in cattle, horses, cats and dogs for perioperative analgesia as well as for soft tissue injuries (Armstrong et al., 1999). Even after long-term administration, carprofen is defined by a low incidence of side effects in addition to having a long duration of effect and good analgesic properties (Gaynor & Muir, 2015; Mathews, 2002; McKellar et al., 1991). As a pharmaceutical active, carprofen is a chiral drug with two enantiomers, the R(-) and S(+) carprofen, within the same formulation. Both enantiomers exhibit significant pharmacodynamic and pharmacokinetic differences. The drug can be administered IV, IM, as well as PO (McKellar et al., 1991). The COX-1 and COX-2 inhibition is characterised by species-specific differences. While it is a preferential COX-1 inhibitor in humans, it is non-selective in horses, and COX-2 preferential in dogs and cats (Lees et al., 2004b; Streppa et al., 2002). Based on its favourable characteristics and additional feedback from wildlife veterinarians reporting satisfying clinical results after analgesic medication with carprofen (J. Marais, personal communication, 2016), carprofen was chosen to be further evaluated in this study for the potential use in white rhinoceros.

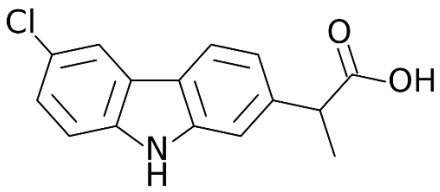


Figure 2-6: Molecular structure of carprofen

Carprofen in the horse, the closest relative to the rhino, is characterised by a $t_{1/2}$ of 21.9 hours (McKellar et al., 1991). The volume of distribution is relatively low (0.23 l/kg) (McKellar et al., 1991) and the clearance (Cl) varies between 58.9 ml/min (Lees et al., 1994) and 44.9 ml/min (McKellar et al., 1991). Remarkably, even when serum drug concentrations have decreased, the tissue drug concentrations maintain a high therapeutic level due to the high degree of protein binding and ion trapping (Moses & Bertone, 2002). The enantio-selective glucuronidation of the S(+)-carprofen leads to a lower plasma concentration of the S(+)-enantiomer than of the R(-)-enantiomer (Soraci et al., 1995). Even though quantitatively, the S(+)-enantiomer is significantly lower, it is more potent than the R(-)-enantiomer and mainly responsible for the pharmacologic properties of the drug (Evans, 1992).

With the aim to compare the analgesic effect of different NSAIDs in the horse, Schatzman et al. (1990) investigated the peripheral pain inhibition after the administration of flunixin-meglumine and carprofen in horses. The study showed that carprofen administered IV at a dose of 0.7 mg/kg body weight resulted in peripheral analgesia for 24 hours and suggests that the plasma concentration needs to reach at least 1.5 micrograms/ml in order to maintain the analgesic effects (Schatzmann et al., 1990). Lees et al. (1994) recorded a carprofen plasma concentration above 1.5 µg/ml only for up to 12 hours in their model of acute non-immune inflammation. However, a mean exudate concentration of 1.38 µg/ml was measured at 24 hours, which indicates an accumulation of carprofen in the exudate of the acute inflammation site and a slower clearance at the site of inflammation than in the plasma. Presumably, in case of inflammation, the accumulation of the drug in the exudate increases the length of effectiveness in addition to the long half-time life of carprofen (Lees et al., 1994). Further, Johnson et al. (1993) assessed the analgesic effect of phenylbutazone (4 mg/kg, n= 72), flunixin (1 mg/kg, n=68) and carprofen (0.7 mg/kg, n= 63) in horses after surgery and reported the analgesic effect of carprofen to last for 11.7 (+/-6.9) hours.

2.7 Cytochrome P450 enzymes

Is the rhino just a big horse? From a phylogenetic point of view, the horse is the closest related species to the rhinoceros (Price & Bininda-Emonds, 2009). However, as pharmacokinetic and pharmacodynamic properties may vary significantly, one cannot simply conclude that the rhino can be medicated and treated like a large horse. Thus far, the horse is often used as a model for rhino and elephant (Mortenson, 2001), but interspecies differences in drug metabolism are likely and may render the horse an inappropriate model. A better understanding of interspecies differences or similarities may be provided through further investigation of the drug metabolising enzymes. The most important ones are the cytochrome P450 (CYP), a diverse family of haemoproteins (monooxygenases), which catalyse the transfer of an oxygen atom to the substrate in order to facilitate degradation and elimination of drugs (Fink-Gremmels, 2008). Their discovery goes back to the 1950s, when Klingenberg found and first described the carbon monoxide binding pigment with its absorbance maximum around 450 nm (Estabrook, 2003; Klingenberg, 1958). Already in the 1960s, their function as drug and steroid metabolising enzymes was revealed (Nebert & Russell, 2002). To the present day, the functions of the CYP450s have been studied in more depths and range from the synthesis of steroid hormones (Payne & Hales, 2004) and an endogenous epithelial relaxation factor (Fisslthaler et al., 1999) to the metabolism of xenobiotics (Anzenbacher & Anzenbacherova,

2001). Overall, there are over 1000 identified P450 enzymes, however, their substrates remain in large parts unresolved (Anzenbacher & Anzenbacherova, 2001).

The nomenclature of the various P450 enzymes (CYP450), e.g. CYP2E1 is based on arabic numbers and letters. CYP450 enzymes, which share a minimum of 40% identity are divided into families assigned with an arabic number. The classification into subfamilies, characterised by letters, is based on a more than 55% identical primary structure (Nelson, 2006; van der Weide & Hinrichs, 2006). The individual isoenzymes differ by a minimum of 3% and are characterised by a second arabic number at the end (Anzenbacher & Anzenbacherova, 2001).

Even though the structure as well as the general mode of action of the enzymes resemble each other, major differences exist in the individual function of the single enzymes and in the composition of their active sites (Anzenbacher & Anzenbacherova, 2001). Additionally, the numbers of isoenzymes across different species vary considerably. While 57 functional CYP450 enzymes have been detected in humans, 108 functional CYP450 have been found in the mouse, 89 in the rat, 59 have been identified in cattle and pigs, and only 15 in the horse so far (UCSC genome browser getaway, 2017; Orr, 2016; Zanger & Schwab, 2013). Besides species specific differences in CYP450 isoenzymes, sex, age, drug-drug interactions, hormonal state and disease influence CYP450 activity and further result in intra- and interspecies differences (Zanger & Schwab, 2013). In humans, the CYP3A4 enzyme is the most important and abundant isoenzyme in the liver, metabolising around 50% of the commonly employed drugs (Anzenbacher & Anzenbacherova, 2001; Guengerich, 1997; Orr, 2016). In the horse, the CYP3A93 bears the greatest resemblance to the human CYP3A4 (Orr, 2016).

While human CYP450s functionality and substrates have been studied more extensively, such information for other species is lacking. However, the few studies in domestic animals indicate that interspecies differences are prominent. Even though the CYP450 system originates from one single ancestral gene and even though closely related species contain conserved regions of amino-acid residues, small differences in the amino acid sequence may lead to considerable changes in substrate specificity and catalytic activity (Martignoni et al., 2006; Nelson et al., 2013). Just a single change in amino acid sequences is sufficient to possibly alter substrate specificity (Lindberg & Negishi, 1989). Thus, even closely related species seem to vary significantly as they exhibit different CYP450 isoenzymes and varying liver enzyme contents (Fink-Gremmels, 2008; Martignoni et al., 2006; Nelson et al., 2013). While few CYP450 have been well conserved across species, the majority of CYP450s differ and cannot

be reliably extrapolated across species. Guengerich (1997) suggested a provisional classification according to ‘catalytic preservation’, meaning the existence of a fair degree of catalytic similarity between related CYP450 enzymes. According to Guengerich (1997), the only CYP450 which can be extrapolated across species is the CYP2E1, cautiousness needs to be applied for CYP1A1, 1A2 and 17A. Even more caution is required for the extrapolation of CYP2D and 3A while the extrapolation of 2A, 2B and 2C leads to flawed results.

2.8 Conclusion

While the poaching crisis has killed thousands of rhinos over the past years and has rendered them a threatened species once again, the need for an effective treatment for severely wounded victims is emerging. However, studies evaluating antimicrobial and analgesic drugs for the use in rhinos are lacking. For this study, the antimicrobial enrofloxacin and the NSAID carprofen were selected as potentially useful medications in the rhinos. The antimicrobial drug was selected based on the principles of treatment of infected wounds, considering pharmacokinetics, MICs as the most important pharmacodynamic parameter, the immune status of the animal, time- versus concentration-dependent killing mechanisms, the location of the infection and potential adverse effects. The NSAID was selected due to its analgesic potency, its superior safety profile (Gaynor & Muir, 2015) and its long duration of effect (McKellar et al., 1991), all in the absence of a sedative effect.

3. Chapter 3: Enrofloxacin in white rhinoceros (*Ceratotherium simum)* – a treatment option for injured poaching victims?

Marion Leiberich[1], Ralph Krebber[2], Michael Hewetson[1], Johan Marais[3], Vinny Naidoo[4]

[1] Department of Companion Animal Clinical Studies, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[2] Bayer Crop Science AG, Research & Development - Regulatory Science - Human Safety – Residue Analysis, Monheim am Rhein, Germany

[3] Onderstepoort Veterinary Academic Hospital, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[4] Department of Paraclinical Science, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

3.1 Abstract

South Africa is currently losing over 1000 white rhinoceros (*Ceratotherium simum*) each year to poaching incidents and numbers of severely injured victims found alive have increased dramatically. However, little is known about the antimicrobial treatment of wounds in rhinoceros. For this reason, this study explores the applicability of enrofloxacin for the treatment of rhinoceros with infected wounds. The pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin were evaluated in five white rhinoceros after intravenous (IV) and after successive IV and oral administration of 12.5 mg/kg enrofloxacin. After IV administration, the half-life, area under the curve (AUC_{tot}), clearance and the volume of distribution were 12.41 ± 2.62 hours, 64.5 ± 14.44 µg*h/ml, 0.19 ± 0.04 L/h*kg and 2.09 ± 0.48 L/kg, respectively. Ciprofloxacin reached 26.42 ± 0.05% of the enrofloxacin plasma concentration. After combined IV and oral enrofloxacin administration oral bioavailability was 33.30 ± 38.33%. After IV enrofloxacin administration, the efficacy marker AUC_{24}: MIC exceeded the recommended ratio of 125 against bacteria with an MIC of 0.5 µg/mL. Subsequent intravenous and oral enrofloxacin administration resulted in a low Cmax: MIC ratio of 3.1. The results suggest that intravenous enrofloxacin could be a useful drug with bactericidal properties in rhinoceros.

However, the maintenance of the drug plasma concentration at a bactericidal level through additional oral enrofloxacin does not seem feasible.

Keywords: Enrofloxacin, fluoroquinolone, antimicrobial drug, white rhinoceros, poaching

3.2 Introduction

The white rhinoceros (*Ceratotherium simum*), one of Africa's iconic species, is in danger of extinction due to unscrupulous poaching. The illegal killing is driven by the demand for rhino horn used in traditional Chinese medicine, for ceremonial purposes and as a status symbol mainly in Asian countries (Challender & MacMillan, 2014). Figures published in 2018 report 1215 deaths in 2014, up from 1004 and 668 in 2013 and 2012, respectively. In 2015 and 2016, another 1175 and 1054 rhinos were killed for their horn (Save the Rhino, 2018). Furthermore, in addition to the dramatic increase in killed rhinoceros, the number of rhinos escaping immediate death has been on the rise, with an estimated 200 animals needing veterinary assistance per year (J. Marais, personal communication, 2016). Injuries seen in these animals include limb wounds caused by snares such as abrasions, tearing of the skin, swelling and muscle damage. Deep gun-shot wounds in the limbs, the head or the torso are common with resultant blood loss, anaemia, hypovolemia, fractures, septic joints and soft tissue secondary infections. Extensive facial wounds with resultant exposed frontal and nasal sinuses after the brutal removal of the horns, are found more and more often (Cooper & Cooper, 2013).

Injured animals require immediate veterinary treatment, which involves stabilizing the patient, haemostatic measures, various diagnostic measures such as radiography and typically wound management including surgical lavage and wound dressing. Analgesic and antimicrobial support is vitally important in all these rhinos. Unfortunately, despite the necessity for proper therapeutic measures, pharmaceutical agents active against infection and pain are yet to be evaluated. As a result, current therapies are extrapolated from other veterinary species. Species-specific knowledge is needed urgently; however, the research of drug pharmacokinetics and pharmacodynamics in non-domesticated species is challenging due to the inability to safely get into close contact, the difficulties with frequent drug re-administration and the need for large drug volumes.

The focus of this study was to optimize the antimicrobial treatment of rhinos by having at least one scientifically evaluated antimicrobial drug available. Initial criteria set for this optimal agent were as follows: the ideal drug should be broad spectrum to allow for treatment in the field where culture and antibiograms are not always feasible and should have a prolonged

mean residence time to prevent frequent re-administration. It should be commercially available as a sufficiently concentrated formulation in order to reduce the dosing volume required (reducing the number of injections per administration). Furthermore, it should be available as an oral, water soluble medication so that treatment can be continued in the drinking water or feed while the animal recovers in an enclosure with minimal human contact (minimise stress and injury from requiring re-immobilisation of the already compromised animal for re-administration).

In the course of the drug selection process, we also screened a database of previously evaluated white rhinoceros bacterial culture results from the bacteriology laboratory of the Department of Tropical Diseases, University of Pretoria obtained between 2008 and beginning of 2015. Of the 33 recorded cases (excluding faecal samples), 15 samples underwent antimicrobial susceptibility testing and revealed that enrofloxacin was one of the antimicrobials with a high susceptibility rate (60%). Based on this criteria and the promising pharmacokinetic characteristics we selected enrofloxacin, a second-generation fluroquinolone for further study. Enrofloxacin, the first fluoroquinolone developed for veterinary purposes, is a broad spectrum antimicrobial, particularly effective against gram-negative bacteria, and most importantly exhibits a rapid bactericidal, concentration dependent effect, which would allow a once daily treatment. Another major advantage is that the product is already available as an oral and parenteral formulation (Lode et al., 1998; Lopez-Cadenas et al., 2013) at a relatively high concentration of 100 mg/ml which could allow the stress-free oral administration of the drug in the drinking water or feed.

3.3 Materials and methods

3.3.1 Experimental design

The study, which was approved by the Animal Ethics Committee of the University of Pretoria (permit number: V074-15), was divided into two phases. For the first phase, five rhinoceros were administered a single intravenous dose of enrofloxacin at 12.5 mg/kg (Baytril, Injectable, Bayer Animal Health, 100 mg/ml) with an IM injection of 1 mg/kg of racemic carprofen (Rimadyl Injection, 50 mg/kg Zoetis) as concurrent anti-inflammatory treatment (results to be presented in a different article). The second phase began after a washout period of eight weeks. All animals were again treated with a single intravenous dose of enrofloxacin at 12.5 mg/kg (Baytril, Injectable, Bayer Animal Health, 100 mg/ml) and a single intramuscular dose of carprofen at 1 mg/kg (Rimadyl, Zoetis, 50 mg/ml). The parenteral drug administration was followed by a single administration of per os enrofloxacin at 12.5 mg/kg (Baytril, Bayer

Animal Health, 10% oral solution, indicated for the use in chickens, turkeys and rabbits). The oral solution was administered in the feed. The liquid enrofloxacin was diluted with an equal volume of water and poured over about two scoops of pellets. After absorption of the enrofloxacin-water mixture by the pellets, the medicated pellets were mixed with two scoops of non-medicated pellets and two handfuls of lucerne (*Medicago sativa*). To mask the bitter taste, a small amount of molasses was added and the ingredients were blended thoroughly until evenly mixed. The total amount of food was weighed before being fed to the animals in order to be able to calculate the exact amount of ingested feed. The results from the first phase have been partially described in the manuscript on the allometric scaling of enrofloxacin in the white rhinoceros (submitted for consideration to PLOS ONE).

3.3.2 Animals

Five habituated white rhinoceros (one female, four males) from the 'The Rhino Orphanage' in South Africa were used for the study (S 3-1). The minimum age was 13 months and the average weight of the animals was 623 kg and 670 kg in the first and second phase, respectively. The rhinoceros graze in groups in large enclosures during daytime and sleep in large enclosed paddocks or the attached night-rooms. Besides the grazing, the animals receive additional feed consisting of teff (*Eragrostis teff*), lucerne (*Medicago sativa*), and pellets (Driehoek Standard Game Cubes South Africa) twice daily and water *ad libitum*. Rhino I and rhino II also received a milk feed of one litre, twice daily during the first phase of the trial. For the period of each trial, the animals were kept in a boma in groups of two to three animals with free access to water and to their daily feeds. Prior to the start of the study, the animals were trained (positive operant conditioning training) to tolerate the touching of their ears for the sample collection through the catheter. To reduce stress during the blood collection phase of the study, animals were administered a single dose of the long acting tranquiliser zuclopenthixol acetate (Clopixol-Acuphase, 50 mg/ml, Lundbeck) at 50 mg/animal intramuscular (Kock & Burroughs, 2012).

3.3.3 Experimental procedures

3.3.3.1 Blood sampling

The plasma concentration of enrofloxacin and its active metabolite ciprofloxacin were evaluated over a period of 72 hours. In the first and the second phase of the study, blood samples were collected prior to administration and around 5, 15, 30, 45 minutes and 2, 6, 12, 24, 48, 72 hours after administration of enrofloxacin. Due to difficulties in approaching the animals for

direct venepuncture, the rhinoceros had to be sedated for the placement of a catheter. Further information on the placement of the catheter can be found in the supplementary information (S 3-2). In phase I, thiafentanil (Thianil, 10 mg/ml, Wildlife Pharmaceuticals RSA), etorphine (M99, 9.8 mg/ml, Novartis) and diazepam (Pax, 10 mg/2ml, Aspen Pharmacare) were used as the sedative. Etorphine or ketamine (Kyron RSA, 1 mg in 20 ml vial) was added when the responsible veterinarian believed that further sedation was needed. Based on the experiences from phase I, for phase II the animals were sedated with a combination of etorphine and thiafentanil, with ketamine or medetomidine (20 mg/ml, Kyron RSA) being used when further sedation was required. After the 12-hour bleed, blood was collected directly from the cephalic vein. For this, the rhinoceros were sedated either with a mixture of thiafentanil and ethorphine in the first phase and butorphanol (Dolorex, MSD animal health, 10 mg/ml) in the second phase. Diprenorphine (M5050, 12 mg/ml, Novartis) was used to partially reverse the effect of etorphine and thiafentanil at the end of the initial sedation or to improve the breathing during the sedation, if necessary. S 3-3, S 3-4, S 3-5 and S 3-6 summarise the drugs used for each rhino during the first and second immobilisation, respectively. In all cases, the immobilization process closely followed that of field management of rhino in South Africa.

3.3.3.2 Analysis of the Enrofloxacin and Ciprofloxacin Plasma Concentrations via Online-Solid Phase Extraction/ Tandem Mass Spectrometry

All blood samples were placed on ice immediately after collection and centrifuged at 3000 rpm for 15 minutes within 4 hours of collection. Plasma samples were stored at -20°C for a maximum of 8 days at the study site prior to being transferred into the -80°C freezers of the University of Pretoria. For evaluation, samples were shipped to Germany on dry ice (World Courier) for analysis by Bayer Animal Health (CITES export permit number: 152722) and analysed by a previously validated method, namely the online – solid phase extraction/ tandem mass spectrometry (online-SPE-MS/MS). The measurement conditions in general have been described by Krebber et al. (2009), with the only modification being the replacement of trifluoroacetic acid by heptafluorobutyric acid as described by Bousova et al. (2013). Briefly, the samples were first thawed and centrifuged. Subsequently, 300 µL clear sample fluid were transferred into an autosampler vial and 300 µl water and 6 µL of the internal standard solution containing enrofloxacin-d_5 hydrochloride (100 µg/L) and ciprofloxacin-d_8 hydrochloride (100 µg/L) (Sigma-Aldrich, Taufkirchen, Germany) were added. After mixing for approximately 5 seconds by means of a vortex mixer, 5 µL of the sample was injected into the online-SPE-MS/MS system while in loading position. Matrix components contained in the injected sample were separated from the retained analytes on an extraction column suited for pre-treatment of

samples at high flow rates. After switching to the elution position, the analytes were transferred to an analytical column. The quantitative determination was performed in a tandem mass spectrometric detector.

Analyses were performed using an HPLC system with two binary pumps (1290 binary pump G4220A and 1200 binary pump G1312A, Agilent Technologies, Waldbronn, Germany), CTC HTS PAL Autosampler with two six port valves (CTC Analytics AG, Zwingen, Switzerland) operated by CHRONOS Software (Axel Semrau, Sprockhövel, Germany). For SPE an Oasis HLB, 2.1mm x 20 mm, 25µm column (Waters, Eschborn, Germany) and for separation a Chromolith Speed Rod, 50 mm x 4.6 mm RP 18e column (Merck, Darmstadt, Germany) were used. Detection and quantification was made on a Sciex API 4000 (Sciex, Darmstadt, Germany) triple-quadrupole tandem mass spectrometer.

The limit of quantification for enrofloxacin and ciprofloxacin was 0.020 mg/L. The method was validated in the range from 0.02 to 25 mg/L for enrofloxacin. For enrofloxacin, the mean recovery rates for the individual fortification levels were between 92 and 113%. The mean overall was 101%, representing an accuracy of +1% with a precision (coefficient of variation) of 6.3%. For ciprofloxacin, the mean recovery rates for the individual fortification levels were between 96 and 106%. The mean overall was 100%, representing an accuracy of 0% with a precision (coefficient of variation) of 5.5%. The linearity was checked in the range from 0.005 to 1 mg/L, the correlation coefficients were ≥ 0.999.

3.3.3.3 Assessment of the Pharmacokinetics of Enrofloxacin and Ciprofloxacin

The plasma concentration of enrofloxacin and its active metabolite ciprofloxacin were determined for each individual at the different points of time. All pharmacokinetic calculations were undertaken in Kinetica 5.0 (Thermo). The following pivotal non-compartmental parameters were calculated for enrofloxacin and ciprofloxacin: The maximum plasma concentration (C_{max}) and the time to maximum concentration (T_{max}) were read directly of the concentration versus time plasma profile. The area under curve to the last quantifiable time point (AUC_{last}) was determined using the linear trapezoidal rule ($AUC_{last} = \sum_{i=1}^{n} 0{,}5 * ((C_i + C_{i+1}) * \Delta t)$). The total area under curve extrapolated to infinity (AUC_{tot}) was calculated as follows: $AUC_{tot} = AUC_{last} + AUC_{extra} = AUC_{last} + C_{Last}/\lambda$ with C_{last} being the computed last measured concentration and λ being the terminal elimination rate constant. The area under the moment curve from the time point zero to the last measured time point ($AUCM_{last}$) was calculated as $AUMC_{last} = \sum_{i=1}^{n} 0{,}5 * (t_i * C_i + t_{i+1} * C_{i+1}) * \Delta t$. The half-life ($t_{1/2}$), clearance (Cl) and volume of distribution during terminal phase (V_z) and volume of distribution at steady

state (V_{ss}) and the mean residence time (MRT) were determined as $t_{1/2} = \ln(2)/\lambda$; $V_z = Cl/\lambda = Dose/(AUC*\lambda)$; $V_{ss} = (Dose*MRT)/AUC$, $Cl = dose/AUC_{tot}$ and $MRT = 1/\lambda$. The oral bioavailability of enrofloxacin was calculated as $F = (AUC_{PO}/Dose_{PO})/(AUC_{IV}/Dose_{IV})$, where the $Dose_{PO}$ was the dose of the orally administered enrofloxacin and AUC_{IV} and $Dose_{IV}$ were the AUC_{tot} and the dose of the intravenously administered enrofloxacin. The AUC_{PO} was estimated as the AUC_{tot} of the first phase subtracted from the AUC_{tot} of the second phase.

3.3.3.4 Assessment of the Pharmacodynamics of Enrofloxacin and Ciprofloxacin

In order to predict the therapeutic use of enrofloxacin, the surrogate markers AUC_{24}: MIC and C_{max}: AUC after IV administration were evaluated. With enrofloxacin being partially transformed to the active metabolite ciprofloxacin, the total AUC_{24} was determined as $AUC_{24enro} + AUC_{24cipro}$. The MIC value of 0.5 used for the calculation of the ratio represents the susceptibility breakpoint for enrofloxacin published by the CLSI (CLSI, 2015). Furthermore, the change in slope of the semilogarithmic plot of the enrofloxacin concentration was used as a brief indicator for the pseudo Cmax of the addititve curve after subsequent intravenous and oral enrofloxacin administration.

3.4 Results

3.4.1 Side effects

No adverse effects were observed during the first phase of the study. During the second phase, four out of five rhinos developed a band-like swelling at the base of the ear in which the enrofloxacin was injected. The swelling appeared within the first six hours after the injection through the auricular catheter and consisted of a painless oedema around the base of the ear. The swelling decreased in all affected individuals within 24 hours and either disappeared or was significantly reduced towards the end of the study, after 72 hours. Apart from the swelling at the base of the ear, the rhinoceros showed no further side effects and did not seem affected by the reaction. All rhino ate within 12 hours after immobilisation and exhibited their normal physiological behaviour. One rhinoceros developed a thrombophlebitis in the auricular vein where the long stay catheter was placed. It was discovered one month after the end of the study. It was assessed by the local veterinarian, it was kept clean and healed without further complications.

3.4.2 Blood sampling

Despite every effort to facilitate blood collection at the scheduled intervals, this was not accurately possible due to the challenges of working with wild animals. On average, the blood

sampling during the first trial took place prior and 8.8, 23.2, 37.4, 52.6 minutes and 2.11, 6.37, 12.33, 24.94, 48.30 und 71.45 hours after the injection of enrofloxacin. For the second trial, the five animals received an enrofloxacin treatment as in the first trial (12.5 mg enrofloxacin/kg body weight IV) followed by an oral single dose of enrofloxacin medication in the feed of 12.5 mg enrofloxacin/kg body weight. The treated food was ingested on average 10.06 ± 1.74 h after intravenous enrofloxacin administration. All individuals ingested the full portion of food with the complete amount of enrofloxacin, indicating that the method of dosing was acceptable. The blood sampling took place before and 7.6, 21.2, 33.4, 48.4 minutes and 2.2, 6.28, 11.92, 22.89, 47.95 and 72.76 hours after enrofloxacin injection. The actual times of collection were used in the subsequent pharmacokinetic analysis.

Physiological parameters such as heart rate, respiratory rate and temperature were monitored during the initial immobilisation. Table 3-1 presents the recorded heart rates and respiratory rates at the time of the first handling, after 10 and 20 minutes and presents the average of the recorded values between 20 minutes and 100 minutes after the initial handling of the animal.

Table 3-1: Heart rate (HR) and respiratory rate (RR) during the immobilisation with an etorphine-thiafentanyl combination and additional ketamine, medetomidine and diprenorphine

Time (min)	0		10		20		20-100	
Animal	**HR (beats /min)**	**RR (breaths/min)**	**HR (beats /min)**	**RR (breaths/min)**	**HR (beats /min)**	**RR (breaths/min)**	**HR (beats /min)**	**RR (breaths/min)**
R I	64	5			52	7	59.14	7.43
R II	132	12	72	6	66	8	97.3	8.5
R III	144	12	142	8	68	7	61.71	11.14
R IV	124	12	88	8	52	8.5	60	10.89
R VI	124	12	144	12	72	8	70.29	8.43
Average	**117.6**	**10.6**	**111.5**	**8.5**	**62**	**7.7**	**69.69**	**9.28**

The values of the heart rate and the respiratory rate when the animals were first handled (0), after 10 minutes and after 20 minutes were recorded. From 20 to 100 minutes, the average value of all points of evaluation was formed.

3.4.3 Pharmacokinetics of enrofloxacin and ciprofloxacin after intravenous enrofloxacin administration (Phase I)

All data is reported as geometric mean (Gmean) and standard deviation **(**± SD**)** for both phases. An enrofloxacin plasma concentration of 13.9 ± 3.70 µg/ml was recorded at the first sampling point post enrofloxacin injection after 8.8 ± 2.4 minutes. Due to challenges during the blood collection, at the last sampling point 71.45 ± 0.8 hours post enrofloxacin injection, only four rhinoceros could be sampled. Of the four rhinoceros, one exhibited an enrofloxacin concentration below the limit of quantification (LOQ < 0.02 µg/ml), while the other three rhinoceros exhibited an average enrofloxacin plasma concentration of 0.054 ± 0.02 µg/ml. Enrofloxacin was characterised by a long half-life of elimination ($t_{1/2}$) of 12.41 ± 2.62 hours. The area under the curve extrapolated to infinity (AUC_{tot}) was 64.5 ± 14.44 µg*h/ml. The clearance (Cl) was slow with a value of 0.19 ± 0.04 L/h*kg. The volume of distribution at steady state (V_{ss}) was 2.09 ± 0.48 L/kg. The mean residence time (MRT) in the plasma was 18.28 ± 4.53 hours. The formation of the active metabolite ciprofloxacin began rapidly. At the first sampling point post enrofloxacin injection, ciprofloxacin concentration was 0.15 ± 0.05 µg/ml and reached its maximum (C_{max}) of 0.92 ± 0.11 µg/ml after 2.1 ± 0.18 hours. At the last blood sampling point after 71.45 hours, ciprofloxacin concentrations of three rhinoceros were below the limit of quantification while one rhinoceros showed a quantifiable concentration of 0.03 µg/ml. The half-life ($t_{1/2}$) was 11.62 ± 1.28 hours. The AUC_{tot} was 17.04 ± 3.84 µg*h/ml. The plasma ciprofloxacin concentration reached 26.42 ± 0.05% of the plasma enrofloxacin concentration. The results of the pharmacokinetic analysis of enrofloxacin and its active metabolite ciprofloxacin after a single intravenous enrofloxacin injection (12.5 mg/kg) are summarized in Table 3-2 and Table 3-3. The mean plasma concentration versus time curves of enrofloxacin and its active metabolite ciprofloxacin are depicted in Figure 3-1 and the individual plasma concentration versus time profiles are depicted in S 3-7.

Table 3-2: Pharmacokinetic parameters of enrofloxacin for each rhinoceros after intravenous administration (12.5 mg/kg) in phase I

Parameter	Units	Animal					Mean	Gmean	SD
		I	II	III	IV	VI			
Λ	h^{-1}	0.05	0.07	0.04	0.07	0.05	0.06	0.05	0.01
t1/2	h	14.22	10.27	15.9	9.71	13.04	12.63	12.41	2.62
C_{max}	µg/ml	14.81	10.30	11.51	19.81	14.90	14.27	13.90	3.70
T_{max}	h	0.1	0.15	0.2	0.17	0.12	0.148	0.14	0.04
AUC_{last}	µg*h/ml	57.95	53.94	87.87	68.30	54.60	64.53	63.40	14.26
AUC_{tot}	µg*h/ml	58.61	54.36	89.65	68.48	57.10	65.64	64.50	14.44
AUC_{extra}	µg*h/ml*h	0.64	0.42	1.78	0.18	2.50	1.10	0.73	0.99
AUC_{extra}	%	1.76	1.25	3.17	0.42	7.02	2.72	1.83	2.60
$AUMC_{last}$	µg*(h)²/ml	577.40	583.01	979.13	547.18	478.99	633.14	612.76	197.79
Clearance	L/h*kg	0.21	0.23	0.14	0.18	0.22	0.20	0.19	0.04
Vz	L/kg	4.38	3.41	3.2	2.56	4.12	3.53	3.47	0.73
Vss	L/kg	2.32	2.62	1.78	1.5	2.47	2.14	2.09	0.48
MRT	h	20	14.29	25	14.29	20	18.71	18.28	4.53

λ, terminal elimination rate constant; $t_{1/2}$, half-life; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{last}, area under the curve until the last time point; AUC_{tot}, area under the curve extrapolated to infinity; AUCextra, area under the curve from the last quantifiable measurement to infinity; $AUCM_{last}$, area under the moment curve from t =0 to the last measured time point; Cl, clearance; Vz, apparent volume of distribution during the terminal phase; Vss, apparent volume of distribution in steady state; MRT, mean residence time

Table 3-3: Pharmacokinetic parameters of ciprofloxacin after intravenous enrofloxacin administration (12.5 mg/kg) for each rhinoceros in phase I

Parameter	Units	Animal					Mean	GMean	SD
		I	II	III	IV	VI			
λ	h^{-1}	0.06	0.06	0.05	0.07	0.06	0.06	0.06	0.01
t1/2	h	12.55	10.77	13.48	10.56	11.01	11.67	11.62	1.28
C_{max}	µg/ml	0.99	0.87	1.08	0.94	0.78	0.93	0.92	0.11
T_{max}	h	1.98	1.82	2.27	2.22	2.27	2.11	2.10	0.18
AUC_{last}	µg*h/ml	17.03	17.82	21.81	17.66	11.02	17.07	16.67	3.87
AUC_{tot}	µg*h/ml	17.25	17.99	22.38	17.82	11.60	17.41	17.04	3.84
AUC_{extra}	µg*h/ml	0.21	0.18	0.57	0.17	0.58	0.34	0.29	0.22
AUC_{extra}	%	1.99	1.58	4.09	1.49	8.07	3.44	2.74	2.79
$AUMC_{last}$	µg*$(h)^2$/ml	228.25	261.84	369.50	241.37	138.43	247.88	236.37	82.75
MRT	h	16.67	16.67	20	14.29	16.67	16.86	16.76	2.04

λ, terminal elimination rate constant; $t_{1/2}$, half-life, C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{last}, area under the curve until the last time point; AUC_{tot}, area under the curve extrapolated to infinity, AUC_{extra}, area under the curve from the last quantifiable measurement to infinity; $AUCM_{last}$, area under the moment curve from t =0 to the last measured time point; MRT, mean residence time

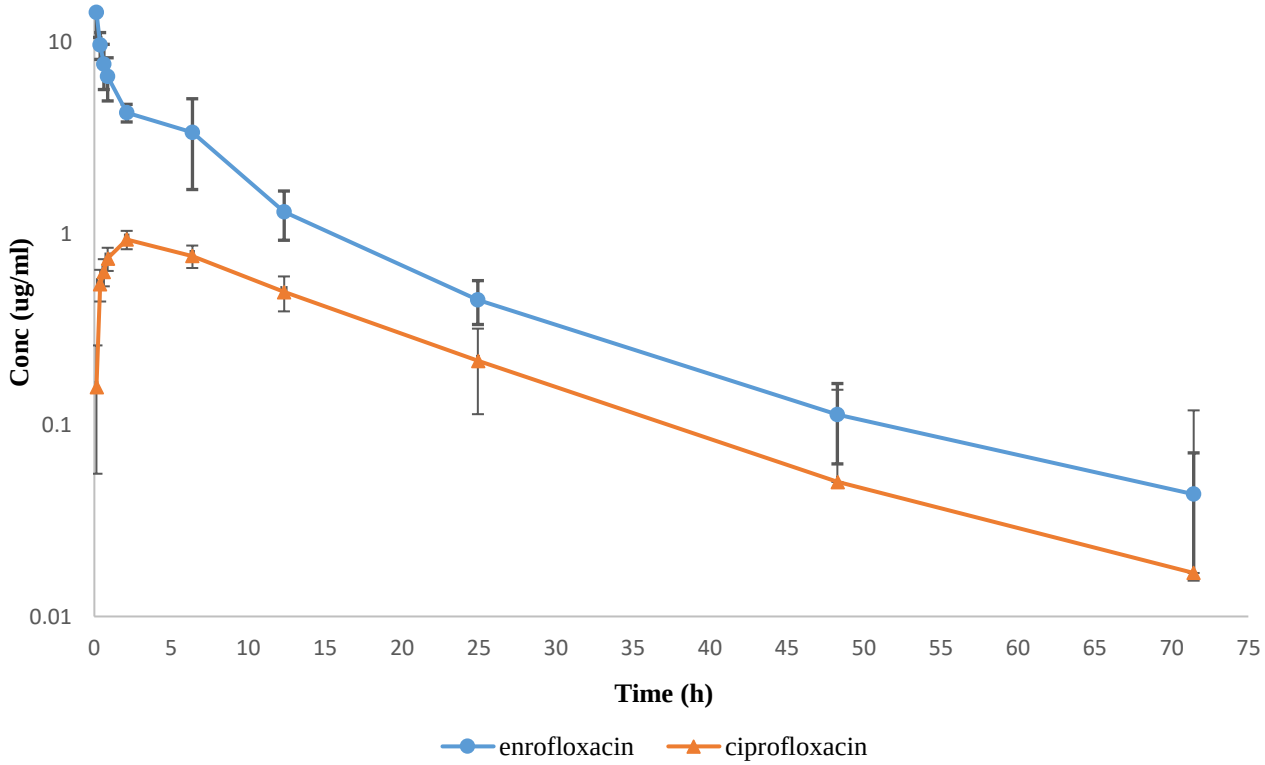


Figure 3-1: Average plasma concentration versus time profile with standard deviation (bars) of all 5 rhinoceros after IV administration of enrofloxacin (circle) at 12.5 mg/kg and its ciprofloxacin (triangle) metabolite

3.4.4 Pharmacokinetics of enrofloxacin and ciprofloxacin after intravenous and oral enrofloxacin administration (Phase II)

In the second phase of the study, enrofloxacin was administered intravenously and after an average of 10.16 ± 1.74 hours, a second dose of enrofloxacin (12.5 mg/kg) was given to the animals per os in a limited quantity of food. In all cases, the animals consumed the entire portion of medicated pellets. Enrofloxacin plasma concentration 7.8 ± 1.8 minutes post enrofloxacin administration was 19.64 ± 8.05 µg/ml. At the last sampling point, after 72.76 ± 1.41 hours, the average plasma concentration was 0.07 ± 0.02 µg/ml and all animals exhibited an enrofloxacin plasma concentration above the limit of quantification (0.02 µg/ml). The half-life ($t_{1/2}$) of enrofloxacin was 11.5 ± 0.84 hours and the MRT was 16.59 ± 1.18 hours. The AUC_{tot} was 92.38 ± 12.14 µg*h/ml. The mean Cl was 0.14 ± 0.02 L/h*kg and the apparent V_{ss} was 2.05 ± 0.14 L/kg. The estimated fraction of absorption of enrofloxacin was 33.3 ± 38.34%.

At the first sampling point post enrofloxacin injection, after 7.8 ± 1.8 minutes, ciprofloxacin concentrations reached in average 0.13 ± 0.03 µg/ml. The maximum ciprofloxacin concentration (C_{max}) of 0.71 ± 0.11 µg/ml was reached after 2.2 ± 2.1 hours. At the last sampling point (72.76 ± 1.41 hours), ciprofloxacin concentrations in one rhinoceros were below the limit of quantification (0.02 µg/ml), while in the remaining four animals the average concentration was 0.034 ± 0.01 µg/ml. The $t_{1/2}$ was 14.89 ± 1.32 hours. The MRT of ciprofloxacin was 20.91 ± 2.24 hours and the AUC_{tot} was 20.27 ± 3.42 µg*h/ml. Ciprofloxacin plasma concentrations reached 21.95% of the plasma concentration of the parent drug as compared to 26.42% in the first phase. The results of the kinetic analysis are summarized in Table 3-4 and Table 3-5. The mean plasma concentration versus time curve of enrofloxacin and its active metabolite ciprofloxacin is depicted in Figure 3-2 and the enrofloxacin and ciprofloxacin concentration versus time curves for each individual are presented in S 3-8.

Table 3-4: Pharmacokinetic parameters of enrofloxacin after intravenous and oral enrofloxacin administration (12.5 mg/kg) for each rhinoceros in phase II

Parameter	Unit	Animal					Mean	GMean	SD
		I	II	III	IV	VI			
λ	h^{-1}	0.067	0.063	0.057	0.058	0.057	0.060	0.060	0.005
t1/2	h	10.31	10.98	12.26	11.95	12.11	11.52	11.50	0.84
C_{max}	µg/ml	18.50	23.70	33.30	13.60	14.70	20.76	19.64	8.05
T_{max}	h	0.13	0.10	0.10	0.13	0.17	0.13	0.12	0.03
AUC_{last}	µg*h/ml	87.49	110.30	95.70	79.12	86.72	91.86	91.28	11.86
AUC_{tot}	µg*h/ml	88.24	111.77	97.24	80.07	87.62	92.99	92.38	12.14
AUC_{extra}	µg*h/ml	0.75	1.47	1.54	0.96	0.90	1.13	1.08	0.36
AUC_{extra}	%	1.37	2.11	2.54	1.91	1.65	1.92	1.88	0.45
$AUMC_{last}$	µg*$(h)^2$/ml	1190.58	1770.24	1375.74	971.15	1321.40	1325.82	1300.56	293.30
Cl	L/h*kg	0.14	0.11	0.13	0.16	0.14	0.14	0.14	0.02
Vz	L/kg	2.11	1.77	2.27	2.69	2.49	2.27	2.24	0.35
Vss	L/kg	2.02	1.90	2.00	2.06	2.29	2.05	2.05	0.14
MRT	h	14.93	15.87	17.54	17.24	17.54	16.63	16.59	1.18

λ, terminal elimination rate constant; $t_{1/2}$, half-life; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{last}, area under the curve until the last time point; AUC_{tot}, area under the curve extrapolated to infinity, AUC_{extra}, area under the curve from the last quantifiable measurement to infinity; $AUCM_{last}$, area under the moment curve from t =0 to the last measured time point; MRT, mean residence time

Table 3-5: Pharmacokinetic parameters of ciprofloxacin after intravenous and oral enrofloxacin administration (12.5 mg/kg) for each rhinoceros in phase II

Parameter	Units	Animal					Mean	Gmean	SD
		I	II	III	IV	VI			
1	h^{-1}	0.05	0.05	0.04	0.05	0.05	0.05	0.05	0.00
t1/2	h	14.90	15.29	16.54	15.09	12.88	14.94	14.89	1.32
C_{max}	µg/ml	0.63	0.81	0.83	0.72	0.58	0.71	0.71	0.11
T_{max}	h	0.90	6.33	2.00	2.10	2.13	2.69	2.20	2.10
AUC_{last}	µg *h/ml	18.87	24.58	20.33	19.29	15.85	19.78	19.59	3.16
AUC_{tot}	µg*h/ml	19.49	25.56	21.36	19.97	16.12	20.50	20.27	3.42
AUC_{extra}	µg*h/ml	0.62	0.98	1.03	0.68	0.27	0.72	0.65	0.31
AUC_{extra}	%	5.07	6.14	7.74	5.45	2.70	5.42	5.13	1.83
$AUMC_{last}$	µg*$(h)^2$/ml	353.54	486.28	395.22	367.11	299.19	380.27	375.48	68.78
MRT	h	20	20	25	20	20	21	20.91	2.24

λ, terminal elimination rate constant; $t_{1/2}$, half-life; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{last}, area under the curve until the last time point; AUC_{tot}, area under the curve extrapolated to infinity, AUC_{extra}, area under the curve from the last quantifiable measurement to infinity; $AUCM_{last}$, area under the moment curve from t =0 to the last measured time point; MRT, mean residence time

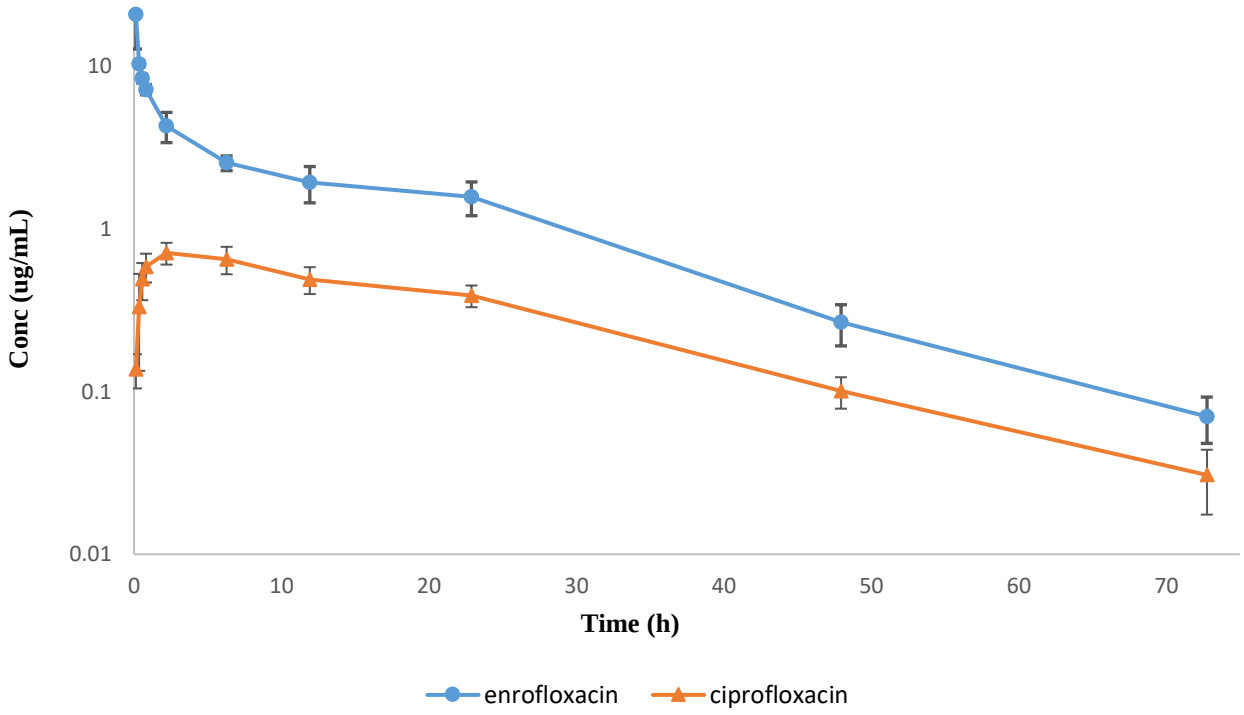


Figure 3-2: Average plasma concentration versus time profile with standard deviation (bars) of all 5 rhinoceros after successive IV and oral administration of enrofloxacin (circle) at 12.5 mg/kg and its ciprofloxacin (triangle) metabolite

3.4.5 Pharmacodynamics of enrofloxacin

The AUC_{24} (AUC_{enro24} + $AUC_{cipro24}$) after administration of 12.5 mg enrofloxacin/kg was 69.88 ± 14.94 µg*h/mL and 76.8 ± 8.86 µg*h/mL following intravenous and intravenous + oral enrofloxacin administration, respectively. Using the susceptibility breakpoint of 0.5 as the MIC value, the AUC_{24}: MIC ratio was 137.32 and 152.83, respectively. The C_{max}: MIC ratio in phase I and II was 28.54 and 41.52, respectively. The AUC_{24} and the AUC_{24}: MIC ratio after oral enrofloxacin administration could not be calculated. However, the semi-logarithmic plot (Figure 3-2) depicts a change in slope after 22.89 hours, which represents the pseudo C_{max} of 1.53 ± 0.37 µg/ml of the additive curve after subsequent intravenous and oral enrofloxacin administration. Thus, the estimated Cmax: MIC ratio of the additive curve is 3.06.

3.5 Discussion

For this study we set out to determine the pharmacokinetics of enrofloxacin and ciprofloxacin in white rhinoceros. Unfortunately, due to the difficulties with the blood flow through the catheters, we had to resort to needle stick bleeds from the cephalic vein for blood draws after 24, 48 and 72 hours. Despite making use of animals habituated to human care in order to maximise the likelihood of sample collection, the endeavour proved too difficult to accomplish without sedation of the animals. As a result, the rhinoceros had to be sedated repeatedly with opioids for the last three sampling points. Opioids are known for their negative effects on the cardiorespiratory system, causing depression of the respiratory centres, hypertension, tachycardia, hypercapnia in combination with respiratory and metabolic acidosis (Buss et al., 2015; Heard et al., 1992; Leblanc et al., 1987; Wenger et al., 2007). Since the sedative agent most likely influenced blood pressure negatively and therewith the pharmacokinetics of the drugs, it may be argued that the profiles attained are not valid. However, we believe that the profiles are indeed valid for clinical pharmacological application in the particular species. It mirrors the situation in the field where the management of wounds of severely injured rhinoceros always requires chemical immobilisation. Also to this regard, a study conducted by Buss et al. (2015) evaluated the cardiorespiratory effect, blood gas and lactate values during a prolonged immobilisation with etorphine, azaperone and additional butorphanol. In this study, they were able to demonstrate that animals could be immobilised for 100 minutes without negative effects. By comparing the heart rates and respiratory rates from our study to those of Buss et al. (2015), we are confident that the animals we immobilised experienced similar cardiorespiratory effects as one would expect in field immobilised animals (Table 3-6).

Table 3-6: Comparison of heart rate (HR) and respiratory rate (RR) from this study with values previously published for animals immobilised under field conditions.

Time (min) after initial blood sample		present study	Buss et.al (2015)
0	HR (beats/min)	115.33	121.46
	RR (breaths/min)	10.83	7.62
10	HR (beats/min	125.20	86.31
	RR (breaths/min)	10.00	6.31
20	HR (beats/min	76.00	80.31
	RR (breaths/min)	8.42	5.92
20-100	HR (beats/min	72.94	71.4
	RR (breaths/min)	9.55	5.6

The published study included ten animals.

When examining the actual pharmacokinetics after intravenous administration, enrofloxacin was characterised by a geometric mean half-life of 12.41 hours, which makes it the longest half-life following intravenous administration reported for this drug in any mammalian species thus far. In comparison, the half-life recorded in adult horses varies between 4.4 hours (Kaartinen et al., 1997) and 6.15 hours (Peyrou et al., 2006). For this reason, we undertook a more detailed evaluation of interspecies scaling of pharmacokinetic parameters, presented in the article '*Is the White Rhinoceros a Large Horse? The Use of Allometry and Pharmacokinetic Modelling to Evaluate the Importance of Interspecies Differences for One of Africa's Iconic Species'* (manuscript submitted for consideration to PLOS ONE). In the mentioned article, we conclude that the substantially longer half-life of enrofloxacin in the rhino cannot be solely explained by a lower metabolic rate relative to size. We suspect that the rhinoceros expresses a high degree of species-specific metabolic capacity that is neither readily extrapolated to their body size nor to their nearest related species being the horse. This difference would most likely result from distinctions in the cytochrome P450 (CYP450) enzyme content, in either enzyme type and/or relative concentrations.

Following intravenous administration of 12.5 mg enrofloxacin/kg with additional oral administration of 12.5 mg enrofloxacin/kg, the AUC extrapolated to infinity was 92.38 ± 12.14 µg*h/ml. The addition of oral enrofloxacin after an average of 10.16 hours resulted in a slight change in the profile compared to that of intravenous treatment alone. We calculated the fraction of absorption as the difference between the AUC_{tot} of the two phases. From this difference, we estimated the absolute bioavailability at 33.3 ± 38.34%, which was highly variable between the treated animals. While the intrasubject variability is evident amongst other species (Haines et al., 2000; Nielsen & GyrdHansen, 1997), the oral absorption was

substantially lower than that reported in domestic animal species and elephants (Bugyei et al., 1999; Küng et al., 1993; Nielsen & GyrdHansen, 1997; Sanchez et al., 2005). In the horse, the bioavailability varied between 78.29% and 55% (Haines et al., 2000; Peyrou et al., 2006), in pigs between approximately 101% in fasted and 83% in fed animals (Nielsen & GyrdHansen, 1997), while in dogs it varied between 63.22% and 100% (Bidgood & Papich, 2005; Küng et al., 1993).

At present, we cannot completely explain the poor oral bioavailability observed. However, based on conventional pharmacokinetic theory, low permeability of the gastrointestinal wall, metabolism of the drug in the gut wall, chemical degradation, physical inactivation, microbial transformation and hepatic first pass effect (Kwan, 1997; Peyrou et al., 2006) could have all contributed to a lowered oral bioavailability. Consequently, we propose the following as the reason for the poor absorption: With regard to the pharmaceutical factors, the poor bioavailability could have resulted from non-specific binding to the food or instability of the drug, neither of which would fit. Previous findings in horses and elephants indicate that enrofloxacin is not affected by non-specific binding to fibre (Lucerne) or molasses. A study in Asian elephants (*Elephas maximus*) showed that the animals treated with 2.5 mg enrofloxacin/kg in the feed consisting of pellets and grain or hay, exhibit an $AUC_{0\text{-}36h}$ of 34.93 ± 10.2 µg*h/mL (Sanchez et al., 2005). Despite the high fibre diet and a dose of only 20% of that given to the rhinoceros, the $AUC_{0\text{-}36h}$ in elephants was greater than 37% of the AUC in the rhinoceros. It is therefore unlikely that fibre binding of enrofloxacin results in the low bioavailability. The same can be said for molasses, as Peyrou et al. (2006) recorded similar AUC values for the fluoroquinolone marbofloxacin mixed with molasses in horses compared to studies which did not use molasses as a vehicle (Bousquet-Melou et al., 2002). Another typical characteristic of non-specific binding of drugs to fibre is a biphasic absorption. In such a case, the plasma concentration versus time profile illustrates a first rapid absorptive phase from the small intestine 1 to 2 hours after feeding and a second delayed absorption phase approximately 8 to 10 hours from the large intestine when the bound drug is released during fibre digestion in the caecum. This feature has been observed in hindgut fermenters such as the horse after phenylbutazone administration, however, was not apparent in this study (Maitho et al., 1986; van Duijkeren et al., 1994).

With regard to animal factors, we doubt that hepatic metabolism could have a major impact on the low bioavailability. In the horse, the hepatic first pass effect results in only 35% of the drug being removed. Furthermore, with the intravenous profile of the rhino clearly showing that the rhino is a slower metaboliser of enrofloxacin, the liver is unlikely to rapidly

remove drugs absorbed through the GIT (Peyrou et al., 2006). In a similar manner, we doubt that the microbial bacterial population could have been responsible for a lower absorption in the rhino than in the horse, as the type and numbers of microbial gut flora are determined by diet (Ilett et al., 1990). If the poor bioavailability was a result of the diet, one should have similar oral bioavailability between the horse and rhino (both hindgut-fermenter feeding on similar diets), which is not the case. This would therefore leave active transport and intestinal membrane permeability as potential reasons for the low bioavailability. The latter have been shown to be actively involved in the secretion of the quinolones into the intestinal lumen (Alvarez et al., 2008). More specific, the ATP-binding cassette (ABC) transporter responsible for the efflux of enrofloxacin is the breast cancer resistant protein (BCRP), which has been identified in species such as humans and horses (Alvarez et al., 2008; El-Kattan & Varma, 2012; Tyden et al., 2010). Further research would be needed to confirm these presumptions and to establish whether the BCRP is found in the gastro-intestinal wall of the rhinoceros and if there is a difference in ABC transporters between the latter and other species with a higher oral bioavailability.

An important feature in the pharmacokinetics of enrofloxacin is the partial transformation into its active metabolite ciprofloxacin, which leads to a simultaneous circulation of both antimicrobials and an additive antimicrobial activity against certain bacteria such as *Pseudomonas aeruginosa* (Blondeau et al., 2012; Lautzenhiser et al., 2001). In the rhino, plasma ciprofloxacin concentration reached 26.42 ± 0.05% and 21.95 ± 0.02% of the plasma concentration of the parent drug. This compared favourably with the horse (20-35%) (Kaartinen et al., 1997), sheep (26%) (Otero et al., 2009) and goat (34%) (Rao et al., 2002). It was however higher than the 10% ciprofloxacin formation reported for the pig and the very low ciprofloxacin formation observed in the elephant (Nielsen & GyrdHansen, 1997; Sanchez et al., 2005). Despite the apparent similarity to the horse, an important difference can be seen with T_{max} of ciprofloxacin, which was in average 0.44 ± 0.06 hours in the horse (Kaartinen et al., 1997) versus the substantially longer 2.1 ± 0.18 hours in the rhinoceros. This indicates once again that while the rhino has the requisite enzyme to metabolise enrofloxacin to ciprofloxacin, this enzyme system either occurs at lower levels in the rhino or consists of enzymes with slower kinetics. Further support for the limitation in metabolic capacity can be seen with the half-life of elimination of ciprofloxacin (11.62 ± 1.28 hours) which was considerably longer than the 5.1 ± 2.1 hours reported for the horse (Kaartinen et al., 1997).

Besides the assessment of the pharmacokinetic properties of enrofloxacin in rhinoceros, pharmacodynamic indices are valuable for the prediction of the ideal dose of the drug and are

used to forecast antimicrobial success. For this evaluation, the AUC_{24}, C_{max} and the time above the MIC are the most relevant three pharmacokinetic parameters, while the most important pharmacodynamic parameter is the MIC (Hyatt et al., 1995). Efficacy marker such as AUC_{24}: MIC and C_{max}: MIC and T > MIC have been identified for the assessment of the treatment outcome. For the concentration dependent fluoroquinolones, the AUC_{24}: MIC and C_{max}: MIC ratios have been found to be correlated with the success of an antimicrobial treatment (Hyatt et al., 1995). Generally, three AUC_{24}: MIC breakpoint values have been determined for the assessment of their efficacy. An AUC_{24}: MIC value below 100 leads to a bacteriostatic effect and can only result in bacterial killing if the host immune system is capable of fighting the infection. Due to the risk of selecting for resistant bacteria, the use of fluoroquinolone doses resulting in a low ratio below 100 have been controversially discussed (Schentag et al., 2003; Thomas et al., 1998). A breakpoint value of 100 – 250 leads to a bactericidal effect at a slow rate, after approximately seven days of treatment. The reaching of the latter is generally recommended in order to ensure a successful treatment outcome. Ratios above the third breakpoint of 250 are preferred and lead to a rapid bactericidal effect and to bacterial eradication within 24 hours (Schentag et al., 2003). For the C_{max}: MIC ratio, a value greater than 8:1 and ideally a ratio greater than 12:1 is required in order to reduce the bacterial numbers by 99%, to inhibit the bacterial regrowth after 24 hours and to reduce the risk of resistance (Blaser et al., 1987).

Toutain (2002), as well as Forrest et al. (1993), postulated the AUC_{24}: MIC ratio to be the most appropriate surrogate marker of activity. In contrast, Drusano et al. (1993) concluded that C_{max}: MIC ratios ≥ 10 or > 20 were the better indicator of a successful outcome, while for C_{max}: MIC ratios < 10, the AUC_{24}: MIC ratio was well correlated to the outcome. As a general MIC value for the evaluation of the efficacy marker in the rhinoceros, the published susceptibility breakpoint for enrofloxacin of 0.5 as determined by the CLSI (CLSI, 2015) was used. At this level, the AUC_{24}: MIC ratio was 137.32 and 152.83 after intravenous and combined intravenous and oral enrofloxacin administration. These findings indicate that in both cases, enrofloxacin administration at a dose of 12.5 mg/kg exceeds the recommended ratio of 100 – 125 and leads to a bactericidal activity against susceptible bacteria. The C_{max}: MIC ratios after a single intravenous enrofloxacin injection and after the combined enrofloxacin treatment were 28.54 and 41.52, respectively. Those results largely exceed the recommended breakpoint values for a successful antimicrobial treatment. With both these surrogate markers being favourably, we conclude that intravenous enrofloxacin treatment would result in effective plasma concentrations. The oral curve did not add enough data for the calculation of the AUC_{24}:

MIC ratio resulting from oral enrofloxacin administration only. However, the pseudo-C_{max} value of the additive curve estimated after subsequent intravenous and oral enrofloxacin administration was 1.53 ± 0.37 µg/ml, leading to a very low C_{max}: MIC ratio of 3.1:1. This ratio is much lower than the recommended ratio of 10 to 12 (Blaser et al., 1987) and indicates that the maintenance of the drug plasma concentration at a therapeutic level through additional oral enrofloxacin administration of 12.5 mg/kg is not feasible if one is aiming for a rapid, bactericidal effect with a low risk of emerging resistance. Previous studies have shown that a C_{max}: MIC ratio below 4 renders a clinical and microbiologic cure more unlikely (Forrest et al., 1993). Moreover, we would also not recommend a single intravenous dose treatment with enrofloxacin. This far, single dose treatment has only been used for bovine and swine respiratory disease (Bayer Veterinary Services, 2003). In contrast, the treatment of skin infections with enrofloxacin in species such as dogs require 5 to 10 days of treatment and a minimum of 3 days has been recommended for cattle and swine (IVS desk reference, 2013).

One potential option for the use of oral enrofloxacin would be to increase the drug dose significantly in order to reach recommended AUC_{24}: MIC ratios. However, such an increase in the drug dose may come with additional risks of adverse drug reactions. As an example, gastrointestinal side effects due to the interference of enrofloxacin with the enteric microflora (Keen & Livingston, 1983; Woodward, 2005) cannot be ruled out and a considerable increase of the dose would require careful clinical evaluation. Overall, due to the surprisingly low bioavailability in rhinoceros, the food or water based medication does not seem to be the solution for a continued antimicrobial treatment. For the best and most reliable therapeutic outcome, a rhinoceros in a captive situation or one that can be kept in an enclosure for follow-up treatment could be re-sedated in form of a low dose butorphanol-based, standing sedation and enrofloxacin could then be re-injected intravenously, provided venous access is possible.

Conclusion

In this study, we assessed the pharmacokinetic properties and efficacy markers of enrofloxacin in white rhinoceros with the aim to evaluate the use of enrofloxacin for the treatment of poaching victims in particular, and any other white rhinoceros requiring antimicrobial treatment. The results were surprisingly different to those in domestic animal species. The outstanding half-life, which is longer than any recorded half-life in mammalian species and the considerably different oral bioavailability, support the conclusion that the rhino is not a ‘big horse’ and should not be treated as such. Plasma concentrations after intravenous enrofloxacin administration at 12.5 mg/kg result in surrogate markers above the recommended

ratio of 125 whereas the maintenance of the drug plasma concentration at a bactericidal level through additional oral enrofloxacin administration does not seem feasible.

3.6 Acknowledgements

We thank the 'The Rhino Orphanage', it's founder Mr. Arrie van Deventer who allowed us to carry out the field work and data collection at 'The Rhino Orphanage' and the staff, in particular Laura Ellison and Jamie Traynor, who helped with the implementation of the field work and the data collection. Furthermore, we would like to thank Dr. Robin Gieling, Aminisha Somers and Klaudia Pruczkowska for their assistance during the field work and Dr. Pierre Bester for the chemical immobilisation and the monitoring of the rhinoceros during the trial. A sincere thank you also goes to the German Academic Exchange Service (DAAD), the University of Pretoria, the South African Veterinary Association (SAVA) and Bayer Animal Health for their financial and material support.

3.7 Supplementary information

S 3-1: Characteristics of the five white rhinoceros used in the pharmacokinetic study listed according to their age

Age (months) First/second trial	Rhino	Sex	Weight (kg) First/ second trial	Dose of enrofloxacin administered (mg/kg)
13/15	Rhino I	Female	527/ 556	12.5
13/15	Rhino II	Male	477/ 538	12.5
17/19	Rhino IV	Male	505/ 551	12.5
18/20	Rhino III	Male	522/ 573	12.5
28/30	Rhino VI	Male	846/ 902	12.5

S 3-2: Placement of the catheters

After the onset of effect of the immobilising drugs, the rhinoceros were blindfolded and walked onto a scale for the accurate determination of their body weights and the calculation of the drug dose. The animals were then walked into air-conditioned night rooms for the better control of the animals' body temperatures. A long-stay catheter (extended use MILACATH, 18ga x 6.25cm/2.5 in, radiopaque polyurethane IV catheter, MILA International Inc) was placed into the auricular vein of the right ear. It was held in place by two single skin sutures (#0 nylon,

reverse cutting needle, Ethicon, Ethilon) and was additionally secured with some tape. An extension set (MILA International Inc) was attached to the catheter and additionally secured with a piece of tape sutured to the ear. A second catheter (18ga, JELCO IV Catheter Radiopaque) was placed in one auricular vein of the left ear for the injection of the intravenous enrofloxacin (12.5 mg/kg) and was removed post enrofloxacin administration. To standardise the injection time, 20 ml of enrofloxacin were administered over a period of roughly 30 seconds. The rhinos were monitored continuously and kept immobilised for a total of two to three hours.

S 3-3: Immobilisation phase I

	Initial drug combination (IM)		**Diazepam**	**Top up**	**Zuclopen-thixol Acetate**	**Dipre-norphine**
	Thia-fentanil	**Etorphine**				
Rhino I	0.3 mg	0.4 mg	10 mg IV after 0.7h	Etorphine 0.25 mg after 0.77h	50 mg IM after 1.13h	3 mg IV after 2.65h
Rhino II	0.3 mg	0.4 mg	10 mg IV after 0.63h	Etorphine 0.3 mg after 0.73h	50 mg IM after 1.2h	0.25 mg IM after 1.35h 3 mg IV after 2.85h
Rhino III	0.5 mg	0.5 mg	10 mg IV after 1.02h	Ketamine 50 mg IV after 1.2h	50 mg IM after 2.43h	0.2 mg IM after 0.35h 3 mg IM after 2.38h
Rhino IV	0.5 mg	0.5 mg			50 mg IM after 2.32h	0.2 mg IV after 0.15h 3 mg IM after 2.3h
Rhino VI	0.33 mg	0.66 mg	10 mg IV after 1.68h		50 mg IM after 2.03h	0.2 mg IV after 0.7h

S 3-4: Immobilisation phase II

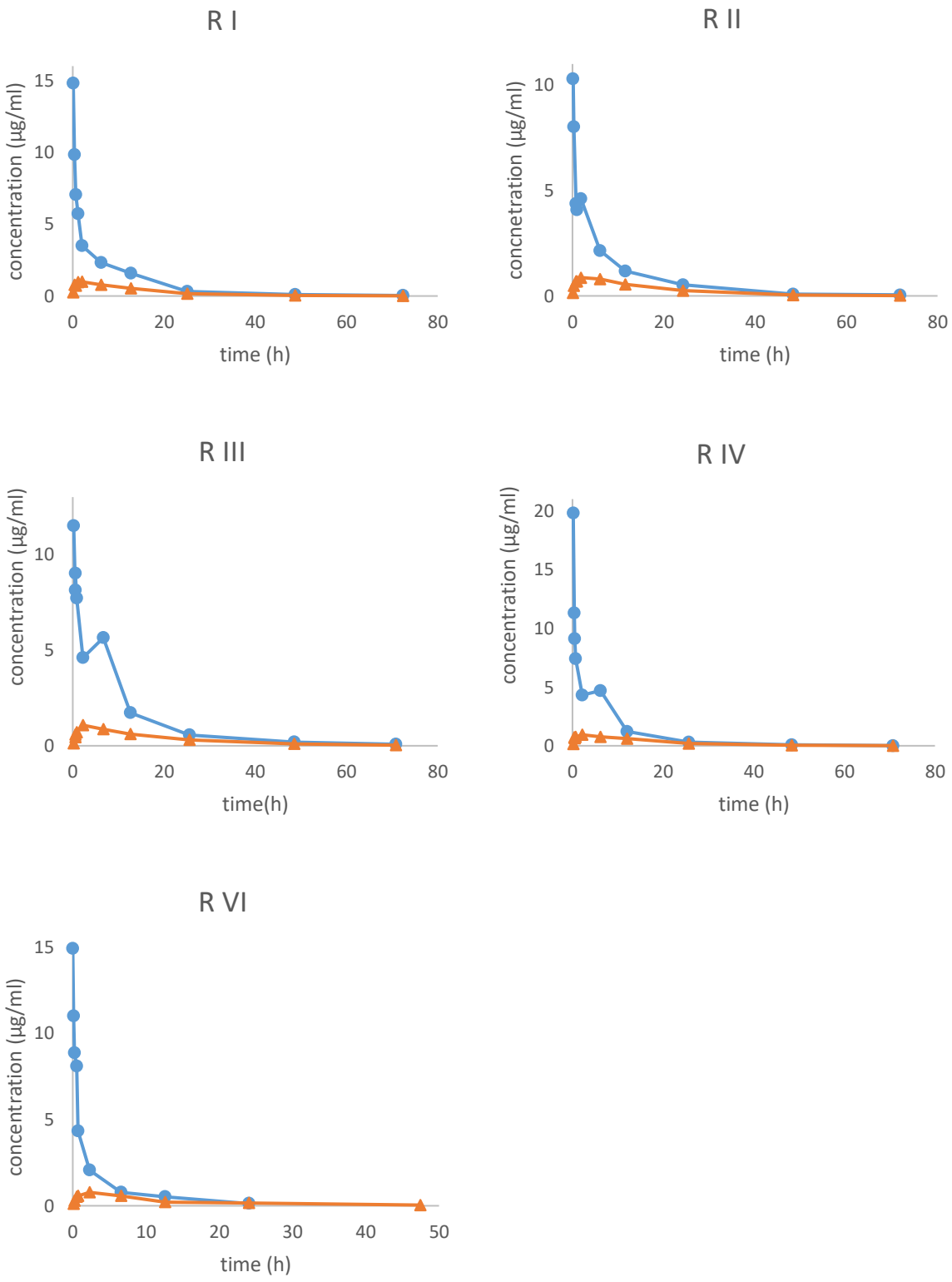
	Initial drug combination (IM)		Top up	Acuphase	Diprenorphine
	Thia-fentanil	**Etorphine**			
Rhino I	0.25 mg	0.5 mg	X	50 mg IM after 1.5h	0.2 mg IV after 0.32h 5 mg IV after 1.88h
Rhino II	0.25 mg	0.5 mg	2 mg Medetomidine IV after 1.68h	50 mg IM after 1.43h	0.2 mg IV after 0.3h 5 mg IV after 1.95h
Rhino III	0.5 mg	0.5 mg	200 mg Ketamine IV after 1.08h 2 mg Medetomidine IV after 1.18h	50 mg IM after 1.72h	0.3 mg IV after 0.33h 0.2 mg IV after 0.5h 5 mg IV after 2.52h
Rhino IV	0.5 mg	0.5 mg	X	50 mg IM after 1.77h	0.2 mg IV after 0.55h 5 mg IV after 2.45h
Rhino VI	0.25 mg	0.5 mg	X	50 mg IM after 2.17h	0.3 mg IV after 0.4h 5 mg IM after 2.12h

S 3-5: Phase I – re-immobilisation for blood sampling after 24, 48, 72 hours

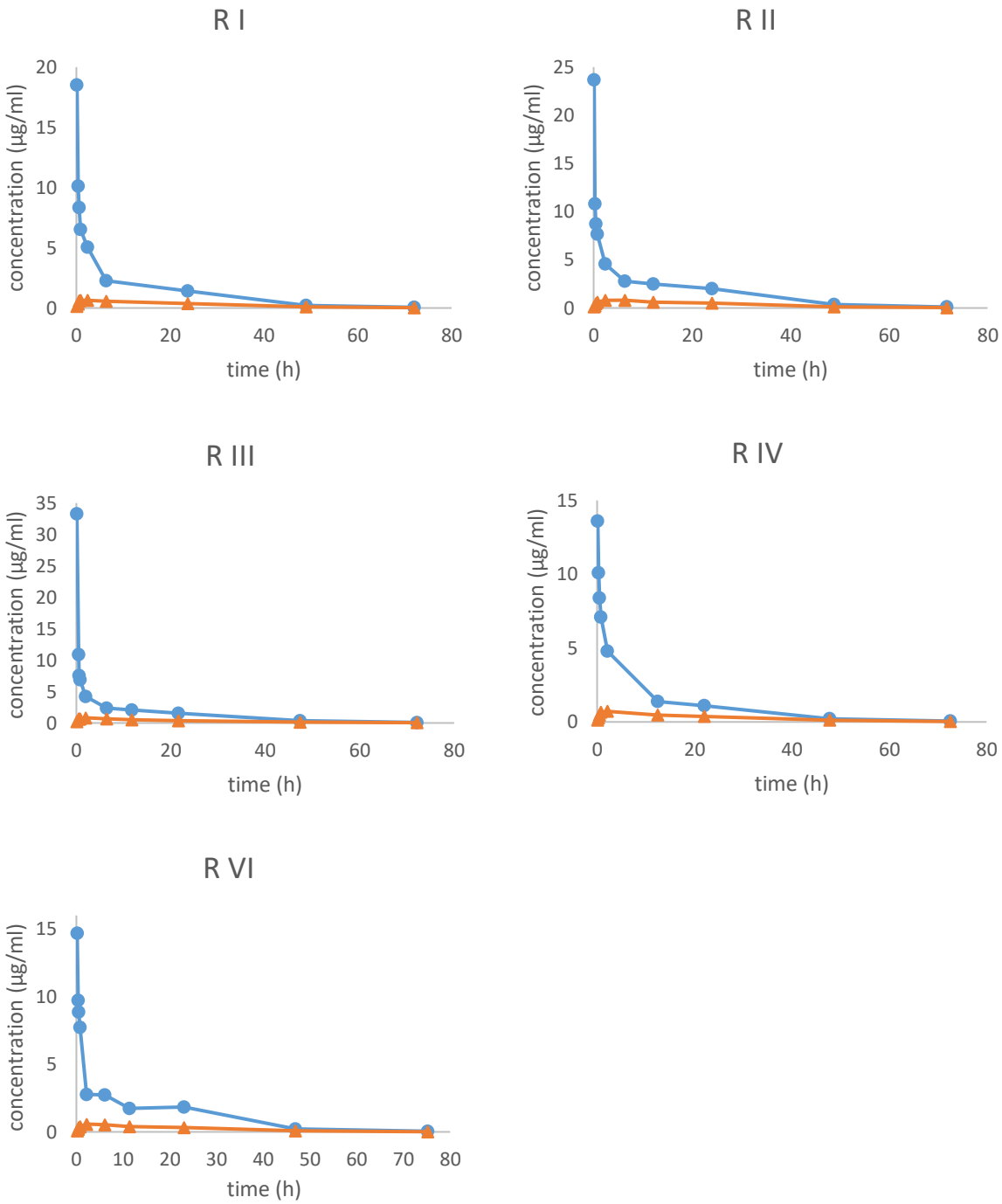
		Sample 9 (24 h)	**Sample 10 (48 h)**	**Sample 11 (72 h)**
Rhino I	Immobilisation	0.5 thiafentanil	0.25 mg thiafentanil 0.5 mg of ethorphine	0.25 mg thiafentanil 0.5 mg etorphine
	Reversal	1 mg of diprenorphine after 0.33h + 5 mg diprenorphine after 0.43h	5 mg diprenorphine after 0.3h	3 mg of diprenorphine after 0.3h
Rhino II	Immobilisation	Without sedation/immobilisation	0.25 mg thiafentanil 0.5 etorphine	0.25 mg thiafentanil 0.5 etorphine
	Reversal		3 mg diprenorphine after 0.35h	3 mg diprenorphine after 0.2h
Rhino III	Immobilisation	0.25 mg thiafentanil 0.5 mg etorphine	0.25 mg thiafentanil 0.5 mg etorphine	0.33 mg thiafentanil 0.66 mg etorphine
	Reversal	3 mg diprenorphine IV after 0.32h	3 mg diprenorphine IM after 0.38h	25 mg naltrexone IM after 0.23h
Rhino IV	Immobilisation	0.25 mg thiafentanil 0.5 mg ethorphine	0.25 mg thiafentanil 0.5 mg ethorphine	0.33 mg thiafentanil 0.66 mg ethorphine
	Reversal	3 mg diprenorphine IV after 0.5h	3 mg diprenorphine IM after 0.28h	25 mg naltrexone IM after 0.23h
Rhino VI	Immobilisation	0.5 mg thiafentanil 0.5 mg etorphine	0.33 mg thiafentanil 0.66 mg etorphine	No sample
	Reversal	3 mg diprenorphine after 0.43h	25 mg naltrexone after 0.3h	

S 3-6: Phase II - sedation for blood sampling after 24, 48, 72 hours with all samples drawn from the cephalic vein

	Sample 9 (24 h)	**Sample 10 (48 h)**	**Sample 11 (72 h)**
Rhino I	10 mg butorphanol IV	15 mg butorphanol IM (catheter not functional)	15 mg butorphanol IM (catheter not functional)
Rhino II	15 mg butorphanol IV, 5 mg top up IV 0.5h later	15 mg butorphanol IV	12 mg butorphanol IV
Rhino III	15 mg butorphanol IV	10 mg butorphanol IV	10 mg butorphanol IM (catheter not functional)
Rhino IV	15 mg butorphanol IV	10 mg butorphanol IV	10 mg butorphanol IV
Rhino VI	30 mg butorphanol IV	20 mg butorphanol IV	15 mg butorphanol IV



S 3-7: Plasma concentration versus time profile for each rhinoceros after IV administration of enrofloxacin (circle) at 12.5 mg/kg and its ciprofloxacin (triangle) metabolite



S 3-8: Plasma concentration versus time curve for each rhinoceros after IV and subsequent oral administration of 12.5 mg/kg enrofloxacin (circle) and its ciprofloxacin metabolite (triangle)

4. Chapter 4: Is the White Rhinoceros a Large Horse? The Use of Allometry and Pharmacokinetic Modelling to Evaluate the Importance of Interspecies Differences for One of Africa's Iconic Species

Allometric pharmacokinetic scaling of enrofloxacin for the white rhinoceros

Marion Leiberich[1*], Ralph Krebber[2], Michael Hewetson[1], Johan Marais[3], Vinny Naidoo[4]

[1] Department of Companion Animal Clinical Studies, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[2] Bayer Crop Science AG, Research & Development - Regulatory Science - Human Safety – Residue Analysis, Monheim am Rhein, Germany

[3] Onderstepoort Veterinary Academic Hospital, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[4] Department of Paraclinical Science, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

*Corresponding author

E-mail: u13404106@tuks.co.za (ML)

4.1 Abstract

Africa is characterised by its abundant and unique species of wildlife, which are of huge conservation importance. However, very little veterinary information is available to support the treatment of enigmatic species such as rhinoceros (*Rhinocerotidae*), despite their need for intervention due to poaching and other injuries. For this study, we investigated the value of allometric scaling for the extrapolation of pharmacokinetic parameters from different species to the rhinoceros. We used enrofloxacin as our study drug and compared the in-vivo pharmacokinetics obtained from six subadult white rhinoceros (*Ceratotherium simum*) to predictive values based on previously published allometric equations, to values from generated predictive equations based on monogastric and herbivorous species and to species with a similar enrofloxacin conversion rate to the white rhino. Therefore, the pharmacokinetic data for seven monogastric species, eight herbivorous species and four species with a similar enrofloxacin metabolism to the rhino obtained from the literature was correlated to the body weight using the function $Y= a*W^b$, where "Y" represents the parameter of interest. "W" is the body weight,

"a" is the allometric coefficient and "b" is the allometric exponent. The observed half-life (11.89 ± 2.96 h), clearance (0.2 ± 0.03 L/h/kg), volume of distribution at steady state (2.12 ± 0.48 L/kg) and volume of distribution during terminal phase (3.34 ± 0.9 L/kg) in the rhinoceros could not be accurately predicted by any of the applied models. No specific grouping of species by features such as physiological traits improved the predictability. Despite the goodness of fit of the model to the data from monogastric species, the predicted half-life of 7.2 h in the rhinoceros was underestimated by 39%. This leads to the conclusion that allometric scaling is not all that helpful for the white rhinoceros as it exhibits a species specific metabolic capacity, which appears to be non-scalable.

4.2 Introduction

Despite growing human populations and the re-tasking of land towards agricultural and industrial developments in the place of natural habitats, Africa is still characterised by its abundant and unique species of wildlife. While the most popular species associated with Africa are the "Big Five" including elephant (*Loxadanta Africana Africana*), leopard (*Panthera pardus*), buffalo (*Syncerus caffer*), lion (*Panthera leo*) and rhinoceros (*Rhinocerotidae*), Africa is home to many more species of various sizes and habitats. Alarmingly, this diverse range of species is at different risks of extinction (Emslie et al., 2016). The rapid decline of wildlife and the extinction of many wildlife species emphasise the necessity of veterinary interventions in order to ensure wildlife health, to correct ecosystem dysfunction and to promote species survival. This is of great importance for the preservation of biodiversity, for conservation purposes, but also to support a healthy wildlife industry in order to ensure the sustainable development of the continent. The breeding of wildlife species for game ranching and in captive facilities, the existence of rehabilitation centres for injured animals and institutional and private zoological collections has also increased the need of veterinary attention and treatment of various wild animal species in human care. Unfortunately, current veterinary medicine is not necessarily optimised for wild species, with the result that many of these wild species are treated using information extrapolated from domestic species. The literature indicated, that the white rhinoceros is currently one such important and threatened species. The so-called charismatic mega-herbivore is the second biggest living terrestrial species, attracting many local and foreign tourists every year (Lehohla, 2015). Once abundant, the white rhinoceros was close to extinction at the end of the 19th century with less than 100 animals left in KwaZulu-Natal, South Africa due to malicious poaching of the animals for their horns (Amin et al., 2006).

However, intense rescue and conservation efforts and translocations of individuals were then effectively implemented to save the species. The endeavour was successful in re-establishing the dwindling population to a level that the number of white rhinos reached 20,170 at the end of 2010 (Emslie, 2012). Unfortunately, the poaching started again, with thousands of rhinos killed in the last ten years, and has reached such an extent that the white rhino is once again threatened. In addition to the injudicious slaughter of the animal for their horn, rhinos are often left alive and injured by the poachers after their horns have been hacked off. Victims of poaching incidents often suffer from gun-shot wounds or from wounds situated on the frontal and nasal bone after the brutal removal of the horn and require veterinary treatment against both, infection and pain (J. Marais, personal communication, 2016). Despite the need to treat these rhinos for their injuries, very little is known about their veterinary management and the correct application of drug dosages. For the rhinoceros, and for most other wildlife species, much needed information on medication is not available in the literature.

Unlike for domestic animal species, where pharmaceutical companies are willing to invest substantially in optimising treatment, such information is lacking for most of Africa's wildlife with the result that in most cases the dosages from domestic species are applied. For the rhinoceros, is it generally assumed that they would be closely related to the horse due to the similar anatomy and common physiology of their gastrointestinal tract (Clauss et al., 2005; Oftedal et al., 1996). However, differences in body mass, metabolism and clearance rates could result in inaccurate calculations leading to overdosing and toxic effects, or potentially to a reduced therapeutic effect of the drug. A better means of predicting the effective dose in wildlife, which is both feasible and cost effective, needs to be established. One method by which this may be achieved would be to use a combination of antimicrobial pharmacodynamics theory and allometric scaling. In pharmacodynamic theory, the efficacy of the antimicrobials is linked to the concentration of the drug in the plasma (Craig, 1998). In general, there are two types of effects. For time dependent drugs, the important factor is the time the drug stays above the MIC (T>MIC). In concentration dependent drugs, the effect is linked to total concentrations achieved in plasma in a defined period of time relative to the MIC (AUC24: MIC). Currently, the pharmacodynamic properties of the commonly used antimicrobials have been established. As evident from above, the plasma concentrations of the antimicrobial in question need to be established and could be predicted through allometric pharmacokinetic scaling. In allometry, biological functional systems are evaluated and described mathematically by using allometric equations (Sharma & McNeill, 2009). Pharmacokinetic scaling is based on the idea that the pharmacokinetics of a drug are not linear to the weight of the animal, but that the change in a

pharmacokinetic parameter correlates with the change in weight. Thus, instead of scaling the drug dose, pharmacokinetic parameters are scaled allometrically in relation to the weight.

For this study, we assessed the value of allometric scaling as a predictive tool in the white rhinoceros. We specifically selected the treatment of wounds as our aim, as poaching results in large wounds requiring regular veterinary management. While information on rhino wound infections is lacking, limited samples submitted to the bacteriology laboratory of the Faculty of Veterinary Science, University of Pretoria, indicate that infections in rhinoceros may be caused by *Staphylococcus aureus* and other *Staphylococcus* spp, *Escherichia coli*, *Salmonella enteridis*, *Streptococcus* spp, *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Enterobacter cloacae* and *Arcanobacterium pyogenes.* From the literature, we believe that the second generation fluoroquinolone enrofloxacin would be a suitable antibiotic as the drug is particularly effective against Gram-negative bacteria and exhibits a rapid bactericidal, concentration dependent effect. The published MIC_{90} values range from 0.03 to 0.125 µg/ml for *E.coli* (Giguère & Dowling, 2013), 1 µg/ml for *Streptococcus* spp (Boothe et al., 2006) and 0.12 µg/ml for *Staphylococcus* spp (Sanchez et al., 2005). Lastly, enrofloxacin is also available as an oral and parenteral formulation, potentially making it more advantageous for the use in wild species, which are difficult to approach and to medicate.

Overall, allometric scaling of the clearance and the volume of distribution of enrofloxacin across monogastric species resulted in significant correlations between the pharmacokinetic parameters and the animals' body weights. However, despite the goodness of fit of the model, the accurate prediction of the rhinos' pharmacokinetic parameters could not be achieved.

4.3 Results

4.3.1 Pharmacokinetics of enrofloxacin in white rhinoceros

The results of the pharmacokinetic study of enrofloxacin in white rhinoceros presented as mean (± SD) revealed a half-life of elimination ($t_{1/2}$) of 11.89 (± 2.96) hours, a total body clearance (Cl) of 0.20 (± 0.03) L/h/kg and a volume of distribution during terminal phase (V_z) and in steady state (V_{ss}) of 3.34 (± 0.90) L/kg and 2.12 (± 0.48) L/kg, respectively. The pharmacokinetic parameters for each rhino are summarised in S 4-1. The plasma concentration-time profile data for each rhino is depicted in S 4-2. Figure 4-1 illustrates the graphs of the plasma concentration versus time profile for each rhino. The conversion of enrofloxacin to ciprofloxacin based on the AUC_{last} was estimated at 26.42 ± 0.05%.

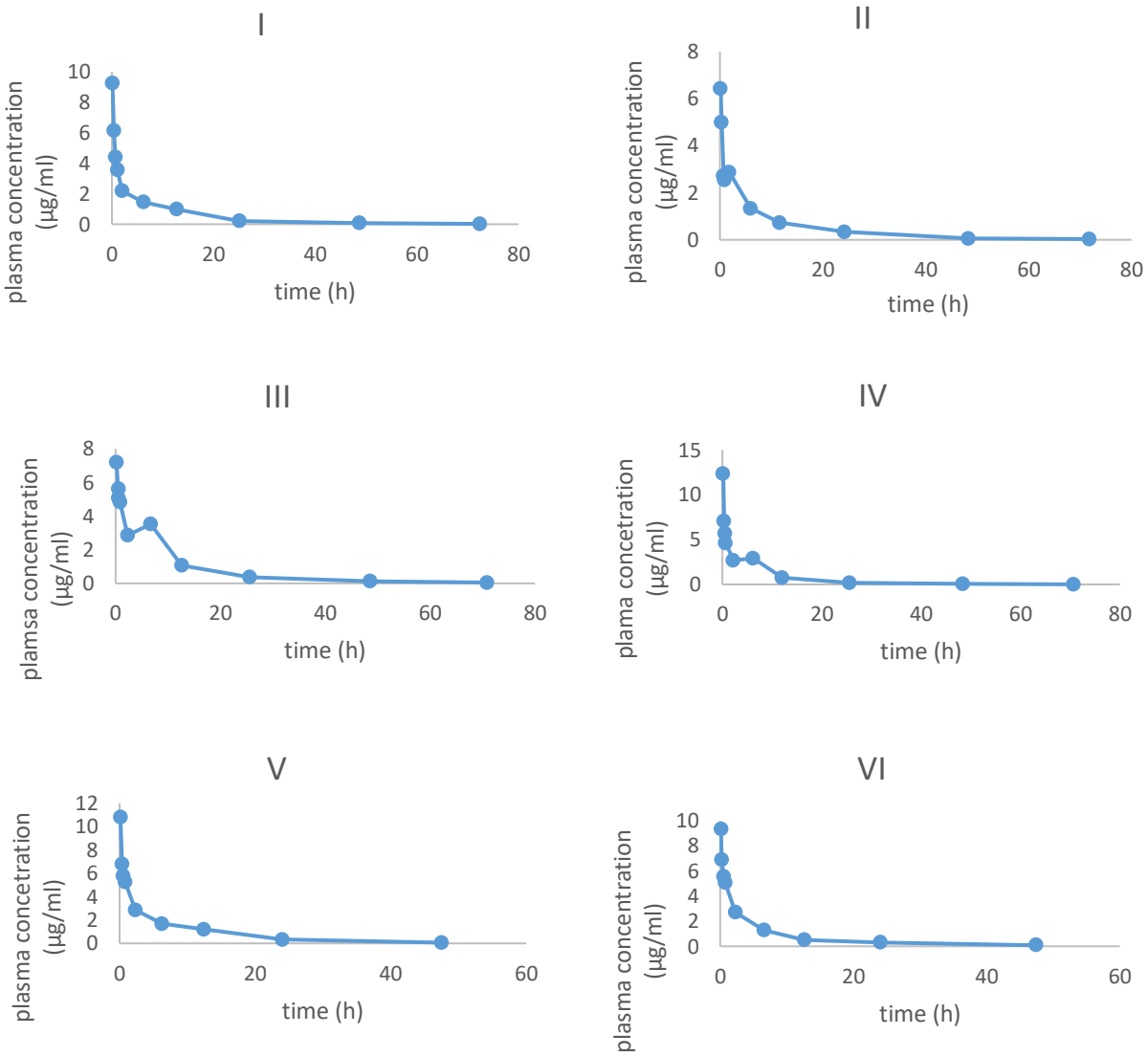


Figure 4-1: Plasma concentration *vs* time profiles of six white rhinoceros (rhino I – rhino VI) after intravenous enrofloxacin injection (corrected to 7.8 mg/kg)

4.3.2 Allometric scaling of enrofloxacin across monogastric species

The double logarithmic graphs of the pharmacokinetic parameter versus the body weight showed a statistically significant relationship ($P < 0.05$) between the clearance ($P < 0.001$), the volume of distribution during terminal phase ($P < 0.001$), the volume of distribution at steady state ($P < 0.001$), and the body weight of the various animals included in the scaling (Figure 4-2). Coefficients of determination (R^2) for the clearance, volume of distribution at steady state and during terminal phase were 0.968, 0.988 and 0.996, respectively. In contrast, the half-life of elimination was characterised by a a coefficient of determination of 0.556 and a p-value of 0.054 in relationship to the body weight. While this was non-significant as per set criteria of $P < 0.05$, this was nonetheless taken as a sufficient indication of significance, due to the closeness of the final value. In addition, there was no correlation between the predicted value and the study's experimental value at the individual animal level (S 4-3). The coefficients and exponents as well as the coefficients of determination and the significance of the allometric equations of all models are summarised in Table 4-1. Based on the equations, the estimated clearance, volume of distribution at steady state, volume of distribution during terminal phase and half-life of elimination in the rhinos were 0.47 (± 0.02) L/h/kg, 4.53 (± 0.07) L/kg, 4.5 (± 0.04) L/kg and 7.25 (± 0.3) h, respectively (Table 4-2). The allometric equations were best characterised as follows: $t_{1/2} = 2.74*W^{0.15}$; $Cl = 1.25*W^{0.85}$; $V_z = 5.66 * W^{0.96}$; $V_{ss} = 3.19*W^{1.05}$ with 'W' being the body weight in kg. The graphs depicting the allometric relationship between the pharmacokinetic parameter half-life of elimination, clearance, volume of distribution at steady state and during terminal phase versus the body weight of seven monogastric species are presented in Figure 4-2. No overlap was present between the 95% confidence interval for the predicted value when compared to the study's experimental values (Figure 4-2).

Table 4-1: Summary of the coefficients and exponents of the allometric equations as well as the coefficient of determination and the calculated significance for the different models based on 11 mammalian species and 15 species including non-mammalian species, 5 mammalian species, herbivore species, monogastric species and species with a similar enrofloxacin conversion rate to the white rhino

Grouping	Parameter	Coefficient	Exponent	Coefficient of Determination (R^2)	Significance (P)
11 mammalian species	Cl	15.9	0.76	0.594	< 0.001
	$t_{1/2}$	4	0.06	0.022	0.415
	Vss	6	0.72	0.650	< 0.001
15 species including non-mammalian species	Cl	7.2	0.94	0.797	< 0.001
	$t_{1/2}$	6.8	-0.06	0.036	0.241
	Vss	4.11	0.80	0.818	< 0.001
5 mammalian species	Cl	47.9	0.82	0.992	<0.01
	$t_{1/2}$	115.6	0.06	0.976	<0.001
	Vz	10.9	0.9	0.988	<0.001
8 herbivore species	Cl	1.91	0.96	0.878	< 0.001
	$t_{1/2}$	1.43	0.13	0.164	0.320
	Vss	5.35	0.91	0.765	0.004
7 monogastric species	Cl	1.25	0.85	0.968	< 0.001
	$t_{1/2}$	2.74	0.15	0.556	0.054
	Vss	3.19	1.05	0.988	< 0.001
	Vz	5.66	0.96	0.996	< 0.001
4 species with similar enrofloxacin conversion rate	Cl	1.54	0.84	0.881	0.060
	$t_{1/2}$	0.72	0.38	0.368	0.390
	Vss	1.39	1.16	0.987	0.007

Table 4-2: Pharmacokinetic parameters (mean ± standard deviation) estimated from an in vivo study in 6 white rhino at a dose of 7.8 mg/kg in comparison to parameters obtained from the different predictive equations and the predictive error (in parenthesis)

Parameter	Experimental values	Predictive equation based on					
		7 monogastric species	8 herbivore species	4 species with similar ciprofloxacin conversion	5 mammalian species	11 mammalian species	15 species including non-mammalian species
$t_{1/2}$ (h)	11.89 ± 2.96	7.25 ± 0.30 (39.02)	3.29 ±0.12 (72.33)	8.09 ± 0.85 (31.96)	2.83 ± 0.05 (76.2)	5.95 ± 0.10 (49.96)	4.57 ± 0.08 (61.56)
Cl (L/h/kg)	0.2 ± 0.03	0.47 ± 0.02 (-135.0)	1.47 ± 0.02 (-635.38)	0.57 ± 0.02 (-183.75)	0.91 ±0.04 (-355.0)	0.21 ± 0.01 (-5.0)	0.29 ± 0.005 (-45.0)
V_z (L/kg)	3.34 ± 0.9	4.5 ± 0.04 (-34.73)			5.73 ± 0.15 (-71.56)		
V_{ss} (L/kg)	2.12 ± 0.48	4.53 ± 0.07 (-113.68)	2.91 ± 0.07 (-37.26)	3.82 ± 0.16 (-80.19)		1.03 ± 0.07 (51.42)	1.17 ± 0.06 (44.81)
Source	Six treated rhino	Estimated in this study	Estimated in this study	Estimated in this study	Bregante et al., 1999	Cox et al., 2004	Cox et al., 2004

Values in parenthesis are the predictive error (%); < 30 % difference is considered acceptable, 31 – 50% as a fair prediction, while errors above 51% were rated as poor predictions (Goteti et al., 2010; Mahmood, 2001). A negative sign indicates that the predicted value was higher than the actual pharmacokinetic value.

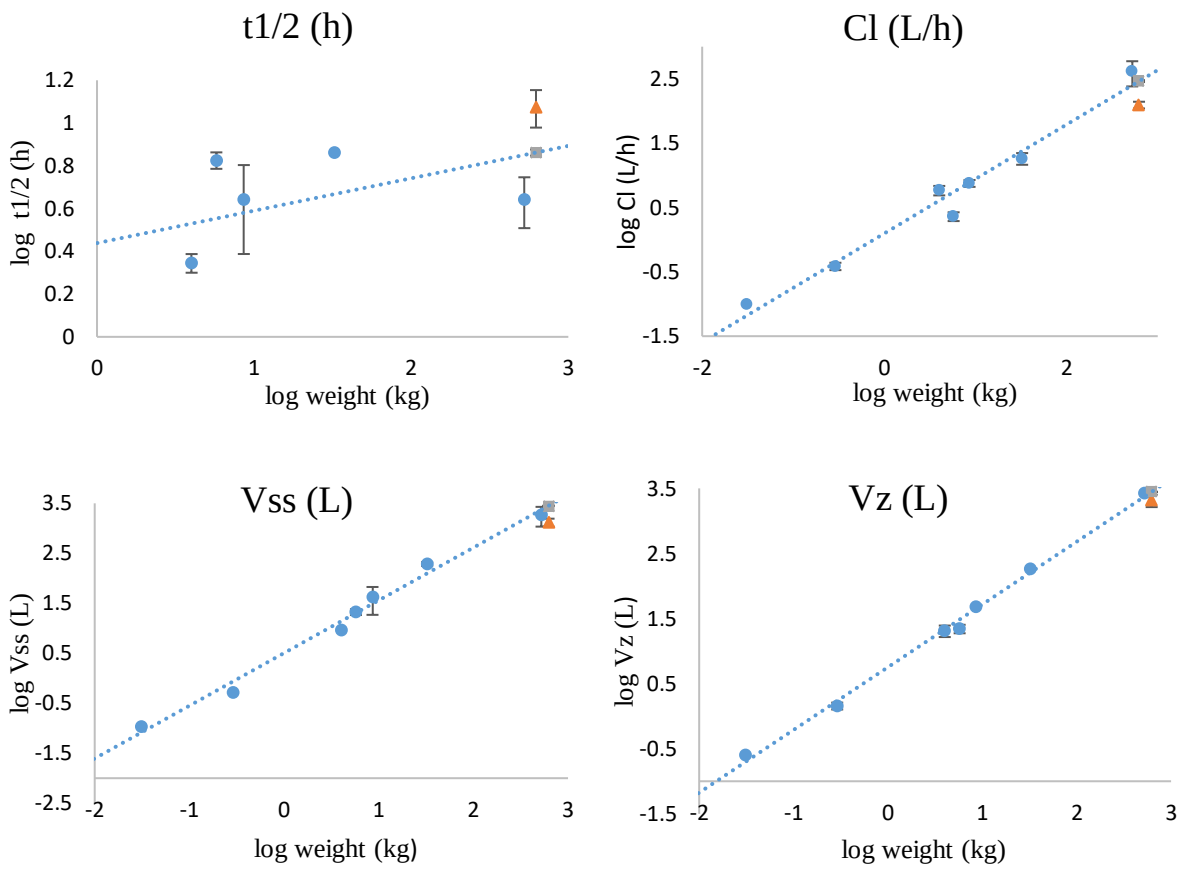


Figure 4-2: Allometric association between half-life of elimination ($t_{1/2}$); clearance (Cl); volume of distribution at steady state (V_{ss}) or volume of distribution during terminal phase (V_z) of enrofloxacin versus body weight of 7 different monogastric species on a double logarithmic plot. The species represented in the graphs (from left to right) are mouse, rat, rabbit, cat, dog, pig and the horse (circles). The best fit equations for $t_{1/2}$, Cl, V_z and V_{ss} were log $t_{1/2}$ = 0.15x + 0.44 (R^2 = 0.556); log Cl = 0.85x + 0.1 (R^2 = 0.968); log V_z = 0.96x + 0.75 (R^2 = 0.996) and log V_{ss} = 1.05x + 0.50 (R^2 = 0.988), respectively. The square is the predictive value for the rhinoceros, while the triangle is the actual pharmacokinetic parameter from the rhinoceros. The error bars depict the 95% confidence interval

4.3.3 Allometric scaling across herbivorous species

The relation between the pharmacokinetic parameters and the body weight of herbivorous species, plotted as double logarithmic scale (Figure 4-3), showed a statistically significant correlation (P< 0.05) between the clearance (P < 0.001) and the body weight and further between the volume of distribution at steady state (P = 0.004) and the body weight. The coefficients of determination (R^2) for the clearance and volume of distribution at steady state were 0.878 and 0.765, respectively. The coefficient of determination of the half-life of elimination was 0.164 and, in accordance with the other scaling approaches, revealed a lack of correlation with the body weight (P = 0.32). The graphs of the best fit equations for Cl, V_{ss} and $t_{1/2}$ are presented in Figure 4-3. Based on the allometric equations estimated for herbivore

species, the value of the clearance, the volume of distribution at steady state and half-life of elimination in the rhinos were 1.47 (± 0.02) L/h/kg, 2.91 (± 0.07) L/kg and 3.29 (± 0.12) h, respectively (Table 4-2). The allometric equations for scaling across herbivorous species can be set up as follows: $t_{1/2} = 1.43*W^{0.13}$; $Cl = 1.91*W^{0.96}$; $V_{ss} = 5.35*W^{0.91}$ with 'W' being the body weight in kg. No overlap was present between the 95% confidence intervals of the predicted and experimental values (Figure 4-3) and no correlation was determined between the predicted value and the study's experimental value at the individual animal level (S 4-3).

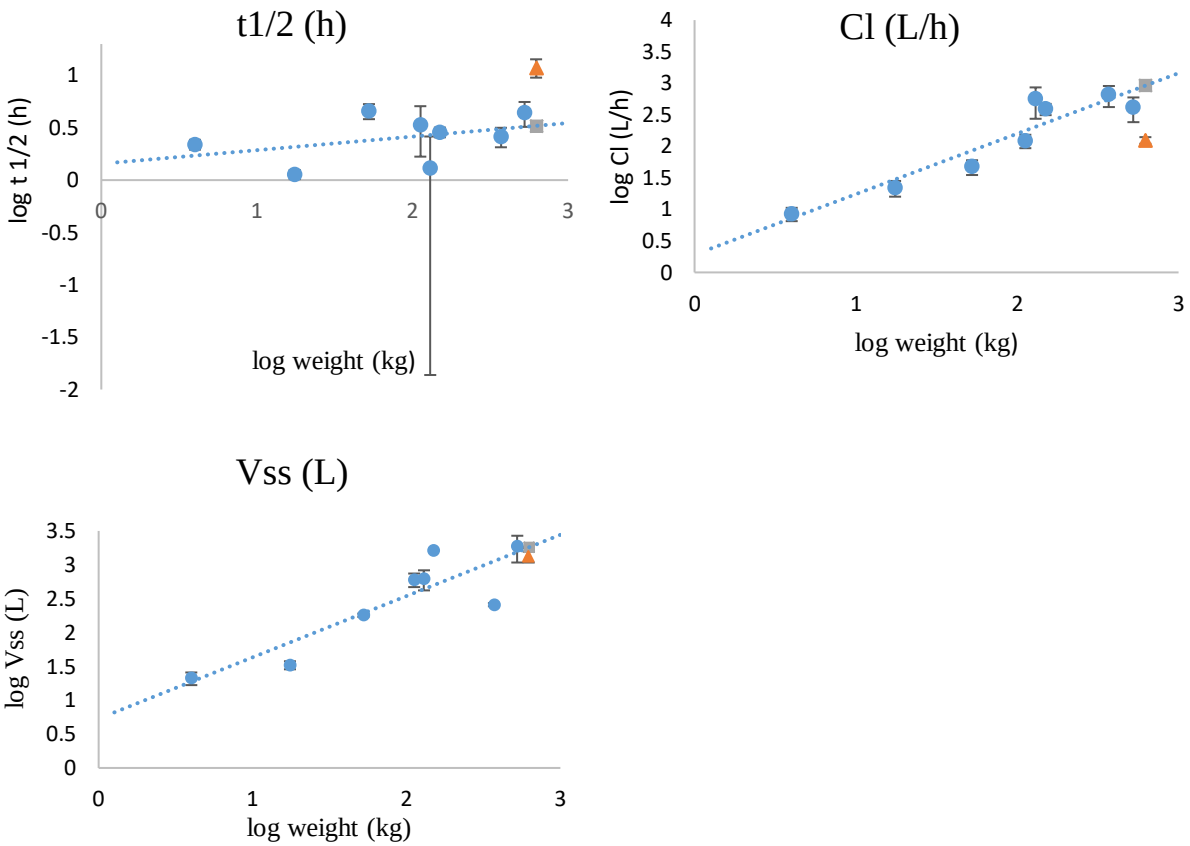


Figure 4-3: Allometric association between half-life of elimination ($t_{1/2}$); clearance (Cl) and volume of distribution at steady state (V_{ss}) of enrofloxacin versus body weight of 8 different herbivore species as double logarithmic plot. Species included in the graphs are from left to right: rabbit, goat, sheep, llama, oryx, buffalo calf, cow, horse (circles). The best fit equations for $t_{1/2}$, Cl, V_{ss} and were log $t_{1/2}$ = 0.13x + 0.16 (R^2 = 0.164), log Cl = 0.96x + 0.28 (R^2 = 0.878) and log V_{ss} = 0.91x + 0.73 (R^2 = 0.765), respectively. The square is the predicted rhinoceros value while the triangle is the actual measured value from rhino. The error bars depict the 95% confidence interval

4.3.4 Allometric scaling of enrofloxacin across species with a similar conversion rate of enrofloxacin

The double logarithmic graph of the pharmacokinetic parameter versus the body weight exhibited significant statistical correlation ($P<0.05$) between the volume of distribution at steady state and the body weight ($P = 0.007$). The correlation between the clearance and the body weight ($P = 0.06$) and further between the half-life of elimination and the body weight ($P = 0.393$) was not statistically significant. The coefficients of determination of the half-life, the clearance and the volume of distribution at steady state were 0.368, 0.881 and 0.987, respectively (Table 4-1). Based on the allometric equations, the estimated $t_{1/2}$, Cl and V_{ss} for the white rhino were 8.09 (± 0.85), 0.57 (± 0.02) and 3.82 (± 0.16), respectively. The allomeric equations for scaling across species with a similar enrofloxacin conversion rate were best characterised as follows: $t_{1/2} = 0.72*W^{0.38}$, $Cl = 1.54*W^{0.84}$, $V_{ss} = 1.39*W^{1.16}$, with 'W' being the body weight in kg. The graphic presentation of the allometric relationship between the pharmacokinetic parameter versus the body weight is depicted in Figure 4-4. No overlap was present between the 95% confidence intervals for the predicted and experimental values. Furthermore, there was no correlation between the predicted value and the study's experimental value at the individual animal level (S 4-3).

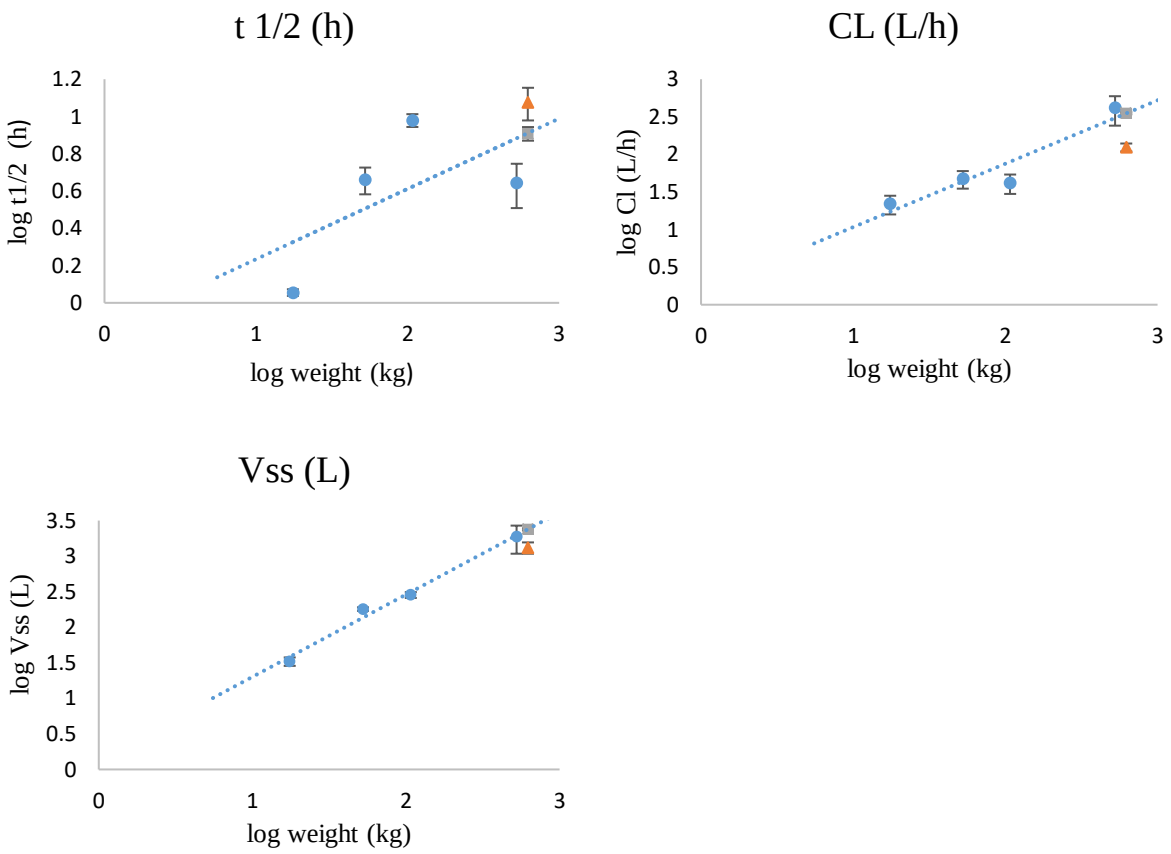


Figure 4-4: Allometric association between half-life of elimination ($t_{1/2}$); clearance (Cl); volume of distribution at steady state (V_{ss}) of enrofloxacin versus body weight of 4 different species with similar enrofloxacin conversion rates to the rhino on a double logarithmic plot. The species represented in the graphs (from left to right) are goat, sheep, donkey and the horse (circles). The best fit equations for $t_{1/2}$, Cl, V_z and V_{ss} were log $t_{1/2}$ = 0.38x – 0.14 (R^2 = 0.368); log Cl = 0.84x + 0.19 (R^2 = 0.881) and log V_{ss} = 1.16x + 0.14 (R^2 = 0.987), respectively. The square represents the predictive value for the rhinoceros, while the triangle represents the actual pharmacokinetic parameter from the rhinoceros. The error bars depict the 95% confidence interval.

4.3.5 Comparison of the different allometric scaling approaches, published and calculated

The estimated and actual pharmacokinetic parameters and predictive errors are presented in Table 4-3 for the three published equations of Cox et al. (2004) and Bregante et al. (1999). Neither scaling across the five species of Bregante et al. (1999) nor the 15 species of Cox et al. (2004) led to any accurate prediction of the rhinos' pharmacokinetic parameters, while scaling across mammalian species (Cox et al., 2004) led to an accurate prediction of the clearance (prediction error 5%). Scaling across only monogastric species led to a fair prediction of the volume of distribution during terminal phase (prediction error 34.73%) and the half-life of elimination (prediction error 39.02%). Despite the greater degree of relationship of the rhinos to herbivores, scaling across herbivore species did not lead to a high predictability of the pharmacokinetic parameter, with only the predicted volume of distribution at steady state being near acceptable (37.26% prediction error). Scaling across species which exhibit a similar enrofloxacin conversion rate to the white rhino revealed a fair prediction of the half-life of elimination (31.96% prediction error), but failed to predict the clearance as well as the volume of distribution accurately (Table 4-2).

Table 4-3: The different published allometric equations for enrofloxacin used in the following study

Method	$t_{1/2}$	V_{ss} (L)	V_z (L)	Cl (ml/min)	Reference
Scaling across 5 mammalian species	$= 115.6*W^{0.06}$ (min) $R^2 = 0.976$		$= 10.87*W^{0.9}$ $R^2 = 0.988$	$= 47.9*W^{0.82}$ $R^2 = 0.992$	Bregante et al., 1999
Scaling across 11 mammalian species	$= 4*W^{0.062}$ (h) $R^2 = 0.022$	$= 6*W^{0.724}$ $R^2 = 0.650$		$= 15.9*W^{0.764}$ $R^2 = 0.594$	Cox et al., 2004
Scaling across 15 species incl. non-mammalian species	$= 6.8*W^{-0.062}$ (h) $R^2 = 0.036$	$= 4.11*W^{0.803}$ $R^2 = 0.818$		$= 7.2*W^{0.939}$ $R^2 = 0.797$	Cox et al., 2004

The scaling approach across five mammalian species included cows, sheep, rabbits, rats and mice in the calculations. The scaling approach across 11 mammalian species was based on the cow, pig, sheep, rabbit, dog, Llama, cat, camel, horse, goat and buffalo. The scaling approach across 15 species including non-mammalian species additionally comprised chicken, emu, Houbara bustard and Atlantic salmon. The R^2 values indicate the goodness of fit of the published model.

4.4 Discussion

As a result of an increase in human inflicted injuries (poaching, snares and gunshot wounds) in wildlife and the necessity to treat captive-held, sick or injured individuals in zoological gardens, rehabilitation facilities or in their natural habitat, measures need to be in place to optimise treatment and veterinary knowledge on drug doses. Unfortunately, despite the need for this optimisation, the treatment of wild animal species represents a big challenge due to the difficulty to simply approach, sample and handle these individuals. Stress, caused by close human interaction, physical restraint and chemical immobilisation can alter the study results. For rhinoceros, veterinarians often extrapolate doses using the horse as the prototype species. In other cases, doses have been guessed or have been based on experiences. While this can be an effective manner of dose determination, this rough estimation can be potentially disastrous. This is best illustrated with the tragic story of Tusko, the Asian elephant, who was given lysergic acid diethylamide (LSD). Estimated from the dose recommended for cats (1.5 mg/kg), he was administered 1 mg/kg of LSD, thus a total dose of 297 mg. Tusko died after the injection in a stage of convulsions (Mahmood, 2007). With many of these high value and endangered wildlife species currently being managed in South Africa, rough estimations of the dose are no longer appropriate. In order to facilitate better estimations, three different methods are available, namely linear extrapolation, metabolic scaling and allometric scaling.

Linear scaling extrapolates a dose for one species across species linearly with the increase in body weight without considering species-specific pharmacologic differences. Metabolic scaling is based on the ratio of a known physiologic process or anatomic feature such as the metabolic rate or body surface area of two species for the calculation of a dosage estimate in the new species. In contrast to linking the dosage to the body weight, this method links the dosage in a species to a physiologic function under the assumption that the interspecies differences in pharmacokinetic and pharmacodynamic properties are not clinically relevant. This method has not been validated for veterinary medicine nor has it been used for human drug extrapolations. It implies that the metabolic rate can be applied to most drugs and oversimplifies the relationship between the basal metabolic rate and the drug pharmacokinetics (Hunter, 2008). Furthermore, dose conversion using the body surface area has been described in the literature. The doses-by-factor method uses the exponent for body surface area (0.67), which accounts for the differences in metabolic rate to convert doses between animals and humans. It has been used to determine the initial dose as a safe starting point for human dose trials (Nair & Jacob, 2016). However, this method has not been validated for the calculation of a therapeutic drug dose in human medicine nor in veterinary medicine.

Thus far, published literature (Hunter, 2008; Mahmood, 2007; Cox et al., 2004; Goteti, 2010) has shown that allometric scaling based on the assumption that the pharmacokinetics of a drug correlate allometrically to the change in weight is the most accurate approach for the prediction of pharmacokinetic parameters and ultimately drug doses. In addition to simple allometric scaling, Mahmood (2007) assessed different approaches in order to improve the predictive performance of allometric scaling. Species weight and maximum life span potential and further a two-term power equation based on the brain weight and body weight were taken into consideration. While the prediction of the clearance was improved in humans by incorporating the maximum lifespan potential or the brain weight, the correction factors did not improve the predictability in large animal species. The use of correction factors in large animals often led to a bigger error than the use of simple allometry. As a result, simple scaling still remains the preferred manner of determining pharmacokinetic parameters in veterinary medicine.

Allometric scaling has the potential to be used as an effective and affordable way of acquiring the information on appropriate drug dosage in unstudied species in situations where the species pharmacokinetics can't be studied and the information is needed rapidly in order to treat an individual accordingly. In retrospect, if the extrapolated dose Tusko was given was compared to dose calculations based on the surface law he would have been given only 3 mg compared to 297 mg. If allometric scaling was used (Boxenbaum & Dilea, 1995) estimated dose would have been 5.4 mg (Hunter, 2008; Harwood, 1963; Boxenbaum & Dilea, 1995).

Allometric scaling cannot, however, be employed unconditionally as accuracy and success of the scaling depends on the drug type. Extensively metabolised drugs are most likely not suitable for pharmacokinetic scaling (Sharma & McNeill, 2009). Antibiotics are considered to be suitable for allometric scaling (Riviere et al., 1997; Sharma & McNeill, 2009) as they are generally not extensively metabolised, but renally excreted and because the efficacy of the drug is well correlated to the plasma drug concentration. Other considerations depend on species-specific physiologic characteristics. As an example, accurate predictions can be hampered by the ruminants' content of the gastrointestinal tract forming a large part of the body weight without being actual bodyweight. The advantage or disadvantage of including different species has also not been fully resolved. However, several attempts have been made to account for interspecies differences. An example is the assembly of species to the five Hainsworth energy groups, which implies that basal metabolic rates are species specific (Hunter & Isaza, 2008). Scaling from a mammalian species to a non-mammalian species should also not be undertaken without considering major physiological differences. Lastly, scaling may be inaccurate if

sufficiently large animals (typically horses, camels and cattle) are not included in the scaling. In order to minimize errors and inaccuracies, Mahmood (2007) and Calder et al. (1981) also recommended that in scaling, body weights should vary by 3 orders of magnitude with at least one large species included. Furthermore, including three or more species in the scaling increases the accuracy of the prediction remarkably (Goteti et al., 2010).

For our study, we made use of the need for an effective antimicrobial to treat infections in injured rhino as a test for the effectivity of allometric scaling in this large herbivore. Enrofloxacin was selected for several reasons including the fact that the drug is easily available, obtainable as a high concentration injectable solution and that its half-life of elimination is long in numerous species. To ascertain the value of predictive pharmacokinetics in rhinoceros, the actual pharmacokinetic parameters half-life of elimination, clearance and volume of distribution from six healthy animals treated with enrofloxacin were compared to the parameters calculated with published allometric pharmacokinetic equations (Bregante et al., 1999; Cox et al., 2004) and with the allometric equations based on similar gastrointestinal anatomy, diet and enrofloxacin conversion rates. The reason we selected these three additional groupings (gastrointestinal anatomy, diet, metabolism) was based on the higher degree of relationship between those species, on the comparable anatomy and physiology of the gastrointestinal tract (monogastric), on their diet (herbivores) or their similar metabolic rate as seen with the conversion rates to ciprofloxacin. As an example, the horse, a herbivore and monogastric species, is regarded as the most suitable species for the establishment of diets for rhinoceros (Oftedal et al., 1996). Clauss et al. (2005) confirmed this for the Indian rhinoceros whose digestive parameters are best comparable to the ones of the horse, despite the large variation in body mass and ingesta retention time. Furthermore, a study on the ‘Quality and Digestibility of White Rhinoceros Food’ (Kiefer, 2002) also stated that the digestive physiology of the white rhinoceros corresponds best to that of the horse. The selection of closely related species for the different scaling approaches was furthermore motivated by previous research findings stating that, due to substantial variation between lineages, the metabolic rate of mammals cannot be predicted with a universal predictive approach. Instead, the allometric exponents differ between the mammalian lineages. Research results indicate that there is no overall relationship between the metabolic rate and body weight and that the allometric exponent is subject to the phylogeny (Capellini et al., 2010; Sieg et al., 2009; Symonds & Elgar, 2002). The presented data set had to be based on mean data values, as the individual animal data of all studies in all species was not available. However, the use of mean values is routinely applied as per other publications on pharmacokinetic scaling (Cox et al., 2004; Riviere et al.,

1997). Thus, we believe that it is an accurate implementation of the methods described for allometric scaling.

In order to generate the complete profiles for the rhino, we used sedated subadult white rhinoceros (between 13 and 28 months old) in the study with the assumption that at the specific age of treatment, the animals would have the equivalent metabolic capacity as full adults. As all our study animals were older than 12 months, we expected the metabolism to be equivalent to that of an adult rhinoceros. While it may be argued that the 25% coefficient of variation for the parameter half-life of elimination may indicate age specific change, no linear relationship was present between the age and the extent of the parameter. As a result, we believe that the pharmacokinetic parameters calculated in our study animals is a fair indication of those of fully grown rhinoceros. We are also confident that the pharmacokinetic parameters obtained from the sedated animals will translate to applicable field parameters, as it is routine practice to sedate wild rhinoceros before treatment is administered.

To assess the accuracy of allomeric scaling in an unusual animal species such as the rhinoceros, we used two methods of determining the validity of the model. For the first step, the mean parameters from the different species were plotted against weight and coefficients of determination were calculated. R^2 values above 0.50 were considered acceptable to indicate a sufficient fitting for all three evaluated parameters. The latter was less strict than the 0.74 selected by Riviere et al. (1997), but accommodates for the sample sizes less than 15 for the total number of species available in the model. Based on these criteria, the following models were deemed acceptable for prediction. Once a model was deemed to be predictive, the mathematical models were compared to the actual pharmacokinetic parameters and the prediction errors were calculated based on the criteria by Mahmood (2001) and Goteti et al. (2010). Up to 30% difference between the predicted and the experimentally determined clearance was still be seen as acceptable (Mahmood, 2001) and an error of 31 – 50% was interpreted as a fair prediction, while errors above 51% were rated as poor predictions (Goteti, 2010). When the parameters are looked at individually only the clearance derived from 11 mammalian species was able to predict the parameter with 5 % error (clearance of 0.21 L/h/kg). None of the equations were predictive for V_{ss}, V_z or $t_{1/2}$ with an error of less than 30%. More surprisingly, the equations of Bregante et al. (1999), which showed good correlation when scaling across cows, sheep, rabbits, rats and mice, were completely non-predictive for rhino. While the same poor predictability was in general evident when data was fitted to the allosteric equations obtained from only monogastric animals, V_z and $t_{1/2}$ met the criteria as fair predictors. Like the scaling approach across monogastric species, the prediction of the rhinos'

pharmacokinetic parameters based on scaling across herbivores also did not lead to good predictions. Only the volume of distribution in steady state met the criteria for a fair prediction (prediction error 37.26%). Also, compared to the high correlation between the body weight and the clearance and volume of distribution seen in monogastric species, the pharmacokinetic parameters and the body weight of the herbivores did not exhibit the same high degree of correlation. Furthermore, scaling across species with a similar percentage conversion of enrofloxacin to ciprofloxacin was thought to possibly provide an accurate way of prediction, as the rate of conversion and thus metabolism was partially accounted for between species. Nonetheless, even under these conditions, only the half-life of elimination met the criteria as a fair predictor (31.96%).

Not surprisingly, the half-life of elimination of enrofloxacin in rhino was relatively longer in relation to the weight of the animal and supported previous findings that the metabolic capacity of large animal species is lower than that of small animal species viz. lower metabolism will result in a longer time for the drug to be cleared from the plasma. This corresponded to findings of Sanchez et al. (2005), who described a half-life of 18.23 hours in the Asian elephant (*Elephas maximus*) after oral drug administration, which is more than twice as long as the half-life in horses (Langston et al., 1996). Interestingly, the half-life of elimination of enrofloxacin in the rhino is the longest recorded so far after intravenous administration to adult mammalian species (Lopez-Cadenas et al., 2013). The reasoning for this is linked to small endothermic species having a relatively larger surface area compared to their body mass with resultant larger relative heat loss. They thus require a higher relative amount of energy for the maintenance of a constant core temperature and exhibit a greater basal metabolic rate than large animal species (Sharma & McNeill, 2009). Rodrigues et al. (1994) also observed that despite the various liver and body sizes of different species, comparable total quantities of cytochrome P450 enzymes were present in the livers i.e. the relative amount of cytochrome P450 per gram body weight in small animal species is bigger than in larger animal species.

However, the unexpected finding for this study was that in all cases allometric scaling predicted a more rapid half-life of elimination than the actual estimates. With the model being deemed acceptable ($R^2 > 0.50$), we believe that this would indicate that the reason for the poor predictability is at the species level. While speculative, we believe that this could be due to differences in the cytochrome P450 (CYP450) enzyme content of the rhino liver, in both enzyme type and concentrations. The CYP450 enzymes are a diverse group of haemoproteins (monooxygenases), mainly responsible for the phase I metabolism of drugs and other xenobiotics. While the substrate specificity of the CYP450 enzymes is usually selective, there

are overlaps depending on the isoform (Martignoni et al., 2006; Sharma & McNeill, 2009). Even though the CYP450 system originates from one single ancestral gene with enzymes of different species being closely related containing conserved regions of amino acid residues, small differences in the amino acid sequence may lead to considerable changes in substrate specificity and catalytic activity (Martignoni et al., 2006; Nelson et al., 2013). One change in the amino acid sequence is sufficient to possibly change substrate specificity (Lindberg & Negishi, 1989). Even between closely related species with similar physiological characteristics, the cytochrome P450 enzyme activity is not correlated. From different studies, it has been demonstrated that the enzyme liver pattern of herbivore and carnivore species, between monogastric species and ruminants and even within a species such as cattle have been found to differ significantly (Fink-Gremmels, 2008). Mahmood (2001) was also able to show that even if the specific isoenzyme involved in the metabolism is known, allometric scaling could lead to inaccurate predictions of the clearance.

Of importance to support metabolism as a limiting factor is the potential for tissue binding/partitioning, which would slow down the overall rate of elimination. For the volume of distribution during terminal phase and at steady state of a drug, which distributes mainly through the body water, the exponent should be equal to one as the body water and the body weight are directly proportional. A drug, which remains in the extravascular fluids is characterised by an exponent of 0.67. For the volume of distribution at steady state, which takes vascular, extravascular and total body fluid into consideration, the exponent should be between 0.67 and 1 (Riviere, 2011). The exponents for the volume of distribution during terminal phase and at steady state for monogastric species being 0.96 and 1.05, respectively and the exponent for the volume of distribution in steady state being 0.91 in herbivores and 1.16 in species with a similar enrofloxacin conversion rate to the rhino, are indicative of a low potential for accumulation of enrofloxacin in the extravascular spaces and suggests that the mean residence time is rather linked to metabolism. The drug possibly being dependent on metabolism within the three different groupings and the rhinoceros having a longer half-life of elimination than predicted would indicate that they, as a species, show limitations in their metabolism of the drug, which are higher than expected.

Conclusion

For this study, we evaluated the potential of allometric scaling in determining the relevant pharmacokinetic parameters for dose optimisation of enrofloxacin in the white rhinoceros. The calculated, predictive values were compared to the actual pharmacokinetic

parameters of enrofloxacin generated from the white rhinoceros. Despite the goodness of fit of the model to the data from monogastric species, the model was not highly predictive for the rhinoceros. This leads to the conclusion that allometric scaling may not be all that helpful for the rhino as they exhibit a species-specific metabolic capacity, which is not scalable.

4.5 Materials and methods

4.5.1 Pharmacokinetics of enrofloxacin

4.5.1.1 Animals and treatments

Six white rhinoceros (one female and five males) with average body weight of 623 ± 179 kg from the 'The Rhino Orphanage', a registered non-profit organisation in the Limpopo Province of in South Africa, were used for the pharmacokinetic study. The specialist centre founded by Arrie van Deventer cares for orphaned and injured young rhinoceros, whose mothers have been poached, and rehabilitates the young rhinoceros to be released into the wild once old enough. Permission to conduct the research at 'The Rhino Orphanage' was given by the founder Arrie van Deventer and the study was approved by the Animal Ethics Committee of the University of Pretoria (Approval number: V074-15). The study animals were chosen based on their age, calmness around people and clean bill of health. During daytime, the rhinoceros graze in groups in large enclosures of several hectares. Overnight, they sleep in large enclosed paddocks or attached nightrooms. Besides the grazing, the animals receive additional feed consisting of teff (*Eragrostis teff*), lucerne (*Medicago sativa*), pellets (Driehoek Standard Game Cubes South Africa) twice daily and water ad libitum. Additionally, rhino I and rhino II, were still partially dependent on a milk feed of one litre twice daily. For the period of the trial, the rhinoceros were restricted to their paddock where they were artificially fed. Prior to sample collection, animals were habituated to the handling of their ears through the use of positive operant conditioning training in order to minimise stress during the trial. For the study, five rhinoceros received a single intravenous dose of 12.5 mg enrofloxacin/kg body weight (Baytril, Injectable, Bayer Animal Health, 100 mg/ml) in the auricular vein. Due to a calculation error, one rhinoceros received a dose of 7.8 mg enrofloxacin/kg body weight. The detailed characteristics of the rhinoceros used in the study are summarised in Table 4-4. After completion of the study, the six white rhinoceros remained at 'The Rhino Orphanage' and underwent further rehabilitation to be released into safe nature reserves.

Table 4-4: Characteristics of the six white rhinoceros used in the pharmacokinetic study listed according to their age

Age (months)	Rhino	Sex	Weight (kg)	Dose of enrofloxacin administered (mg/kg)
13	Rhino I	Female	527	12.5
13	Rhino II	Male	477	12.5
17	Rhino IV	Male	505	12.5
18	Rhino III	Male	522	12.5
28	Rhino V	Male	860	7.8
28	Rhino VI	Male	846	12.5

The rhinoceros also received 1 mg carprofen/kg body weight as part of another aspect of the study (results not presented). Blood samples were collected in lithium heparin tubes prior to the enrofloxacin injection and in average 8.8, 23.2, 37.4, 52,6 minutes and 2.11, 6.37, 12.33, 24.94, 48.30 and 71.45 hours after the injection of enrofloxacin from a polyethylene long-stay catheter (MILACATH, 18ga x 6.25cm/2.5 in, MILA International Inc) placed in the auricular vein or directly from the cephalic vein. For the placement of the catheter and the first five frequent blood draws, the animals were sedated with thiafentanil (Thianil, 10 mg/ml, Wildlife Pharmaceuticals RSA), etorphine (M99, 9.8 mg/ml, Novartis) and diazepam (Pax, 10 mg/2ml, Aspen Pharmacare). Furthermore, etorphine or ketamine (Kyron RSA, 1 mg in 20 ml vial) was added when the responsible veterinarian believed that further sedation was required. The remainder of the blood samples were collected without sedation when possible. However, if needed, the animals were sedated again with a mixture of thiafentanil and ethorphine for the scheduled sampling after 24, 48 and 72 hours. Naltrexone (Trexonil, 50 mg/ml, Wildlife Pharmaceuticals) was used to reverse and diprenorphine (M5050, 12 mg/ml, Novartis) was used to partially reverse the effect of etorphine and thiafentanil at the end of the sedation or to improve the breathing during the sedation, if necessary. Further details of the chemical immobilisation are presented in S 4-4 and S 4-5**.** Samples were placed on ice immediately after collection and centrifuged at 3000 rpm for 15 minutes. The plasma was stored at -20°C for a maximum of 8 days until the end of the field work and transferred into a – 80°C freezer until analysis.

4.5.1.2 Plasma enrofloxacin analysis

The plasma concentrations of enrofloxacin were analysed by online solid phase extraction with tandem mass spectrometric detection (online-SPE-MS/MS) using measurement conditions as described by Krebber et al. (2009). The only modification was the replacement of trifluoroacetic acid by heptafluorobutyric acid similar as reported by Bousova, Senyuva & Mittendorf (2013).

The samples were thawed and centrifuged. Subsequently 300 µL clear sample fluid were transferred into an autosampler vial and 300 µl water and 6 µL of the internal standard solution containing enrofloxacin-d_5 hydrochloride (100 µg/L) and ciprofloxacin-d_8 hydrochloride (100 µg/L) (Sigma-Aldrich, Taufkirchen, Germany) were added. After mixing for approximately 5 seconds by means of a vortex mixer, 5 µL of the sample was injected into the online-SPE-MS/MS system while in loading position. Matrix components contained in the injected sample were separated from the retained analytes on an extraction column suited for pre-treatment of samples at high flow rates. After switching to the elution position, the analytes were transferred to an analytical column. The quantitative determination was performed in a tandem mass spectrometric detector.

Analyses were performed using an HPLC system with two binary pumps (1290 binary pump G4220A and 1200 binary pump G1312A, Agilent Technologies, Waldbronn, Germany), CTC HTS PAL Autosampler with two six port valves (CTC Analytics AG, Zwingen, Switzerland) operated by CHRONOS Software (Axel Semrau, Sprockhövel, Germany). For SPE an Oasis HLB, 2.1mm x 20 mm, 25µm column (Waters, Eschborn, Germany) and for separation a Chromolith Speed Rod, 50 mm x 4.6 mm RP 18e column (Merck, Darmstadt, Germany) were used. Detection and quantification was made on a Sciex API 4000 (Sciex, Darmstadt, Germany) triple-quadrupole tandem mass spectrometer.

The limit of quantification for enrofloxacin and ciprofloxacin was 0.020 mg/L. The method was validated in the range from 0.02 to 25 mg/L for enrofloxacin. The mean recovery rate for enrofloxacin was 101% (± 6.3%). The linearity was checked in the range from 0.005 to 1 mg/L, the correlation coefficients were ≥ 0.999.

4.5.1.3 Pharmacokinetic analysis

All pharmacokinetic calculations were undertaken in Kinetica 5.0 (Thermo) by means of non-compartmental analysis. Prior to calculation, all plasma concentrations were equilibrated to 7.8 mg enrofloxacin/kg body weight to match the lower dose administered to one rhino. Therefore, the plasma concentrations were divided by 12.5 mg and multiplied by 7.8 mg. The following pivotal pharmacokinetic parameters were determined: The maximum plasma concentration (C_{max}) and the time to the maximum concentration (T_{max}) were read directly of the concentration versus time plasma profile. The area under curve to the last quantifiable time point (AUC_{last}) was determined using the linear trapezoidal rule $AUC_{last} = \sum_{i=1}^{n} 0{,}5 * ((C_i + C_{i+1}) * \Delta t)$. The total area under curve extrapolated to infinity (AUC_{inf}) was calculated as follows: $AUC_{inf} = AUC_{last} + AUC_{extra} = AUC_{last} + C_{Last}/Lz$ with C_{last} being the

computed last measured concentration. The area under the moment curve from the time point zero to the last measured time point ($AUCM_{last}$) was calculated as $AUMC_{last} = \sum_{i=1}^{n} 0{,}5 * (t_i * C_i + t_{i+1} * C_{i+1}) * \Delta t$. The half-life of elimination ($t_{1/2}$), clearance (Cl) and volume of distribution during terminal phase (V_z) and volume of distribution at steady state (V_{ss}) were determined as $t_{1/2}$= ln(2)/Lz; V_z = Cl/Lz = Dose/(AUC*Lz); V_{ss} = (Dose*MRT)/AUC and Cl = dose/AUC_{tot}.

4.5.2 Allometric interspecies scaling

To determine the appropriateness of allometric scaling, we used the equations of Cox et al. (2004), who provided two sets of predictive equations derived from known pharmacokinetic parameters for enrofloxacin from only mammalian species (n=11) or from a combination of mammalian and non-mammalian species such as birds and fish (n=15). Comparison were also made to predictive equations derived by Bregante et al. (1999), who included cows, sheep, rats, rabbits and mice in the calculations (Table 4-3).

We also estimated our own allometric equations based on a more anatomical approach of rhino being a monogastric species and large herbivores, as well as based on species with a similar conversion of enrofloxacin to ciprofloxacin. For scaling across monogastric species, information was included from intravenous results of the horse, pig, dog, cat, rabbit, rat and mouse (S 4-6) (Bregante et al., 1999; Kaartinen et al., 1997; Monlouis et al., 1997; Nielsen & GyrdHansen, 1997; Seguin et al., 2004); for the large herbivores we included the horse (Kaartinen et al., 1997), cow (Varma et al., 2003), oryx (Gamble et al., 1997), llama (Christensen et al., 1996), buffalo calf (Sharma et al., 2003), goat (Rao et al., 2002), sheep (Otero et al., 2009) and the rabbit (Cabanes et al., 1992) (S 4-7). Scaling across species with similar enrofloxacin conversion rates included sheep (Otero et al., 2009), goat (Rao et al., 2002), horse (Kaartinen et al., 1997) and donkey (Sekkin et al., 2012) with conversion rates of 26%, 34%, 20-35% and 20%, respectively (S 4-8). Pharmacokinetic modelling was based on enrofloxacin concentrations only.

Given the high variability of the pharmacokinetic parameters of the white rhino, the relevance of the size of the residuals was assessed by calculating a 95% confidence interval for each species for each model, where possible. The 95% confidence interval of each parameter (where applicable) is presented as error bars for the applicable species on the relevant plots. In addition, the correlation between the actual value and the predicted value of each parameter of each model at the individual animal level was assessed.

In cases where a pharmacokinetic parameter wasn't available, it was calculated from the given

parameters using standard pharmacokinetic equations ($t_{1/2}$ = ln(2)/Lz.; V_z = Cl/Lz = Dose/(AUC*Lz); V_{ss} = (Dose*MRT)/AUC; Cl = dose/AUC_{tot}). For optimal comparison of the results, all parameters that involved AUC were dose corrected to 7.8 mg/kg. For further standardisation, only pharmacokinetic parameters derived from HPLC analysis were used. For the scaling, the half-life, the volume of distribution and the clearance of monogastric and herbivore species as well as species with a similar enrofloxacin conversion rate to the rhino were plotted against the body weight (W). The best fit equation was established as log P = c + b * (log W), where "P" was the parameter and "W" the weight of the animal (in kg) in Microsoft Excel. The body weight for each species was also taken as published for the particular study. Based on those equations, the allometric equation $P = a*W^b$, with "a" being the allometric coefficient and antilogarithm of "c", and "b" being the allometric exponent could be established (Cox et al., 2004; Hunter & Isaza, 2008).

Based on the equations generated, the predictive values of the clearance, the volume of distribution and the half-life of elimination were calculated for each individual rhinoceros and the mean and 95% confidence intervals were established. The discrepancy between the observed and the predicted values for the clearance, the volume of distribution and the half-life of elimination was calculated as % prediction error = ((observed-predicted) *100)/observed.

4.6 Acknowledgements

This study was made possible through the help of ‘The Rhino Orphanage’, it’s founder Mr. Arrie van Deventer, who allowed us to carry out the field work and data collection at ‘The Rhino Orphanage’ and the staff, in particular Jamie Traynor and Laura Ellison, who helped with the implementation of the field work and the data collection. Furthermore, we would like to thank Dr. Robin Gieling, Aminisha Somers and Klaudia Pruczkowska for their assistance during the field work. We thank Dr Pierre Bester for the chemical immobilisation and the monitoring of the rhinoceros during the trial. The bacteriology laboratory of the Faculty of Veterinary Science, University of Pretoria is also thanked for allowing access to their historical results.

4.7 Supplementary information

S 4-1: Pharmacokinetic parameters for each rhinoceros after intravenous enrofloxacin injection (dose equilibrated to 7.8 mg/kg)

Parameter	Units	Animal						Mean	GMean	SD
		I	II	III	IV	V	VI			
Lz	h^{-1}	0.05	0.07	0.04	0.07	0.08	0.05	0.06	0.06	0.02
t1/2	h	14.38	10.35	15.89	9.59	8.27	12.85	11.89	11.58	2.96
C_{max}	µg/ml	9.24	6.43	7.18	12.36	10.80	9.30	9.22	8.99	2.21
T_{max}	h	0.10	0.15	0.20	0.17	0.13	0.12	0.14	0.14	0.04
AUC_{last}	µg*h/ml	35.67	33.05	54.37	42.35	42.74	33.55	40.29	39.66	8.10
AUC_{tot}	µg*h/ml	36.09	33.32	55.48	42.47	43.43	35.03	40.97	40.34	8.20
AUC_{extra}	µg*h/ml	0.41	0.27	1.11	0.11	0.69	1.49	0.68	0.49	0.53
AUC_{extra}	%	1.14	0.83	2.00	0.27	1.58	4.25	1.68	1.22	1.39
$AUMC_{last}$	µg*$(h)^2$/ml	358.93	366.03	612.29	347.91	380.28	295.44	393.48	382.75	111.07
Clearance	L/h/kg	0.22	0.23	0.14	0.18	0.18	0.22	0.20	0.19	0.03
Vz	L/kg	4.48	3.50	3.22	2.54	2.14	4.13	3.34	3.23	0.90
Vss	L/kg	2.38	2.74	1.82	1.55	1.74	2.50	2.12	2.07	0.48
MRT	h	11.01	11.70	12.91	8.42	9.69	11.23	10.83	10.73	1.57

S 4-2: Plasma concentration – time profile for each individual after intravenous enrofloxacin injection (dose equilibrated to 7.8 mg/kg)

Rhino I		Rhino II		Rhino III	
Time (h)	**Conc (µg/ml)**	**Time (h)**	**Conc (µg/ml)**	**Time (h)**	**Conc (µg/ml)**
0.10	9.24	0.15	6.43	0.20	7.18
0.42	6.13	0.35	5.00	0.55	5.07
0.72	4.40	0.77	2.73	0.57	5.62
1.12	3.56	0.97	2.55	0.85	4.82
1.98	2.19	1.82	2.88	2.27	2.87
6.25	1.45	6.03	1.34	6.73	3.53
12.75	0.99	11.60	0.74	12.62	1.07
25.13	0.20	24.27	0.33	25.58	0.36
48.73	0.06	48.38	0.05	48.52	0.12
72.38	0.02	71.83	0.03	70.87	0.05

Rhino IV		Rhino V		Rhino VI	
Time (h)	**Conc (µg/ml)**	**Time (h)**	**Conc (µg/ml)**	**Time (h)**	**Conc (µg/ml)**
0.17	12.36	0.13	10.80	0.12	9.30
0.37	7.05	0.33	6.80	0.25	6.86
0.55	5.70	0.55	5.79	0.52	5.53
0.70	4.62	0.82	5.27	0.75	5.04
2.22	2.69	2.35	2.86	2.27	2.70
6.23	2.93	6.27	1.68	6.60	1.29
12.07	0.75	12.42	1.22	12.62	0.49
25.63	0.20	23.98	0.32	24.07	0.32
48.42	0.04	47.48	0.06	47.43	0.08
70.70	0.01				

S 4-3: Correlation between the study`s experimental value and the predicted value based on the different models at the individual animal level

model	parameter	correlation between actual and predicted value
monogastric species	t1/2	-0.33
	CL	-0.075
	Vss	-0.18
	Vz	0.17
herbivore species	t1/2	-0.33
	CL	-0.07
	Vss	0.17
species with similar ciprofloxacin conversion	t1/2	-0.33
	CL	-0.07
	Vss	-0.18

S 4-4: Sedatives used for the chemical immobilisation of the six rhinoceros for the initial catheter placement and first frequent blood draws

	Initial drug combination (IM)		Diazepam	Top up	Zuclopen-thixol Acetate	Dipre-norphine
	Thia-fentanil	Etorphine				
Rhino I	0.3 mg	0.4 mg	10 mg IV after 0.7h	Etorphine 0.25 mg after 0.77h	50 mg IM after 1.13h	3 mg IV after 2.65h
Rhino II	0.3 mg	0.4 mg	10 mg IV after 0.63h	Etorphine 0.3 mg after 0.73h	50 mg IM after 1.2h	0.25 mg IM after 1.35h 3 mg IV after 2.85h
Rhino III	0.5 mg	0.5 mg	10 mg IV after 1.02h	Ketamine 50 mg IV after 1.2h	50 mg IM after 2.43h	0.2 mg IM after 0.35h 3 mg IM after 2.38h
Rhino IV	0.5 mg	0.5 mg			50 mg IM after 2.32h	0.2 mg IV after 0.15h 3 mg IM after 2.3h
Rhino V	0.33 mg	0.66 mg	10 mg IV after 1.77h		50 mg IM after 2.07h	0.2 mg IV after 0.3h
Rhino VI	0.33 mg	0.66 mg	10 mg IV after 1.68h		50 mg IM after 2.03h	0.2 mg IV after 0.7h

S 4-5: Re-sedation for blood sampling after 24, 48 and 72 hours

		Sample 9 (24 h)	Sample 10 (48 h)	Sample 11 (72 h)
Rhino I	**Immobilisation**	0.5 thiafentanil	0.25 mg thiafentanil 0.5 mg of etorphine	0.25 mg thiafentanil 0.5 mg etorphine
	Reversal	1 mg of diprenorphine after 0.33h + 5 mg diprenorphine after 0.43h	5 mg diprenorphine after 0.3h	3 mg of diprenorphine after 0.3h
Rhino II	**Immobilisation**	Without sedation/immobili-sation	0.25 mg thiafentanil 0.5 etorphine	0.25 mg thiafentanil 0.5 etorphine
	Reversal		3 mg diprenorphine after 0.35h	3 mg diprenorphine after 0.2h
Rhino III	**Immobilisation**	0.25 mg thiafentanil 0.5 mg etorphine	0.25 mg thiafentanil 0.5 mg etorphine	0.33 mg thiafentanil 0.66 mg etorphine
	Reversal	3 mg diprenorphine IV after 0.32h	3 mg diprenorphine IM after 0.38h	25 mg naltrexone IM after 0.23h
Rhino IV	**Immobilisation**	0.25 mg thiafentanil 0.5 mg ethorphine	0.25 mg thiafentanil 0.5 mg ethorphine	0.33 mg thiafentanil 0.66 mg ethorphine
	Reversal	3 mg diprenorphine IV after 0.5h	3 mg diprenorphine IM after 0.28h	25 mg naltrexone IM after 0.23h
Rhino V	**Immobilisation**	0.5 mg thiafentanil 0.5 mg etorphine	0.33 mg thiafentanil 0.66 mg etorphine	Without sedation/immobilisat ion
	Reversal	3 mg diprenorphine after 0.22h	25 mg naltrexone after 0.27h	
Rhino VI	**Immobilisation**	0.5 mg thiafentanil 0.5 mg etorphine	0.33 mg thiafentanil 0.66 mg etorphine	No sample
	Reversal	3 mg diprenorphine after 0.43h	25 mg naltrexone after 0.3h	

S 4-6: Pharmacokinetic parameters of different monogastric species after intravenous administration of enrofloxacin. The pharmacokinetic data was used for the derivation of allometric equations for monogastric species

Species	**Horse**	**Pig**	**Dog**	**Cat**	**Rabbit**	**Rat**	**Mouse**
Dose (mg/kg)	5	5.1	5.8	5	7.5	7.5	10
Weight (kg)	525	32.5	8.6	5.75	4	0.29	0.031
$t_{1/2}$ (h)	4.4	7.3	4.4	6.7	2.22	1.8	1.48
V_{ss} (L/kg)	2.31	3.9	3.7	2.37	2.27	1.75	4.49
V_z (L/kg)	3.24	3.7	4.15	2.46	4.94	4.78	10.5
Cl (L/h/kg)	0.51	0.37	0.65	0.26	1.41	1.28	4.09
Reference	Kaartinen et al. (1997)	Nielsen & GyrdHansen (1997)	Monlouis et al. (1997)	Seguin et al. (2004)	Bregante et al. (1999)	Bregante et al. (1999)	Bregante et al. (1999)

S 4-7: Summary of pharmacokinetic parameters of herbivore species following intravenous administration of enrofloxacin. The pharmacokinetic data was used for the derivation of allometric equations for herbivore species

Species	**Horse**	**Oryx**	**Cow**	**Buffalo calf**	**Llama**	**Sheep**	**Goat**	**Rabbit**
Dose (mg/kg)	5	1.3	5	5	5	2.5	5	5
Weight (kg)	525	130	370	150	112.25	52.5	17.5	4
$t_{1/2}$ (h)	4.4	1.31	2.61	2.87	3.38	4.57	1.14	2.19
Vdss (L/kg)	2.31	0.8	0.45	6.9	3.46	1.1	1.21	3.4
Cl (L/h/kg)	0.51	0.72	1.14	1.67	0.7	0.29	0.81	1.37
Reference	Kaartinen et al. (1997)	Gamble et al. (1997)	Varma et al (2003)	Sharma et al. (2003)	Christensen et al. (1996)	Otero et al. (2009)	Rao et al. (2002)	Cabanes et al. (1992)

S 4-8: Summary of pharmacokinetic parameters of four species with a similar enrofloxacin conversion rate to the white rhinoceros species following intravenous administration of enrofloxacin The pharmacokinetic data was used for the derivation of allometric equations for species with a similar enrofloxacin conversion rate to the white rhinoceros

Species	**Horse**	**Sheep**	**Goat**	**Donkey**
Dose (mg/kg)	5	2.5	5	5
Weight (kg)	525	52.5	17.5	107.5
$t_{1/2}$ (h)	4.4	4.57	1.14	9.54
Vdss (L/kg)	2.31	1.1	1.21	1.7
Cl (L/h/kg)	0.51	0.29	0.81	0.25
Reference	Kaartinen et al. (1997)	Otero et al. (2009)	Rao et al. (2002)	Sekkin et al. (2012)

5. Chapter 5: A Study of the Pharmacokinetics and Thromboxane Inhibitory Activity of a Single Intramuscular Dose of Carprofen as a Means to Establish its Potential Use as an Analgesic Drug in White Rhinoceros

Marion Leiberich[1*], Ralph Krebber[2], Michael Hewetson[1], Johan Marais[3], Vinny Naidoo[4]

[1] Department of Companion Animal Clinical Studies, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[2] Bayer AG - Crop Science Division, Research & Development - Regulatory Science - Human Safety – Residue Analysis, Monheim am Rhein, Germany

[3] Saving the Survivors, Pretoria, South Africa

[4] Department of Paraclinical Science, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

*Corresponding author

5.1 Abstract

The alleviation of pain and prevention of suffering are key aspects of animal welfare. Unfortunately, analgesic drugs are not available for all species. White rhinoceros (*Ceratotherium simum*), representing one of such species, which survive poaching attempts inflicted with severe facial injuries and gunshot wounds, nonetheless require analgesic support. In order to improve treatment conditions, this study explored the use of carprofen for the treatment of pain and inflammation in white rhinoceros. The pharmacokinetics of 1 mg/kg intramuscular carprofen were evaluated in six healthy white rhinoceros. The half-life of lambda_z and mean residence time were 105.71 ± 15.67 and 155.01 ± 22.46 hours, respectively. The area under the curve and the maximum carprofen concentration were 904.61 ± 110.78 µg/ml*h. and 5.77 ± 0.63 µg/ml, respectively. Plasma TXB2 inhibition demonstrated anti-inflammatory properties and indicated that carprofen may be effective for a minimum of 48 hours in most animals. With its long half-life further indicating that a single dose could be

effective for several days, we suggest that carprofen may be a useful drug for the treatment of white rhinoceros.

Keywords: Analgesia, carprofen, NSAID, pharmacokinetics, white rhinoceros

5.2 Introduction

Pain has been described as “a more terrible lord of mankind than even death itself" by Dr. Albert Schweitzer (Brabazon, 2000). However, despite the recent recognition of pain in animal species and the now widely accepted importance of pain management in veterinary medicine, not all veterinary patients are easily treatable. The Southern white rhinoceros (*Ceratotherium simum simum)*, one of such species, has only recently come to the forefront as a species requiring appropriate analgesic treatment, all as a result of poaching and the illegal acquisition of rhino horn (Emslie et al., 2016). In most cases, this results in the brutal killing of the animals (Emslie et al., 2016), however some individuals miraculously survive the attack and are found alive often suffering from tremendous facial injuries, bone loss and severe haemorrhaging (Cooper & Cooper, 2013). Roughly, 200 animals per year are in need of veterinary assistance due to poaching and poaching related injuries (J. Marais, personal communication, 2016). Besides the facial wounds, injuries encountered in those animals include gunshot wounds and wounds caused by snares (Cooper & Cooper, 2013). In order to alleviate pain and reduce the suffering of those individuals, appropriate analgesic treatment is needed. Among the different pain relieving drugs in animal species, NSAIDs are one of the most widely used for perioperative analgesia in horses, cats and dogs, as well as for chronic pain relief in osteoarthritis in dogs (Lees et al., 2002; Schatzman et al., 1990; Lipscomb et al., 2002). NSAIDs are also readily applied in cattle for the reduction of acute inflammatory reaction in respiratory diseases (Balmer et al., 1997) and further for the treatment of peracute and acute bovine mastitis (Papich, 2010).

For this study, carprofen (±6-chloro-α-methyl-carbazole-2-acetic acid), a propionic acid derivative, was chosen for further evaluation as a potentially effective and appropriate NSAID for the use in white rhinoceros. The choice was influenced by wildlife veterinarians who reported obtaining satisfactory analgesia for two to three days following the administration of carprofen to rhino suffering from lameness (J. Marais, personal communication, 2016). Furthermore, carprofen is defined by its low incidence of side effects and high therapeutic index in most veterinary species, with the possible exception of the vulture (Fourie et al., 2015). The wide safety margin seen in most species may be explained by the relatively weak to moderate inhibition of the COX-1 and COX-2 isoforms observed in species like the horse (Lees et al.,

2002), calf (Delatour et al., 1996), cat (Taylor et al., 1996) and dog (McKellar et al., 1990) given at clinically recommended drug doses. Furthermore, carprofen exhibits excellent analgesic properties for soft tissue injuries, perioperative pain management as well as after orthopaedic procedures (Mathews, 2002; Grisneaux et al., 1999) and can be administered intravenously (IV) intramuscularly (IM) as well as subcutaneously (SC) and per os (PO) (McKellar et al., 1991, 1994; Taylor et al., 1996). Carprofen is characterised by a low volume of distribution in steady state of 0.093 L/kg in sheep (Welsh et al., 1992), 0.14 L/kg in the dog (McKellar et al., 1990) and 0,091 L/kg in the cow (Lohuis et al., 1991). Furthermore, it is known for its long half-life of elimination in the horse (21.9 hours) (McKellar et al., 1991), the cow (30.7 hours) (Lohuis et al., 1991) and sheep (26.1 h) (Welsh et al., 1992). The clearance ranges between 0.75 ml/kg/h in horses (McKellar et al., 1991), 2.4 ml/kg/h in cattle and 2.5 ml/kg/h in sheep (Welsh et al., 1992). When looking at the different enantiomers of the chiral drug carprofen as opposed to the total carprofen concentration, the R(-) carprofen plasma concentrations exceeds those of the S(+) carprofen. Furthermore, in species such as the horse, the mean residence time and elimination half-life have been found to be three times longer for R(-) carprofen than for S(+) carprofen when administered at 0.7 mg/kg, while the clearance of the S(+) carprofen was 5.7 times more rapid (Lees et al., 2002). The enantiomer concentrations were found to increase in direct proportion to the administered dose. Even though quantitatively, the R(-) enantiomer is predominant, the effect of racemic carprofen can be almost completely ascribed to the S(+) enantiomer (Lees et al., 2004c), which is characterised by a significantly higher potency.

Due to the complexity of working with wild, endangered animals, we evaluate carprofen as it would be administered in clinical practice, which is in conjunction with an antimicrobial, while the animals are immobilised.

5.3 Materials and methods

5.3.1 Animals

The study proposal was reviewed and approved by the Animal Ethics Committee of the University of Pretoria (Approval number: V074-15). Six white rhinoceros, one female and five males from ‘The Rhino Orphanage’ in South Africa were included in the study. The animals were between 15 and 30 months old, weighing in average 670 (538 – 902) kg. The detailed characteristics of the rhinoceros are summarised in Table 5-1.

Table 5-1: Characteristics of the six white rhinoceros used in the study listed according to their age

Rhino	Age (months)	Sex	Weight (kg)
Rhino I	15	Female	556
Rhino II	15	Male	538
Rhino IV	19	Male	551
Rhino III	20	Male	573
Rhino V	30	Male	900
Rhino VI	30	Male	902

5.3.2 Experimental design and sampling procedure

Six white rhinoceros received a single IM injection of racemic carprofen (±6-chloro-α-methyl-carbazole-2-acetic acid) at 1 mg/kg (Rimadyl Injectable Solution, 50 mg/kg, Zoetis) into the gluteal muscle. No more than 18 ml of carprofen were injected at one injection site. Carprofen was administered in conjunction with a single dose of IV enrofloxacin (12.5 mg/kg, Baytril, Injectable, Bayer Animal Health, 100 mg/ml), followed by a subsequent dose of oral enrofloxacin (12.5 mg/kg, Baytril, Bayer Animal Health, 10% oral solution) after 10.45 ± 0.76 hours (enrofloxacin results not included in this publication). Enrofloxacin was evaluated in combination with carprofen as many wounds require concurrent antimicrobial therapy. The study of enrofloxacin at the same time, thus simulated not only expected clinical use but also allowed for the maximum use of the study animals, which are not easy to source and work with.

The carprofen and thromboxane B2 (TXB2) plasma concentrations were evaluated over a period of roughly 72 hours from a maximum of 11 blood samples per animal. The blood samples were taken prior to the drug administration and in average after 4.68, 18.45, 31.1, 45.77 minutes and 2.11, 6.17, 11.87, 22.8, 47.65, 73.07 hours post carprofen injection. A total of 18 ml of blood were collected in lithium heparin tubes and partly used for the analysis of the carprofen plasma concentration. Additional two to four ml of blood were collected in indomethacin spiked serum tubes for the measurement of plasma TXB2 activity. For the indomethacin treatment of the tubes, 0.05 ml of a 0.04M indomethacin solution and 0.95 ml of the EDTA buffer (pH of 7.4) were mixed for every 10 ml of blood. Syringes and Vacutainer tubes were coated according to their appropriate volume. For the EDTA buffer, 2 g disodium EDTA (purity > 99%, Sigma Aldrich) and 0.8 g sodium chloride (purity >99%, Merck) were mixed with distilled water to a total volume of 100 ml and adjusted with NaOH (purity 98%, UnivAR) to the pH of 7.4. The 0.04M indomethacin solution consisted of 50 mg of indomethacin (purity ≥ 99% (TLC), Sigma Aldrich) dissolved in 3.5 ml absolute ethanol (purity 99.9%, ILLOVO).

5.3.3 Immobilisation of the rhinoceros

The placement of the auricular catheter, the administration of the drugs and the first five blood samples were not possible without chemical immobilisation of the animals. Thus, the six rhinoceros were sedated using a mixture of etorphine (M99, 9.8 mg/ml, Novartis) and thiafentanil (Thianil, 10 mg/ml, Wildlife Pharmaceuticals RSA). All rhinos also received 50 mg of zuclopenthixol acetate (Clopixol-Acuphase, 50 mg/ml) within 2.5 hours of the initial immobilisation. Diprenorphine (M5050, 12 mg/ml, Novartis) was given at a dose of 0.2 or 0.5 mg during the immobilisation to improve the breathing and at a higher dose of 5 mg per animal at the end of the immobilisation as a partial reversal, if necessary. Additionally, medetomidine (20 mg/ml, Kyron RSA) and ketamine (50 mg/ml, Kyron RSA) were used to improve anaesthetic depth when needed. The details of the sedation of each individual are summarised in S 5-1. The blood samples scheduled at 24, 48 and 72 hours were collected under a low-dose butorphanol (Dolorex, MSD animal health, 10 mg/ml) based standing sedation directly from the cephalic vein. The details regarding the butorphanol based low-dose sedation are summarised in S 5-2.

5.3.4 Analytical techniques

5.3.4.1 Processing of the blood samples

All samples were placed on ice immediately after blood collection and centrifuged within four hours at 3000 rpm for 15 minutes. Plasma samples for pharmacokinetic evaluation were frozen at -20°C for a maximum of 8 days prior to storage in a -80°C freezer. The blood collected in indomethacin spiked serum tubes for the pharmacodynamic evaluation of carprofen was flash-frozen in liquid nitrogen and transferred to the -80°C freezer after a maximum of eight days.

5.3.4.2 Analysis of the carprofen plasma concentrations via tandem mass spectrometry – liquid chromatography

The samples for the pharmacokinetic analysis of carprofen were shipped to Germany (dry ice, World Courier) for analysis by Bayer Animal Health (CITES export permit number: 152722) and analysed by high performance liquid chromatography/ tandem mass spectrometry (HPLC-MS/MS).

The plasma samples were mixed with the internal standard carprofen-d_3 and deproteinised. Therefore, 100 µL of plasma were added to 900 µL of a mixture containing 100 mL of 0.40 g ammonium acetate in 1L water, 1 mL formic acid and 600 mL acetonitrile. The

samples were placed on a vibratory mixer for 30 seconds prior to centrifugation. An aliquot of 5 µL was then injected into the HPLC system. The chromatographic separation was performed with a ZORBAX SB Phenyl column, 2.1 x 50 mm, 1.8 µm (Agilent Technologies, Waldbronn, Germany) using the mobile phase A (water, 10 mMol ammonium formate and 0.12 mL/L formic acid) and the mobile phase B (methanol, 10 mMol ammonium formate, and 0.12 mL/L formic acid). Gradient elution with a flow rate of 0.5 ml/min started at 10% B for 0.5 min, eluent strength was then increased and, after 1.5 minutes, reached 95% B for one minute. Thereafter, the eluent strength decreased to 10% B after three minutes and was equilibrated for one minute. The quantitative determination was performed by HPLC with detection by tandem mass spectrometry using an API 5500 mass spectrometer (Sciex, Darmstadt, Germany). The retention time for carprofen was 1.7 min. Carprofen was detected in the positive ionisation mode using the transition from its precursor ion at $^{m}/_{z}$ 274 to its product ion at $^{m}/_{z}$ 228 or $^{m}/_{z}$ 193. The internal standard carprofen-d_3 was detected in the positive ionisation mode using the transition from its precursor ion at $^{m}/_{z}$ 277 to its product ion at $^{m}/_{z}$ 231. The limit of quantification was 0.01 mg/L. Since the amount of available rhino control plasma was limited, the method validation was performed using four validation levels (0.01/0.1 /1 /10 µg/mL) with three replicates each. The mean recovery rates for the individual fortification levels were between 81 and 97%. The mean overall was 93%, representing an accuracy of -7% with a precision (coefficient of variation) of 10%. The method is a variation of our routine plasma method and shows a high specificity as a result of the selective determination by tandem mass spectrometry. Matrix effects are compensated by the use of an internal standard. The limit of detection was set to the lowest standard concentration used for the measurement, which corresponds to 0.005 µg/mL.

5.3.4.3 Thromboxane B2 analysis

The inhibition of TXB2, the stable metabolite of thromboxane A2 (TXA2), was used as an indicator for the extent and the time dependent course/duration of COX-1 inhibition (Lees et al., 2002). The ‘Thromboxane B2 Express EIA Kit - Monoclonal’ (Cayman Chemical) was used to measure the TXB2 concentrations in the plasma. Plasma samples were purified prior to TXB2 analysis via ELISA and all procedures were carried out according to the instructions provided by Cayman Chemical. However, the test was not validated by the manufacturer for the use of rhino plasma. Briefly, the concentration of TXB2 from the plasma sample binding to a limited amount of TXB2–specific antibodies in the well determines the intensity of the enzymatic reaction, which is assessed photospectrometrically (410 nm).

For the calculation of the TXB2 concentrations based on the absorbance readings, a standard curve was generated with eight standards of known concentrations. The ratio of standard bound/maximal bound (B/B0) for all standards was plotted against the TXB2 concentration as a semi-logarithmic plot and a linear visual fit was carried out. With the equation generated from the standard curve and the B/B0 ratio (sample bound/maximal bound) of each sample, the TXB2 concentration of each plasma sample was calculated.

5.3.4.4 Pharmacokinetic analysis

All pharmacokinetic parameters were calculated with Kinetica 5.0 (Thermo) using a non-compartmental model. The maximum plasma concentration (C_{max}) and the time to maximum concentration (T_{max}) were read directly of the concentration versus time plasma profile. The area under curve to the last quantifiable time point (AUC_{last}) was determined using the linear trapezoidal rule ($AUC_{last} = \sum_{i=1}^{n} 0{,}5 * ((C_i + C_{i+1}) * \Delta t)$. The total area under curve extrapolated to infinity (AUC_{tot}) was calculated as $AUC_{tot} = AUC_{last} + AUC_{extra} = AUC_{last} + C_{Last}/\lambda$ with C_{last} being the computed last measured concentration and λ being the terminal elimination rate constant. The area under the moment curve from the time point zero to the last measured time point ($AUCM_{last}$) was calculated as $AUMC_{last} = \sum_{i=1}^{n} 0{,}5 * (t_i * C_i + t_{i+1} * C_{i+1}) * \Delta t$. The half-life of lambda_z ($t_{1/2}$), clearance (Cl/F), volume of distribution during terminal phase (V_z/F) and volume of distribution at steady state (V_{ss}/F) and the mean residence time (MRT) were determined as $t_{1/2} = \ln(2)/\lambda$; $V_z = Cl/\lambda = Dose/(AUC_{tot} * \lambda)$; $V_{ss} = (Dose*MRT)/AUC$, $Cl = dose/AUC_{tot}$ and $MRT = AUMC_{tot}/AUC_{tot}$.

5.4 Results

5.4.1 Side effects

No adverse effects were observed at the site of injection after carprofen administration. All six rhinoceros ate within 12 hours after immobilisation and displayed normal physiological behaviour. In four out of six animals, a band like swelling was observed at the base of the ear in which the intravenous enrofloxacin was administered. The adverse reaction appeared within the first six hours after drug administration and formed a painless oedema around the base of the ear. The swelling decreased in all affected individuals within 24 hours and disappeared or was significantly reduced towards the end of the study, after 72 hours.

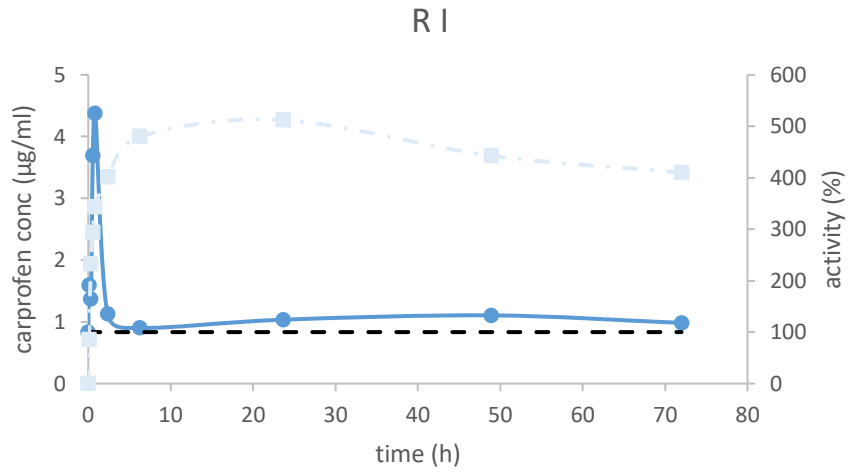
5.4.2 Pharmacokinetic analysis

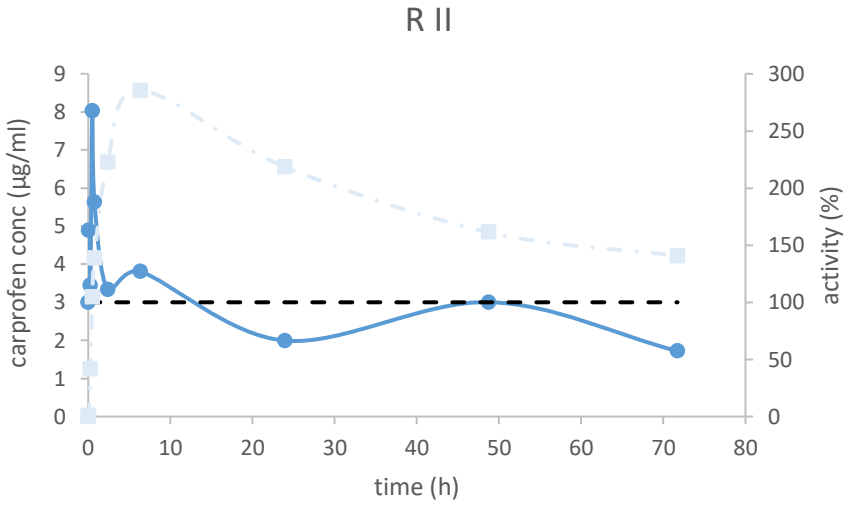
All data is reported as geometric means (Gmean) and standard error of the mean (± SEM). After the single IM injection of 1 mg/kg, a carprofen concentration of 0.088 ± 0.11 µg/ml was reached after 4.68 ± 0.54 minutes. The maximum carprofen concentration (C_{max}) of 5.77 ± 0.63 µg/ml was reached after 9.51 ± 3.53 hours. At the time of the last blood draw, after 73.07 ± 0.63 hours, carprofen plasma concentrations were still at 3.69 ± 0.2 µg/ml. Carprofen half-life of lambda_z was estimated at 105.71 ± 15.67 hours and the mean residence time (MRT) in plasma at 155.01 ± 22.46 hours. The area under the curve extrapolated to infinity (AUC_{tot}) was 904.61 ± 110.78 µg*h/ml. The mean clearance of carprofen was 0.0011 ± 0.0001 L/h*kg. The apparent volume of distribution at steady state and at terminal phase was 0.17 ± 0.01 L/kg. The pharmacokinetic parameters for all individuals are summarised in Table 5-2. The carprofen plasma concentration-time curve for each individual is shown in Figure 5-1. The average plasma concentration–time curve and the standard error of the mean for all individuals is presented in Figure 5-2.

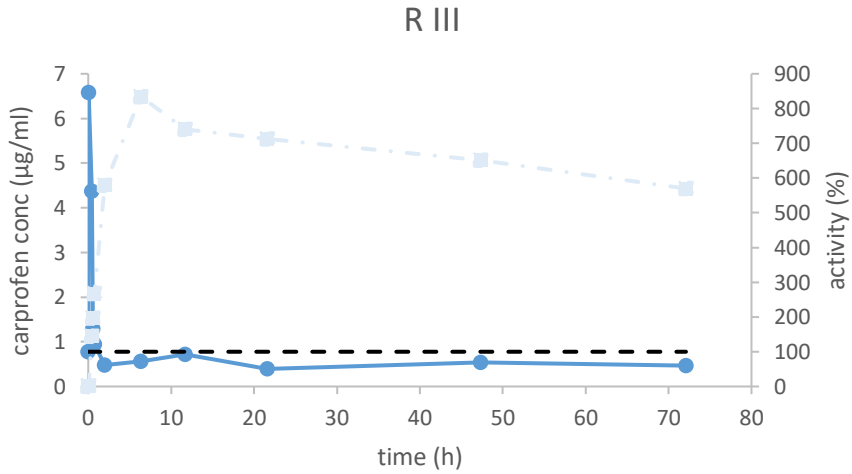
Table 5-2: Pharmacokinetic parameters of carprofen in six white rhinoceros after single intra muscular injection of 1 mg/kg

Parameter	Units	Animal						Mean	GMean	SEM
		I	II	III	IV	V	VI			
λ	h^{-1}	0.005	0.008	0.004	0.010	0.008	0.006	0.007	0.007	0.00
t1/2	h	150.31	81.62	162.60	66.92	92.30	113.23	111.16	105.71	15.67
C_{max}	µg/ml	4.27	8.56	6.49	5.40	6.19	4.64	5.93	5.77	0.63
T_{max}	h	23.67	6.32	6.35	22.02	5.88	6.03	11.71	9.51	3.53
AUC_{last}	µg*h/ml	275.22	408.76	371.66	310.39	337.05	304.59	334.61	331.72	19.91
AUC_{tot}	µg*h/ml	1009.15	897.78	1421.93	616.62	806.73	855.11	934.55	904.61	110.78
AUC_{extra}	µg*h/ml	733.93	489.02	1050.27	306.23	469.68	550.52	599.94	556.95	106.26
AUC_{extra}	%	72.73	54.47	73.86	49.66	58.22	64.38	62.22	61.57	4.02
$AUMC_{last}$	µg*$(h)^2$/ml	9650.94	13019.90	12886.80	10462.30	11649.80	11006.90	11446.00	11380.00	547.01
Cl/F	L/h*kg	0.0010	0.0011	0.0007	0.0016	0.0012	0.0012	0.0011	0.0011	0.00
Vz/F	L/kg	0.21	0.13	0.16	0.16	0.17	0.19	0.17	0.17	0.01
Vss/F	L/kg	0.22	0.13	0.17	0.16	0.17	0.19	0.17	0.17	0.01
MRT	h	219.63	117.71	235.58	100.95	135.58	166.44	162.65	155.01	22.46

λ, terminal elimination rate constant; $t_{1/2}$, half-life; C_{max}, maximum plasma concentration; T_{max}, time to maximum plasma concentration; AUC_{last}, area under the curve until the last time point; AUC_{tot}, area under the curve extrapolated to infinity; AUCextra, area under the curve from the last quantifiable measurement to infinity; $AUCM_{last}$, area under the moment curve from t =0 to the last measured time point; Cl/F, clearance corrected for bioavailability; Vz/F, apparent volume of distribution during the terminal phase corrected for bioavailability; Vss/F, apparent volume of distribution in steady state corrected for bioavailability; MRT, mean residence time


R I
carprofen conc (µg/ml)
activity (%)
time (h)
0
1
2
3
4
5
0
100
200
300
400
500
600
0
10
20
30
40
50
60
70
80


R II
carprofen conc (µg/ml)
activity (%)
time (h)
0
1
2
3
4
5
6
7
8
9
0
50
100
150
200
250
300
0
10
20
30
40
50
60
70
80


R III
carprofen conc (µg/ml)
activity (%)
time (h)
0
1
2
3
4
5
6
7
0
100
200
300
400
500
600
700
800
900
0
10
20
30
40
50
60
70
80

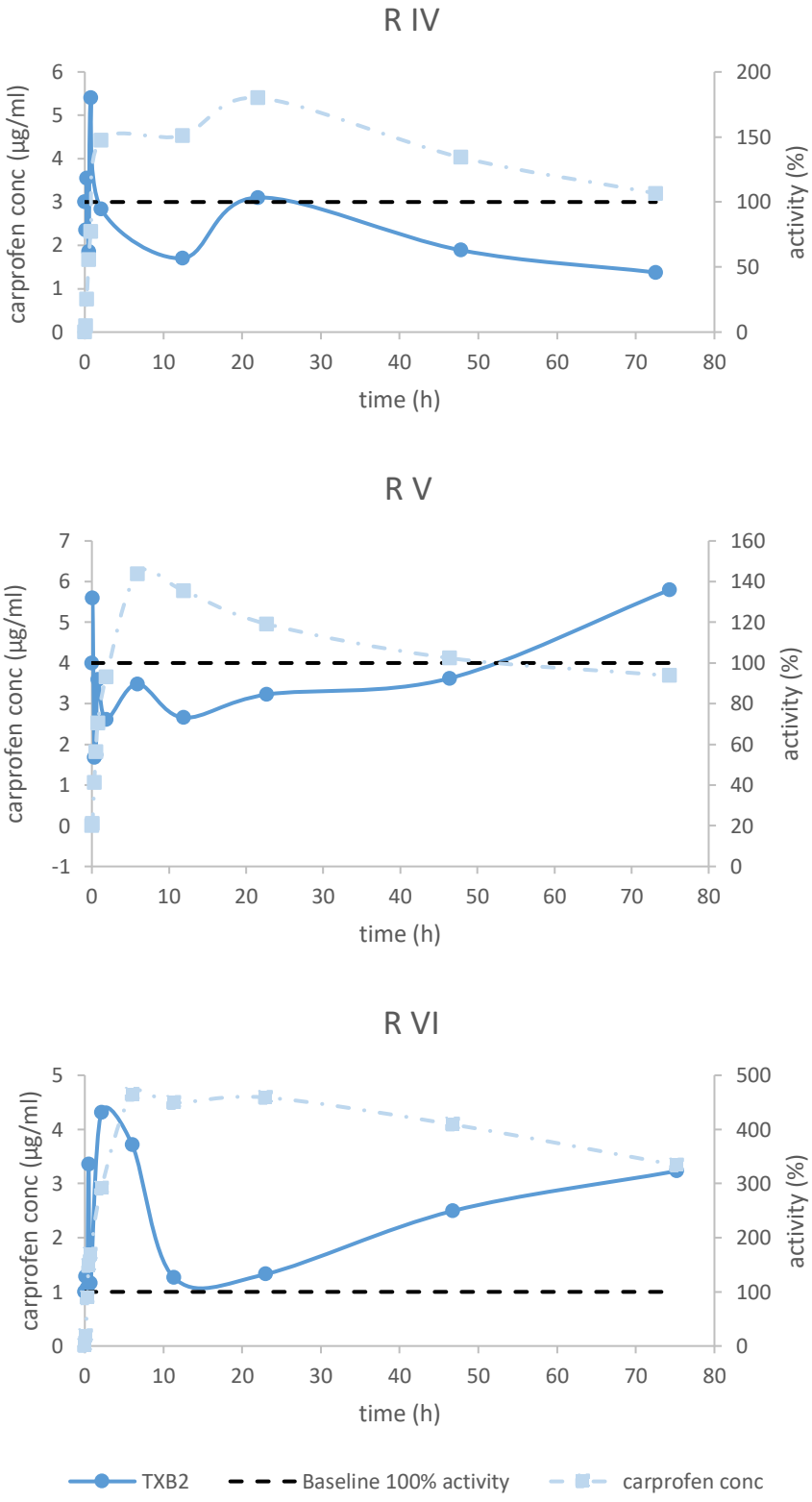


Figure 5-1: Time versus plasma drug concentration (primary axis) for carprofen (square) and % activity (as a percentage of the zero hour sample, secondary axis) of TBX (circle) versus time after a single intramuscular injection of 1 mg/kg carprofen in rhino I (R I) to rhino VI (R VI)

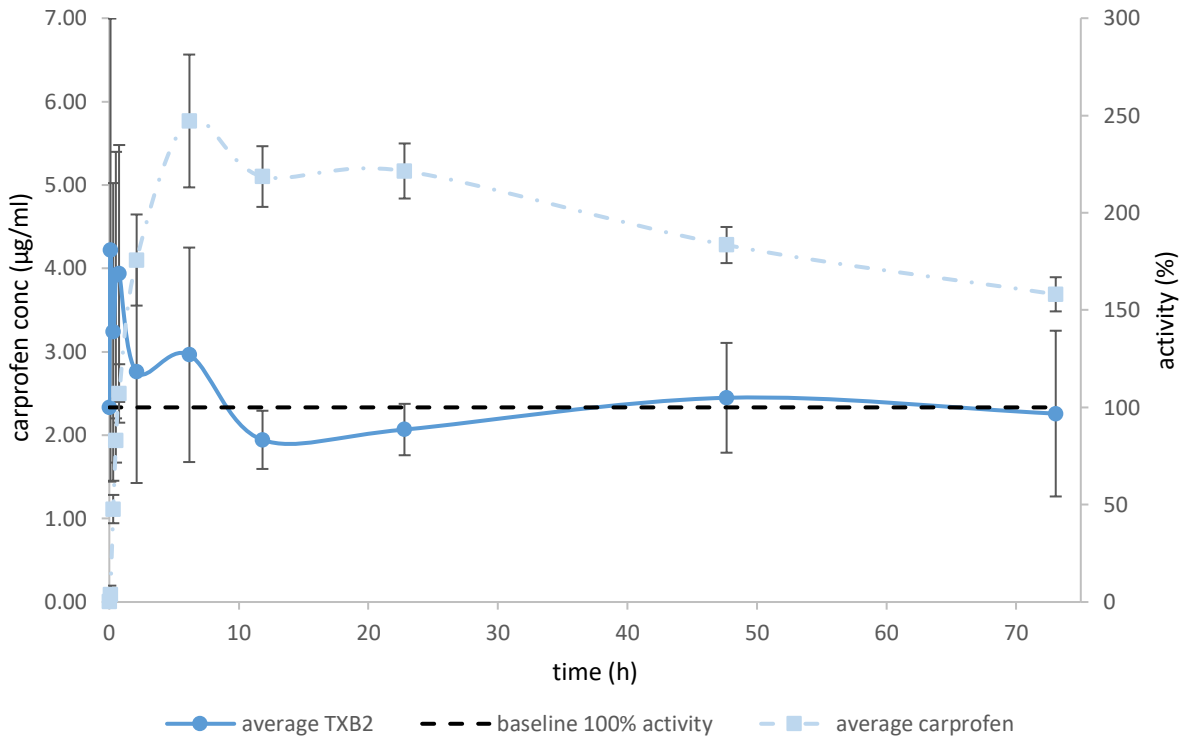


Figure 5-2: Time versus average plasma drug concentration (RI-RVI, primary axis) for carprofen (square) and % activity (as a percentage of the zero hour sample, secondary axis) of average TBX (RI-RVI, circle) versus time

5.4.3 Pharmacodynamic analysis

TXB2 plasma concentrations were determined by ELISA and were characterised by high inter-animal differences (Figure 5-1, Figure 5-2). In all individuals, TXB2 concentrations increased after carprofen administration and reached a peak concentration after 22.3 ± 18.46 minutes. The mean carprofen plasma concentration at the time of the peak was 0.6 ± 0.59 µg/ml (Figure 5-2). In rhino I, II and III the TXB2 activity decreased rapidly after reaching the peak and remained around the initial TXB2 concentration measured prior to the administration of carprofen. In rhino IV, the TXB2 concentration continuously decreased after 22.02 hours until the end of the study (72.57 hours). In contrast, while the TXB2 activity in rhino V and VI decreased after reaching the peak concentration, TXB2 concentrations started to increase again after 11.88 hours (rhino V) and 11.32 hours (rhino VI) until the end of the study. The carprofen plasma concentration versus time profiles, as well as the TXB2 versus time profiles for each individual and as the Gmean (±SEM) of all individuals are depicted in Figure 5-1 and Figure 5-2, respectively.

5.5 Discussion

Carprofen, a drug with antipyretic, anti-inflammatory and analgesic properties, is widely used in horses, cats and dogs for the management of skeletal, muscular and soft tissue injuries (Lees et al., 2002) as well as for the management of post-surgical pain. Resting upon its favourable characteristics in domestic species and the positive feedback of wildlife veterinarians reporting long analgesic effects in rhinoceros suffering from lameness (J. Marais, personal communication, 2016), it was deemed a potentially promising drug for the treatment of injured poaching victims. Based on the pharmacokinetic profile, we evaluated if carprofen administered IM at a dose of 1 mg/kg could be of value for the treatment of injuries in rhino. The dose of 1 mg/kg was selected based on the dose of 0.7 mg/kg in the horse. It was chosen slightly higher to accommodate for potential interspecies differences and to be able to scale down rather than to scale up in case of a required dose adjustment. Carprofen was administered in conjunction with intravenous and oral enrofloxacin in order to mimic the field situation, where injured poaching victims generally require analgesic as well as antimicrobial treatment.

Carprofen was characterised by a fairly long absorptive phase with a T_{max} of 9.51 ± 3.53 hours (corresponding Cmax of 5.77 ± 1.55 µg/ml). The absorptive phase compares favourably with the horse, which exhibits a comparable T_{max} of 10.6 ± 1.6 hours, albeit at a lower maximum concentration of 2.2 ± 0.4 µg/ml following a single dose of 0.7 mg carprofen/kg (McKellar et al., 1991). While we did not specifically evaluate the reason for the slow absorption of the drug from the site of injection, we believe that it may be attributed to two possible causes. The first being the ability of the drug to precipitate or crystallise at the site of administration. This process has been evaluated in the horse, where an increase in creatinine kinase (CK) activity, a marker of muscle cell damage, was noted after IM carprofen injection (McKellar et al., 1991). From pharmacokinetic theory it is known that localised irritation at the administration site can slow absorption (McKellar et. al., 1991), which is used in formulation chemistry to create long lasting effects for certain antibiotic formulations (Xia et al., 1983). The second reason for the slow absorption may be a drug interaction between etorphine and carprofen. From previous studies. vasoconstrictive effects have been reported following etorphine administration in both rhino and goats (Heard et al., 1992; 1996). As such, the change in blood supply to the site of administration may be responsible for the delayed absorptive phase. Of the two causes, the latter is more likely, as no injection site reactions were visible clinically in any of the treated animals.

Another major finding for this study is the exceptionally long half-life of 105.71 ± 15.67 hours with large inter-animal variability. The half-life in the white rhino is substantially longer

than the 29.4 ± 3 hours following a single IM administration at 0.7 mg/kg in the horse (McKellar et al., 1991); or the 30.7 ± 2.3 hours in the cow (Lohuis et al., 1991) and 26.1 ± 1.1 hours in sheep (0.7 mg/kg IV) (Welsh et al., 1992). To our knowledge, the half-life in the white rhinoceros is the longest recorded half-life amongst all mammalian species. This in itself is not a very surprising finding, as previous studies we have undertaken with enrofloxacin have also shown the rhino to be a slow metaboliser, even when their size is taken into consideration. As a result, we speculate that the rhino is metabolically constrained.

Along with the metabolic restrictions, the slower clearance and prolonged half-life of carprofen may result from a drug-drug interaction. Enrofloxacin, a fluoroquinolone, has been reported to interact with the pharmacokinetics of other drugs such as the methylxanthine theophylline (Intorre et al., 1995) and NSAIDs on several occasions (Abo-El-Sooud & Al-Anati, 2011; Ogino et al., 2005; Rahal et al., 2008). The reason for the interaction of enrofloxacin with the pharmacokinetics of certain drugs is thought to be linked to the inhibition of particular drug metabolising cytochrome P450 (CYP) enzymes, such as CYP1A1 and CYP1A2 (Intorre et al., 1995; Rahal et al., 2008; Sasaki & Shimoda, 2015; Vancutsem & Babish, 1996; Regmi et al., 2005). While enrofloxacin seems to increase the half-life of elimination of theophylline, the effect on the metabolism of NSAIDs seems to be less consistent (Intorre et al., 1995). Flunixin in conjunction with enrofloxacin (5 mg/kg SC) in dogs leads to a significant increase in the elimination half-life by 29% (Ogino et al., 2005), while the administration of diclofenac with enrofloxacin in sheep results in a slowed absorption and a prolonged elimination half-life (57%) (Rahal et al., 2008). In contrast, in calves enrofloxacin induces a 27% decrease in the half-life of elimination of flunixin (Abo-El-Sooud & Al-Anati, 2011). In horses, however, the concomitant administration of enrofloxacin at a dose of 5 mg/kg with firocoxib does not change the pharmacokinetics of firocoxib (Cox et al., 2012). With specific information on interaction of fluoroquinolones and carprofen currently unavailable, it is difficult to establish if the long half-life of carprofen results from an interaction or a general metabolic constraint in the rhinoceros. Nonetheless, based on the extremely long half-life of carprofen in the rhino, enzyme inhibition alone would probably not explain the latter. To definitely answer this question, the pharmacokinetics of carprofen would need to be established in a single drug study.

To evaluate the duration of benefits of carprofen we assessed its effects on TXB2 plasma concentrations. In general, the NSAIDs are known to in part function through the inhibition of the cyclooxygenase 1 and 2 (COX-1 and 2) leading to a reduced production of eicosanoids, and as a result, inhibiting inflammatory reactions, pain and hypersensitivity

(Moses & Bertone, 2002). The constitutively expressed COX-1 plays a less significant role in cases of tissue injury and inflammation than COX-2, however, increases by 2 to 3 fold after tissue injuries and plays a role in pain transmission (Brooks et al., 1999). In contrast, COX-2 enzymes are induced and upregulated during inflammatory processes and in case of tissue damage. Their concentrations can raise 20 fold above the baseline values (Brooks et al., 1999; Lee et al., 1992).

In order to assess the degree of COX-1 inhibition of NSAIDs, TXB2 is commonly used as a surrogate marker (Lees et al., 2004c).We were expecting TXB2 to decline as described in species such as the horse and the cat (Taylor et al., 1996; Lees et al., 1994). In horses, studies demonstrated a moderate inhibition of TXB2 at low doses of carprofen (0.7 mg/kg) and complete inhibition at high carprofen doses (4 mg/kg) (Lees et al., 1994, 2002). However, as with field-type trials, we experienced an unexpected adverse reaction for the concurrently administered enrofloxacin. The enrofloxacin administration caused a visible, localised adverse reaction in four out of six rhinoceros (rhino I, III, IV, VI). It manifested in the form of a band like swelling at the base of the ear in which enrofloxacin was injected. This was evident as a rapid increase in plasma TXB2 concentrations with the peak of activity after 22.3 ± 18.46 minutes, which was followed by a rapid decline. Using this unexpected reaction, we are able to provide an indication of the expected duration of effect of carprofen. Firstly, the early decline of the TXB2 concentration after 22.3 ± 18.46 minutes indicates a rapid onset of effect of carprofen, which is consistent with the results from a study by Borer et al. (2003). Using an acute synovitis model, Borer et al. (2003) assessed the analgesic and anti-inflammatory properties of carprofen in dogs and recorded an analgesic effect after 2.6 hours.

Besides assessing the plasma drug concentration, an important consideration when looking at the duration of effect of the NSAIDs, is the duration of enzyme inhibition. Studies by Lees et al. (1994) in horses have shown no identifiable relationship between the carprofen plasma concentration and the effect on the COX enzymes. This has led to the concept of a pharmacodynamic half-life, which describes the relation between the effect (E) of a drug and the time (t). A mathematical relationship can be established between the pharmacokinetic and pharmacodynamic half-life and shows that for high drug concentrations, the pharmacodynamic half-life is considerably longer than the pharmacokinetic half-life, leading to a longer lasting drug effect (Keller et al., 1998). As an example, the concept of the pharmacodynamic half-life could explain why carprofen in dogs exhibits a relatively short half-life of 7.99 ± 2.89 hours, while its postsurgical analgesic effect lasts for about 24 hours (McKellar et al., 1990; Shih et al., 2008). Based on the effective concentration and the secondary peak of TXB2 production in

rhino V and VI after 48 hours, we believe that the drug would be effective for a minimum of 48 hours after single carprofen administration. Nonetheless, these results have to be assessed with caution as the interpretation of the data is complicated by the lack of obvious correlation between the degree of TXB2 inhibition and carprofen plasma concentrations, as described by Lees et al. (1994) in the horse.

While we are confident of the anti-inflammatory effect of carprofen in white rhino, we cannot conclusively show that it will be effective as an analgesic. To try to ascertain the value of the pharmacokinetic parameters, the concentrations achieved were compared to results from Schatzman et al. (1990) who assessed the peripheral pain inhibition of carprofen in horses. Using the heating element model, the study demonstrated that 0.7 mg carprofen/mg successfully inhibited pain for roughly 24 hours. The analgesic effect of carprofen was correlated to a plasma concentration of at least 1.5 µg/ml. When comparing the effective carprofen plasma concentration of at least 1.5 µg/ml in the horse to the rhino pharmacokinetic profile, the plasma concentration of 1.5 µg/ml was achieved as early as 31 ± 0.98 minutes after carprofen administration and was maintained beyond the 72-hour monitoring period, with the carprofen plasma concentration still above 3.68 ± 0.2 µg/ml. While we suggest that actual pain monitoring needs to be conducted in rhino treated with carprofen, we would expect the drug to be effective in most animals for a minimum of 48 hours.

Conclusion

Wildlife veterinarians have documented good analgesic effects when using carprofen in injured white rhinoceros in the field (J. Marais, personal communication, 2016). In order to scientifically evaluate carprofen for a safe and efficient application in white rhinoceros, this first of its kind study investigated the pharmacokinetic and pharmacodynamic properties after a single dose injection. We were able to demonstrate that the pharmacokinetic profile of carprofen in rhinoceros differed significantly from that of the horse and any other species. Carprofen was characterised by a remarkable long half-life of lambda_z, which is longer than the reported half-lives in any mammalian species. Based on the evaluation of the surrogate marker TXB2, the drug also appeared to be anti-inflammatory for a minimum of 48 hours after administration in most animals, making it a promising drug to consider and further evaluate for the management of pain and inflammation in white rhinoceros.

5.6 Acknowledgements

This study was made possible through the help of ‘The Rhino Orphanage’, it’s founder Mr. Arrie van Deventer who allowed us to carry out the field work and data collection at ‘The Rhino Orphanage’ and the staff, in particular Laura Ellison and Jamie Traynor, who helped with the implementation of the field work and the data collection. Furthermore, we would like to thank Dr. Robin Gieling, Aminisha Somers and Klaudia Pruczkowska for their assistance during the field work. We thank Dr Pierre Bester for the chemical immobilisation and the monitoring of the rhinoceros during the trial. Furthermore, a sincere thank you goes to the German Academic Exchange Service (DAAD), the University of Pretoria, the South African Veterinary Association (SAVA) and Bayer Animal Health for their financial and material support.

5.7 Supplementary information

S 5-1: Immobilisation with etorphine and thiafentanil

	Initial drug combination (IM)		Top up	Acuphase	Diprenorphine
	Thia-fentanil	Etorphine			
Rhino I	0.25 mg	0.5 mg	X	50 mg IM after 1.5h	0.2 mg IV after 0.32h 5 mg IV after 1.88h
Rhino II	0.25 mg	0.5 mg	2 mg Medetomidine IV after 1.68h	50 mg IM after 1.43h	0.2 mg IV after 0.3h 5 mg IV after 1.95h
Rhino III	0.25 mg	0.5 mg	200 mg Ketamine IV after 1.08h 2 mg Medetomidine IV after 1.18h	50 mg IM after 1.72h	0.3 mg IV after 0.33h 0.2 mg IV after 0.5h 5 mg IV after 2.52h
Rhino IV	0.25 mg	0.5 mg	X	50 mg IM after 1.77h	0.2 mg IV after 0.55h 5 mg IV after 2.45h
Rhino V	0.25 mg	0.5 mg	X	50 mg IM after 2 h	0.3 mg IV after 0.43h 5 mg IM after 2.48h
Rhino VI	0.25 mg	0.5 mg	X	50 mg IM after 2.17h	0.3 mg IV after 0.4h 5 mg IM after 2.12h

S 5-2: Sedation for blood sampling after 24, 48, 72 hours with all samples drawn from the cephalic vein

	Sample 9 (24 h)	**Sample 10 (48 h)**	**Sample 11 (72 h)**
Rhino I	10 mg butorphanol IV	15 mg butorphanol IM (catheter not functional)	15 mg butorphanol IM (catheter not functional)
Rhino II	15 mg butorphanol IV, 5 mg top up IV 0.5h later	15 mg butorphanol IV	12 mg butorphanol IV
Rhino III	15 mg butorphanol IV	10 mg butorphanol IV	10 mg butorphanol IM (catheter not functional)
Rhino IV	15 mg butorphanol IV	10 mg butorphanol IV	10 mg butorphanol IV
Rhino V	30 mg butorphanol IV	20 mg butorphanol IV	15 mg butorphanol IV
Rhino VI	30 mg butorphanol IV	20 mg butorphanol IV	15 mg butorphanol IV

6. Chapter 6: Phylogenetic analysis of the cytochrome P450 (CYP450) nucleotide sequence of the horse and predicted CYP450 of the white rhinoceros (*Ceratotherium simum*) and other mammalian species

Marion Leiberich[1]*, Johan Marais[2], Vinny Naidoo[3]

[1] Department of Companion Animal Clinical Studies, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

[2] Saving the Survivors, Pretoria, South Africa

[3] Department of Paraclinical Science, University of Pretoria, Faculty of Veterinary Science, Pretoria, South Africa

*Corresponding author

6.1 Abstract

Background. The plight of the white rhinoceros (*Ceratotherium simum*) and the increasing need of treatment options for injured poaching victims led to the necessity to expand the knowledge on applicable drugs in this endangered species. With very little information available on drug pharmacokinetics in rhino, veterinarians have to rely on information generated from other species. The horse, being a closely related species, has served as the model for dose extrapolations. However, from recent research on enrofloxacin and carprofen, the white rhino showed considerable differences in the pharmacokinetic properties of these drugs in comparison to the horse. While the reason for the distinctions is unknown, a likely cause may be a difference in present cytochrome P450 (CYP450), that is, the rhino may be genetically deficient in certain enzyme families.

Methods. For this paper, we assess the degree of similarity of the CYP gene sequences across the different species using BLAT (BLAST-like alignment tool) for the alignment of the nucleotide sequences of the equine CYP450 with potential homologous nucleotide sequences of the published database from white rhinos and other mammalian species (cow, pig, dog, sheep, elephant, mouse, and human).

Results. The white rhino nucleotide sequences were 90.74% identical to the equine sequences. This was higher than the degree of similarity between any of the other evaluated species sequences. While no specific CYP family was lacking in the published rhino genome, the horse genome contained additional genetic sequences for a larger number of iso-enzymes, which were not present in the rhino.

Discussion. In pharmacokinetic study, it is well known that the absence of a metabolic enzyme will result in constraints in drug metabolism and elimination. While this was our speculation, the comparison to the horse and other mammalian species indicates that all described CYP genes required for metabolism are present within the rhino genome. Thus, the difference in metabolic capacity between the horse and the rhino cannot be directly attributed to an absolute deficiency of a CYP enzyme family. With the difference possibly being the absence of specific isoenzymes in the rhino, this could suggest that the horse is a more efficient metabolizer. The higher iso-enzyme expression would translate to an overall higher metabolic capacity. Since this was not a functional study, no comment can be made on the effective expression of any enzyme system in this study. However, the ineffective expression would also explain the slower drug metabolic capacity evident in the rhino.

6.2 Introduction

The escalating rhino crisis and the brutal poaching of thousands of rhinos over the last few years (Save the Rhino, 2018; Emslie et al., 2016) have drawn special attention to the plight of this threatened species. With the increasing numbers of rhinos requiring urgent medical help, the need to optimise the treatment of severely injured poaching victims is substantial. Unfortunately, as the need of appropriate drugs is a relatively recent problem, scientifically evaluated data for the treatment of rhino is lacking. Furthermore, research in wildlife medicine is often complicated by the animals' size and untamed nature, by the lack of sufficient numbers of study animals and is often challenging due to their status as endangered species.

As a result, wildlife veterinarians have resorted to using the horse (*Equus caballus*) as a model for the treatment of white rhino (*Ceratotherium simum*). While, from a general phylogenetic point of view, the horse is regarded as one of the closest related species to the rhinoceros (Tougard et al., 2001), we questioned whether it is correct to treat rhinos like large horses. Studies (Leiberich et al., 2018; unpublished data, M Leiberich) have recently been

undertaken in order to elucidate this issue. Enrofloxacin and carprofen, two potential drugs for the antimicrobial and analgesic treatment of poaching victims and other injured white rhinos, were evaluated in plasma pharmacokinetic studies. These studies revealed significant differences between drug pharmacokinetic parameters in the white rhino and the horse. Caprofen was characterised by a half-life of elimination of 105.71 ± 15.67 h, which is by far the longest recorded for any animal species (Leiberich et al., 2018). Furthermore, interspecies allometric scaling of enrofloxacin was able to show that the difference in the half-life of enrofloxacin in white rhino was not only due to the relative differences in their body size and that the latter was not allometrically scalable. Overall, these studies suggest that the rhino is a slow metaboliser of some drugs and that the latter is not simply comparable with the horse. While speculative, it was believed that the interspecies differences result at least partially from differences in drug metabolising enzymes and possibly from a lack of an important drug metabolising enzyme family. However, very little is known about the metabolic capacity of different animal species and about the causes of variation in drug metabolism. Consequently, the assessment of the drug metabolising units is needed.

The most important drug metabolising enzymes are the cytochrome P450s (CYP450), a diverse family of heme containing monooxygenases (Ioannides, 2006; Toutain et al., 2010), which play a major role in phase I reactions (Smith, 2009). The discovery of CYP450 enzymes dates back to the 1950s, when Klingenberg first described the carbon monoxide binding pigment with its absorbance maximum around 450 nm (Estabrook, 2003; Klingenberg, 1958). Already in the 1960s, the CYP450s were known to be linked to the drug and steroid metabolism (Nebert & Russell, 2002). Nowadays, the diverse functions have been further elucidated and range from the synthesis of steroid hormones (Payne & Hales, 2004) and endogenous epithelial relaxation factor (Fisslthaler et al., 1999) to the metabolism of xenobiotics (Anzenbacher & Anzenbacherova, 2001). For classification purposes, the CYP450 enzymes have been divided into families sharing a primary structure which is at least 40% identical. The classification into subfamilies, characterised by letters, is based on a more than 55% identical primary structure (van der Weide & Hinrichs, 2006). The individual isoenzymes differ by a minimum of 3% and are characterised by a second arabic number at the end (Anzenbacher & Anzenbacherova, 2001; Nelson, 2006). The major CYP450 families involved in drug metabolism entail the CYP1, CYP2 and CYP3 families and account for more than 90% of drug oxidation in humans (Ioannides, 2006; Zanger & Schwab, 2013). While cytochrome P450 enzymes have been widely studied in humans, information for animal species is scarce (Fink-Gremmels, 2008). Despite the interest in adequate animal models for

human drug development and the interest in the prediction of drug residue levels in production animal species, the scientific knowledge in this field is still in its infancy (Fink-Gremmels, 2008; Ioannides, 2006; Martignoni et al., 2006).

The aim of this study was to assess the degree of *in silico* phylogenetic similarity between the CYP enzyme sequences published for the horse and other mammalian species with the gene sequences of the rhino. The main objective was to ascertain if the white rhino could be genetically deficient in any particular CYP enzyme families, which would provide insight for observed clinically prominent differences in drug metabolism. The phylogenetic relationship of the CYP enzymes of the horse and the gene sequence of selected species including the white rhinoceros, the cow (*Bos taurus*), the dog (*Canis lupus familiaris*), the pig (*Sus scrofa*), the elephant (*Loxodanta africana*), the sheep (*Ovis aries*), the mouse (*Mus musculus*) and the human (*Homo sapiens*) was assessed.

6.3 Materials and methods

A data mining strategy was applied to match the cytochrome P450 gene sequences of the horse to the gene sequences of selected species (Table 6-1) in order to determine the existence and the degree of homologous sequences amongst the different species. Fifteen identified CYP genes of the horse, namely CYP11A1, CYP17A1, CYP19A1, CYP27B1, CYP2A13, CYP2C113, CYP2C92, CYP2D50, CYP2E1, CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96 and CYP3A97 (Wade et al., 2009) were used to perform a BLAT (BLAST like alignment tool) (Kent, 2002) search against the NCBI genome assemblies for the selected species (Archibald et al., 2010 a,b; Jiang et al., 2014; Lindblad-Toh et al., 2005; Uenishi et al., 2012; Zimin et al., 2009). Sequences with less than 15% alignment were taken out automatically. Using the Molecular Evolutionary Genetics Analysis (MEGA) (Kumar et al., 2016), the evolutionary relationship between the CYP450 genes of the horse and the corresponding gene sequences of the other species were inferred. Furthermore, a phylogenetic tree was constructed depicting the evolutionary relationship between the cytochrome P450 enzymes of the horse and the matching gene sequences of the selected species.

Table 6-1: Selected species, corresponding accession ID numbers and additional sample information included in the comparison to the gene sequences of CYP enzymes

Species	Accession ID	Additional sample information
Horse (*Equus caballus*)	GCA_000002305.1	Female, thoroughbred, isolate Twilight
White rhinoceros (*Ceratotherium simum*)	GCA_000283155.1	Female
Cow (*Bos taurus*)	GCA_000003055.4	Pooled male and female samples, Hereford, tissue blood
Dog (*Canis lupus familiaris*)	GCA_000002285.2	Female Boxer
Pig (*Sus scrofa domesticus*)	GCA_000003025.4	Female, Duroc, isolate TL Tabasco
Elephant (*Loxodonta africana*)	GCA_000001905.1	Female
Sheep (*Ovis aries*)	GCA_000298735.1	Male and female, Texel
Mouse (*Mus musculus*)	GCA_000001305.2	Strain C57BL/6J
Human (*Homo sapiens*)	GCA_000001305.2	Genome Reference Consortium Human GRCh38

A detailed description on how to build a phylogenetic tree from molecular data with MEGA is given by Hall (2013). Briefly, the sequence alignment was performed using MUSCLE (Multiple Sequence Comparison by Log Expectation) (Edgar, 2004). Subsequently, different substitution models were assessed for the goodness of fit measured by the Bayesian information criterion (BIC) (Schwarz, 1978). Based on the lowest BIC value, the Kimura 2-parameter model (Kimura, 1980) (CYP2E1, CYP3A89, CYP3A96) and the Tamura 3-parameter model (Tamura, 1992) (CYP11A1, CYP17A1, CYP19A1, CYP27B1, CYP2A13, CYP2C113, CYP2C92, CYP2D50, CYP3A93, CYP3A94, CYP3A95, CYP3A97) were chosen to assess the evolutionary distance based on the Maximum Likelihood method. The initial trees for the heuristic search were constructed automatically. Therefore, the Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances, which were estimated using a maximum composite likelihood approach. The topology with the best log likelihood value was chosen. Additionally, in order to model the evolutionary rate differences amongst sites (5 categories), a discrete gamma distribution was applied (Yang, 1994). Codon positions included were 1st+2nd+3rd+Noncoding. Positions with less than 95% site coverage were eliminated.

The bootstrap consensus tree based on 1000 replicates (Felsenstein, 1985) was built in order to assess the reliability of a phylogenetic tree. The percentage of the recovery of the same nodes throughout the bootstrap analysis is indicated next to the branches. The analysis was based on nine nucleotide sequences from the different species. Another bootstrap

consensus tree (1000 replicates) of all equine CYP450s and the matching gene sequences of the chosen species was computed and displayed in the circular view. The estimation of the evolutionary tree was based on the Maximum Likelihood method. It includes 135 nucleotide sequences from nine different species and was constructed using the Tamura-3-parameter model. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The initial trees for the heuristic search were constructed automatically. Therefore, the Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances, which were estimated using a maximum composite likelihood approach. The topology with the best log likelihood value was chosen. In order to model the evolutionary rate differences amongst sites (5 categories, parameter = 1.8250), a discrete gamma distribution was applied (Yang, 1994). The rate variation model allowed for some sites to be evolutionary invariable (2.6181% sites). Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 95% site coverage were eliminated.

Furthermore, the evolutionary distance between the nucleotide sequences of the equine CYP3A enzymes and the matching gene sequences of the white rhino and of the other species were computed using the pairwise distance function in MEGA (Kumar et al., 2016). The nucleotide differences between each pair of sequences were calculated to facilitate the assessment of the degree of similarity among the sequences.

6.4 Results

The individual phylogenetic trees for each P450 enzyme detected in the horse (CYP11A1, CYP17A1, CYP19A1, CYP27B1, CYP2A13, CYP2C113, CYP2C92, CYP2D50, CYP2E1, CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96 and CYP3A97) are depicted in Figure 6-1 and Figure 6-2. The phylogenetic trees highlight the evolutionary relationship of the equine gene sequences and the gene sequences of the white rhino, the cow, the dog, the pig, the elephant, the sheep, the mouse and the human. In all cases, the alignment of the CYP genes of the horse with the gene sequences of the other species showed that the equine CYP enzymes are most closely related to the sequences of the white rhino. The degree of similarity between the known equine CYP450 genes and the sequences identified in the genome of the white rhino ranged from 87.8 to 94.1% (Table 6-2). On average, the white rhino nucleotide sequences were 90.74% identical to the equine CYP450 gene sequences. Figure 6-3 illustrates the relationship between all CYP450 genes of the horse and the matching nucleotide sequences of the other species (also named CYP450s in the

phylogenetic trees). Gene sequences of all CYP enzyme families identified in the horse seem to be present in the white rhino.

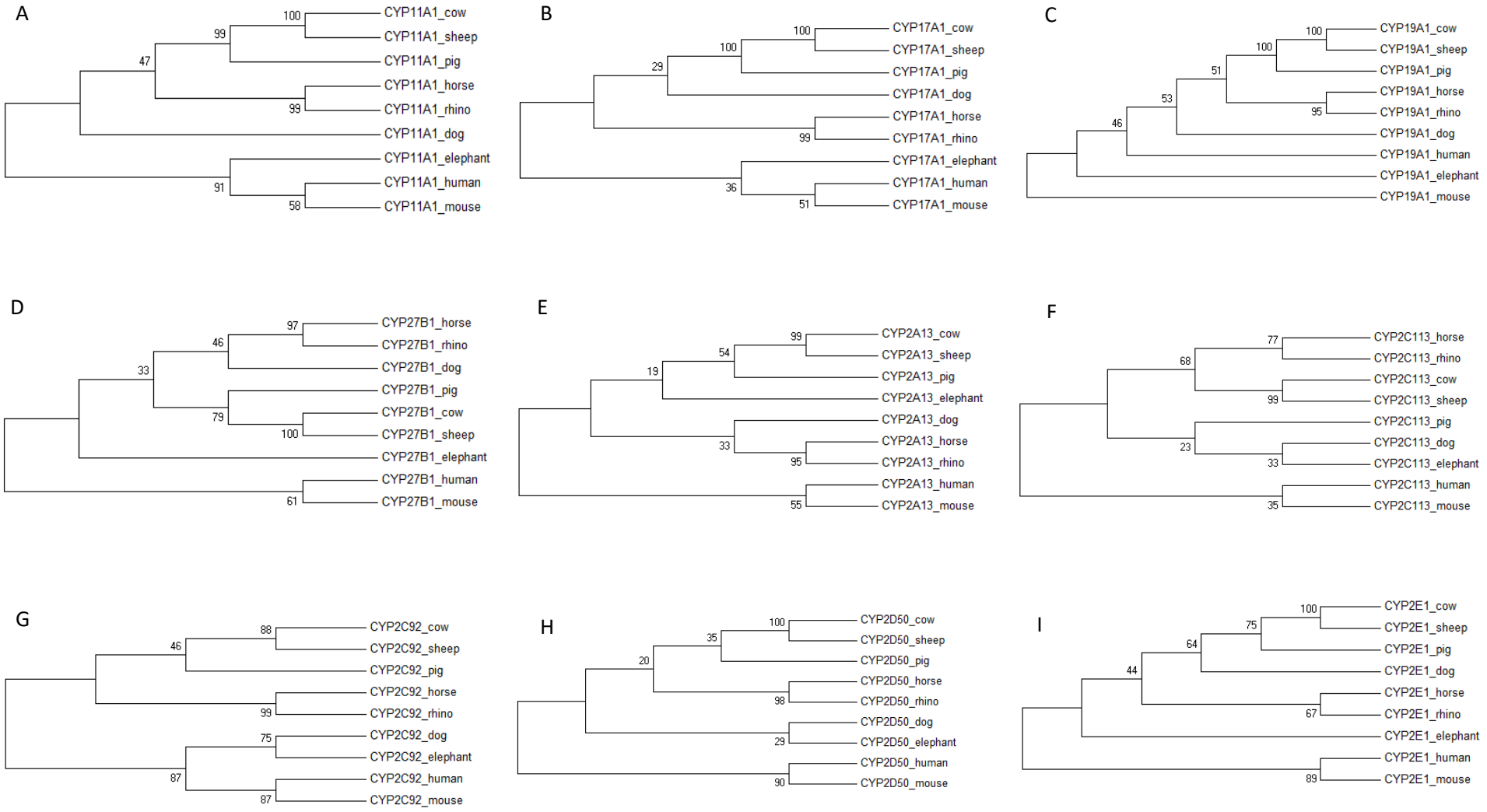


Figure 6-1: Molecular phylogenetic relationship of the CYP11A1 (A), CYP17A1 (B), CYP19A1 (C), CYP27B1 (D) CYP2A13 (E), CYP2C113 (F), CYP2C92 (G), CYP2D50 (H), CYP2E1 (I), across 9 different species (horse, white rhinoceros, cow, sheep, dog, pig, elephant, mouse and human) by Maximum Likelihood (ML) method

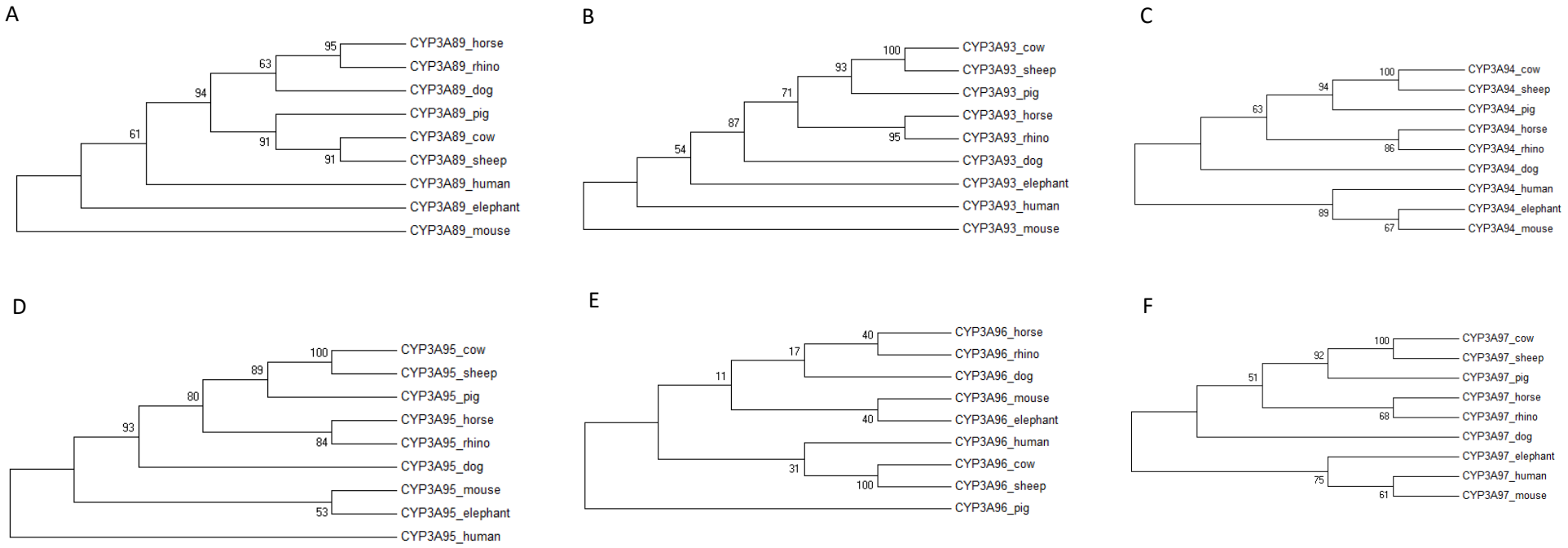


Figure 6-2: Molecular phylogenetic relationship of the CYP3A89 (A), CYP3A93 (B), CYP3A94 (C), CYP3A95 (D), CYP3A96 (E) and CYP3A97 (F) across 9 different species (horse, white rhinoceros, cow, sheep, dog, pig, elephant, mouse and human) by Maximum Likelihood (ML) method

Table 6-2: Degree of similarity (%) between the equine CYP450 genes and the gene sequences of the white rhino

CYP450 of the horse	Similarity (%) between the gene sequences of the white rhino and the horse
CYP11A1 (NM_001082521)	89.8
CYP17A1 (NM_001082523)	91
CYP19A1 (NM_001081805)	89
CYP27B1 (NM_001163957)	94.1
CYP2A13 (NM_001111337)	91.3
CYP2C113 (NM_001291302)	90.7
CYP2C92 (NM_001101652)	87.8
CYP2D50 (NM_001111306)	92.4
CYP2E1 (NM_001111303)	91.2
CYP3A89 (NM_001101651)	90.6
CYP3A93 (NM_001190938)	91.7
CYP3A94 (NM_001190939)	93
CYP3A95 (NM_001190940)	90.5
CYP3A96 (NM_001146163)	89.2
CYP3A97 (NM_001146164)	88.8

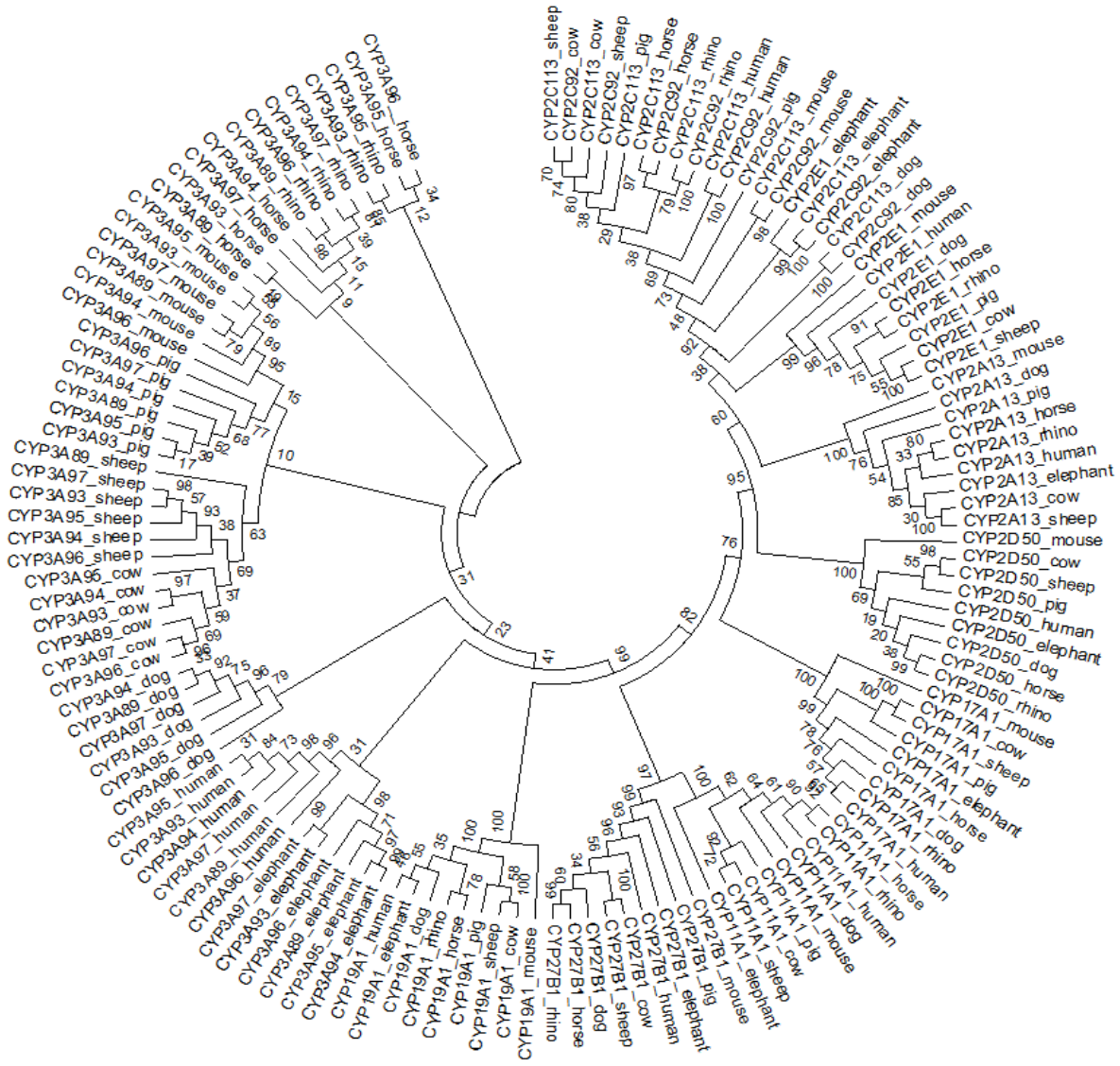


Figure 6-3: Circular phylogenetic tree depicting the relationship of 135 nucleotide sequences consisting of all CYP450 genes of the horse (CYP11A1, 17A1, 19A1, CYP27B1, CYP2A13, CYP2C113, CYP2C92, CYP2D50, CYP2E1, CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96 and CYP3A97) and the matched nucleotide sequences of the white rhinoceros, cow, sheep, dog, pig, elephant, mouse and human (also named CYP450)

The calculation of the pairwise distance between the nucleotide sequences of the rhino (Table 6-3) revealed that the CYP3A gene sequences were identical or highly similar with distance indexes of as little as 0.023 between the CYP3A95 and the CYP3A93 nucleotide sequence and 0 between the CYP3A96 and CYP3A89 nucleotide sequences. Further investigations showed that the sequences matching all the equine CYP3A genes were found at the same location in the genome of the rhino and overlapped each other in most cases. Additionally, the calculation of the pairwise distance of the CYP3A nucleotide sequences of

the cow, dog, pig, human and sheep demonstrate a very high degree of similarity of up to 100% identity amongst each other (S 6-1).

Table 6-3: **Estimates of evolutionary divergence between the nucleotide sequences of the white rhino which matched the equine CYP3A sequences.** Presented as numbers of base differences per site between each pair of sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA (Kumar et al., 2016)

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.066					
CYP3A94	0.056	0.059				
CYP3A95	0.059	0.023	0.036			
CYP3A96	0.000	0.066	0.056	0.059		
CYP3A97	0.056	0.056	0.043	0.046	0.056	

6.5 Discussion

While large numbers of different species, diseases and conditions require medical attention and treatment, wildlife veterinarians often face the lack of approved and scientifically evaluated drugs for zoo and wildlife species. A study published by Tana et al. (2010) stated that only eight to ten compounds are approved for the use in zoo and wildlife in the USA, in contrast to close to 300 in cattle. The white rhino represents one of the species where basic medical knowledge is not yet readily available. However, the poaching crisis and the increasing numbers of injured individuals requiring urgent medical help, especially for the treatment of wounds, has been on the rise. To overcome limitations in species specific information, the medical management of the rhino tends to be based on information available for the horse. However, the findings of a recent study we undertook in rhinos showed that the half-life of elimination for carprofen was more than three fold longer than that in the horse (Leiberich et al., 2018). This made us question the validity of the horse as a model.

The differences in drug metabolism could generally arise from distinctions in anatomy, physiology, behaviour and biotransformation and metabolism by CYP450 enzymes. With the horse and the rhino showing a closely related digestive physiology and anatomy of the gastrointestinal tract and similar feeding habits, the differences in drug metabolism would most likely be ascribed to distinctions in their drug metabolising enzymes. To assess this assumption, the gene sequences of the CYP450s of the horse, the rhino and other selected species were compared as a first step in ascertaining if any of the genes coding for the major drug metabolising families were absent in the rhino genome. From the gene analysis it would appear that the complete genetic deletion of a major CYP enzyme family was not the cause of the

evident limitations in drug metabolism, leaving functional differences in enzyme activity as the likely reason. The latter was evident in the subsequent relatedness analysis. While showing the horse to be the most closely related species to the white rhino, only 90.74% similarity were evident across all the equine CYP enzymes. This finding would indicate that the genetic differences between the horse and the rhino are sufficient to be the cause of the major differences in drug metabolism. This would render drug prediction between the species unreliable at the clinical level.

Similar conclusions have been previously drawn by Fink-Gremmels (2008), Martignoni et al. (2006), Nelson et al. (2013) and Toutain et al. (2010) who declared that small differences in the amino acid sequence of the CYP enzyme can lead to marked changes in substrate specificity and catalytic activity. Thus, not even closely related species with similar physiological characteristics exhibit similar cytochrome P450 enzyme activity. Even a single change in amino acid sequences is sufficient to possibly alter substrate specificity (Lindberg & Negishi, 1989) and different CYP450 enzymes may metabolise the same substrate (Guengerich, 1997). In more serious cases, the genetic difference could result in stop codons being present in the incorrect area within the mRNA sequence, leading to abnormal termination of the enzyme translation (McAdam, Goundis & Reid, 1988). Contrary to general expectations, the enzyme liver pattern of herbivore and carnivore species, of monogastric species and ruminants and even within a species such as cattle, differs markedly (Fink-Gremmels, 2008). Guengerich (1997) suggested a provisional classification according to ‘catalytic preservation’ of the CYP450 enzymes. Accordingly, the only CYP450 enzyme which can be accurately extrapolated across species is the CYP2E1. The extrapolation of the CYP1A1, 1A2 and 17A enzymes needs to be conducted carefully. Even more caution is required for the extrapolation of CYP2D and 3A, whereas the extrapolation of CYP2A, 2B and 2C enzymes across species shows no catalytic preservation.

Another important finding in this study relates to the CYP3A family. In humans, it represents about 30% of the total liver CYP content and metabolises around 50% of all marketed drugs. Its most important drug metaboling isoenzyme is the CYP3A4 (Furge & Guengerich, 2006). While detailed information on the importance of the different isoenzymes and their contribution to drug metabolism in animals is not yet available, one would assume that the important drug metabolising CYP families are the same as in humans. The importance of these enzymes is further evident by the number of isoenzymes within the group. Four have been identified in man (CYP3A4, CYP3A5, CYP3A7 and CYP3A43) and six in the horse

(CYP3A89, CYP3A93, CYP3A94, CYP3A95, CYP3A96, CYP3A97), with the equine CYP3A89 exhibiting the highest similarity to the human CYP3A4 (Schmitz et al., 2010).

The pairwise distance analysis between the CYP3A nucleotide sequences of the white rhino revealed high levels of similarity (Table 6-3). Additionally, the nucleotide sequences were all limited to the same location (JH767858: 353657-1001765) in the genome of the rhino. This finding suggests that the CYP3A family in the rhino genome has no isoenzymes, and consequently, that the white rhino has fewer isoenzymes than the horse. Overall, with the CYP3A subfamily being of major importance for the drug metabolism, a lack of isoenzymes may explain the observed constraint in drug metabolism in white rhino. However, similar observations were made based on the pairwise comparison of the CYP3A gene sequences in the other species. In most cases, the sequences of the pig, cow, dog, human and sheep which were matched to the different CYP3A gene sequences of the horse did not reveal any sequence difference, and thus, seemed to represent one single nucleotide sequence as in the rhino (S 6-1). Alternatively, those findings may imply that the white rhino, like other species may have its own, species specific set of drug metabolising CYP3A isoenzymes, which differ from those in the horse. Rather than reflecting a true non-existence of CYP3A isoenzymes, those may have just not yet been identified in the rhino.

Similarly to the rhino, drug doses for elephants, another species belonging to the hindgut fermenters, are often extrapolated from pharmacokinetic data available for horses (Hunter, Isaza, & Koch, 2003; Mortenson, 2001). However, the comparison of the CYP3A gene sequences also showed that unlike in the rhino, the elephants' CYP3A gene sequences seem to be the closest to those of the mouse and not closest related to the horse nor to the white rhino (Figure 6-2). This finding may further indicate that drug dose extrapolation from horses to other hindgut fermenters and mega-herbivores such as the elephant needs to be conducted with caution and cannot be based solely on the fact that they share similar physiological characteristics.

6.6 Conclusion

In conclusion, the rhino as a species was not overly deficient for any of the genes coding for the major drug metabolizing enzymes. While the white rhino CYP450 gene sequences were most similar to those of the horse, this was overall only at the 90% level. Despite appearing to be a minor distinction, even smaller differences are known to have a major effect on drug metabolism. As a result, despite the close anatomical relationship, the rhino shouldn‘t simply be treated like a big horse.

6.7 Acknowledgements

The authors thank Dr. Emmanuel Adawaren for his introduction to the program MEGA and Dr. Micheal Hewetson for editing the manuscript.

6.8 Supplementary information

S 6-1: Estimates of evolutionary divergence between the CYP3A nucleotide sequences of the cow (A), dog (B), pig (C), human (D), sheep (E), which were matched to the equine CYP3A sequences. Presented as numbers of base differences per site between each pair of sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Evolutionary analyses were conducted in MEGA (Kumar et al., 2016)

A

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.000					
CYP3A94	0.000	0.000				
CYP3A95	0.000	0.000	0.000			
CYP3A96	0.000	0.000	0.000	0.000		
CYP3A97	0.000	0.000	0.000	0.000	0.000	

B

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.000					
CYP3A94	0.000	0.000				
CYP3A95	0.007	0.007	0.007			
CYP3A96	0.000	0.000	0.000	0.007		
CYP3A97	0.000	0.000	0.000	0.007	0.000	

C

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.000					
CYP3A94	0.000	0.000				
CYP3A95	0.000	0.000	0.000			
CYP3A96	0.000	0.000	0.000	0.000		
CYP3A97	0.000	0.000	0.000	0.000	0.000	

D

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.102					
CYP3A94	0.102	0.000				
CYP3A95	0.102	0.000	0.000			
CYP3A96	0.099	0.05	0.05	0.05		
CYP3A97	0.102	0.000	0.000	0.000	0.05	

E

	CYP3A89	CYP3A93	CYP3A94	CYP3A95	CYP3A96	CYP3A97
CYP3A89						
CYP3A93	0.000					
CYP3A94	0.000	0.000				
CYP3A95	0.000	0.000	0.000			
CYP3A96	0.053	0.053	0.053	0.053		
CYP3A97	0.000	0.000	0.000	0.000	0.053	

7. Chapter 7: General discussion

7.1 The potential for the use of enrofloxacin in white rhino

The current rhino poaching crisis necessitates adequate treatment options to be available for the alleviation of pain and inflammation and for the management of infections in injured poaching victims in order to enhance their chance of survival. With no knowledge readily available in this field, the aim of the study was to take a first step towards treatment optimisation and to provide some groundwork and insight via pharmacokinetic studies in white rhinoceros. Therefore, one antimicrobial (enrofloxacin) and one anti-inflammatory drug (carprofen) were assessed as potential treatment options. The broad-spectrum antimicrobial properties of enrofloxacin, the long duration of effect recorded in other species, its rapid concentration dependent bactericidal effect and the different routes of administration rendered it a promising drug for the treatment of white rhino. Carprofen, known for its long elimination half-life, good safety profile and analgesic effect in different species (Gaynor & Muir, 2015; Mathews, 2002; McKellar et al., 1991) was evaluated as a potential option for pain relief in white rhino.

The intravenous administration of enrofloxacin (12.5 mg/kg) was well tolerated in the rhinoceros except for some swelling occurring in the second phase of the study at the base of the ear in which the drug was administered. In all animals, sufficient samples were collected to allow for the complete characterisation of the intravenous pharmacokinetic profile. The findings revealed a surprisingly long elimination half-life (12.41 h), which was much longer than in any other studied mammalian species, including the horse at 4.4 hours (Kaartinen et al., 1997) and 6.15 hours (Peyrou et al., 2006). When the intravenous dose was followed by a single oral treatment after recovery from immobilisation, enrofloxacin was characterised by a low bioavailability of 33.3%. While speculative, the latter may have resulted from the efflux of the drug into the gastrointestinal tract by the actions of the ATP transporter BCRP, which has been described to occur in the intestinal wall of humans and horses (Alvarez et al., 2008; El-Kattan & Varma, 2012; Tyden et al., 2010). Further research is however required to ascertain the presence and the role of active transport mechanisms in white rhino.

To ascertain the value of enrofloxacin in treatment, pharmacodynamic assessment was subsequently undertaken on the obtained pharmacokinetic data. Using the efficacy markers AUC_{24}: MIC and Cmax: MIC, the results suggested that intravenous enrofloxacin at 12.5 mg/kg body weight exceeded the recommended values of 125 and 12:1, respectively. These findings indicate a successful treatment outcome viz. a successful clinical and microbiological cure of infection. However, for treatment to be effective by the intravenous route, once daily

administration would be needed, which makes IV treatment alone an unlikely therapeutic option. Since this failure was considered in the original study design, intravenous enrofloxacin administration in phase 2 was followed by oral enrofloxacin administration once the animals ate after chemical immobilisation. Unfortunately, even under these conditions, therapeutic drug levels achieved through the additional oral drug dose failed to reach the target values of the efficacy markers. As a result, we conclude that neither oral enrofloxacin administration nor the single dose treatment with enrofloxacin is recommended, if one is aiming for a rapid, bactericidal effect with a low risk of emerging resistance, unless the animal can be repeatedly re-sedated for enrofloxacin administration. In the case of habituated rhino, low doses of butorphanol may represent an adequate option for repeated sedation.

As we experienced problems with the blood draws from the auricular catheters after more than 12 hours post catheter placement, the samples at 24, 48 and 72 hours could not be drawn as intended. As a result, we had to resort to sedating the animals for blood collection. Butorphanol was therefore administered as a direct sedative at low doses of 16.63 to 33.33 µg/kg (10-30 mg/animal) IV via the auricular catheter. Butorphanol produced an acceptable level of sedation and allowed the handling of the rhinos and the blood collection within 15 minutes of administration. While the chemical immobilisation with etorphine generally results in recumbency of the animals, the low doses of butorphanol led to dose responsive sedative effects and standing sedation. Thus far, butorphanol is mostly used to alleviate respiratory depression during chemical immobilisation with etorphine. However, the ability to lightly sedate the study animals without using etorphine results in a cheaper immobilisation. Furthermore, it offers a greater operator and patient safety through reduced side effects such as respiratory depression and hypoxia and may be of growing importance given the increase of poaching victims and orphaned rhino calves.

7.2 The potential for the use of carprofen in white rhino

For this part of the study, the pharmacokinetics of carprofen were evaluated following a single IM treatment (1 mg/kg) concurrently administered with enrofloxacin, as it would be facilitated under field conditions. In all cases, carprofen was well tolerated without any visible adverse reactions. Similar to enrofloxacin, it was characterised by an exceptionally long half-life of elimination (105.71 hours), which was more than 3.5 times the half-life in a horse (29.4 hours), and by a very slow clearance of 0.001 L/h*kg. The long half-life of elimination of carprofen in the rhino compared to other mammalian species led to the presumption of a constrained metabolism in the white rhinoceros, which may have been exacerbated by the

enzyme inhibitory effects of enrofloxacin. The latter is however speculative and is based on the known ability of enrofloxacin to prolong the half-life of elimination of theophylline and certain NSAIDs through the inhibition of the CYP450 enzymes, such as CYP1A1 and CYP1A2 (Intorre et al., 1995; Rahal et al., 2008; Regmi et al., 2005; Vancutsem & Babish, 1996, Abo-El-Sooud & Al-Anati, 2011; Ogino et al., 2005). To properly explain if the slower half-life derived from enzyme inhibition, we would recommend that the pharmacokinetics of carprofen get evaluated following single drug administration.

The efficacy of carprofen was tentatively assessed through the COX-1 inhibitory potential of the administered dose and related plasma concentration. Against initial expectations of a decrease in TXB2 concentrations, the latter rapidly increased with the peak of activity after 22.3 ± 45.2 minutes, which was followed by a rapid decline. TXB2 tended to reach pre-treatment levels by 48 hours. The unexpected increase of TXB2 concentrations was attributed to the adverse reaction caused by the concurrently administered enrofloxacin, which led to an inflammatory reaction and therewith to the increase in COX-1 marker substrates. In all cases, a pharmacokinetic-pharmacodynamic model could not be fitted to the data due to the prolonged plasma versus time profile achieved. Nonetheless, we were able to use the change in plasma concentrations over time to provide an indication of the expected duration of effect of carprofen. From the early decline in TXB2 we conclude that carprofen has a rapid onset of effect, while the prolonged plasma concentration would indicate the potential for the drug to be effective as an anti-inflammatory for a minimum of 48 hours. Based on the long half-life of elimination and the potential for a prolonged effect, carprofen appears to be a valuable drug for a single dose treatment in white rhino. While the results indicate the value of carprofen as an anti-inflammatory in white rhino, the analgesic effect and safety profile of the drug in the rhino still needs to be evaluated. Thus far, veterinarians already applying carprofen have indicated that they are satisfied with the analgesic effect they are seeing under clinical use (J. Marais, personal communication, 2016).

7.3 Metabolic constraints of the white rhinoceros

Both, carprofen and enrofloxacin administration was characterised by the unexpected finding of a long half-life of elimination, especially when compared to the horse. This leads to the speculation that the white rhinoceros is metabolically constrained. In order to demonstrate that the slower metabolism was present as a result of metabolic constraints as opposed to size related slower metabolism, the pharmacokinetic parameters of enrofloxacin were further evaluated by allometric scaling. Cox et al. (2004) indicated that the drugs' pharmacokinetic

parameters clearance and volume of distribution could be scaled between species and numerous models were published. Following the allometric scaling of pharmacokinetic parameters across different groupings of species (monogastric species, herbivores, mammalian species and all species), a good model fit was achieved for the clearance and the volume of distribution, and a moderate model fit was achieved for the half-life of elimination. In all cases, the models in use predicted shorter half-lives of elimination than seen for the actual calculated parameter. Based on this result, it is concluded that the metabolism of enrofloxacin in the rhino is much lower than what can be explained by the size alone. Thus, the rhinoceros is metabolically constrained as opposed to exhibiting a slower metabolism related to its larger size. This also leads us to conclude that the use of scaling to obtain the correct dose from horses may result in gross underestimation of the half-life of elimination and an increased potential for toxicity.

When looking at the metabolism of drugs, an important system is the cytochrome P450 group of enzymes (Anzenbacher & Anzenbacherova, 2001; Fink-Gremmels, 2008). The current knowledge shows that interspecies differences in the CYP450 enzymes expressed are responsible for differences seen in the metabolism of xenobiotics. For the last part of the study, we assessed the genome of the white rhinoceros published by the Broad Institute (National Center for Biotechnology Information , 2012) to establish if the rhino may be overtly deficient in any of the CYP enzyme systems. For this comparison, the currently known CYP enzyme gene sequences of the horse (Wade et al., 2009) were compared to the genome of the white rhinoceros and of other selected species (cow, pig, sheep, mouse, dog, elephant and human) using the BLAT alignment program. The aim was to identify if similar sequences were present in the different species. Phylogenetic trees were subsequently constructed based on the degree of homology between the equine CYP gene sequences and the matched gene sequences of the chosen species.

Despite the significant differences in drug pharmacokinetics, the phylogenetic analysis showed that from the gene sequences compared, the white rhino was the closest related to the horse. However, this genetic similarity does not implicate that the horse is a suitable model for the medication of white rhinoceros as indicated above. Despite the closeness of the relationship between the horse and rhino, the species were between 87.8 and 94.1% similar, which could explain the evident differences in metabolism.

Another interesting finding was present for the CYP3A genes, one of the important drug metabolising enzyme families in humans (Furge & Guengerich, 2006). While six different CYP3A isoenzymes have been identified in the horse, only one CYP3A gene sequence was

found in the rhinoceros i.e. the rhinoceros only had one sequence matching all the equine CYP3A genes. This finding suggests that the rhino has fewer isoenzymes than the horse, and may thus explain the constraints in the rhinos' ability to metabolise drugs. In this case, the differences in drug metabolism between the horse and the rhino may result in a lower number of the total different isoenzymes (relative capacity). Whether the differences in drug metabolism between the rhino, the horse and other species occur due to a reduced count of each isoenzyme or due to differences in the relative capacity will require further assessment of levels of expression, catalytic activity and substrate specificity of the different CYP3A isoenzymes.

Based on the findings from the *in silico* aspects of the study, it would appear that the rhinoceros is characterised by a considerably slower metabolism of drugs than expected for an animal of its size. This leads to the conclusion that it may be dangerous to directly extrapolate rhino drug doses based upon published data for the horse, even though they are closely related species.

8. Chapter 8: References

Abo-El-Sooud, K. & Al-Anati, L. (2011) Pharmacokinetic study of flunixin and its interaction with enrofloxacin after intramuscular administration in calves. *Veterinary World*, 4(10), 449-454. 10.5455/vetworld.2011.449-454

Abramson, S., Edelson, H., Kaplan, H., Ludewig, R., & Weissmann, G. (1984). Inhibition of neutrophil activation by nonsteroidal anti-inflammatory drugs. *American Journal of Medicine, 77*(4B), 3-6.

Adkesson, M. J., Junge, R. E., Allender, M. C., & Martin-Jimenez, T. (2012). Pharmacokinetics of a long-acting ceftiofur crystalline-free acid formulation in Asian elephants (Elephas maximus). *American Journal of Veterinary Research, 73*(10), 1512-1518.

Alvarez, A. I., Perez, M., Prieto, J. G., Molina, A. J., Real, R., & Merino, G. (2008). Fluoroquinolone efflux mediated by ABC transporters. *Journal of Pharmaceutical Sciences, 97*(9), 3483-3493. 10.1002/jps.21233

Amin, R., Thomas, K., Emslie, R. H., Foose, T. J., & Van Strien, N. (2006). An overview of the conservation status of and threats to rhinoceros species in the wild. *International Zoo Yearbook, 40*, 96-117. 10.1111/j.1748-1090.2006.00096.x

Andreasen, A. S., Krabbe, K. S., Krogh-Madsen, R., Taudorf, S., Pedersen, B. K., & Moller, K. (2008). Human endotoxemia as a model of systemic inflammation. *Current Medicinal Chemistry, 15*(17), 1697-1705. 10.2174/092986708784872393

Andrews, J. (2001). Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy, 48*, 5-16.

Antithrombotic Trialists' Collaboration (2002). Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. *British Medical Journal, 324*, 71-86.

Anzenbacher, P., & Anzenbacherova, E. (2001). Cytochromes P450 and metabolism of xenobiotics. *Cellular and Molecular Life Sciences, 58*(5-6), 737-747. 10.1007/PL00000897

Archibald, A. L., Bolund, L., Churcher, C., Fredholm, M., Groenen, M. A. M., Harlizius, B., Lee, K.-T., Milan, D., Rogers, J., Rothschild, M. F., Uenishi, H., Wang, J., Schook, L.B. & Swine Genome Sequencing Consortium. (2010a). Pig genome sequence - analysis and publication strategy. *BMC Genomics, 11*(438).

Archibald, A. L., Cockett, N. E., Dalrymple, B. P., Faraut, T., Kijas, J. W., Maddox, J. F., McEwan, J. C., Hutton Oddy, V., Raadsma, H. W., Wade, C., Wang, J., Wang, W., Xun, X & Int Sheep Genomics Consortium. (2010b). The sheep genome reference sequence: A work in progress. *Animal Genetics, 41*(5), 449-453. 10.1111/j.1365-2052.2010.02100.x

Argentieri, D. C., Ritchie, D. M., Ferro, M. P., Kirchner, T., Wachter, M. P., Anderson, D. W., Rosenthale, M. E. & Capetola, R. J. (1994). Tepoxalin - a dual cyclooxygenase 5-lipoxygenase inhibitor of arachidonic-acid metabolism with potent antiinflammatory activity and a favorable gastrointestinal profile. *Journal of Pharmacology and Experimental Therapeutics, 271*(3), 1399-1408.

Armstrong, S., Tricklebank, P., Lake, A., Frean, S., & Lees, P. (1999). Pharmacokinetics of carprofen enantiomers in equine plasma and synovial fluid - a comparison with ketoprofen.

Journal of Veterinary Pharmacology and Therapeutics, 22(3), 196-201. 10.1046/j.1365-2885.1999.00202.x

Aronoff, D. M., & Neilson, E. G. (2001). Antipyretics: Mechanisms of action and clinical use in fever suppression. *American Journal of Medicine, 111*(4), 304-315. 10.1016/S0002-9343(01)00834-8

Balmer, T. V., Williams, P., & Selman, I. E. (1997). Comparison of carprofen and flunixin meglumine as adjunctive therapy in bovine respiratory disease. *Veterinary Journal, 154*(3), 233-241. 10.1016/S1090-0233(97)80028-7

Bauwens, L., Vroey, C. d., & Meurichy, W. d. (1996). A case of exfoliative dermatitis in a captive Southern white rhinoceros (Ceratotherium simum simum). *Journal of Zoo and Wildlife Medicine, 27*(2), 271-274.

Bayer Veterinary Services (2003). *Bovine Respiratory disease (BRD) - Treatment with Batril.* Retrieved from https://www.baytril.com/en/farm-animals/cattle/bovine-respiratory-disease-brd/

Bechert, U., & Christensen, J. M. (2007). Pharmacokinetics of orally administered ibuprofen in African and Asian elephants (Loxodonta africana and Elephas maximus). *Journal of Zoo and Wildlife Medicine, 38*(2), 258-268. 10.1638/1042-7260(2007)038[0258:POOAII]2.0.CO;2

Bechert, U., Christensen, J. M., Nguyen, C., Neelkant, R., & Bendas, E. (2008). Pharmacokinetics of orally administered phenylbutazone in African and Asian elephants (Loxodonta africana and Elephas maximus). *Journal of Zoo and Wildlife Medicine, 39*(2), 188-200. 10.1638/2007-0139R.1

Bertone, A., Tremaine, W., Macoris, D., Simmons, E., Ewert, K., Herr, L., & Weisbrode, S. (2000). Effect of long-term administration of an injectable enrofloxacin solution on physical and musculoskeletal variables in adult horses. *Journal of the American Veterinary Medical Association, 217*(10), 1514-1521. 10.2460/javma.2000.217.1514

Bidgood, T., & Papich, M. (2005). Plasma and interstitial fluid pharmacokinetics of enrofloxacin, its metabolite ciprofloxacin, and marbofloxacin after oral administration and a constant rate intravenous infusion in dogs. *Journal of Veterinary Pharmacology and Therapeutics, 28*(4), 329-341. 10.1111/j.1365-2885.2005.00664.x

Biggs, D., Courchamp, F., Martin, R., & Possingham, H. P. (2013). Legal trade of Africa's rhino horns. *Science, 339*(6123), 1038-1039. 10.1126/science.1229998

Blaser, J., Stone, B., Groner, M., & Zinner, S. (1987). Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. *Antimicrobial Agents and Chemotherapy, 31*(7), 1054-1060.

Blikslager, A., & Jones, S. (2005). NSAIDs. *Journal of Equine Veterinary Science, 25*(3), 98-102. 10.1016/j.jevs.2005.02.004

Blondeau, J. M., Borsos, S., Blondeau, L. D., & Blondeau, B. J. (2012). In vitro killing of Escherichia coli, Staphylococcus pseudintermedius and Pseudomonas aeruginosa by enrofloxacin in combination with its active metabolite ciprofloxacin using clinically relevant drug concentrations in the dog and cat. *Veterinary Microbiology, 155*(2-4), 284-290. 10.1016/j.vetmic.2011.08.015

Blood, D. C., Studdert, V. P., & Gay, C. C. (2007). *Saunders Comprehensive Veterinary Dictionary* (3rd ed.). Edinburgh ; New York: Elsevier Saunders.

Boeckh, C., Buchanan, C., Boeckh, A., Wilkie, S., Davis, C., Buchanan, T., & Boothe, D. (2001). Pharmacokinetics of the bovine formulation of enrofloxacin (Baytril 100) in horses. *Veterinary Therapeutics, 2*(2), 129-134.

Boothe, D. (1994). Enrofloxacin revisited. *Veterinary Medicine, 89*(10), 744-753.

Boothe, D. M., Boeckh, A., Simpson, R. B., & Dubose, K. (2006). Comparison of pharmacodynamic and pharmacokinetic indices of efficacy for 5 fluoroquinolones toward pathogens of dogs and cats. *Journal of Veterinary Internal Medicine, 20*(6), 1297-1306. 10.1892/0891-6640(2006)20[1297:COPAPI]2.0.CO;2

Borer, L. R., Peel, J. E., Seewald, W., Schawalder, P., & Spreng, D. E. (2003). Effect of carprofen, etodolac, meloxicam, or butorphanol in dogs with induced acute synovitis. *American Journal of Veterinary Research, 64*(11), 1429-1437. 10.2460/ajvr.2003.64.1429

Botting, R., & Ayoub, S. S. (2005). COX-3 and the mechanism of action of paracetamol/acetaminophen. *Prostaglandins Leukotrienes and Essential Fatty Acids, 72*(2), 85-87. 10.1016/j.plefa.2004.10.005

Bousova, K., Senyuva, H., & Mittendorf, K. (2013). Quantitative multi-residue method for determination antibiotics in chicken meat using turbulent flow chromatography coupled to liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1274*, 19-27. 10.1016/j.chroma.2012.11.067

Bousquet-Melou, A., Bernard, S., Schneider, M., & Toutain, P. L. (2002). Pharmacokinetics of marbofloxacin in horses. *Equine Veterinary Journal, 34*(4), 366-372. 10.2746/042516402776249191 ER

Boxenbaum, H., & Dilea, C. (1995). First-time-in-human dose selection - allometric thoughts and perspectives. *Journal of Clinical Pharmacology, 35*(10), 957-966.

Brabazon, J. (2000). *Albert Schweitzer – A biography* (2nd ed), Syracuse, NY: Syracuse University Press.

Bregante, M., Saez, P., Aramayona, J., Fraile, L., Garcia, M., & Solans, C. (1999). Comparative pharmacokinetics of enrofloxacin in mice, rats, rabbits, sheep, and cows. *American Journal of Veterinary Research, 60*(9), 1111-1116.

Brooks, P., Emery, P., Evans, J. F., Fenner, H., Hawkey, C. J., Patrono, C., Smolen, J., Breedveld, F., Day, R., Dougados, M., Ehrich, E. W., Gijon-Baños, J., Kvien, T.K., Van Rijswijk, M. H., Warner, T., Zeidler H. (1999). Interpreting the clinical significance of the differential inhibition of cyclooxygenase-1 and cyclooxygenase-2. *Rheumatology, 38*(8), 779-788. 10.1093/rheumatology/38.8.779

Brown, S. A. (1996). Fluoroquinolones in animal health. *Journal of Veterinary Pharmacology and Therapeutics, 19*(1), 1-14. 10.1111/j.1365-2885.1996.tb00001.x ER

Bryant, B., Blyde, D., Eamens, G., & Whittington, R. (2012). Mycobacterium avium subspecies paratuberculosis cultured from the feces of a Southern black rhinoceros (Diceros bicornis minor) with diarrhea and weight loss. *Journal of Zoo and Wildlife Medicine, 43*(2), 391-393. 10.1638/2010-0161.1 ER

Bugyei, K., Black, W., & McEwen, S. (1999). Pharmacokinetics of enrofloxacin given by the oral, intravenous and intramuscular routes in broiler chickens. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire, 63*(3), 193-200.

Bush, M., Raath, J. P., deVos, V., & Stoskopf, M. K. (1996). Serum oxytetracycline levels in free-ranging male African elephants (Loxodonta africana) injected with a long-acting formulation. *Journal of Zoo and Wildlife Medicine, 27*(3), 382-385.

Bush, M., Stoskopf, M., Raath, J., & Papich, M. (2000). Serum oxytetracycline concentrations in African elephant (Loxodonta africana) calves after long-acting formulation injection. *Journal of Zoo and Wildlife Medicine, 31*(1), 41-46.

Buss, P., Olea-Popelka, F., Meyer, L., Hofmeyr, J., Mathebula, N., Kruger, M., .Brüns, A., Martin, L., Miller, M. (2015). Evaluation of cardiorespiratory, blood gas, and lactate values during extended immobilization of white rhinoceros (Ceratotherium simum). *Journal of Zoo and Wildlife Medicine, 46*(2), 224-233.

Cabanes, A., Arboix, M., Anton, J., & Reig, F. (1992). Pharmacokinetics of enrofloxacin after intravenous and intramuscular injection in rabbits. *American Journal of Veterinary Research, 53*(11), 2090-2093.

Calder, W. A. (1981). Scaling of physiological processes in homeothermic animals. *Annual Review of Physiology, 43*, 301-322. 10.1146/annurev.ph.43.030181.001505

Capellini, I., Venditti, C., & Barton, R. A. (2010). Phylogeny and metabolic scaling in mammals. *Ecology, 91*(9), 2783-2793. 10.1890/09-0817.1

Cester, C. C., & Toutain, P. L. (1997). A comprehensive model for enrofloxacin to ciprofloxacin transformation and disposition in dog. *Journal of Pharmaceutical Sciences, 86*(10), 1148-1155. 10.1021/js9603461

Challender, D. W. S., & MacMillan, D. C. (2014). Poaching is more than an enforcement problem. *Conservation Letters, 7*(5), 484-494. 10.1111/conl.12082

Chan, T. A., Morin, P. J., Vogelstein, B., & Kinzler, K. W. (1998). Mechanisms underlying nonsteroidal antiinflammatory drug-mediated apoptosis. *Proceedings of the National Academy of Sciences of the United States of America, 95*(2), 681-686. 10.1073/pnas.95.2.681

Chandrasekharan, N. V., Dai, H., Roos, K. L. T., Evanson, N. K., Tomsik, J., Elton, T. S., & Simmons, D. L. (2002). COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: Cloning, structure, and expression. *Proceedings of the National Academy of Sciences of the United States of America, 99*(21), 13926-13931. 10.1073/pnas.16248699

Christensen, J., Smith, B., Murdane, S., & Hollingshead, N. (1996). The disposition of five therapeutically important antimicrobial agents in llamas. *Journal of Veterinary Pharmacology and Therapeutics, 19*(6), 431-438. 10.1111/j.1365-2885.1996.tb00079.x

Clausen, B., & Ashford, W. A. (1980). Bacteriologic survey of black rhinoceros (Diceros bicornis). *Journal of Wildlife Diseases, 16*(4), 475-480.

Clauss, M., Polster, C., Kienzle, E., Wiesner, H., Baumgartner, K., Houwald, F. v., Ortmann S., Streich, W. J. & Dierenfels, E. S. (2005). Studies on digestive physiology and feed digestibilities in captive Indian rhinoceros (Rhinoceros unicornis). *Journal of Animal Physiology and Animal Nutrition, 89*(3/6), 229-237. 10.1111/j.1439-0396.2005.00546.x ER

CLSI (2015). Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals. *CLSI supplement VET01S* (3rd ed.). Wayne, PA, USA: Clinical and Laboratory Standards Institute.

Cooper, J. E., & Cooper, M. E. (Eds.). (2013). Javan rhinoceros survey. *Wildlife forensic investigation: Principles and practice*. Boca Raton, FL: CRC Press.

Cox, S. K., Cottrell, M. B., Smith, L., Papich, M. G., Frazier, D. L., & Bartges, J. (2004). Allometric analysis of ciprofloxacin and enrofloxacin pharmacokinetics across species. *Journal of Veterinary Pharmacology and Therapeutics, 27*(3), 139-146. 10.1111/j.1365-2885.2004.00560.x

Cox, S., Dudenbostel, L., Sommardahl, C., Yarbrough, J., Saleh, M. & Doherty, T. (2012) Pharmacokinetics of firocoxib and its interaction with enrofloxacin in horses. *Journal of Veterinary Pharmacology and Therapeutics*, 35(6), 615-617. 10.1111/j.1365-2885.2011.01362.x ER

Craig, W. A. (1998). Pharmacokinetic/pharmacodynamic parameters: Rationale for antibacterial dosing of mice and men. *Clinical Infectious Diseases, 26*(1), 1-10. 10.1086/516284

Cromsigt, J. P. G. M., & te Beest, M. (2014). Restoration of a megaherbivore: Landscape-level impacts of white rhinoceros in Kruger National Park, South Africa. *Journal of Ecology, 102*(3), 566-575. 10.1111/1365-2745.12218

Davis, J. L., Salmon, J. H., & Papich, M. G. (2005). Pharmacokinetics and tissue fluid distribution of cephalexin in the horse after oral and i.v. administration. *Journal of Veterinary Pharmacology and Therapeutics, 28*(5), 425-431. 10.1111/j.1365-2885.2005.00683.x

Delatour, P., Foot, R., Foster, A., Baggot, D., & Lees, P. (1996). Pharmacodynamics and chiral pharmacokinetics of carprofen in calves. *British Veterinary Journal, 152*(2), 183-198. 10.1016/S0007-1935(96)80073-X

DeManuelle, T. C., Ihrke, P. J., Brandt, C. M., Kass, P. H., & Vulliet, P. R. (1998). Determination of skin concentrations of enrofloxacin in dogs with pyoderma. *American Journal of Veterinary Research, 59*(12), 1599-1604.

Dinerstein, E. (2011). Family Rhinocerotidae (Rhinoceroses). In D. E. Wilson, & R. A. Mittermeier (Eds., pp. 144-181), *Handbook of the mammals of the world, volume 2: Hoofed mammals*. New York, Lynx.

Dorfman, M., Barsanti, J., & Budsberg, S. C. (1995). Enrofloxacin concentrations in dogs with normal prostate and dogs with chronic bacterial prostatitis. *American Journal of Veterinary Research, 56*(3), 386-390.

Drusano, G. L., Johnson, D. E., Rosen, M., & Standiford, H. C. (1993). Pharmacodynamics of a fluoroquinolone antimicrobial agent in a neutropenic rat model of pseudomonas sepsis. *Antimicrobial Agents and Chemotherapy, 37*(3), 483-490.

Dumonceaux, G., Isaza, R., Koch, D. E., & Hunter, R. P. (2005). Pharmacokinetics and i.m. bioavailability of ceftiofur in Asian elephants (*Elephas maximus*). *Journal of Veterinary Pharmacology and Therapeutics, 28*(5), 441-446. 10.1111/j.1365-2885.2005.00686.x

Duval, J. M., & Budsberg, S. C. (1995). Cortical bone concentrations of enrofloxacin in dogs. *American Journal of Veterinary Research, 56*(2), 188-192.

Edgar, R. C. (2004). MUSCLE: A multiple sequence alignment method with reduced time and space complexity. *Bmc Bioinformatics, 5*, 1-19. 10.1186/1471-2105-5-113

El-Kattan, A., & Varma, M. (2012). Oral absorption, intestinal metabolism and human oral bioavailability. In Paxton J. (Ed.) *Topics on Drug Metabolism* (pp. 1-34). Rijeka, Croatia: Intech.

Emslie, R. (2012). *Ceratotherium simum*. The IUCN Red List of Threatened Species 2012: e.T4185A16980466. Retrieved from http://www.iucnredlist.org/details/4185/0.

Emslie, R.H., Milliken, T., Talukdar, B., Ellis, S., Keryn, A. & Knight, M.H. (2016). African and Asian Rhinoceroses - Status, Conservation and Trade. *A Report from the IUCN Species Survival Commission (IUCN SSC) African and Asian Rhino Specialist Groups and TRAFFIC to the CITES Secretariat Pursuant to Resolution Conf. 9.14 (Rev. CoP15)*. Retrieved from https://cites.org/sites/default/files/eng/cop/17/WorkingDocs/E-CoP17-68-A5.pdf

Epstein, M. (2002). Non-steroidal anti-inflammatory drugs and the continuum of renal dysfunction. *Journal of Hypertension, 20*, S17-S23.

Estabrook, R. W. (2003). A passion for P450s (remembrances of the early history of research on cytochrome P450). *Drug Metabolism and Disposition, 31*(12), 1461-1473. 10.1124/dmd.31.12.1461

Estes, L. (1998). Review of pharmacokinetics and pharmacodynamics of antimicrobial agents. *Mayo Clinic Proceedings, 73*(11), 1114-1122. http://dx.doi.org/10.4065/73.11.1114

Evans, A. M. (1992). Enantioselective pharmacodynamics and pharmacokinetics of chiral nonsteroidal antiinflammatory drugs. *European Journal of Clinical Pharmacology, 42*(3), 237-256.

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*(4), 783-791. 10.2307/2408678

Fink-Gremmels, J. (2008). Implications of hepatic cytochrome P450-related biotransformation processes in veterinary sciences. *European Journal of Pharmacology, 585*(2-3), 502-509. 10.1016/j.ejphar.2008.03.013

Fisslthaler, B., Popp, R., Kiss, L., Potente, M., Harder, D. R., Fleming, I., & Busse, R. (1999). Cytochrome P4502C is an EDHF synthase in coronary arteries. *Nature, 401*(6752), 493-497.

Flach, E. J., Walsh, T. C., Dodds, J., White, A., & Crowe, O. M. (2003). Treatment of osteomyelitis in a greater one-horned rhinoceros (*Rhinoceros unicornis*). *Erkrankungen Der Zootiere: Verhandlungsbericht des 41.Internationalen Symposiums über die Erkrankungen Der Zoo- und Wildtiere, (*pp. 195-201), Rome, Italy.

Forrest, A., Nix, D. E., Ballow, C. H., Goss, T. F., Birmingham, M. C., & Schentag, J. J. (1993). Pharmacodynamics of intravenous ciprofloxacin in seriously ill-patients. *Antimicrobial Agents and Chemotherapy, 37*(5), 1073-1081.

Fourie, T., Cromarty, D., Duncan, N., Wolter, K. & Naidoo, V. (2015) The Safety and Pharmacokinetics of Carprofen, Flunixin and Phenylbutazone in the Cape Vulture (Gyps coprotheres) following Oral Exposure. *Plos One*, 10(10), e0141419. 10.1371/journal.pone.0141419

Furge LL, Guengerich F. P. (2006). Cytochrome P450 enzymes in drug metabolism and chemical toxicology. *Biochemistry and Molecular Biology Education*, 34(2),66-74. 10.1002/bmb.2006.49403402066

Gamble, K., Boothe, D., Jensen, J., Heatley, J., & Helmick, K. (1997). Pharmacokinetics of a single intravenous enrofloxacin dose in Scimitar-Horned Oryx (Oryx dammah). *Journal of Zoo and Wildlife Medicine, 28*(1), 36-42.

Gaynor, J. S., & Muir, W. W. (2015). *Handbook of Veterinary Pain Management* (3rd ed.). St. Louis, Missouri: Elsevier.

Giguère, S., Sweeney, R., & Belanger, M. (1996). Pharmacokinetics of enrofloxacin in adult horses and concentration of the drug in serum, body fluids, and endometrial tissues after repeated intragastrically administered doses. *American Journal of Veterinary Research, 57*(7), 1025-1030.

Giguère, S., Sweeney, R., Habecker, P., Lucas, J., & Richardson, D. (1999). Tolerability of orally administered enrofloxacin in adult horses: A pilot study. *Journal of Veterinary Pharmacology and Therapeutics, 22*(5), 343-347.

Giguère, S. (2013). Principles of antimicrobial drug selection and use. In S. Giguère, J. F. Prescott & P. M. Dowling (Eds.), *Antimicrobial therapy in veterinary medicine* (5th ed., pp. 105-115). Ames, Iowa: WILEY Blackwell.

Giguère, S., & Dowling, P. M. (2013). Fluoroquinolones. In S. Giguère, J. F. Prescott, J. D. Baggot, R. D. Walker & P. M. Dowling (Eds.), *Antimicrobial therapy in veterinary medicine* (4th ed., pp. 295-314). Ames, Iowa: WILEY Blackwell.

Goteti, K., Garner, C. E., & Mahmood, I. (2010). Prediction of human drug clearance from two species: A comparison of several allometric methods. *Journal of Pharmaceutical Sciences, 99*(3), 1601-1613. 10.1002/jps.21926

Grisneaux, E., Pibarot, P., Dupuis, J. & Blais, D. (1999) Comparison of ketoprofen and carprofen administered prior to orthopedic surgery for control of postoperative pain in dogs. *Journal of the American Veterinary Medical Association*, 215(8), 1105-1110.

Guengerich, F. P. (1997). Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. *Chemico-Biological Interactions, 106*(3), 161-182. 10.1016/S0009-2797(97)00068-9

Haines, G. R., Brown, M. P., Gronwall, R. R., & Merritt, K. A. (2000). Serum concentrations and pharmacokinetics of enrofloxacin after intravenous and intragastric administration to mares. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire, 64*(3), 171-177.

Hall, B. G. (2013). Building phylogenetic trees from molecular data with MEGA. *Molecular Biology and Evolution, 30*(5), 1229-1235. 10.1093/molbev/mst012

Harizi, H., Corcuff, J., & Gualde, N. (2008). Arachidonic-acid-derived eicosanoids: Roles in biology and immunopathology. *Trends in Molecular Medicine, 14*(10), 461-469. 10.1016/j.molmed.2008.08.005

Harwood, P. (1963). Therapeutic dosage in small and large mammals. *Science, 139*(355), 684-685. 10.1126/science.139.3555.684

Heard, D. J., Olsen, J. H., & Stover, J. (1992). Cardiopulmonary changes associated with chemical immobilization and recumbency in a white rhinoceros (Ceratotherium simum). *Journal of Zoo and Wildlife Medicine, 23*(2), 197-200.

Heard, D.J., Nichols, W.W., Buss, D. & Kollias, G.V. (1996) Comparative cardiopulmonary effects of intramuscularly administered etorphine and carfentanil in goats. *American Journal of Veterinary Research,* 57(1), 87-96.

Hitchins, P. M., & Keep, M. E. (1970). Observations on skin lesions of the black rhinoceros (Diceros bicornis linn.) in the Hluhluwe Game Reserve, Zululand. *Lammergeyer, 12*, 56-65.

Holgate, S. T., Peters-Golden, M., Panettieri, R. A., & Henderson, W. R. J. (2003). Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *The Journal of Allergy and Clinical Immunology, 111*(1 Suppl), S18-36.

Hudson, S. (2017). *Mechanism of action for analgesia*. Retrieved from http://www.frca.co.uk/article.aspx?articleid=101336

Hunter, R. P., Isaza, R., & Koch, D. E. (2003). Oral bioavailability and pharmacokinetic characteristics of ketoprofen enantiomers after oral and intravenous administration in Asian elephants (Elephas maximus). *American Journal of Veterinary Research, 64*(1), 109-114. 10.2460/ajvr.2003.64.109

Hunter, R. P., & Isaza, R. (2008). Concepts and issues with interspecies scaling in zoological pharmacology. *Journal of Zoo and Wildlife Medicine, 39*(4), 517-526. 10.1638/2008-0041.1

Hyatt, J. M., MCKinnon, P. S., Zimmer, G. S., & Schentag, J. J. (1995). The importance of pharmacokinetic-pharmacodynamic surrogate markers to outcome - focus on antibacterial agents. *Clinical Pharmacokinetics, 28*(2), 143-160.

Ilett, K. F., Tee, L. B. G., Reeves, P. T., & Minchin, R. F. (1990). Metabolism of drugs and other xenobiotics in the gut lumen and wall. *Pharmacology & Therapeutics, 46*(1), 67-93. 10.1016/0163-7258(90)90036-2

Intorre, L., Mengozzi, G., Maccheroni, M., Bertini, S. & Soldani, G. (1995) Enrofloxacin-theophylline interaction: influence of enrofloxacin on theophylline steady-state pharmacokinetics in the Beagle dog. *Journal of Veterinary Pharmacology and Therapeutics*, 18(5), 352-356. 10.1111/j.1365-2885.1995.tb00603.x

Ioannides, C. (2006). Cytochrome P450 expression in the liver of food-producing animals. *Current Drug Metabolism, 7*(4), 335-348. 10.2174/138920006776873544

IVS desk reference (2013). *MIMS IDR* (12th ed.). Saxonwold, South Africa: MIMS.

Jiang, Y., Xie, M., Chen, W., Talbot, R., Maddox, J. F., Faraut, T., Wu, C., Muzny, D.M., Li, Y., Zhang, W., Stanton, J., Brauning, R., Barris, W.C., Hourlier, T., Aken, B.L., Searle, S.M.J., Adelson, D.L., Bian, C., Cam, G.R., Chen, Y., Cheng, S., DeSilva, U., Dixen, K., Dong, Y., Fan, G., Franklin, I.R., Fu, S., Fuentes-Utrilla, P., Guan, R., Highland, M.A., Holder, M.E., Huang, G., Ingham, A.B., Jhangiani, S.N., Kalra, D., Kovar, C.L., Lee, S.L., Liu, W., Liu, X., Lu, C., Lv, T., Mathew, T., McWilliam, S., Menzies, M., Pan, S., Robelin, D., Servin, B., Townley, D., Wang, W., Wei, B., White, S.N., Yang, X., Ye, C., Yue, Y., Zeng, P., Zhou, Q., Hansen, J.B., Kristiansen, K., Gibbs, R.A., Flicek, P., Warkup, C.C., Jones, H.E., Oddy, V.H., Nicholas, F.W., McEwan, J.C., Kijas, J.W., Wang, J., Worley, K.C., Archibald, A.L., Cockett, N., Xu, X., Wang, W. & Dalrymple, B. P. (2014). The

sheep genome illuminates biology of the rumen and lipid metabolism. *Science, 344*(6188), 1168-1173. 10.1126/science.1252806

Johnson, C. B., Taylor, P. M., Young, S. S., & Brearley, J. C. (1993). Postoperative analgesia using phenylbutazone, flunixin or carprofen in horses. *Veterinary Record, 133*(14), 336-338.

Jones, D. M., & Thomsett, L. R. (1972). A short review of the diseases of rhinoceros skin with case reports on an exudative dermatitis of the white rhinoceros (Ceratotherium simum). *Diseases of Zoo Animals.XIVth International Symposium, 1972, Wroclaw.*, 227-231.

Kaartinen, L., Salonen, M., Alli, L., & Pyorala, S. (1995). Pharmacokinetics of enrofloxacin after single intravenous, intramuscular and subcutaneous injections in lactating cows. *Journal of Veterinary Pharmacology and Therapeutics, 18*(5), 357-362. 10.1111/j.1365-2885.1995.tb00604.x

Kaartinen, L., Panu, S., & Pyorala, S. (1997). Pharmacokinetics of enrofloxacin in horses after single intravenous and intramuscular administration. *Equine Veterinary Journal, 29*(5), 378-381.

Kahn, C. M., & Line, S. (2010). *The Merck Veterinary Manual* (10th ed.). Whitehouse Station, N.J., Wiley.

Keen, P., & Livingston, A. (1983). Adverse reactions to drugs. *In Practice, 5*(5), 174-80.

Keller, F., Czock, D., Zellner, D. & Giehl, M. (1998) Relationship between pharmacokinetic half-life and pharmacodynamic half-life in effect-time modeling. *International Journal of Clinical Pharmacology and Therapeutics*, 36, 168-175.

Kent, W. J. (2002). BLAT - the BLAST-like alignment tool. *Genome Research, 12*(4), 656-664. 10.1101/gr.229202

Kiefer, B. (2002). *Qualitaet and Verdaulichkeit der vom Breitmaulnashorn aufgenommenen Nahrung* (Doctoral dissertation), Ludwig Maximilians University of Munich, Germany. Retrieved from http://www.rhinoresourcecenter.com/pdf_files/141/1416207741.pdf.

Kimura, M. (1980). A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide-sequences. *Journal of Molecular Evolution, 16*(2), 111-120. 10.1007/BF01731581

King, J. N., & Gerring, E. L. (1989). Antagonism of endotoxin-induced disruption of equine bowel motility by flunixin and phenylbutazone. *Equine Veterinary Journal.Supplement,* (7), 38-42.

Kis, B., Snipes, J. A., & Busija, D. W. (2005). Acetaminophen and the cyclooxygenase-3 puzzle: Sorting out facts, fictions, and uncertainties. *Journal of Pharmacology and Experimental Therapeutics, 315*(3), 1415-1416. 10.1124/jpet.105.094870

Kleiber, M. (1947). Body size and metabolic rate. *Physiological Reviews, 27*(4), 511-541.

Klingenberg, M. (1958). Pigments of rat liver microsomes. *Archives of Biochemistry and Biophysics, 75*(2), 376-386. 10.1016/0003-9861(58)90436-3

Knapp, D. W., Richardson, R. C., Chan, T. C. K., Bottoms, G. D., Widmer, W. R., DeNicola, D. B., Teclaw, R., Bonney, P. L., & Kuczek , T. (1994). Piroxicam therapy in 34 dogs with transitional cell carcinoma of the urinary bladder. *Journal of Veterinary Internal Medicine, 8*(4), 273-278.

Knight, E. V., Kimball, J. P., Keenan, C. M., Smith, I. L., Wong, F. A., Barrett, D. S., Dempster, A. M., Lieuallen, W. G., Panigrahi, D., Powers, W. J., & Szot, R. J. (1996). Preclinical toxicity evaluation of tepoxalin, a dual inhibitor of cyclooxygenase and 5-lipoxygenase, in sprague-dawley rats and beagle dogs. *Fundamental and Applied Toxicology, 33*(1), 38-48. 10.1006/faat.1996.0141

Kock, M. D., & Burroughs, R. (Eds.). (2012). *Chemical and physical restraint of wild animals: A training and field manual for African species* (2nd ed.). South Africa: IWCS.

Krebber, R., Hoffend, F. J., & Ruttmann, F. (2009). Simple and rapid determination of enrofloxacin and ciprofloxacin in edible tissues by turbulent flow chromatography/tandem mass spectrometry (TFC-MS/MS). *Analytica Chimica Acta, 637*(1/2), 208-213. 10.1016/j.aca.2008.11.006 ER

Küng, K., Riond, J.-L., & Wanner, M. (1993). Pharmacokinetics of enrofloxacin and its metabolite ciprofloxacin after intravenous and oral administration of enrofloxacin in dogs. *Journal of Veterinary Pharmacology and Therapeutics, 16*(4), 462-468. 10.1111/j.1365-2885.1993.tb00212.x

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution, 33*(7), 1870-1874. 10.1093/molbev/msw054

Kwan, K. (1997). Oral bioavailability and first-pass effects. *Drug Metabolism and Disposition, 25*(12), 1329-1336.

Langston, V., Sedrish, S., & Boothe, D. (1996). Disposition of single-dose oral enrofloxacin in the horse. *Journal of Veterinary Pharmacology and Therapeutics, 19*(4), 316-319. 10.1111/j.1365-2885.1996.tb00057.x

Laufer, S., Tries, S., Augustin, J., & Dannhardt, G. (1994). Pharmacological profile of a new pyrrolizine derivative inhibiting the enzymes cyclooxygenase and 5-lipoxygenase. *Arzneimittel-Forschung/drug Research, 44-1*(5), 629-636.

Lautzenhiser, S., Fialkowski, J., Bjorling, D., & Rosin, E. (2001). In vitro antibacterial activity of enrofloxacin and ciprofloxacin in combination against Escherichia coli and staphylococcal clinical isolates from dogs. *Research in Veterinary Science, 70*(3), 239-241. 10.1053/rvsc.2001.0466

Leblanc, P. H., Eicker, S. W., Curtis, M., & Beehler, B. (1987). Hypertension following etorphine anesthesia in a rhinoceros (Diceros simus). *Journal of Zoo Animal Medicine, 18*(4), 141-143. 10.2307/20094825

Lee, S. H., Soyoola, E., Chanmugam, P., Hart, S., Sun, W. Q., Zhong, H., Liou, S., Simmons, D., & Hwang, D. (1992). Selective expression of mitogen-inducible cyclooxygenase in macrophages stimulated with lipopolysaccharide. *Journal of Biological Chemistry, 267*(36), 25934-25938.

Lees, P., & Higgins, A. J. (1985). Clinical-pharmacology and therapeutic uses of non-steroidal anti-inflammatory drugs in the horse. *Equine Veterinary Journal, 17*(2), 83-96. 4

Lees, P., May, S. A., & McKellar, Q. A. (1991). Pharmacology and therapeutics of nonsteroidal antiinflammatory drugs in the dog and cat .1 General pharmacology. *Journal of Small Animal Practice, 32*(4), 183-193. 10.1111/j.1748-5827.1991.tb00541.x

Lees, P., McKellar, Q., May, S.A. & Ludwig, B. (1994) Pharmacodynamics and Pharmacokinetics of Carprofen in the Horse. *Equine Veterinary Journal*, 26(3), 203-208.

Lees, P., Aliabadi, F. S., & Landoni, M. F. (2002). Pharmacodynamics and enantioselective pharmacokinetics of racemic carprofen in the horse. *Journal of Veterinary Pharmacology and Therapeutics, 25*(6), 433-448. 10.1046/j.1365-2885.2002.00436.x

Lees, P., Cunningham, F. M., & Elliott, J. (2004a). Principles of pharmacodynamics and their applications in veterinary pharmacology. *Journal of Veterinary Pharmacology and Therapeutics, 27*(6), 397-414. 10.1111/j.1365-2885.2004.00620.x

Lees, P., Landoni, M. F., Giraudel, J., & Toutain, P. L. (2004b). Pharmacodynamics and pharmacokinetics of nonsteroidal anti-inflammatory drugs in species of veterinary interest. *Journal of Veterinary Pharmacology and Therapeutics, 27*(6), 479-490. 10.1111/j.1365-2885.2004.00617.x

Lees, P., Giraudel, J., Landoni, M.F. & Toutain, P.L. (2004c) PK-PD integration and PK-PD modelling of nonsteroidal anti-inflammatory drugs: principles and applications in veterinary pharmacology. *Journal of Veterinary Pharmacology and Therapeutics*, 27(6), 491-502. 10.1111/j.1365-2885.2004.00618.x

Lehohla, P. (2015). *Tourism satellite account for South Africa, final 2011 and provisional 2012 and 2013*. Retrieved from http://www.statssa.gov.za/publications/Report-04-05-07/Report-04-05-072013.pdf

Leiberich, M., Krebber, R., Hewtson, M., Marais, J., Naidoo, V. (2018). A Study of the Pharmacokinetics and Thromboxane Inhibitory Activity of a Single Intramuscular Dose of Carprofen as a Means to Establish its Potential Use as an Analgesic Drug in White Rhinoceros. *Veterinary Pharmacology and Therapeutics.* 10.1111/jvp.12508

Leone, S., Ottani, A., & Bertolini, A. (2007). Dual acting anti-inflammatory drugs. *Current Topics in Medicinal Chemistry, 7*(3), 265-275. 10.2174/156802607779941341

Li, S., Wang, Y., Matsumura, K., Ballou, L. R., Morham, S. G., & Blatteis, C. M. (1999). The febrile response to lipopolysaccharide is blocked in cyclooxygenase-2(-/-), but not in cyclooxygenase-1(-/-) mice. *Brain Research, 825*(1-2), 86-94. 10.1016/S0006-8993(99)01225-1

Lindberg, R. L. P., & Negishi, M. (1989). Alteration of mouse cytochrome-P450coh substrate-specificity by mutation of a single amino-acid residue. *Nature, 339*(6226), 632-634. 10.1038/339632a0

Lindblad-Toh, K., Wade, C. M., Mikkelsen, T. S., Karlsson, E. K., Jaffe, D. B., Kamal, M., Clamp, M., Chang, J. L., Kulbokas, E. J., Zody, M.C., Mauceli, E., Xie, X. H., Breen, M., Wayne, R. K., Ostrander, E. A., Ponting, C. P., Galibert, F., Smith, D. R., deJong, P. J., Kirkness, E., Alvarez, P., Biagi, T., Brockman, W., Butler, J., Chin, C.W., Cook, A., Cuff, J., Daly, M. J., DeCaprio, D., Gnerre, S., Grabherr, M., Kellis, M., Kleber, M., Bardeleben, C., Goodstadt, L., Heger, A., Hitte, C., Kim, L., Koepfli, K. P., Parker, H. G., Pollinger, J. P., Searle, S. M. J., Sutter, N. B., Thomas, R., Webber, C., Broad Institute Genome Sequencing Platform & Lander, E. S., (2005). Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature, 438*(7069), 803-819. 10.1038/nature04338

Lindsey, P. A., Alexander, R., Mills, M. G. L., Romanach, S., & Woodroffe, R. (2007). Wildlife viewing preferences of visitors to protected areas in South Africa: Implications for the role of ecotourism in conservation. *Journal of Ecotourism, 6*(1), 19-33. 10.2167/joe133.0

Lipscomb, V. J., AliAbadi, F. S., Lees, P., Pead, M. J., & Muir, P. (2002). Clinical efficacy and pharmacokinetics of carprofen in the treatment of dogs with osteoarthritis. *Veterinary Record, 150*(22), 684-689. 10.1136/vr.150.22.684

Lode, H., Borner, K., & Koeppe, P. (1998). Pharmacodynamics of fluoroquinolones. *Clinical Infectious Diseases, 27*(1), 33-39. 10.1086/514623

Lodwick, L. J., Dubach, J. M., Phillips, L. G., Brown, C. S., & Jandreski, M. A. (1994). Pharmacokinetics of amikacin in African elephants (Loxodonta-africana). *Journal of Zoo and Wildlife Medicine, 25*(3), 367-375.

Lohuis, J.A.C.M., Vanwerven, T., Brand, A., Vanmiert, A.S.J.P.A.M., Rohde, E., Ludwig, B., Heizmann, P. & Rehm, W.F. (1991) Pharmacodynamics and Pharmacokinetics of Carprofen, a Nonsteroidal Antiinflammatory Drug, in Healthy Cows and Cows with Escherichia-Coli Endotoxin-Induced Mastitis. *Journal of Veterinary Pharmacology and Therapeutics,* 14(3), 219-229. 10.1111/j.1365-2885.1991.tb00830.x

Lopez-Cadenas, C., Sierra-Vega, M., Garcia-Vieitez, J. J., Jose Diez-Liebana, M., Sahagun-Prieto, A., & Fernandez-Martinez, N. (2013). Enrofloxacin: Pharmacokinetics and metabolism in domestic animal species. *Current Drug Metabolism, 14*(10), 1042-1058.

Mahmood, I. (2001). Interspecies scaling: Is a priori knowledge of cytochrome p450 isozymes involved in drug metabolism helpful in prediction of clearance in humans from animal data? *Drug Metabolism and Drug Interactions, 18*(2), 135-47.

Mahmood, I. (2007). Application of allometric principles for the prediction of pharmacokinetics in human and veterinary drug development. *Advanced Drug Delivery Reviews, 59*(11), 1177-1192. http://dx.doi.org/10.1016/j.addr.2007.05.015

Maitho, T. E., Lees, P., & Taylor, J. B. (1986). Absorption and pharmacokinetics of phenylbutazone in Welsh Mountain ponies. *Journal of Veterinary Pharmacology and Therapeutics, 9*(1), 26-39. 10.1111/j.1365-2885.1986.tb00009.x

Martel-Pelletier, J., Lajeunesse, D., Reboul, P., & Pelletier, J. P. (2003). Therapeutic role of dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-inflammatory drugs. *Annals of the Rheumatic Diseases, 62*(6), 501-509. 10.1136/ard.62.6.501

Martignoni, M., Groothuis, G. M. M., & de Kanter, R. (2006). Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology, 2*(6), 875-894. 10.1517/17425255.2.6.875

Martinez, M., McDermott, P., & Walker, R. (2006). Pharmacology of the fluoroquinolones: A perspective for the use in domestic animals. *The Veterinary Journal, 172*(1), 10-28. http://dx.doi.org/10.1016/j.tvjl.2005.07.010

Mathews, K. A. (2002). Non-steroidal anti-inflammatory analgesics: A review of current practice. *Journal of Veterinary Emergency and Critical Care, 12*(2), 89-97. 10.1046/j.1435-6935.2002.00007.x

McAdam, R. A., Goundis, D., & Reid, K. B. M (1988). A homozygous point mutation results in a stop codon in the C1q B-chain of a C1q-deficient individual. *Immunogenetic*s, 27(4), 259-264.

McKellar, Q. A., Pearson, T., Bogan, J. A., Gaibraith, E. A., Lees, P., Ludwig, B., & Tiberghien, M. P. (1990). Pharmacokinetics, tolerance and serum thromboxane inhibition of carprofen in the dog. *Journal of Small Animal Practice, 31*(9), 443-448. 10.1111/j.1748-5827.1990.tb00510.x

McKellar, Q. A., Bogan, J. A., von Fellenberg, R. L., Ludwig, B., & Cawley, G. D. (1991). Pharmacokinetic, biochemical and tolerance studies on carprofen in the horse. *Equine Veterinary Journal, 23*(4), 280-284.

McKellar, Q. A., Delatour, P., & Lees, P. (1994). Stereospecific pharmacodynamics and pharmacokinetics of carprofen in the dog. *Journal of Veterinary Pharmacology and Therapeutics, 17*(6), 447-454. 10.1111/j.1365-2885.1994.tb00276.x

Miller, M. A., & Buss, P. E. (2014). Rhinoceridae (Rhinoceroses). In Miller, R.E. & Fowler, M.E., (Eds.), *Fowler's Zoo and Wild Animal Medicine (*Vol. 8, pp. 538-547). St.Louis, Missouri: Elsevier.

Milliken, T., & Shaw, J. (2012). *The South Africa – Viet Nam rhino horn trade nexus: A deadly combination of institutional lapses, corrupt wildlife industry professionals and Asian crime syndicates.* Retrieved from: http://www.trafficj.org/publication/12_The_SouthAfrica-VietNam_RhinoHorn_Trade_Nexus.pdf

Monlouis, J. D., Jong, A. d., Limet, A., & Richez, P. (1997). Plasma pharmacokinetics and urine concentrations of enrofloxacin after oral administration of enrofloxacin in dogs. *Journal of Veterinary Pharmacology and Therapeutics, 20*(Supplement 1), 61-63. 10.1046/j.1365-2885.1997.00041.x ER

Mortenson, J. (2001). Determining dosages for antibiotic and anti-inflammatory agents. In Csuti, B., Sargent E. L. & Bechert U. S. (Eds.), *The elephant's foot: Prevention and care of foot conditions in captive Asian and African elephants* (pp. 141-144). Ames, USA: Iowa State University Press,

Moses, V. S., & Bertone, A. L. (2002). Nonsteroidal anti-inflammatory drugs. *Veterinary Clinics of North America-Equine Practice, 18*(1), 21-37. 10.1016/S0749-0739(01)00002-5

Nair, A. B., & Jacob, S. (2016). A simple practice guide for dose conversion between animals and human. *Journal of Basic and Clinical Pharmacy, 7*(2), 27-31. 10.4103/0976-0105.177703

National Center for Biotechnology Information, U.S. National Library of Medicine (2012). *Ceratotherium simum simum (Southern white rhinoceros)*. Retrieved from: https://www.ncbi.nlm.nih.gov/bioproject/PRJNA74583/)

Nebert, D. W., Adesnik, M., Coon, M. J., Estabrook, R. W., Gonzalez, F. J., Guengerich, F. P., Gunsalus, I. C., Johnson, E., F., Kemper, B., Levin, W., Phillips, R.S. & Waterman M. R. (1987). The P450 gene superfamily - recommended nomenclature. *DNA-a Journal of Molecular & Cellular Biology, 6*(1), 1-11. 10.1089/dna.1987.6.1

Nebert, D. W., & Russell, D. W. (2002). Clinical importance of the cytochromes P450. *Lancet, 360*(9340), 1155-1162. 10.1016/S0140-6736(02)11203-7

Nelson, D. R. (2006). Cytochrome P450 nomenclature, 2004. *Methods in Molecular Biology, 320*, 1-10.

Nelson, D. R., Goldstone, J. V., & Stegeman, J. J. (2013). The cytochrome P450 genesis locus: The origin and evolution of animal cytochrome P450s. *Royal Society Philosophical Transactions Biological Sciences, 368*(1612), 1-22.

Nielsen, P., & GyrdHansen, N. (1997). Bioavailability of enrofloxacin after oral administration to fed and fasted pigs. *Pharmacology & Toxicology, 80*(5), 246-250.

Oftedal, O. T., Baer, D. J., & Allen, M. E. (1996). The feeding and nutrition of herbivores. In D. G. Kleiman, M. E. Allen, K. V. Thompson & S. Lumpkin (Eds.), *Wild mammals in captivity: Principles and techniques* (pp. 129-138). Chicago: The University of Chicago Press.

Ogino, T., Mizuno, Y., Ogata, T., & Takahashi, Y. (2005). Pharmacokinetic interactions of flunixin meglumine and enrofloxacin in dogs. *American Journal of Veterinary Research, 66*(7), 1209-1213. 10.2460/ajvr.2005.66.1209

Ogletree, M. L. (1987). Overview of physiological and pathophysiological effects of thromboxane-A2. *Federation Proceedings, 46*(1), 133-138.

Orr, C. (2016). *Characterisation of equine cytochrome p450* (Doctoral Dissertation, University of Nottingham, UK).
Retrieved from http://eprints.nottingham.ac.uk/33315/1/Thesis%20corrections.pdf

Otero, J. L., Mestorino, N., & Errecalde, J. O. (2009). Pharmacokinetics of enrofloxacin after single intravenous administration in sheep. *Revue Scientifique Et Technique/ Office International Des Epizooties, 28*(3), 1129-1142.

Page, C. D., Mautino, M., Derendorf, H. D., & Anhalt, J. P. (1991). Comparative pharmacokinetics of trimethoprim-sulfamethoxazole administered intravenously and orally to captive elephants. *Journal of Zoo and Wildlife Medicine, 22*(4), 409-416.

Papich, M. G., Van Camp, S. D., Cole, J. A., & Whitacre, M. D. (2002). Pharmacokinetics and endometrial tissue concentrations of enrofloxacin and the metabolite ciprofloxacin after i.v. administration of enrofloxacin to mares. *Journal of Veterinary Pharmacology and Therapeutics, 25*(5), 343-350. 10.1046/j.1365-2885.2002.00434.x

Papich, M. G. (Ed.). (2010). *Saunders Handbook of Veterinary Drugs* (3rd ed.). Philadelphia, USA: Saunders Elsevier.

Parameswaran, K., Cox, G., Radford, K., Janssen, L. J., Sehmi, R., & O'Byrne, P. M. (2002). Cysteinyl leukotrienes promote human airway smooth muscle migration. *American Journal of Respiratory and Critical Care Medicine, 166*(5), 738-742. 10.1164/rccm.200204-291OC

Parker, C. W. (1987). 5-lipoxygenase, leukotrienes, and regulation of inflammatory responses. *Drug Development Research, 10*(4), 277-293. 10.1002/ddr.430100408

Payne, A., & Hales, D. (2004). Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. *Endocrine Reviews, 25*(6), 947-970. 10.1210/er.2003-0030

Peyrou, M., Bousquet-Melou, A., Laroute, V., Vrins, A., & Doucet, M. Y. (2006). Enrofloxacin and marbofloxacin in horses: Comparison of pharmacokinetic parameters, use of urinary and metabolite data to estimate first-pass effect and absorbed fraction. *Journal of Veterinary Pharmacology and Therapeutics, 29*(5), 337-344. 10.1111/j.1365-2885.2006.00752.x

Piddock, L. J. V. (1998). Fluoroquinolone resistance - overuse of fluoroquinolones in human and veterinary medicine can breed resistance. *British Medical Journal, 317*(7165), 1029-1030.

Save the Rhino (2018). *Poaching statistics*. Retrieved from: https://www.savetherhino.org/rhino_info/poaching_statistics

Prescott, J. F., & Yielding, K. M. (1990). Invitro susceptibility of selected veterinary bacterial pathogens to ciprofloxacin, enrofloxacin and norfloxacin. *Canadian Journal of Veterinary Research-Revue Canadienne De Recherche Veterinaire, 54*(1), 195-197.

Price, S. A., & Bininda-Emonds, O. R. P. (2009). A comprehensive phylogeny of extant horses, rhinos and tapirs (perissodactyla) through data combination. *Zoosystematics and Evolution, 85*(2), 277-291. 10.1002/zoos.200900005

Rainsford, K. D. (1987). The effects of 5-lipoxygenase inhibitors and leukotriene antagonists on the development of gastric-lesions induced by nonsteroidal antiinflammatory drugs in mice. *Agents and Actions, 21*(3-4), 316-319. 10.1007/BF01966502

Rahal, A., Kumar, A., Ahmad, A. H., & Malik, J. K. (2008). Pharmacokinetics of diclofenac and its interaction with enrofloxacin in sheep. *Research in Veterinary Science, 84*(3), 452-456. 10.1016/j.rvsc.2007.06.002

Rao, G. S., Ramesh, S., Ahmad, A. H., Tripathi, H. C., Sharma, L. D., & Malik, J. K. (2002). Disposition kinetics of enrofloxacin and ciprofloxacin following intravenous administration of enrofloxacin in goats. *Small Ruminant Research, 44*(1), 9-15. 10.1016/S0921-4488(02)00003-2 ER

Reddy, B. S., Tokumo, K., Kulkarni, N., Aligia, C., & Kelloff, G. (1992). Inhibition of colon carcinogenesis by prostaglandin synthesis inhibitors and related-compounds. *Carcinogenesis, 13*(6), 1019-1023. 10.1093/carcin/13.6.1019

Regmi, N. L., Abd El-Aty, A. M., Kuroha, M., Nakamura, M., & Shimoda, M. (2005). Inhibitory effect of several fluoroquinolones on hepatic microsomal cytochrome P-450 1A activities in dogs. *Journal of Veterinary Pharmacology and Therapeutics, 28*(6), 553-557. 10.1111/j.1365-2885.2005.00698.x

Reuter, B. K., Asfaha, S., Buret, A., Sharkey, K. A., & Wallace, J. L. (1996). Exacerbation of inflammation-associated colonic injury in rat through inhibition of cyclooxygenase-2. *Journal of Clinical Investigation, 98*(9), 2076-2085. 10.1172/JCI119013

Rhino Resource Center (n.d.). Retrieved from http://www.rhinoresourcecenter.com/species/

Ripple, W. J., Newsome, T. M., Wolf, C., Dirzo, R., Everatt, K. T., Galetti, M., Hayward, M.W., Kerley, G. I. H., Levi, T., Lindsey, P. A., Macdonald, D.W., Malhi, Y., Painter, L.E., Sandom, C.J., Terborgh, J., Van Valkenburgh, B. (2015). Collapse of the world's argest herbivores. *Science Advances, 1*(4), e1400103-e1400103. 10.1126/sciadv.1400103

Riviere, J. E., MartinJimenez, T., Sundlof, S., & Craigmill, A. (1997). Interspecies allometric analysis of the comparative pharmacokinetics of 44 drugs across veterinary and laboratory animal species. *Journal of Veterinary Pharmacology and Therapeutics, 20*(6), 453-463. 10.1046/j.1365-2885.1997.00095.x

Riviere, J. E., & Papich, M. G. (2009). *Veterinary Pharmacology and Therapeutics* (9th ed.). Ames, Iowa: Wiley-Blackwell.

Riviere, J. E. (Ed). (2011). Interspecies extrapolations. *Comparative Pharmacokinetics, Principles, Techniques & Applications* (2nd ed., pp. 399-412). Raleigh, North Carolina: Wiley-Blackwell.

Rodrigues, A. D., Ferrero, J. L., Amann, M. T., Rotert, G. A., Cepa, S. P., Surber, B. W., Machinist, J. M., Tich, N. R., Sullivan, J. P., Garvey, D. S., Fitzgerald, M. & Arneric, S. P. (1994). The in-vitro hepatic-metabolism of ABT-418, a cholinergic channel activator, in rats, dogs, cynomolgus monkeys, and humans. *Drug Metabolism and Disposition, 22*(5), 788-798.

Rosin, E., Schultzdarken, N., Perry, B., & Teare, J. A. (1993). Pharmacokinetics of ampicillin administered orally in Asian elephants (Elaphus maximus). *Journal of Zoo and Wildlife Medicine, 24*(4), 515-518.

Sanchez, C., Murray, S., Isaza, R., & Papich, M. (2005). Pharmacokinetics of a single dose of enrofloxacin administered orally to captive Asian elephants (Elephas maximus). *American Journal of Veterinary Research, 66*(11), 1948-1953. 0.2460/ajvr.2005.66.1948

Saper, C. B., & Breder, C. D. (1994). The neurologic basis of fever. *New England Journal of Medicine, 331*(19), 1309-1309.

Sasaki, K., & Shimoda, M. (2015). Possible drug-drug interaction in dogs and cats resulted from alteration in drug metabolism: A mini review. *Journal of Advanced Research, 6*(3), 383-392. 10.1016/j.jare.2015.02.003

Savides, M. C., Oehme, F. W., Nash, S. L., & Leipold, H. W. (1984). The toxicity and biotransformation of single doses of acetaminophen in dogs and cats. *Toxicology and Applied Pharmacology, 74*(1), 26-34. 10.1016/0041-008X(84)90266-7

Schatzmann, U., Gugelmann, M., Voncranach, J., Ludwig, B. M., & Rehm, W. F. (1990). Pharmacodynamic evaluation of the peripheral pain inhibition by carprofen and flunixin in the horse. *Schweizer Archiv Fur Tierheilkunde, 132*(9), 497-504.

Schentag, J. J., Meagher, A. K., & Forrest, A. (2003). Fluoroquinolone AUIC break points and the link to bacterial killing rates - part 1: In vitro and animal models. *Annals of Pharmacotherapy, 37*(9), 1287-1298. 10.1345/aph.1C199

Schmassmann, A., Peskar, B. M., Stettler, C., Netzer, P., Stroff, T., Flogerzi, B., & Halter, F. (1998). Effects of inhibition of prostaglandin endoperoxide synthase-2 in chronic gastrointestinal ulcer models in rats. *British Journal of Pharmacology, 123*(5), 795-804. 10.1038/sj.bjp.0701672

Schmidt, M. J. (1978). Penicillin-G and amoxicillin in elephants - study comparing dose regimens administered with serum levels achieved in healthy elephants. *Journal of Zoo Animal Medicine, 9*(4), 127-136. 10.2307/20094393

Schmitz, A., Demmel, S., Peters, L. M., Leeb, T., Mevissen, M., & Haase, B. (2010). Comparative human-horse sequence analysis of the CYP3A subfamily gene cluster. *Animal Genetics, 41,* 72-79. 10.1111/j.1365-2052.2010.02111.x

Schwarz, G. (1978). Estimating dimension of a model. *Annals of Statistics, 6*(2), 461-464. 10.1214/aos/1176344136

Sedgwick, C. J. (1993). Allometric scaling and emergency care: The importance of body size. In M. E. Fowler (Ed.), *Zoo and Wild Animal Medicine: Current Therapy* (3rd ed, pp. 34-37). Philadelphia: Saunders.

Seguin, M. A., Papich, M. G., Sigle, K. J., Gibson, N. M., & Levy, J. K. (2004). Pharmackokinetics of enrofloxacin in neonatal kittens. *American Journal of Veterinary Research, 65*(3), 350-356. 10.2460/ajvr.2004.65.350

Sekkin, S., Gokbulut, C., Kum, C., & Karademir, U. (2012). Plasma disposition of enrofloxacin following intravenous and intramuscular administration in donkeys. *Veterinary Record, 171*(18), 447. 10.1136/vr.100653 ER

Semrad, S. D., Hardee, G. E., Hardee, M. M., & Moore, J. N. (1987). Low-dose flunixin meglumine - effects on eicosanoid production and clinical signs induced by experimental endotoxemia in horses. *Equine Veterinary Journal, 19*(3), 201-206.

Senthilkumar, K., Senthilkumar, A., & Jayathangaraj, M. G. (2014). Clinical management of chronic abscess in an Asian elephant (Elephas maximus). *Journal of Advanced Veterinary and Animal Research, 1*(2), 73-74.

Sessions, J. K., Reynolds, L. R., & Budsberg, S. C. (2005). In vivo effects of carprofen, deracoxib, and etodolac on prostanoid production in blood, gastric mucosa, and synovial fluid in dogs with chronic osteoarthritis. *American Journal of Veterinary Research, 66*(5), 812-817. 10.2460/ajvr.2005.66.812

Shafiuzama, M., Prasad, A. A., Senthilkumar, K., & William, B. J. (2012). Therapeutic management of foot rot in an Asian elephant (Elephas maximus). *Intas Polivet, 13*(2), 482-483.

Sharma, P., Ahmad, A., Sharma, L., & Varma, R. (2003). Pharmacokinetics of enrofloxacin and the rate of formation of its metabolite ciprofloxacin following intravenous and intramuscular single dose administration to male buffalo calves. *Veterinary Journal, 166*(1), 101-104. 10.1016/S1090-0233(02)00261-7

Sharma, V., & McNeill, J. H. (2009). To scale or not to scale: The principles of dose extrapolation. *British Journal of Pharmacology, 157*(6), 907-921. 10.1111/j.1476-5381.2009.00267.x

Shih, A. C., Robertson, S., Isaza, N., Pablo, L., & Davies, W. (2008). Comparison between analgesic effects of buprenorphine, carprofen, and buprenorphine with carprofen for canine ovariohysterectomy. *Veterinary Anaesthesia and Analgesia, 35*(1), 69-79. 10.1111/j.1467-2995.2007.00352.x

Sieg, A. E., O'Connor, M. P., McNair, J. N., Grant, B. W., Agosta, S. J., & Dunham, A. E. (2009). Mammalian metabolic allometry: Do intraspecific variation, phylogeny, and regression models matter? *American Naturalist, 174*(5), 720-733. 10.1086/606023

Smith, H. S. (2009). Opioid metabolism. *Mayo Clinic Proceedings, 84*(7), 613-624.

Soraci, A., Benoit, E., Jaussaud, P., Lees, P., & Delatour, P. (1995). Enantioselective glucuronidation and subsequent biliary excretion of carprofen in horses. *American Journal of Veterinary Research, 56*(3), 358-361.

South African National Biodiversity Institute (2015). *SA's vast biological diversity*. Retrieved from: http://www.sanbi.org/about.

Spivey, J. M. (1992). The postantibiotic effect. *Clinical Pharmacy, 11*(10), 865-875.

St-Louis, I., Singh, M., Brasseur, K., Leblanc, V., Parent, S., & Asselin, E. (2010). Expression of COX-1 and COX-2 in the endometrium of cyclic, pregnant and in a model of

pseudopregnant rats and their regulation by sex steroids. *Reproductive Biology and Endocrinology, 8*, 103. 10.1186/1477-7827-8-103

Streppa, H. K., Jones, C. J., & Budsberg, S. C. (2002). Cyclooxygenase selectivity of nonsteroidal anti-inflammatory drugs in canine blood. *American Journal of Veterinary Research, 63*(1), 91-94. 10.2460/AJVR.2002.63.91

Symonds, M. R. E., & Elgar, M. A. (2002). Phylogeny affects estimation of metabolic scaling in mammals. *Evolution, 56*(11), 2330-2333.

Tamura, K. (1992). Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Molecular Biology and Evolution, 9*(4), 678-687.

Tana, L. M., Isaza, R., Koch, D. E., & Hunter, R. P. (2010). Pharmacokinetics and intramuscular bioavailability of a single dose of butorphanol in Asian elephants (Elephas maximus). *Journal of Zoo and Wildlife Medicine, 41*(3), 418-425. 10.1638/2009-0073.1

Taylor, P. M., Landoni, F. M., Deal, C., Pickett, C., Aliabadi, F. S., Foot, R., & Lees, P. (1996). Pharmacodynamics and enantioselective pharmacokinetics of carprofen in the cat. *Research in Veterinary Science, 60*(2), 144-151. 10.1016/S0034-5288(96)90009-0

Thomas, J. K., Forrest, A., Bhavnani, S. M., Hyatt, J. M., Cheng, A., Ballow, C. H., & Schentag, J. J. (1998). Pharmacodynamic evaluation of factors associated with the development of bacterial resistance in acutely ill patients during therapy. *Antimicrobial Agents and Chemotherapy, 42*(3), 521-527.

Thun, M. J., Namboodiri, M. M., & Heath, C. W. (1991). Aspirin use and reduced risk of fatal colon cancer. *New England Journal of Medicine, 325*(23), 1593-1596. 10.1056/NEJM199112053252301

Tougard, C., Delefosse, T., Hanni, C., & Montgelard, C. (2001). Phylogenetic Relationships of the Five Extant Rhinoceros Species (Rhinocerotidae, Perissodactyla) based on Mitochondrial Cytochrome b and 12S rRNA Genes. *Molecular Phylogenetics and Evolution, 19*(1), 34-44. 10.1006/mpev.2000.0903

Toutain, P. L. (2002). Pharmacokinetic/pharmacodynamic integration in drug development and dosage-regimen optimization for veterinary medicine. *American Association of Pharmaceutical Scientists, 4*(4), 38.

Toutain, P., Ferran, A., & Bousquet-Melou, A. (2010). Species differences in pharmacokinetics and pharmacodynamics. *Comparative and Veterinary Pharmacology, 199*, 19-48. 10.1007/978-3-642-10324-7_2

Turnidge, J. (1999). Pharmacokinetics and pharmacodynamics of fluoroquinolones. *Drugs, 58*, 29-36. 10.2165/00003495-199958002-00006

Tyden, E., Bjornstrom, H., Tjalve, H., & Larsson, P. (2010). Expression and localization of BCRP, MRP1 and MRP2 in intestines, liver and kidney in horse. *Journal of Veterinary Pharmacology and Therapeutics, 33*(4), 332-340. 10.1111/j.1365-2885.2009.01140.x

UCSC genome browser getaway (2017). Retrieved from: https://genome.ucsc.edu/cgi-bin/hgGateway

Uenishi, H., Morozumi, T., Toki, D., Eguchi-Ogawa, T., Rund, L. A., & Schook, L. B. (2012). Large-scale sequencing based on full-length-enriched cDNA libraries in pigs: Contribution

to annotation of the pig genome draft sequence. *BMC Genomics, 13*, 581. 10.1186/1471-2164-13-581

Vancutsem, P. M., & Babish, J. G. (1996). In vitro and in vivo study of the effects of enrofloxacin on hepatic cytochrome P-450. Potential for drug interactions. *Veterinary and Human Toxicology, 38*(4), 254-259.

van der Weide, J., & Hinrichs, J. W. J. (2006). The influence of cytochrome P450 pharmacogenetics on disposition of common antidepressant and antipsychotic medications. *The Clinical Biochemist.Reviews, 27*(1), 17-25.

van Duijkeren, E., Vulto, A. G., Sloet van Oldruitenborghoosterbaan, M. M., Mevius, D. J., Kessels, B. G., Breukink, H. J., & van Miert, A. S. (1994). A comparative-study of the pharmacokinetics of intravenous and oral trimethoprim sulfadiazine formulations in the horse. *Journal of Veterinary Pharmacology and Therapeutics, 17*(6), 440-446.

Vane, J. R., & Botting, R. M. (1995). New insights into the mode of action of antiinflammatory drugs. *Inflammation Research, 44*(1), 1-10. 10.1007/BF01630479

Varma, R., Ahmad, A., Sharma, L., Aggarwal, P., & Ahuja, V. (2003). Pharmacokinetics of enrofloxacin and its active metabolite ciprofloxacin in cows following single dose intravenous administration. *Journal of Veterinary Pharmacology and Therapeutics, 26*(4), 303-305. 10.1046/j.1365-2885.2003.00480.x

von Houwald, F., Geyer, H., & Eulenberger, K. (2001). Foot problems in captive Indian rhinos (rhinoceros unicornis): Anatomy, pathology, and evaluation of the causes. *Erkrankungen Der Zootiere, 40*, 183-188.

Wade, C. M., Giulotto, E., Sigurdsson, S., Zoli, M., Gnerre, S., Imsland, F., Lear, T. L., Adelson, D. L., Bailey, E., Bellone, R. R., Bloecker, H., Distl, O., Edgar, R. C., Garber, M., Leeb, T., Mauceli, E., MacLeod, J. N., Penedo, M. C. T., Raison, J. M., Sharpe, T., Vogel, J., Andersson, L., Antczak, D.F., Biagi, T., Binns, M. M., Chowdhary, B. P., Coleman, S. J., Della Valle, G., Fryc, S., Guerin, G., Hasegawa, T., Hill, E. W., Jurka, J., Kiialainen, A., Lindgren, G., Liu, J., Magnani, E., Mickelson, J. R., Murray, J., Nergadze, S. G., Onofrio, R., Pedroni, S., Piras, M. F., Raudsepp, T., Rocchi, M., Roed, K. H., Ryder, O. A., Searle, S., Skow, L., Swinburne, J. E., Syvanen, A. C., Tozaki, T., Valberg, S. J., Vaudin, M., White, J. R., Zody, M. C., Broad Institute Genome Sequencing Platform, Broad Institute Whole Genome Assembly Team, Lander, E. S, & Lindblad-Toh, K. (2009). Genome sequence, comparative analysis, and population genetics of the domestic horse. *Science, 326*(5954), 865-867. 10.1126/science.1178158

Wallace, J. L., Reuter, B., Cicala, C., Mcknight, W., Grisham, M. B., & Cirino, G. (1994). Novel nonsteroidal antiinflammatory drug derivatives with markedly reduced ulcerogenic properties in the rat. *Gastroenterology, 107*(1), 173-179.

Welsh, E. M., Baxter, P., & Nolan, A. M. (1992). Pharmacokinetics of carprofen administered intravenously to sheep. *Research in Veterinary Science, 53*(2), 264-266. http://dx.doi.org.uplib.idm.oclc.org/10.1016/0034-5288(92)90123-J

Wenger, S., Boardman, W., Buss, P., Govender, D., & Foggin, C. (2007). The cardiopulmonary effects of etorphine, azaperone, detomidine, and butorphanol in field-anesthetized white rhinoceroses (ceratotherium simum). *Journal of Zoo and Wildlife Medicine, 38*(3), 380-387. 10.1638/2006-0038R.1

Wetzstein, H. G. (2005). Comparative mutant prevention concentrations of pradofloxacin and other veterinary fluoroquinolones indicate differing potentials in preventing selection of

resistance. *Antimicrobial Agents and Chemotherapy, 49*(10), 4166-4173. 10.1128/AAC.49.10.4166-4173.2005

Woodward, K. N. (2005). Veterinary pharmacovigilance. part 3. adverse effects of veterinary medicinal products in animals and on the environment. *Journal of Veterinary Pharmacology and Therapeutics, 28*(2), 171-184. 10.1111/j.1365-2885.2005.00647.x

Xia, W., Gyrd-Hansen, N. & Nielsen, P. (1983) Comparison of Pharmacokinetic Parameters for 2 Oxytetracycline Preparations in Pigs. *Journal of Veterinary Pharmacology and Therapeutics*, 6(2), 113-119. 10.1111/j.1365-2885.1983.tb00387.x

Yang, Z. H. (1994). Maximum-likelihood phylogenetic estimation from dna-sequences with variable rates over sites - approximate methods. *Journal of Molecular Evolution, 39*(3), 306-314. 10.1007/BF00160154

Yoon, J. B., Kim, S. J., Hwang, S. G., Chang, S., Kang, S. S., & Chun, J. S. (2003). Non-steroidal anti-inflammatory drugs inhibit nitric oxide-induced apoptosis and dedifferentiation of articular chondrocytes independent of cyclooxygenase activity. *Journal of Biological Chemistry, 278*(17), 15319-15325. 10.1074/jbc.M212520200

Zanger, U. M., & Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. *Pharmacology & Therapeutics, 138*(1), 103-141. 10.1016/j.pharmthera.2012.12.007

Zhang, M. Z., Wang, J. L., Cheng, H. F., Harris, R. C., & McKanna, J. A. (1997). Cyclooxygenase-2 in rat nephron development. *American Journal of Physiology-Renal Physiology, 273*(6), F994-F1002.

Zimin, A. V., Delcher, A. L., Florea, L., Kelley, D. R., Schatz, M. C., Puiu, D., Hanrahan, F., Pertea, G., Van Tassell, C. P., Sonstegard, T.S., Marçais, G., Roberts, M., Subramanian, P., Yorke, J. A. & Salzberg, S. L. (2009). A whole-genome assembly of the domestic cow, bos taurus. *Genome Biology, 10*(4), R42. 10.1186/gb-2009-10-4-r42