Determination of Safe and Effective Dosing Regimens for Nonsteroidal Anti-inflammatory Drugs in African and Asian Elephants.

by

Jack J. Kottwitz

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Approved by

Dawn M Boothe, Chair, Professor of Physiology and Pharmacology
Sue Hudson Duran, Professor Emeritus of Clinical Sciences
Ya-Xiong Tao, Professor of Physiology
Satyanarayana R. Pondugula, Associate Professor of Physiology
Dennis Schmitt, Professor Emeritus of Reproductive Biology

Abstract

Arthritis and foot disease are well documented conditions affecting captive elephants that are frequently treated with nonsteroidal anti-inflammatory drugs (NSAIDs). An important consideration regarding efficacy and safety of NSAIDs is the cyclooxygenase isoenzyme (COX-1 or 2) targets and pharmacokinetics of the specific drug. The purpose of this study was to determine the COX preference for each drug in Asian elephants, and to determine a dosing regimen for firocoxib based on pharmacokinetics at two doses (0.01 and 0.1 mg/kg) in Asian and African elephants. Single oral dosing of a commercially available tablet or paste determined a preferred dose. This dose underwent further evaluation via a single i.v. dose, and multiple consecutive doses of both formulations. Studies were performed by participating elephant facilities throughout North America. Samples were subjected to firocoxib analysis using HPLC. Pharmacokinetic data was subjected to non-compartmental analysis. Firocoxib was determined to prefer COX-2 whereas flunixin meglumin preferred COX-1 *in vitro* in Asian elephants (*Elephas magnus*). Serum levels of firocoxib were too low at doses of 0.01 mg/kg for pharmacokinetic analysis. Key pharmacokinetic parameters after single dosing of 0.1 mg/kg in African elephants were Cmax (31.3 +/- 6.6 ng/ml for tablets; 44 +/- 12.5 ng/ml for paste), AUC (1588+/-362 H*mg/ml for tablets; 814 H*mg/ml for paste) and elimination half-life (66 hours for tablets; 37 hours for paste). Key parameters for single dosing of 0.1 mg/kg orally in Asian elephan were Cmax (49 +/-3.27 ng/ml for tablets; 62 +/- 14.8 ng/ml for paste), AUC (1332+/-878 H*mg/ml for tablets; 1455+/-634 H*mg/ml for paste) and elimination half-life (34.3 +/-30.3

hours for tablets; 19.9 +/-12.8 hours for paste). After multiple administration, the time to steady-state was 5 days and after 8 days of dosing, the Cmax, (75.8+/-15.5 ng/ml for tablets; 95.5+/-29.3 ng/ml for paste) AUC (6341+/-3003 H*mg/ml for tablets; 5613+/-2262 H*mg/ml for paste) and half-life (84.4+/-32.2 hours for tablets; 62.9+/-2.25 hours for paste) were. All animals tolerated all doses with no apparent adverse events. Based on these data, firocoxib should be an effective, safe and convenient analgesic at 0.1 mg/kg every 24 hours when administered to Asian or African elephants.

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List of Abbreviations

AA	Arachadonic acid
AAZV	American Association of Zoo Veterinarians
ADH	Antidiuretic hormone
ADME	Absorption, distribution, metabolism, and excretion [of a drug]
ACTH	Adrenocorticotropin hormone
AMDUCA	Animal Medicinal Drug Use Clarification Act
AUC	Area under the curve
AUC_{inf}	Area under the curve to infinity
AUC_{all}	Area under the curve from the time of last dose administered to the time of the last observation.
AWA	Animal Welfare Act
CITES	Convention on International Trade in Endangered Species
cGR	Cytosol glucocorticoid receptor
CL	Clearance
CL_s	Systemic clearance
C_{max}	Maximum plasma concentration
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1 isoenzyme
COX-2	Cyclooxygenase 2 isoenzyme

CRH	Corticotropin-releasing hormone
EM	Extreme (fast) metabolizer
ESA	Endangered species act of the United States
F_{abs}	Absolute bioavailability
F_{rel}	Relative bioavailability
%F	Percent bioavailability
GFR	Glomerular filtration rate
HPLC	High-performance liquid chromatography
HPPA	Hypothalamic-pituitary-adrenal axis
IACUC	Institutional Animal Care and Use Committee
IC_{20}	Inhibitory concentration that inhibits 20% of activity
IC_{50}	Inhibitory concentration that inhibits 50% of activity
IC_{80}	Inhibitory concentration that inhibits 80% of activity
K	Elimination rate constant
LLOQ	Lower limits of quantification
LO	Lipoxygenase
LOD	Limit of Detection
LPS	Lipopolysaccharide
mGR	Membrane bound glucocorticoid receptor
MBA	Migratory Bird Act
NOAA	National Oceanic and Atmospheric Administration
NSAID	Non-steroidal anti-inflammatory drug
PD	Pharmacokinetics

PM	Slow (poor) metabolizer
PG	Prostaglandin
PGE_2	Prostaglandin E_2
PGE_2M	Prostaglandin E_2 and metabolites
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PK	Pharmacokinetics
SSP	Species Survival Plan
TAG	Taxon Advisors Group
T1/2	Terminal half life
TDM	Therapeutic Drug Monitoring
Tx	Thromboxane
TxB_2	Thromboxane B_2
UM	Uncharacterized metabolizers
USDA-APHIS	US Department of Agriculture-Animal and Plant Health Inspection Service
US-FWS	United States Fish and Wildlife Service
UV	Ultraviolet
V_c	Volume of the central compartment
VD	Volume of distribution
V_{area}	Volume of distribution calculated by the area method
VD_{ss}	Volume of distribution at steady state

Chapter 1

Principles of Clinical Pharmacology as Applied to Non-Domestic Species

1.1 Introduction

Pharmaceutical compounds have a fundamental and essential place in the medicine of all species. Different drugs are administered for a wide variety of medical conditions, ranging from treatment of infectious diseases, genetic and hormonal abnormalities, contraception, behavior modification, sedation and/or anesthesia, to pain management needed for improving the quality of life of geriatric patients. A drug is simply a chemical or substance that causes a physiological effect or change within the body when it enters the body.[1] Drug administration occurs when a drug first enters a patient's body.[1] Once the drug has been administered, it is released in a predictable and characterizable manner where a fraction of the drug is absorbed from the site of administration either into surrounding tissue, into the body, or both and is then distributed to the site of action where a pharmacological effect occurs if the drug concentration at the specific site of action is equal to or exceeds that patient's minimum effective concentration.[1] The drug may also undergo metabolism during and after absorption that may result in metabolites that have an either desired or potentially harmful effect. The drug is then eliminated via metabolic pathways, excreted unchanged or as metabolites, or otherwise ceases to have an effect as concentrations in target tissues decrease (Figure 1).[2] Elimination of the drug may necessitate re-administration to continue to maintain therapeutic levels as plasma levels decrease over time

It is also important to note that the specific formulation of a drug administered or concurrent administration of other drugs may result in interactions, potentiation, or inhibition of

the effects of a given drug. Drug interactions can occur between simultaneously administered drugs causing either increased drug effect of one or both drugs resulting in drug toxicity or decreased drug effect of one or both drugs resulting in therapeutic failure.[1] Drug action can be characterized by the specificity of the drug for receptors within a body, by the selectivity of a drug for specific receptors, by the affinity for the drug to bind to these receptors, by the potency or the relationship between the drug dose and the magnitude of effect, and finally by the efficacy or the efficacy or response achieved from the drug.[1],[2,3] These general factors all play an important role in the pharmacology of any drug administered to the patient no matter what the species.

1.2 Absorption, distribution, metabolism, and elimination (ADME)

The four key physiological processes that govern the fate of all drugs in any organism are absorption, distribution, metabolism, and elimination (ADME) of the drug (Figure 2).[2,4,5] Despite the obvious differences in physical anatomy and physiology, the fundamental principles of ADME apply to all species of all ages, whether healthy or suffering from disease.[5-8] All organisms treated with a drug must absorb that drug from its respective administration site, be it an oral, topical, or a parenteral route. Specifics of drug administration may affect the outcome of administration of that drug. All organisms treated with a drug must distribute the drug in some way from the site of administration for it to have effects in different areas of the body. All organisms metabolize drugs that have been absorbed and distributed in their body in some way. Finally, all organisms will have some means of eliminating drugs after they have been absorbed, distributed and metabolized. A drug that cannot be metabolized or eliminated by an organism

becomes a toxin if it remains active and at bioactive levels within that organism, with concurrent high risk of harm to that patient.[9]

1.2.1 Drug Absorption

Traditional drug studies center around these parameters in healthy humans and domestic mammals, specifically, dogs, cats, horses, cattle, swine, sheep, and goats.[1,2,5,8,10-12] It is not uncommon for drug doses to be extrapolated to other species, however that extrapolation may not take into account specific nuances of the drug's ADME in that species.[4,10,12,13] In zoological medicine it is important to understand the wide breadth of species encountered, and how that may affect ADME for a given drug.[8,10,11] Zoo veterinarians are expected to have the knowledge and abilities to administer drugs to all species on the planet, other than humans, ranging from sea invertebrates to insects to elephants[4,14,15] The essential necessity for any drug administered is that drugs must be present at the site of action in a tissue at a sufficient concentration for a specific period of time to produce a pharmacological effect.[5] This is especially important in veterinary medicine, specifically zoological medicine, where species differences in ADME processes can significantly affect the effects of the drug and the time course of drug absorption, distribution, metabolism, and excretion.[5,10-12] Understanding each of these processes in detail allows drug choices and treatment plans that better serve the patient. Despite the interspecies variability, in their simplest form, these processes can all be described as nothing more than a drug molecule crossing a series of membranes within an organism.[5]

Drug administration is the very first step in absorption of a drug. Administration methods for drugs can vary widely depending upon the specific drug molecule and the species to which the drug is being administered, but the ultimate goal is the same: the drug must enter an animal's

body where it can begin the journey across membranes as it is absorbed then distributed, metabolized and eliminated by that patient. Typical intravenous, oral, subcutaneous, intramuscular or topical drug administration routes may not be reasonable or even physically possible with some nondomestic species depending upon the patient's respective anatomy, physiology, or other factors.[4,12,16] Success of drug administration may also depends upon specific animal husbandry and management practices and procedures.[16] The method of drug administration plays an important role with domestic animal drug therapy, but it can play a much more important role for medicating zoo animals because difficulty in drug administration directly results in less than desirable drug absorption.

The cardiovascular system plays a key role in drug absorption and distribution, because absorption is the movement of a drug from the site of administration to the general circulation where it can be distributed through the body.[1,5] The fundamentals of cardiovascular anatomy and function in most vertebrates are similar in that the heart as a pump moves blood through arteries to the periphery and returns blood through veins to the heart, where it is then circulated back to the body.[17] While the anatomic structure of the circulatory system is different between vertebrates and invertebrates, even insects and arachnids have organs that distribute hemolymph, their version of blood, which absorbed drugs will enter for distribution throughout their body.[18]

Species differences play a significant role in the ability to access veins for intravenous drug administration. Factors such as hair coat, dermal thickness, subcutaneous fat, and anatomical placement of veins or other circulatory vessels may make intravenous drug administration difficult, if not impossible. For example, intravenous administration of drugs is commonly performed in domestic animals, but can be virtually impossible in large zoo animals such as elephants without major surgical intervention due to their anatomical variations including

a relatively short, thick, heavily muscled neck, overlying thick skin, deep set vessels, anatomical valves within the veins of the legs that may prevent catheter placement, and the inherent danger to the veterinarian attempting such an endeavor.[19-21] Venous access in these species is generally only possible through an ear or saphenous vein.[21,22] It is equally impossible in very small zoo species to obtain venous access due to limitations in the size of available needles relative to the blood vessel's size as well as the overall patient blood volume.[4]

In human medicine, oral drug administration is perhaps the most common form drug delivery, but the animals used for preclinical drug trials differ significantly from humans and from each other anatomically and physiologically.[4,23] These differences, while compensated for in terms of research are extremely important, especially when in a medical field that crosses species boundaries as a zoo veterinarian would do. Rats and rabbits are widely utilized in human research for preclinical drug evaluations, yet these animals represent a very different physiology from that of humans.[23] Humans are monogastric omnivores, without a substantially developed cecum or extended large intestine, while both rats and rabbits used in human research for drug development differ both anatomically and physiologically as they are monogastric herbivores with an enlarged cecum and elongated large intestine necessary for plant digestion.[4,23,24] While this may be often overlooked in drug trials for validation of drugs in human medicine, the wide differences in the gastrointestinal tract and associated effects on drug administration should not be ignored in zoological medicine.[5,10,23] The methods for extrapolation of drug doses traditionally done for pharmaceuticals administered in zoological medicine are likely incorrect and each individual species requires thorough drug studies specific for that species.[4,25] The ultimate goal of interspecies extrapolation should be to accurately predict drug activity or toxicity in a species not previously studied or not studied in as much detail as the first species.[5]

This projective approach to zoological medicine introduces to major sources of error inherent to such extrapolations. The first error is that the drug's absorption, distribution, metabolism, and excretion cannot be generalized between species.[5] There must be some form of adjustment, which may be unknown, to allow for individual species characteristics of metabolism, digestion and excretion of a specific drug molecule.[5] The second is that the specifics of a pharmacodynamic response of a drugs may be different between species and not related to pharmacokinetics, but related to other factors. The most well documented example of this is in avian species with the differences in diclofenac metabolism resulting in renal failure and death in some species, with closely related species to those experiencing toxic adverse events appearing to be unaffected.[26,27]

Oral drug administration may also be a major obstacle in pharmacologic studies and drug administration in zoo species due to interspecies variation, differences in preferred food items, and feeding modalities.[5,8,10,12,28] Oral administration of drugs to zoo animals may require unique or even innovative methods to mask drug taste, texture, or consistency.[4,11,12] Often medications are administered concurrently with a meal or a treat, which may alter drug absorption for some drugs, as gastric acid or digestive enzyme levels may change with digestion or drugs may bind to and be inactivated by the food ingested.[4,11] In the case of some particularly challenging species, such as marine mammals, some species of fish, insect, insectivore, reptiles and some raptors, the only way to administer drugs orally is by using another animal as a drug delivery device.[4,28] For example, if rodents are utilized, the drug administered is injected or otherwise inserted into the rodent's body and the rodent is fed to the patient.[4] This prey delivery device often does not work with insectivores, so the clinician is often left with the choice of feeding the drug in question to the insects and then in turn feeding the insects to the species that is being treated.[4] This method

works well in some situations, such as increasing dietary calcium intake for reptiles and insectivores, but the clinician must consider potential drug metabolism by the food species prior to administration which may alter desired outcomes.[29] In addition to potential difficulties in oral administration of drugs, the anatomical differences between zoological species must be considered to understand drug absorption. True monogastrics, such as canines or felines have a different intestinal physiology than hindgut fermenters (such as rodents, rabbits, horses, rhinoceroses, or elephants), foregut fermenters (such as some species of monkeys, all camelids, kangaroos, and hippopotamus) or ruminants (such as wild cattle, goats, sheep, cervids, giraffe, antelope).[4,30] This means that potential differences between species for absorption after oral administration of a drug are enormous and extrapolation of specific details between species is not reasonable, reliable, or even possible.[4,30-33]

While intramuscular and subcutaneous drug administration may provide a more reliable means than oral drug administration, these methods of drug administration also present their own unique obstacles with zoo animals. It may only be possible in many zoo species to administer a an intramuscular or subcutaneous injections by use of remote dart delivery systems.[4,21] These systems carry an inert risk of injury to the animal being darted, along with limitations as to the volume of drug that can be delivered per individual dart.[34] A recent study in domestic cattle has raised questions about the efficacy of this method of drug delivery in all species, as remote dart delivery demonstrated decreased systemic drug concentrations, increased patient stress responses, and increased injection site inflammation.[35] Operant conditioning or some form of manual restraint is generally required to allow for direct injection and to prevent injury to the patient if a remote system is not utilized.[21,36] Unfortunately, there are no such published comparison studies between remote dart and hand or manual intramuscular or subcutaneous

injection in zoo species. Patient stress is a very important concern in zoo animals as repeated injections may ultimately lead to treatment failure due to patient aversion and lack of cooperation.[36]

A pharmaceuticals bioavailability is uniquely linked to the administration method utilized for the drug and associated absorption of a drug based on its method of administration to a specific species.[12] Bioavailability can be defined as the rate and extent an administered drug reaches the systemic circulation, and as a result is transported throughout the body to the site of action.[1,5,24,37,38] The terms bioavailability and absorption must be distinguished from each other, because absorption is only a step between administration of a drug and it's movement to the site of action.[38] At a glance, defining bioavailability can appear confusing, but it ultimately describes the extent and rate at which a substance, or its active metabolites, are delivered to the active site in a body.[38] If a drug is administered intravenously, bioavailability can approach or reach 100%. Bioavailability is only 100% if the drug is administered by the i.v. route and the active substance reaches the arterial circulation without being metabolized as it passes through the lungs and crosses from venous to arterial circulation.[38] The lungs can be a site of substantial drug metabolism, thus reducing active drug concentration and subsequently reducing drug bioavailability for a drug administered i.v.[38] Bioavailability also may not be 100% for i.v. administration if the drug formulation administered is a pro-drug, which must be converted into an active form prior to the desired drug effect occurring.[38] A drug may be absorbed 100% from an orally administered dose, but have a bioavailability less than 100% due to drug breakdown or first pass effect metabolism occurring in the liver immediately after absorption as the drug leaves the gastro intestinal tract and prior to transport through the body to the site of action.[38]

Bioavailability of a drug is represented as either absolute bioavailability or relative bioavailability. Absolute bioavailability can be defined as the actual percentage of the administered drug dose (from 0 to 100%) that reaches the general circulation and is subsequently transported to the site of drug action.[38] Relative bioavailability can be defined by the comparison of two formulations, or two routes of administration of the same formulation, without reference to i.v. drug administration.[38] The interpretation of relative bioavailability by conducting drug studies that do not include knowledge of absolute bioavailability can be of little use.[38] The determination of drug absolute bioavailability has been described as essential, and that no drug development should performed without i.v. administration data.[38]

In addition other factors such as the presence of food administered with the drug or ingesta in the stomach can affect absorption from the gastrointestinal tract resulting in significant interspecies differences in serum drug levels and drug effects.[5,24,39] Unfortunately, oral administration with food may be the only reliable means of repeatedly administering some medications to some zoo animals. Because some drugs may bind directly to food consumed with the drug, care must be taken in choices of food to give a drug with dependent on the individual properties of the drug.[5,11] Changes in rates of absorption associated with concurrent food administration are not just drug and food consumed dependent, they are also dependent on individual species digestive physiology.[5,11] Obviously a continuously foraging animal, such as a horse, would have different gastric emptying times than a gorging carnivore like a lion which consumes large quantities of food every 2 to 4 days, with intermittent fasting in-between meals, and both are substantially different from a reptile like a giant constrictor which may only consume food once every 6 to 8 weeks. This is important for medical therapy because drug absorption typically occurs in the stomach and small intestine, and the rate of delivery to the

small intestine is governed by the gastric emptying time as the food ingested moves from the stomach.[5,40] Variations in feeding modality can also substantially alter gastric pH, with grazers having a much more stable pH than periodic feeders and gorgers, which may have large variation in gastric pH depending on the absence or presence of food and volume of food present.[5,40] Animals with multiple stomach chambers, such as hippopotamus, camelids, and ruminants require further consideration with oral drug administration, because the individual chambers of the stomach are environments with varying epithelial linings, pH and bacterial flora which may result in slow transit time, or cause structural changes or drug breakdown prior to reaching the glandular stomach where absorption can occur.[5,40]

1.2.2 Drug Distribution

For a drug to have an effect after administration, it must move across at least one membrane, and in some cases may have to overcome physical, chemical, or biological barriers as it is absorbed, to move from the site of administration to the site of desired effect.[1,2,41] This movement is in essence a series of events governed by the chemical structure of the drug and interactions with membranes or membrane transporters in an organism's body. The membrane barriers a drug must cross to be absorbed, distributed, metabolized and eliminated often directly or indirectly define the individual physiologic compartments or other mathematical models that are incorporated in pharmacokinetic modeling of drugs.[5] Drug molecule size, pH, and protein binding of a drug plays an important role in absorption, because each of these can affect the ability of a drug to be absorbed as they subsequently move across membranes.[1,5,42] There are different mechanisms by which a drug moves across a biological membrane, each dependent upon the physical characteristics of the drug and the specifics of the species the drug was

administered to.[41] Despite wide variation, the basic principles of drug absorption hold true for all species.

The phospholipid nature of a cellular membranes within an organism allows for movement of nonpolar lipid soluble compounds and polar-water soluble compounds through the hydrophobic regions of the membrane allowing for distribution to the body.[5,41,43] All cellular membranes are composed of a lipid bilayer in which proteins are imbedded in the bilayer and either transverse the bilayer, reside inside of the cell (intracellular) or reside on the outside of the cell (extracellular).[5,39] This lipid bilayer is arranged so that the hydrophilic (polarized) head groups face the surface and the hydrophobic (nonpolarized) tails of the phospholipids face the interior structure of the bilayer.[5,39] The specific lipid composition of a given cellular bilayer can vary depending on the type of cells in the tissue that it composes.[5] In order for a drug to be absorbed or distributed, it must pass through at least one of these phospholipid membranes.[5,39,43,44] The lipid solubility, or lipophilicity of a drug plays a key role in it being able to transverse the phospholipid bilayer.[39,43,44] Lipid bilayers are more permeable to nonpolar lipid-soluble compounds and polar, water-soluble compounds with sufficient lipid solubility to successfully diffuse through the hydrophobic portion of the membrane.[5,39,43] The innate charge and ionization of a drug plays an important role in its ability to cross the cellular lipid bilayer as cellular membranes are more permeable to an ionized or charged form of a drug than to a drug that is not ionized.[5,42] Drugs are generally a weak organic acid or a weak base, existing in both un-ionized and ionized forms in an aqueous environment.[39,43] A nonionized form is usually lipid soluble (lipophilic) as a result can easily diffuse across cell membranes.[39,43] The ionized form has a low lipid solubility, but has a high water solubility (hydrophilic) and high electrical resistance and thus cannot easily cross a cellular membrane.[39,43] Disease processes, such as

inflammation, have the potential to alter tissue pH, thus affecting the ability of drugs to cross membranes and enter into the respective affected tissue compartment.[10,45]

Passive, or simple, diffusion is a primary means of drugs crossing cellular membranes.[5,39] Passive diffusion requires a degree of lipid solubility for a drug to cross the phospholipid bilayer as the drug moves down the concentration gradient until equilibrium is reached.[1] The rate of diffusion of a drug is directly proportional to the drugs concentration gradient across a membrane, the drugs lipid:water partition coefficient, and the diffusion coefficient of that substance.[5] The diffusion coefficient of a drug is determined by molecular size, molecular conformation, solubility and degree of ionization of the compound.[5] The lipid:water partition coefficient is the relative solubility of the compound in both lipid and water and is a reflection of its ability to penetrate and pass through the lipid membrane.[5] It is possible that if the lipid:water partition coefficient of a drug is too high for a given drug, that drug may become sequestered in a membrane rather than pass across it.[5]

Proteins integral to the phospholipid bilayer play a key play an important role in the uptake and efflux of both endogenous and exogenous compounds, such as pharmaceuticals waste, and toxin, into (influx) or out of (efflux) cells.[46] Facilitated diffusion is a means of protein transporter associated movement across the membrane that requires no energy, but also cannot move against a concentration gradient.[1] This form of membrane transport allows drugs which may not easily cross a membrane's phospholipid bilayer due to charge or lipopholicity to move down a concentration gradient through specific protein transport channels crossing the membrane into or out of a cell.[1,46] Active drug transport also utilizes cellular membrane proteins which cross the phospholipid bilayer, but is a process that utilizes energy to transport the drug or drug metabolites in or out of the cell, and as a result can transport drugs against a concentration

gradient.[1,46] P-Glycoprotein is a specific transmembrane transport protein that functions to remove substances, including toxins, drugs and drug metabolites, from within cells into the cytosol.[46,47] These transport proteins can have significant effects on drug pharmacokinetics and metabolism as they can decrease drug absorption by actively transporting drugs back into the intestinal lumen and they can decrease drug effects by transporting drugs out of the cytosol into the extracellular space thus preventing binding to active sites in the cell. P-glycoprotein proteins play a key role in phenomena such as drug resistance.[46,47] Membrane transport proteins have become an emerging topic in research, with discoveries that membrane proteins play key roles in specific types of cancers and they may have functions like contributing to the super-concentration of inflammatory mediators in tissues and that can be directly inhibited by anti-inflammatory drugs.[48]

Membrane barriers directly or indirectly define the various anatomical compartments in the body, which play a key role in drug distribution as the drug is absorbed from the site of administration through the body.[1,5] The effects of medications on intestinal flora, especially in herbivores like rabbits or guinea pigs, are not without negative outcomes, as oral administration of antimicrobials and other drugs to these species can result in intestinal flora dysbiosis, enteritis, and fatal enterotoxemia due to drug induced changes in normal intestinal flora.[49,50]

Distribution of a drug occurs once it enters the circulatory system and is subsequently transported throughout the body, then leaves the circulatory system, ideally at target tissue sites.[1] The distribution of a drug once it enters the systemic circulation is based upon four factors: chemical properties of the drug (lipid solubility, protein binding, molecular weight), the concentration gradient established between the blood and tissue, the blood flow rate to a specific tissue, and affinity of the drug for tissue constituents.[5] As with absorption, the chemical

properties of the drug determine how easily it will move in or out of a tissue or if it will remain in systemic circulation. Blood flow rates to specific tissues determine the rate of distribution of a drug, but those rates are not consistent across all species.[1] As a general rule, tissues with a high blood flow/mass ratio include organs such as the brain, heart, liver, kidney, and endocrine glands. Tissues with an intermediate blood flow/mass ratio include muscle and the skin. Finally, tissues with a low blood flow/mass ratio, or poor tissue perfusion, include adipose or connective tissues.[5] Flow rates vary across species as well, especially those with the ability to alter tissue perfusion based upon physiological environments, such as marine mammals or reptiles. There can also be variation not only between tissues but within tissues, with some aspects of a tissue having increased blood flow and others having relatively less blood flow, for example, in the renal cortex vs medulla of the kidney or in different types of skeletal muscle tissue.[5,51] Specific organs in the body also have unique anatomical barriers to penetration of foreign substances, such as the connective tissue of the meninges and associated so called blood/brain barrier protects and isolates the central nervous system.[5]

Proteins in blood plasma may also alter drug absorption and distribution by forming complexes with drugs by various chemical mechanisms and slowing or preventing the drug from leaving the systemic circulation.[5] Covalent bonding of a protein to a drug may prevent the drug from leaving the blood stream as covalently bonded protein-drug complexes cannot freely disassociate and must undergo extensive metabolism and breakdown to remove the drug.[5] Noncovalent binding results in a protein-drug complex that can dissociate depending upon the physiologic environment the complex enters.[5] In domestic animals, albumin is a particularly important circulating blood protein for drug-protein complex formation.[5,52] The extent of protein binding with plasma proteins other than albumin, such as vitelogenic proteins of egg laying

reptiles and birds, which can exceed albumin in concentration during specific times of the reproductive cycle, are not known in nondomestic species. Studies of domestic chickens have shown changes in drug pharmacokinetics during egg laying and subsequent drug residues within eggs laid.[52] It is assumed that this is true for all egg laying animals, but specific scientific data is lacking in nondomestic species. Factors such as reproductive status must be considered when treating egg laying species and when conducting basic drug studies.

1.2.3 Drug Metabolism

Drug metabolism occurs by enzymatic processes that change or otherwise modify the drug in various organs in the body. Depending upon the specific drug, metabolism typically occurs in the liver, but also can occur in the gastrointestinal tract, kidneys, lungs, and, for topical medications, the skin.[5,53,54] Multiple metabolic pathways are involved in drug metabolism, including oxidation, reduction, hydrolysis, hydration, and conjunction.[5] These processes can be divided into Phase I and Phase II processes. Phase 1 metabolism are reactions which change the structure of the drug, often deactivating it, by introducing functional groups such as -OH, -COOH, -SH, -O-, or $-NH_2$ to the compound that are necessary for binding with Phase II enzymes prior to drug breakdown.[5,53,55] Phase II enzymatic reactions involve the introduction of hydrophilic groups to the compound through sulfation, glucuronidation and glutathione conjugation, which aid in increasing water solubility for drug excretion/elimination.[53,55] Thus Phase I reactions prepare drugs for Phase II metabolism, which is necessary for final drug deactivation and drug excretion.[5,53]

The liver functions to detoxify and facilitate excretion of xenobiotics, foreign chemicals or drugs by enzymatically converting lipid-soluble compounds to more water-soluble

compounds. Drug metabolism is achieved through either phase I reactions, phase II reactions, or both. The most common phase I reaction is oxidation, which is catalyzed by the CYP enzyme system. CYP enzymes are a hemoprotein that is one of the largest and most functionally diverse protein superfamilies found in nature.[56] These enzymes exist both in plants and animals where they function to catalyze a wide variety of substances, but there appears to be variability between species.[57] This is a very large group of enzymes, with more than 10,000 different CYP proteins described belonging to over 1000 different families in organisms ranging from viruses to humans.[58] Cytochrome P450 isoenzymes have been extensively studied in humans and domestic animals as key enzymes necessary for oxidation and metabolism of drugs with roughly 90% of drugs undergoing some type of metabolism by a CYP450 enzyme.[1,5,53,55,59] Drug metabolism by these enzymes occur in multiple sites in an organism, including the liver, intestinal wall, lungs, kidneys, and plasma.[60] The CYP enzymes are named according to gene sequence similarity and grouped according to family (number), subfamily (letter), and unique gene product (number).[60,61] Because of significant species differences in gene sequence of these enzymes, each species tends to have their unique CYP names, although orthologs or genes derived from the same ancestral gene with the same function that diverged after speciation are not uncommon.[61] Therefore the species must be considered when identify specific CYP enzymes as there can be a similar function by enzymes with different names.[61] Research in human medicine has found that many drugs are metabolized by P450 enzymes specifically in the human CYP1, CYP2, and CYP3 families.[57] The human CYP1 family is associated with estrogen metabolism and therefore associated with estrogen producing endometrial and ovarian cancers in humans.[62] The human CYP1 family is also exhibits hydroxylase activity in oxidation of arachidonic acid associated with the inflammatory processes integral to these types of estrogen associated neoplasia.[62] The

human CYP2 family is diverse and functions as the epoxygenase isoform abundantly expressed in the endothelium, myocardium, and kidney in humans and has an integral role with cardiac protection from xenobiotics, inflammation, and drug metabolism.[63] The human CYP3 family functions are not as well described as CYP1 or CYP2, but there has been drug metabolism identified by members of the CYP3 family also.[64] In humans, several of the P450 genes and enzymes exhibit differential expression, depending on the age, sex, and ethnic background of the individuals examined.[65] In addition, many of these enzymes are induced differently depending on the species being examined. Unfortunately, these variations in the CYP enzymes have not been fully described in domestic animals, much less zoo animals.

Once a drug is absorbed, polymorphisms in the genes responsible for coding for the cytochrome P450 (CYP) enzymes, which are responsible for Phase 1 oxidation, reduction, or hydrolysis of drugs, can lead to differences in drug metabolism, as noted in domestic dogs.[66] There have been at least five P450 enzymes described with polymorphisms in domestic dogs, including CYP1A2, CYP2C41, CYP2D15, CYP2E1, and CYP3A12, each of which contribute to the altered metabolism of different drugs.[66] It seems safe to assume that if a single domestic species has been shown to produce a minimum of 5 known polymorphisms of a given enzyme, such polymorphisms may also be present but undiagnosed in other species.[66]

One of the first identified polymorphisms in domestic dogs was for the metabolism of the cyclooxygenase (COX) 2 inhibitor celecoxib.[67] Celecoxib is a COX-2 specific nonsteroidal anti-inflammatory drug that is metabolized via a single oxidative pathway in domestic dogs and humans[67] Celecoxib is primarily metabolized by CYP2D15 in domestic dogs[67] The methyl group of celecoxib is first oxidized to the hydroxymethyl metabolite, followed by further oxidation of the hydroxyl metabolite to the carboxylic acid analog[67] These differing rates of

metabolism can be divided into 3 groups: extensive metabolizers (EM), poor metabolizers (PM) and uncharacterized metabolizers (UM)[67] In a study population of 242 laboratory raised beagles, 45.0% were of the EM phenotype, 53.5% were of the PM phenotype, and 1.65% could not be adequately characterized.[67] Extensive metabolizers had an elimination half-life of 0.93 - 2.51 h, while poor metabolizers had an elimination half-life of 3.89 - 6.47 h.[67] It is easily demonstrated that EM dogs metabolized celecoxib at a much higher rate than PM dogs did.[67] It is interesting to note that no gender differences were observed in that specific study.[67] The recognition of these polymorphisms in the same species has huge implications on the potential for drastic variability in metabolism when drugs are administered to species where the CYP isoenzymes have not been fully described. In this example alone, there is nearly a 50% chance that the dogs will metabolize the drug very quickly (EM), limiting therapeutic efficacy, while at the same time there is a nearly a 50% chance that a beagle will metabolize it much more slowly (PM), potentially increasing the risk of negative complications or drug toxicity.[67]

Examples of other drug metabolism processes associated with alterations in Phase II metabolism include decreased glucuronidation of the drug acetaminophen, leading to toxicity in domestic cats and mustelids, including. the domestic ferret.[68,69] Domestic cats have a mutation of the gene encoding UGT1A6, which leads to a deficiency in the activity or complete loss of function of the glucuronyl transferase enzyme, which is key to the conjugation of acetaminophen to glucuronic acid.[68,69] Ferrets have not had such a mutation identified, but similarly have exceptionally slow glucuronidation of acetaminophen, making them highly susceptible to acetaminophen toxicity.[68,69] This lack of an identified mutation further demonstrates the issues associated with extrapolation of a single pathway descriptions of Phase II metabolism within and between species.

The concepts of enzyme induction and enzyme inhibition must be considered when analyzing drug administration and metabolism, and the liver is a key organ in this pathway for all vertebrate species.[5] Because the liver's primary function centers on clearing the body of toxins, there are enzyme systems present in this organ that may activate with exposure to a chemical such as a drug, resulting in increased synthesis of enzymatic products and stimulation of metabolic pathways.[66,70] This enzyme activation can result in altered metabolism of a drug, possibly potentiating toxicity.[70] Induction can also act on enzyme pathways not specifically related to the drug that induces these actions, but instead results in changes in metabolism of other drugs.[70] Conversely certain drugs can inhibit enzyme processes, preventing metabolism of themselves or of other drugs.[70] Enzyme induction or inhibition creates a special concern when a drug is administered to a species that has not had these enzymatic pathways described, because these processes may not be consistent across all species. These concerns legitimize the general consensus of "no zoo veterinarian wants to be the first to administer a drug" because of the associated negative outcomes that may occur in the face of an adverse drug event.[4] These nuances of drug metabolism illustrate the attention zoological veterinarians must place on the known aspects of drug metabolism in domestic species, comparable mechanisms in nondomestic species, and the vigilance that must be kept for potential negative and toxic effects of drugs that are being administered without scientifically evaluated doses.

1.2.4 Drug Elimination

Drug and drug metabolites' structure, specifically molecular weight, polarity and lipophilicity are key factors in determining the route of elimination from the body.[30,71-73] Exposure to compounds of increasing molecular weights (150 to greater than 700 g/mol) results

in a shift from renal excretion to hepatic excretion via bile as molecular weight increases[30,71-73] Drugs and their metabolites with low molecular weights of less than 300 g/mol are primarily eliminated glomerular filtration in the species evaluated, while substances with molecular weights greater than 600 g/mol are typically eliminated in the bile by active carrier-mediated transport in mammals.[30,71-73] Despite these findings, it is important to understand that with drugs and other substances with molecular weights between 300 and 600 to approximately 800 g/mol, there can be large species differences for the preferential route of elimination, with one species primarily eliminating a substance via renal pathways and another using hepatic metabolism and biliary excretion.[30,71-73] Animals can be generally grouped in this range of molecular weights as "poor" biliary excreters such as rabbits, guinea-pigs, and humans; "good" biliary excreters such as rats, chickens, and dogs; and "intermediate" biliary excreters such as domestic cats and sheep.[30,71] These species difference arise from species differences in the molecular weight threshold, which is thought to be unique to all species.[71]

Bile is continuously produced by mammalian liver cells, and as drug and drug metabolite transport into bile occurs by active transport, drug saturation limitations must be considered as biliary excretion systems are overwhelmed.[24] Bile moves from the hepatocytes down the biliary tract to the gall bladder, if a species has such an anatomic structure, where it is stored until excretion into bile duct and ultimately the intestine.[24] Most compounds secreted into the bile are finally excreted from the body in feces.[1,5,55,74] Intestinal microflora play an important role in drug breakdown prior to fecal passage.[5,74,75] Some drugs may be reabsorbed from the intestine, and undergo enterohepatic recirculation before being re-secreted into the bile.[1,5,24,55] These re-excreted metabolites may also be reabsorbed into the blood and further metabolized until they are eventually excreted from the body via the urine.[5]

Not all organisms encountered in zoological medicine have the same form of bile metabolism. Bile metabolism in vertebrates can involve both bile alcohols and/or bile acids as the end product of cholesterol metabolism.[76] Bile alcohol sulfates and conjugated bile acids are collectively called "bile salts," which can present some confusion in interpretation of the veterinary literature regarding the specifics of liver metabolism, because these processes are described in some species, but they are not described in all.[76] While bile acids are present in different forms in vertebrates, bile alcohols retain the structure of cholesterol with an 8-carbon (C-8) side chain and a total of 27 carbon atoms (C27).[76,77] Bile alcohols represent a more "primitive" form of bile processing, and are likely the evolutionarily "oldest" form of cholesterol metabolism, most commonly identified in early evolutionary variants of fish and amphibians.[76]

The specifics of liver metabolism, and specifically liver metabolism as it applies to the nuances of drug metabolism, remain unknown in many species at this time. Elephants, mantee and hyrax are closely related mammalian species where bile alcohol metabolism has been identified as the primary form of cholesterol metabolism, meaning that bile is not produced in the same form or via the same mechanism in these species as it is with other mammals.[77] This difference in cholesterol metabolism has significant implications for drug metabolism, and the determination or evaluation of potential deleterious effects associated with drug metabolism and drug elimination in these species. Both bile acids and bile alcohols can be bound to or conjugated to metabolic waste products and drug metabolites, thus resulting in both compounds serving as removal mechanisms of compounds from the body as bile acids and bile alcohols are both excreted into the intestinal tract then exit the body with feces.[77] The general consensus is that animals producing C27 bile alcohols for cholesterol metabolism, like elephants, likely have a simpler and shorter synthesis pathway for those bile alcohols compared to species producing

more advanced bile acids.[77] [78] Bile alcohols do not require side chain cleavage or oxidation of the terminal alcohol for formation, making these the simplest bile salt end product.[78] Enterohepatic circulation, where compounds bound by bile alcohols and excreted into the intestine are reabsorbed from the intestine back into to the blood stream and recirculated through the body has been identified with drug studies in elephants, including the NSAID phenylbutazone, which is primarily metabolized in the liver.[79] The exact nature of enterohepatic circulation is not known in many species, including elephants, however it is thought that in fish producing bile alcohols, some bile alcohols are likely to not be absorbed from the intestine due to the structural shape, limited solubility the unsulfated bile alcohol, and large number of hydroxyl groups while other smaller chain bile alcohols may be reabsorbed.[76] This may also happen in elephants, thus creating a confounding issue in descriptions of drug elimination in this species, as some drugs may be quickly eliminated and others may show variable increasing and decreasing serum levels due to this enterohepatic cycling. Attention must be paid to the drug as well as the drug metabolite's specific structure, as it may variably bind with bile alcohols as it is metabolized. Based on the elephant as an example, assumptions cannot be made for all animals that the liver will metabolize all drugs the same way, and specific nuances of the species in question must be identified and considered. In addition to the specific drug interactions with bile acids or bile alcohols, the presence or absence of a gall bladder may affect drug elimination in that species.[80] In animals that do not have a gall bladder, bile is secreted continuously, flowing from the liver into the duodenum. In animals with a gall bladder, bile instead flows into the gall bladder, where it is concentrated and stored to be excreted in larger quantities at the time of gall bladder contraction, which is associated with the sight, smell, or ingestion of food.[80] This can result in more pronounced changes in serum levels or waxing and waning irregularities in serum

drug levels due to the higher levels of conjugated drug excreted at the time of gall bladder contraction if the drug undergoes enterohepatic circulation and is reabsorbed from the GI tract.[80]

The kidneys are the primary organs involved in elimination of drugs and drug metabolites of smaller molecular weights, less than 300 g/mol.[30,71] Renal elimination of drugs includes glomerular filtration, renal tubular secretion, and tubular reabsorption, with the total renal excretion of a drug being equal to the sum of all three mechanisms[5,55] Excretion by glomerular filtration is unidirectional with non-protein bound drugs and drug metabolites being removed by bulk flow from the renal artery through the glomerulus.[5,81] The rate of drug filtration for glomerular filtration is directly dependent upon both the extent of drug protein binding and the glomerular filtration rate (GFR).[5,55,81]

Species specific anatomical structures and differences in glomerular function can alter glomerular filtration or result in varying rates of glomerular filtration between mammalian species.[5,81,82] Glomerular dysfunction resulting in changes in the negative charge of the epithelial cells of Bowman's capsule in the glomerulus results in changes in membrane selectivity that allows proteins and protein-drug complexes that are normally not filtered to pass into the renal tubule.[2,5] Tubular secretion is an active process in mammals, with ATP fueling Na+-K+ ATPase- coupled transport systems.[5,81] The transport systems across the tubule cells involves two separate pairs of transport proteins, that ultimately create an overall "polarity" between the interstitial fluid and tubular lumen.[5,55,81] The first set of transport proteins in mammals is located at the brush border of the interface with the tubular fluid and the other is located in the basolateral membrane.[5,55] Energy coupling with ATP generally occurs near the mitochondria of the cells which, in secretion, increases intracellular drug concentrations that are then transported to the tubular fluid by concentration-driven facilitated transport carrier

proteins.[5,55] In tubular reabsorption, the reverse of secretion occurs, as the basolateral energy-driven pumps create low intracellular drug concentrations, which in turn promote facilitated carrier-mediated reabsorption through the tubular membrane. The distal renal tubule contains two distinct secretory pathways in mammals, an organic anion transporter for positively charged (acidic compounds) and an organic cation transporter for negatively charged compounds.[5,83] These mechanisms are present to recover essential nutrients, such as glucose, that have been filtered by the glomerulus, and transport them back into the circulatory system.[5,83] The final factor controlling renal drug excretion is non-ionic passive tubular reabsorption.[5] This is a process dependent upon urine flow rate, lipid solubility of the nonionized drug moiety, and urine pH.[5,24] At low urine flow rates, there is greater opportunity for diffusion of drug from the distal tubular fluid back into the blood stream.[5] Diffusion down a concentration gradient is facilitated by a much higher concentration of a drug in the tubular fluid.[5] . Polar compounds with relatively low lipid solubility are not reabsorbed as their charge and lack of lipid solubility prevents them from crossing the lipid membrane.[5] In contrast, lipid-soluble, nonionized drugs are reabsorbed into the blood.[5]

While these anatomic structures and mechanisms are conserved across most mammal species, variation does exist because of species specific differences in urine flow, pH, and concentration.[24] Conversely, renal drug excretion can be vastly different in birds and reptiles compared to mammals due to fundamental differences in renal structure and function.[81,82,84] While the kidneys of birds have anatomical structures in common with the kidneys of both mammals and reptiles, there is a much greater morphological heterogeneity in the nephron of an avian kidney than mammals or reptiles[81] Although the individual avian nephrons have a lower GFR compared to mammalian nephrons, the total number of nephrons per kidney is higher in

bird.[84] This higher number of glomeruli results in a similar overall GFR for birds and mammals.[84]

The greatest anatomical difference between mammals and birds is that all mammalian nephrons have a loop of Henle of the nephron tubule, while the majority (60 to 90 percent depending upon species) of avian nephrons have no loop of Henle. No reptile kidney has this structure.[81,82,84] Avian nephrons can be further divided into cortical nephrons, which have no loop of Henle, and medullary nephrons, which are similar in structure to mammalian nephrons.[82,84] The avian cortical nephrons are described as small nephrons with a short tubule that folds over on itself.[81,82] This anatomical arrangement does not permit the nephron tubule to function as a countercurrent multiplier system creating hyperosmotic urine, which potentially affects both active and passive drug excretion.[81,84] As the urine from these tubules cannot be more concentrated than the plasma, it is possible that renal elimination of some drugs may only occur by passive diffusion, thus potentiating adverse events from increased drug concentrations or prolonged drug activity. In addition to the lack of a loop of Henle, in the avian kidney the collecting ducts that drain the cortical nephrons enter the medullary cones and merge with the collecting ducts that drain the nephrons with loops[81,84] This results in these collecting ducts delivering a large amount of dilute to isosmotic fluid to the avian version of a countercurrent multiplier system that has limited function within the medullary cones of the kidney.[81] The osmotic gradient through which the loop of Henle traverses extends from the cortical-medullary boundary to the tip of the medullary region is primarily made of two solutes, sodium chloride and urea in mammals with differences in other types of animals.[81] In birds and reptiles, uric acid metabolism results in the production of nitrogen as the break down product, and not urea formation, this gradient is made of only sodium chloride.[81] Mammals excrete waste nitrogen as

urea, a compound 40,000 times more soluble in water than the uric acid form of waste nitrogen secreted by birds and reptiles.[81] Because of this difference in solubility, urea requires additional water for excretion.[81] A small portion of the urea is also retained in the renal medulla, where it plays an important role as a component of the intramedullary solute gradient of the loop of Henle in mammals[81] This gradient is required to produce urine that is hyperosmotic to plasma and to concentrate waste materials, such as drugs and drug metabolites, in the urine. This limits the capacity of the avian and reptile kidney to concentrate urine compared to mammalian kidneys, which will subsequently affect the elimination of drugs.[84]

Reptile kidneys have additional anatomical differences from avian or mammalian kidneys, with grossly observable modifications in morphology between terrestrial chelonian, ophidian, and aquatic reptiles.[81,82] Microscopically, the kidney of all reptiles has an external capsule with distinct lobular segmentation.[81] The glomeruli are poorly developed, with a lower number of capillaries per gram of weight than those of birds and mammals.[82] Reptiles also possess the ability to shunt blood returning from the pelvic limb away from the kidney, bypassing renal filtration and remaining in the general circulation.[82] . Lower numbers of capillaries mean a lower bulk blood flow rate, and when combined with this shunting of blood away from the kidney, these circulatory differences can result in decreased renal elimination of drugs and drug metabolites in reptiles. There is no structure comparable to the loop of Henle in reptile kidneys, as all glomeruli collect to short, folded tubules originating on the dorsolateral surface of each lobule, and these tubules are oriented at right angles to the long axis of the kidney[81] These structural differences, combined the uric acid component of renal metabolism of reptiles, results in the inability to excrete hyperosmotic urine. The inability of reptiles to

concentrate urine may have significant effects on drug elimination, as urine drug concentrations will never exceed plasma concentrations.

In humans, it has been shown that there can be a wide variability in response to pharmacological interventions, including variations in drug administration success in desired outcome, presence of adverse events, which may be related to individual anomalies that affect drug metabolism.[9] The dose administered to a human is generally determined by carefully conducted and constructed clinical trials that provide correct drug concentrations and desired actions in the body based upon that drug's ADME.[9,85] The field of pharmacogenomics, or specific tailoring of drug protocols based upon the genetic makeup of an individual, is poised to greatly increase the efficacy and safety of drugs in humans, but we have not yet reached that stage in veterinary medicine regarding domestic species, much less zoological medicine.[9,85] Despite these new discoveries, the basic principles of pharmacokinetics and pharmacodynamics still must be described to fully understand the ADME and effects of a given drug on a specific species. These principals can be considered uniform across all species, as long as nuances of the individual's species are not forgotten or ignored.

1.3 Pharmacokinetics

Pharmacokinetics (PK) is best defined as the use of mathematical models to quantitate the time course of drug absorption, disposition, metabolism and excretion.[86] The mathematical formulas utilized should be relevant for all species. Application of pharmacokinetic calculations allow for a dose of a specific drug to be tailored to that individual, or species, to optimize therapeutic effectiveness, minimize adverse events, and avoid tissue residues in animals consumed for food by humans or by other animals.[86] The goal is not to just select a drug, but to

determine for a given drug a rational dosing regimen involving dose rate, inter-dosing interval, duration of treatment and modalities of administration. The dosing regimen in a given species is dependent on the individual species' anatomy, biochemistry, physiology, and behavior as well as the nature and causes of the medical condition that is being treated.[24]

The mathematical formulas utilized for determining PK parameters should be applicable to most animals for determining the *in vivo* disposition of a drug. These parameters are calculated by identifying the time point for administration of the drug, and then determining the tissue, blood plasma, or serum concentrations of the drug over a period of time.[87] Plasma has been described as being generally more widely analyzed than whole blood or serum, because sample preparation and analysis methods are easier for plasma, however the specific indications for selection of serum vs plasma for blood analysis of drugs is highly dependent on the drug, the specific methodology of analysis utilized, and the availability of specimen[87] A main tenet in pharmacology is that the plasma or serum concentration controls the concentration at the site of action or the biophase, and that a proportional relationship exists between the plasma or serum and biophase concentrations at equilibrium.[37] Problems may arise with nondomestic species because of sparse sampling due to physiological or behavioral limitations to sample collection, not just the specifics of the calculations utilized.[4,88] The specific formulas utilized for pharmacokinetic parameter calculations are summarized in Table 1.

There are specific parameters that describe the ADME of a drug in the body that can be considered key parameters for a PK study. Bioavailability (F or %F) is the fraction of a drug administered that reaches the systemic circulation following a non-parenteral (non-intravenous or i.v.) administration[38,87] Bioavailability can be calculated by determining the area under the curve (AUC) of multiple samples of a single dose of a drug determined over time.[16,38,86,87,89] The

volume of distribution (V or Vd) is a measure of the apparent volume or space in the body which contains the drug[38,87,90,91] Mathematically this proportionality constant has various definitions depending upon the specific proportion being described.[38,91] There are three different Vd typically reported in pharmacological studies: V_c, the volume of the central compartment, V_{area}, the volume of distribution calculated by the area method, and Vd_{ss}, the volume of distribution at steady state.[91] The V_c can be thought of as the volume of maximum plasma concentration or the volume of the drug immediately after administration located within the central compartment of an organism prior to any drug elimination or distribution.[91] The V_c can also be viewed as the apparent volume from which drug elimination occurs because the kidney and liver, both components of the central compartment, are also to of the main organs in an organism for clearance of a drug.[91] The V_{area} is the volume of distribution of a drug when pseudo-equilibrium, or the point where the net exchange (balance) between the plasma (central compartment) and the tissues (peripheral compartments) is zero. so as a result any decrease in plasma drug concentration only occurs because of irreversible drug elimination.[91] The volume of distribution at steady-state (Vd_{ss}) can only be determined following i.v. administration[38,87,89] The Vd_{ss} is defined as a proportionality constant between the total amount of compound present in the body and the concentration of the compound, at equilibrium, that is being measured in a given fluid (plasma or serum)[16,37,38,87,91] The Vd can be considered a direct measure of the extent of distribution of a drug from plasma to tissues, although it is a mathematical concept, and is not necessarily a real, physiological space within the body.[38,87] The Vd determines the specific drug dose administered for an individual species.

Serum or plasma half-life provides a measure of the time it takes for a drug to decrease to half of its peak concentration in plasma or serum after reaching pseudo-equilibrium.[38,87,90] Half-

life can be defined as a terminal half-life of a drug or the time required for plasma concentration of a drug to decrease 50% after reaching pseudo-equilibrium.[88,91] This is different from elimination half-life is the time it takes for the concentration of a drug within the plasma or a within a body to decrease by 50%.[88,91] The elimination half-life determines the dosing interval for a drug for a specific species.[90,92] The terminal half-life (t1/2) of a drug following i.v. administration is determined by the drugs distribution and elimination.[87,90] The method of drug administration may lead to slower absorption of the drug, which leads to differences in terminal half-life.[87] In a process known as flip-flop pharmacokinetics, drug absorption is a limiting factor to serum or plasma drug levels, and as a result, the terminal half-life reflects the rate and extent of absorption, not the elimination process[37,38,90] When absorption is not a limiting factor, half-life can be considered a hybrid parameter controlled by the plasma clearance and extent of distribution of the drug in the body[90] Physiologic features, such as differences in liver or renal function may result in much longer or short dosing intervals for a given species compared to a different one for the same drug due to these differences in liver or renal function resulting in differences in drug metabolism and elimination.[24,89]

Clearance (Cl) provides a measure of the ability of a tissue or of the body as a whole to eliminate a drug[87,89] Clearance is determined by the amount of drug that has been irreversibly removed from the body[87,89]. Plasma clearance is a very important PK parameter because it, along with therapeutic plasma concentration and bioavailability, are the three determinants of a dosage rate, or the dose per dosing interval.[89] Clearance can be considered the sum of each individual organ's clearance of a drug.[87,89] A true estimate of the total body or systemic clearance (CLs) of a compound can only be obtained from its concentration versus time profile after i.v. drug administration[87,89] This is because the systemic clearance of a compound is

defined as its rate of elimination from the body normalized to the concentration of the drug in a specific region of the body or bodily fluid such as serum or plasma.[87] Determination of the plasma or serum concentration versus time following i.v. administration of a compound is critical to accurately determine all the PK parameters described above.[87] That said, clearance can be estimated following non-parenteral routes of administration as long as the drug has 100% bioavailability[38,87] The drug must be completely absorbed and not eliminated prior to reaching the systemic circulation following non i.v. dosing to determine this value[87,89] Unfortunately i.v. drug evaluation for a PK study may not be possible with some zoological species, either due to difficulty of venous access due to size, anatomy, or other factors, concerns over stress to the study participant, danger to humans attempting to administer a drug i.v., human reluctance to give a drug iv, or other factors. This means that PK studies performed in zoological medicine that do not include an i.v. component will be missing a key component of the full description of the pharmacokinetics of that drug.

1.4 Pharmacodynamics

Pharmacodynamics (PD) describes the relationship between the compound concentration at the site of action and the effect produced by the compound, the intensity of therapeutic and adverse effects.[87,92-95] The action of a drug with its target initiates a sequence of events which results in the pharmacological response described.[87,95,96] Pharmacodynamics aim to quantify the drug effects by linking the drug effect and drug concentration at the site of action.[87] Drugs almost always exert an effect in the body due to interactions with proteins within the body.[37] Four specific classes of proteins are targeted by pharmaceuticals: enzymes, carrier proteins, ion channel proteins, and membrane receptors (Figure 3).[37] The effects of these protein interactions

may be determined at the sub-molecular, molecular, cellular, tissue/organ and whole body level.[37,95] *In vitro* evaluations to determine PD elucidate drug effects in isolated cells or tissues outside of the studied species body.[95] *In vivo* studies evaluate drug effects in cells or tissues previously exposed to a drug in the living animal.[95] *Ex vivo* studies measure the response and effect after administration of the drug to a live animal[95] Drugs may act on many target molecules in multiple tissues leading to a primary response which, in turn, may induce secondary responses, that may either enhance or diminish the primary response.[95] Because of this, pharmacodynamic studies are often initially done *in vitro* at cellular or tissue levels, so that the primary drug effects can be better understood without interference from the complexities involved in whole animal studies and to prevent negative outcomes to study subjects until better understanding of the drug function is achieved.[95]

At the most fundamental level, the actions of the majority of drugs are produced by mechanisms which are similar or possibly identical to mechanisms by which naturally occurring chemicals interact with these proteins and by which neurotransmitters and hormones (local and circulating) produce their biological effects within a body.[95] Enzymes are often inhibited by drugs, such as the inhibition of cyclooxygenase with nonsteroidal anti-inflammatory drugs (NSAIDS) which in turn limits the inflammatory cascade[37,94] Carrier proteins or membrane transport proteins and ion channels typically are involved with transport of ions or biological products.[37] Drug interaction with these proteins results in increased or decreased movement facilitated by these proteins, which results in an increased or decreased biological effect.[37] Drugs can bind to a membrane receptor as a ligand that can then act as an agonist, producing a conformational change in the receptor that activate physiological pathways ultimately leading to a biological response characteristic of the receptor[37,95] Conversely an antagonist also binds to a

target receptor in the body, but a conformational change and subsequent biological responses do not occur[95] The magnitude of response produced by a drug that is an agonist is often directly proportional to the number of receptors occupied by this drug (Figure 4).[37,95] More occupied receptors lead to a stronger drug response. It is possible for an agonist to be a partial-agonist, in that it does not elicit as strong of a response as a full agonist, despite binding to the same number of receptors. It is also possible for an agonist to be an inverse agonist, where it binds to a receptor, but elicits an equal, but opposite, or inverse, response of an agonist. Full agonists and antagonists, or drugs eliciting the maximal response or lack of response, represent the extremes of physiological action that can occur.[37,95] There is a spectrum of activity between these extremes in the body depending upon the specifics of the drug and the receptor response that occurs.[95] This variation results in different drug responses from different species, with drugs that may elicit a strong response in one species having relatively little effect for the same dose administered in a different species. Drugs which elicit an intermediate response, between that of full agonists and antagonists, are described as partial agonists.[37,95] Conversely, in some instances, there is background biological activity that occurs in the absence of stimulation by an agonist, as the receptors exist in equilibrium with active and inactive forms.[95] If the drug binds preferentially with the inactive receptor form, there will be a decrease in biological activity, or an inverse agonist response because the drug binds to a receptor, but biological activity is not induced.[37,95]

Affinity, efficacy and potency are key terms utilized in describing the drug/receptor interactions in the body for all drugs in all species. Affinity describes the property or degree of attraction between a drug and the receptor that drug acts upon.[95] The specific fraction of a receptor with drug attached to it at a given point in time is the result of the relative rates of

attachment and detachment a drug has for that receptor.[95] The term efficacy is used to characterize and quantify the ability of a receptor agonist to induce a response after binding to that specific receptor.[3,95] Potency describes and quantifies the amount of drug required to elicit a specific level of response.[3,95]

1.5 Modeling

The data obtained during a PK study can be statistically analyzed and integrated with PB data to optimize dosing regimens for veterinary patients.[92,97] This mathematical integration and statistical analysis of measured outcomes is known as modeling. PK modelling is performed using either noncompartmental or a compartmental methods.[86] Noncompartmental models attempt to estimate the exposure of an organism to a drug by estimating the AUC of a drug concentration vs time graph.[98] Total drug exposure is then estimated by AUC methods, specifically the trapezoidal rule (or numeric integration)[99] Compartmental methods estimate the concentration-time graph using kinetic models as they apply to specific physiological areas or equilibrium compartments within an organism.[86] The equilibrium compartments identified in a compartmental model are defined as representing nonspecific body regions where the rate of drug disappearance are of a similar order of magnitude[86]. The compartments of compartmental analysis are not specific organs or organ regions, instead they are theoretical entities that allow the formulation of mathematical models to describe a drugs behavior over time with respect of the drugs movement through compartments.[86] Noncompartmental methods have become more popular in veterinary medicine over the last few years as they appear to have more versatility in that they do not make assumptions about drug action in associated compartments, and appear produce accurate results in different species.[86] The advantage of compartmental modeling over

noncompartmental modeling is that with compartmental modeling a drug concentration can be predicted at any time so long as the proper compartmental model is utilized.[86]

The final outcome of the transformations that a drug undergoes in an organism and the rules that determine this fate depend on a number of interrelated factors. The one-compartment model is the simplest compartmental description of drug distribution and elimination in an organism.[86,97] The one compartment model assumes that drug distribution is uniform throughout the body and that the drug is eliminated from the body at a constant concentration-dependent rate.[86,97] Another way to state this is, a one compartment model assumes that the drug can freely enter or leave the body and the entire body acts like a single, uniform compartment.[97] When drug concentration vs time is plotted on a semi log scale, a one compartment model will result in a simple linear graph.[92,100] The volume of drug at time zero is the Vd for this drug in a one-compartment model. [92,100,101] The rate of elimination is k, the elimination rate constant.[92,100,101] The CL of a drug in a one compartment model can be calculated from the equation $k \times Vd$.[92,100,101] While the one compartment model is appropriate for i.v. administration of some classes of drugs, like aminoglycosides, generally most drugs administered will follow a nonlinear or 2 compartment or multiple compartment model.[97] A two compartment model assumes that a drug is administered and is distributed homogenously throughout the first compartment, then diffuses freely to a second, different compartment, where it is eliminated from the body.[97,102] Compartments can be added to the 2 compartment model to create a multicompartment model to better describe drug actions in the body.[97] The individual compartments in multicompartment drug modeling have been described both mechanistically and physiologically.[102] Mechanistic modeling does not equate individualized compartments to specific tissues or organs, instead each compartment is homogenous and may be a single tissue

or may be a combination of tissues or organs.[92,102] Physiologically-based pharmacokinetic (PBPK) modeling provides a more physiologically relevant description of the drug concentration over time as the compartments in this type of modeling represent actual tissues and organ spaces within a body.[37,92,103-105] PBPK in essence describe the organism studied as a set of tissue compartments interconnected by blood flow.[103] PBPK modeling can be very accurate for depicting drug doses, predict drug pharmacokinetics under different physiological and pathological conditions, or in different age groups, for drug discovery and development, and in drug clinical trials if the equations used to calculate drug activity in each tissue compartment are accurate for the individuals signalment.[103] PBPK modeling has demonstrated promise in human medicine for determining drug doses, although differences in individuals age, sex, or other physiological parameters may complicate accurate drug dose determination.[103] The short coming of PBPK modeling in zoological medicine is individual species differences and the massive breath of organisms that encompass zoological medicine with a lack of development of specific mathematical formulas to use to build the PBPK model. This variation means that PBPK model that works well for a human of a certain age and sex may not be applicable to a bird or any other zoological species, with a given drug, due to differences in organ perfusion, organ function, and other physiological factors due to the specificity of this type of modeling [37,92,103-105]

1.6 Drug Dose Scaling

Linear drug dose extrapolation, metabolic scaling and allometric scaling have all been utilized as methods of determining drug doses for zoological species.[30,106,107] Leaner extrapolation is the use of a single mg/kg dose established for one species and applied across all other species, irrelevant of the nuances of that species.[30,106,107] A combination of

pharmacokinetic and efficacy studies may be performed for that individual species, typically through an average or representative weight range for that given species. [30,106,107] This system employs simple mathematical calculations and standardization of a single dose for a specific species, but it also has shortcomings. [30,106,107] There is a risk of over dose or underdosage of a drug with this method in the target species if that species shows an extreme range in weights, such as the domestic dog, a well-studied species with ranges of normal dogs from 1 to over 100 kilograms.[30,106,107] Considering the problems that arise from this method with a studied species, the extrapolation to other species, without regard for species specific pharmacological differences, physiological and body weight differences, can provide for doses that are very low in smaller animals and extremely high in large animals, therefore it may not provide a safe or accurate dose depending on animal size. [30,106,107]

Metabolic scaling uses the ratio of a known physiological process or feature such as metabolic rate or body surface area of two different species to estimate a drug dose in a species for which there is limited pharmacokinetic data. [30,106,107] This method links the dose to specific physiological function instead of just an animals body weight. [30,106,107] This association with basal metabolic rate provides for more size appropriate doses than linear scaling. [30,106,107] Seasonal or cyclical changes in some species basal metabolic rates may substantially alter an animals dose requirements resulting in this method overdosing or under dosing depending upon the time of the year. [30,106,107] Metabolic scaling proves to be even more problematic for poikilothermic animals such as reptiles and fish as the calculation of basal metabolic rate can vary widely depending upon environmental habitat parameters.

Allometric scaling in various capacities has been suggested as a means of determining drug doses for zoological species. [30,106,107] Allometric scaling involves measuring a

pharmacokinetic parameter in multiple species and then to plot that data against weight to derive a new allometric equation that can be used to estimate the pharmacokinetic parameter of desire in an unknown species.[30,106,107] This method has been widely used in human clinical pharmacology, with the utilization three to five different species of laboratory animal (typically a rodent- rat or mouse, beagle dog, and nonhuman primate) for initial drug evaluation, then extrapolation to first doses utilized for Phase 1 human drug trials. [30,106,107] Allometric scaling makes the assumption that physiologic drug effect differences are negligible between species and that drug pharmacokinetics will always have a nonlinear (allometric) relationship to the animal of interests weight. [30,106,107] There are risks of mathematical error resulting in the wrong calculated dose for some species using allometric scaling.[30,106-108] Allometric scaling relies upon the assumption that the mathematical equation that describes the log-log relationship between CL and bodyweight is always best described by an equation with a single slope and y intercept.[4,30] This mathematical error and concern over an accurate dose calculation occurs because even if a relationship is linear, a change in the slope of the line can occur by altering the weight range of a given species[30,106,107] Allometric scaling functions under the assumption that the variability of the pharmacokinetic parameters selected are identical across all animal species. [30,106-108] A second source of error with allometric scaling is the selection of the species included in data analysis prior to dose extrapolation. [30,106,107] Body mass appears to play a role with this in zoological collections the largest animals are herbivores, some of which have fundamentally different digestive systems, renal systems and ultimately drug metabolism compared to smaller carnivores in addition to substantial differences in weight.[30,106,107] Ideally species selected as reference species for allometric scaling should share all the major physiologic functions, be closely related and differ only in body size[30,106,107] Animals that are poikilothermic, such as fish

and reptiles, may be exceptionally poor candidates for allometric scaling due to changes, ie increases or decreases, in metabolic rates associated with changes in temperature that will affect drug ADME. Finally specific drug parameters may make allometric scaling better for some drugs than others [30,106,107]   Drugs that have blood-flow dependent clearance with limited hepatic metabolism, such as aminoglycoside antimicrobials, have been described as the best candidates to consider allometric scaling. [30,106,107] Conversely drugs undergoing extensive hepatic metabolism creating active metabolites, such as enrofloxacin or those undergoing limited renal excretion are considered drugs that are not good candidates for allometric scaling. [30,106,107] Overall allometric scaling may provide some useful information, but it should not be considered as the only method of drug dose determination and its shortcomings must be considered with all doses extrapolated.[4]

1.7 Unique Considerations for Zoological Drug Studies

Zoo animals present unique medical challenges for pharmacologic investigations not encountered with domesticated animals, as many species hide disease conditions at all costs, while others may exhibit extreme stress responses when handling that may result in patient injury or death unrelated to drug therapy or effects.[109] Species differences in observable clinical effects of drug therapy combined with species specific variation in drug ADME create a widespread lack of information for nondomestic species.[37] Clinicians currently may utilize drugs approved for both humans and animals under the Animal Medicinal Drug Use Clarification Act (AMDUCA), and extrapolate their use and indications from domestic animals and humans to non-approved species.[110] Unfortunately this is often done with little scientific basis to support treatment decisions made.[4]

Unfortunately, the science of the specific mechanisms and actions of drugs in zoological species has lagged as the scope and breadth of zoo and wildlife medicine has undergone a proverbial knowledge explosion the last two decades.[109] Some of the most widely distributed and most widely used references for drug dosing for zoo animals contain information that has little scientific evidence, instead containing references of drug doses extrapolated from domestic species, derived from reports of use in conference proceedings undergoing minimal or no peer review, anecdotal accounts, or single case reports.[13,111,112] This lag in scientific drug studies is perplexing considering that pharmacology applied to zoological species can trace its roots back to ancient times with archeological evidence of an Indian military hospital dedicated to the care of horses and elephants.[98] Currently, there are fewer than 50 pharmaceutical agents approved for domestic sheep and less than 12 for domestic goats, with none approved for new world camelids.[4] The approved drugs for nondomestic, zoological species number even less, with zoological veterinarians relying on extrapolated doses from approved agents for cattle, sheep, or goats.[4] There is currently only one drug approved in the US for amphibians, four agents approved for use in invertebrates, and less than ten compounds for mammalian zoo and wildlife species. This contrasts dramatically with the nearly 300 drugs approved for domestic cattle.[113] Despite this obvious need for research in pharmaceuticals for administration to zoo animals, many of the studies published are simply single dose pharmacokinetic studies, lacking multiple dose or long term administration, or the pharmacodynamic components of a thorough pharmacological evaluation of a drug for a given species.[109] Zoological species represent an extremely diverse grouping of animals, with unique issues including the inability to obtain relevant samples due to human safety, human concerns for animal welfare, and physiological factors such as sample collection sites and safe sample size. There are some zoological species

where thorough drug studies are needed to treat pandemic diseases such as chytridiomycosis caused by the chytrid fungus (*Batrachochytrium dendrobatidis*) in wildlife and to protect the captive populations.[112]

Prior to engaging in any research project involving zoological species that there are specific nuances to research in facilities housing these animals that may not be encountered with animal studies performed with domestic animals at universities or research facilities. This includes external regulation of species that may be studied and potentially the specifics of study design. Internationally, the Convention on International Trade in Endangered Species (CITES), which came into effect in 1975 as a major international treaty between 183 countries, regulating the international trade in specimens of wild animals, including scientific specimen collection, that were considered a threat to the survival of specific species.[114,115] Other important international treaties which may regulate pharmacologic research in zoological species include the Migratory Bird Act (MBA) of the US and Canada and the Endangered Species Act (ESA) of the US.[114] The fundamental purpose of the ESA was, and still remains, the promotion of measures to protect species threatened by immediate or foreseeable extinction.[114] The ESA is administered by the United States Fish and Wildlife Service (US-FWS) and the National Oceanic and Atmospheric Administration (NOAA).[114] NOAA is primarily responsible for marine species. Atlantic sturgeon, sea turtles, and other species in marine and fresh water environments are jointly managed by the US-FWS and the NOAA and other federal agencies with specific responsibilities of enforcing various state and federal statutes, regulations, and international treaties.[114] Permits for research projects, or for the importation or exportation of samples from studies may be required from each of these entities. This process can be lengthy, requiring submission of complex requests, with repeated revision before permits are granted. The

requirements of these agencies, the time associated with permits from them, and any documentation must be accounted for in the planning of studies because failure to do so can result in seizure and destruction of incorrectly permitted samples or even criminal charges.

Zoo's house animals for a combination of conservation, research, and/or education, and it is possible that there may be limitations on projects involving particular animals at individual zoos due to their involvement with other ongoing projects.[116-118] Research and education are encouraged in accredited institutions as part of the general guidelines for accreditation, however, there can often be a distinct slant with research projects at individual institutions to favor wildlife populations or specific topics within captive animal management, such as reproduction or institution exclusive studies that may exclude external projects.[116-118] As with a university, all zoological facilities in the United States are licensed by the US Department of Agriculture-Animal and Plant Health Inspection Service (USDA-APHIS).[114,119] This licensure grants oversite and review of compliance for the display of animals to the public to ensure that all licensed institutions follow the guidelines set forth by the Animal Welfare Act (AWA) and associated legislature, as they depict specifics of general care and in some cases individual species management.[114,119] These multiple layers of management and oversite in an ideal situation create a form of dual review by the university Institutional Animal Care and Use Committee (IACUC) along with concurrent review by the specific zoological institution's IACUC for collaborative projects. If multiple zoological facilities are involved, this means review by the university and each individual facility with participating animals. This dual review can at times be conflicting and difficult to reach agreement to allow research studies because of the differing backgrounds and goals of each individual committee involved in project approval. The zoological institution is represented by individuals who are experts with the

animals they work with and experts on the specifics of zoological and wildlife management, while the university IACUC may contain individuals with little experience or knowledge of those species, but an in-depth knowledge of fundamental principles of research. While each has its merits, the goals and intent of the participants may not always match.

In addition to essentially what is a dual IACUC review of research studies, there may be further review of research plans by various governmental regulatory bodies such as US-FWS that provide international importation permits for research conducted on specific species.[114,115] There can also be permits required for sample collection or interstate movement of zoological samples within the US.[115] This can result in multiple layers of required permits that may ultimately result in longer approval times for research projects. These possible delays must be planned for and accounted for as applications for permits from these entities are reviewed, edited, and ultimately granted. There may also be specific timelines or limitations to samples collected set forth by these agencies irrespective of IACUC and funding entities. Before samples can be collected, investigators must be certain that all necessary permits for the actual sample collection process have been requested and approved or have a thorough plan of action for required permits to eliminate delay.[115] Unfortunately regulations can change rapidly.[114,115] It is possible for multiple year drug studies to start with one set of regulations, and due to policy changes, finish with substantially different regulations governing specimens collected. This is something rarely encountered with pharmaceutical studies for domestic animals. The research detailed within this dissertation faced all of these concerns, but they were overcome cooperative work between the agencies, universities and due diligence by the researchers involved.

Permission granted for participation in a pharmaceutical study by a privately-owned animal can differ from permission granted for zoological animals, especially in the form of

required documentation and/or follow-up and feedback on the study. This difference stems from the concept of ownership, with a privately-owned animal having a single entity that can be designated as the individual of responsibility to grant permission. These individuals may not exist within a zoological institution, because of the hierarchical arrangement of the zoo administration and the decision of participation being made via committee, not a single individual. Not only that, but because some zoological institutions are publicly owned, as a city or county zoo, determination of "ownership" for permission may not be possible. Despite these differences these multiple steps and layers also create an environment that provides a greater deal of oversight and protection for the animals of zoological institutions and should be looked upon as such. Individuals engaging in projects should be aware of the additional time needed for review by multiple entities and as a result, must be able to plan appropriately for the possibility of delays in a project as approval and permits are reviewed.

The work schedules of staff associated with zoological institutions must also be considered when designing a PK/PD study due to the multiple samples over time that may be required to complete the study. Because zoological species are fundamentally wild animals, they may not readily take medications or allow sample collection, especially repeated sample collections. Even one or two samples may require heavy sedation, the use of specialized restraint devices, or general anesthesia, all of which may complicate pharmacological studies due to alterations in normal activity, physiological responses, and potential drug effects changing normal physiology.[4] In addition to that, these animals can be dangerous, capable of inflicting bodily harm or killing an investigator when threatened, as during blood sample collection where they are being poked with a needle.[4,21,109] The concern of the zoo to prevent injury to their animals and humans collecting samples for research projects can be decreased by identifying

captive wildlife at facilities with active training programs. This operant conditioning and training can facilitate administration of drugs to zoo and wildlife, blood collection, or other diagnostic procedures.[109] Such training has become part of general animal care procedures at many zoos and has become mandatory training for some species as training for injections, oral drug administration, and diagnostic sample collection minimizes human risk and provides better quality veterinary care.[109,120] A remarkable variety of species have been taught to tolerate subcutaneous and intramuscular injections, swallow tablets with or without food, and accept various other forms of drug administration.[109] It must be understood that this training requires a significant time commitment both for capturing/teaching the behaviors as well as for ongoing practice with select trained staff to maintain them by using placebos when the animal is healthy.[109] This also means that a pharmacological study utilizing drug administration and blood collection to determine PK or PD effects of a medication will have to incorporate the staff specifically associated with an individual animal's training in order to collect samples.

All of the blood samples collected within the experiments in this dissertation were collected under the concept of "voluntary participation", in which at any time for any collection point, an elephant could refuse to participate in sample collection. Captive elephants in the US are generally conditioned for blood collection as part of routine training and USDA, SSP/TAG, and AZA guidelines for elephant management. In the studies in this dissertation, elephant staff were instructed to allow a 15 to 30 minute break for the elephant if a sample collection attempt was refused by the elephant or signs of stress, including refusal to line up, ear flapping, vasoconstriction, changes in trunk or tail position or other indicators caretakers had identified as specific to that participant, were noted by caretakers that may indicate a desire to not participate in that sample collection and attempt collection again. There was also a strict "three stick rule"

in that blood collection for a given time could only be attempted 3 times. If it was not successful after the third try, no sample would be collected at that time. This resulted in successful blood collection for almost every time point in all studies. There was one elephant that was slow in taking the firocoxib paste formulation, ingesting approximately 95%, then taking an approximately 6 minute break before returning to and consuming all of the drug and food it was offered with. The sample collections for this elephant did not diverge from the others.

An investigator must consider that these staff work a full time job for the zoological institution aside from a potential project , and understand that there may have to be modifications in drug administration or sample collection times, modifications in total samples collected, or modifications in the specifics of the volume or type of sample collected dependent upon the level of animal training and the keeper, supervisory staff, and veterinary staff of the cooperating institution. There can also be variation in specifics from institution to institution. This mandatory involvement of additional required personnel to complete a project may create a need for extensive preplanning prior to beginning a pharmacological study to accommodate staff scheduling and participating animal routines. It is advisable for an investigator to approach staff with an open mind and actively engage in conversation with all involved individuals if possible as their understanding and insight on the nuances of the study animal's behavior and responses may greatly facilitate sample collection. In this communication with the zoological facility prior to beginning a project, it is logical to distinguish a "point person" who facilitates direct contact with the zoo staff and administration. Many times for pharmacological studies this person will be the zoo veterinarian (or veterinarians) and/or veterinary technical staff. Communication prior to, during, and after sample collection is imperative for success.

Figure 1

ADME: the relationship between drug ingestion, absorption, distribution, metabolism, and elimination. Multiple processes, ranging from factors like method of administration to protein binding of the drug to specific hepatic enzymes for drug metabolism to renal physiology for excretion and many others play important roles in the steps from drug intake to drug elimination.

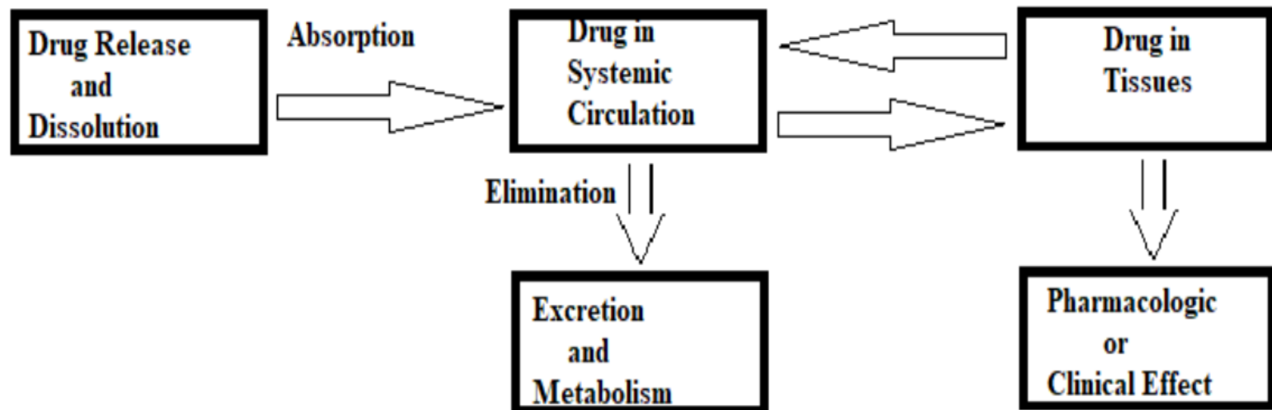


Figure 2

Graphic representation of ADME after administration of a drug in any method other than intravenously. The drug is initially absorbed until a peak plasma concentration is reached. Plasma drug levels begin to decrease as the drug is distributed to the body, undergoes metabolism and ultimately is eliminated. Note that there is often overlap of drug metabolism and elimination, with both possibly occurring simultaneously, depending upon the drug in question. The Area Under the Curve represents total drug exposure over time. If the drug in question undergoes linear pharmacokinetics, with a constant elimination rate constant (K), the AUC is proportional to the total amount of drug absorbed by the body.

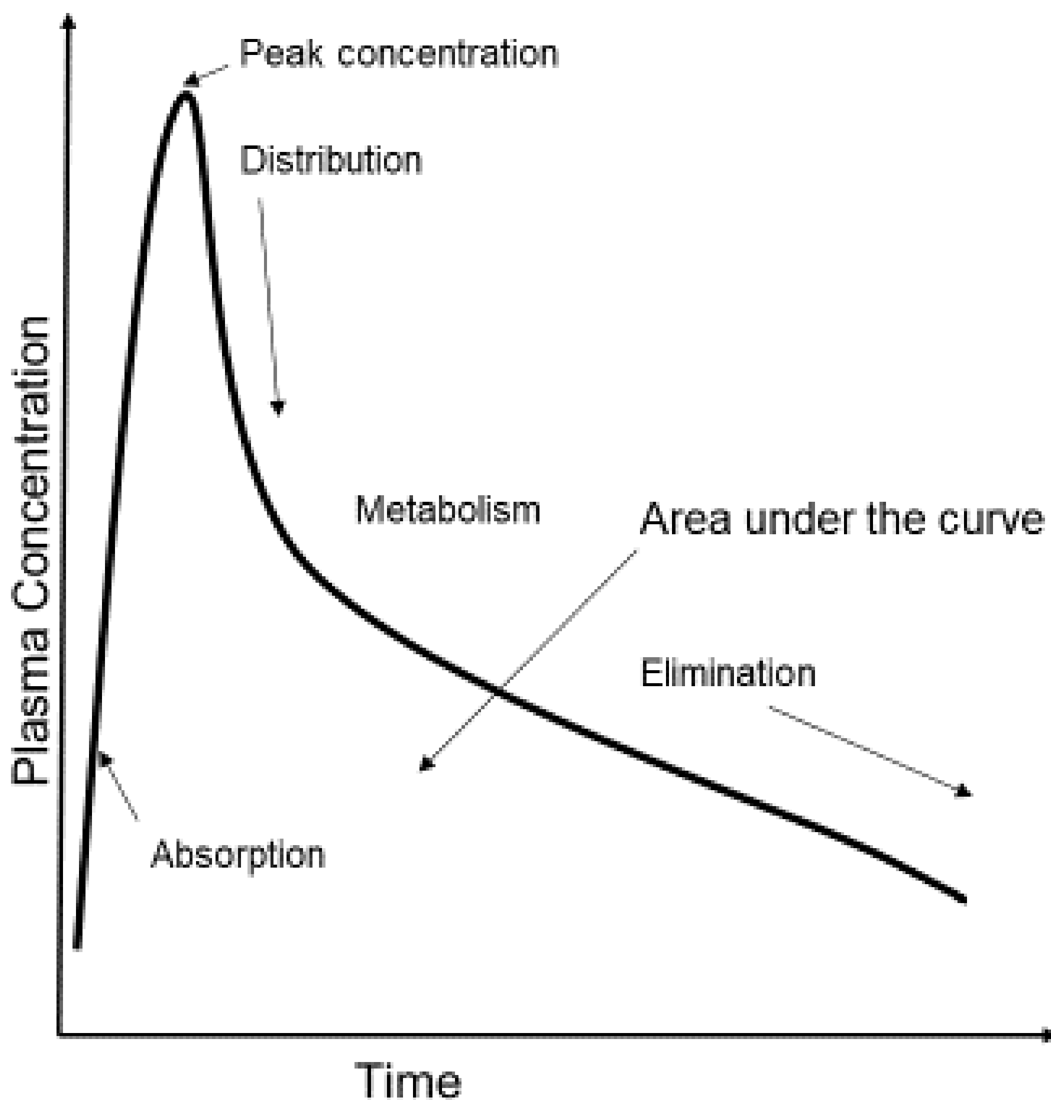


Figure 3

The mechanism of drug action in the body is mediated by biological interaction including activation or deactivation of a protein associated with a specific cell. Different proteins may be targeted by different drugs and as a result initiate different physiological processes.

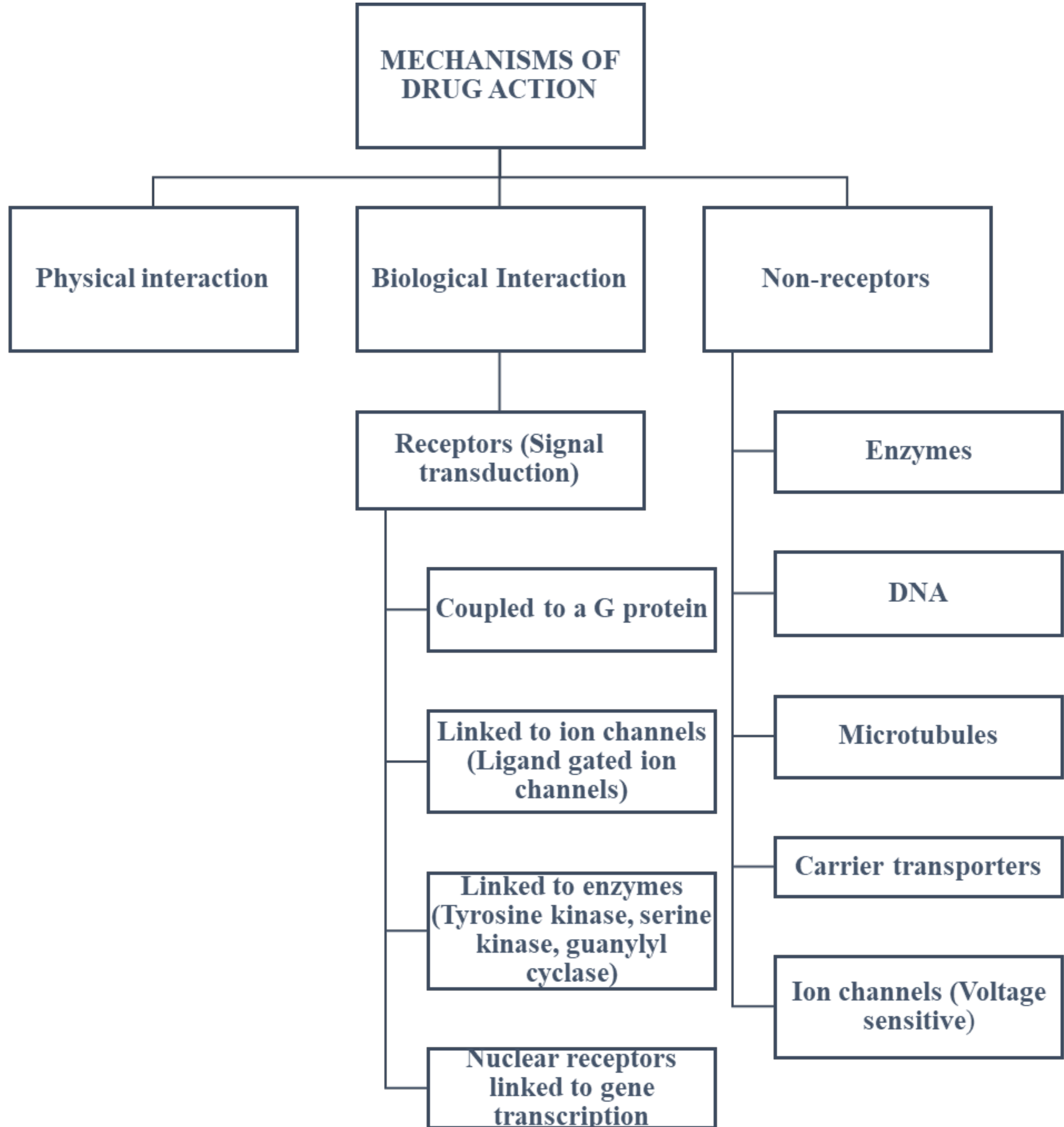


Figure 4

Representation of the spectrum of drug effects ranging from full agonist (maximum positive effect) to full inverse agonisim (maximum negative effect). This extreme negative effect can only be seen if constitutive receptor activity exists which can be inhibited by an inverse agonist. A drug may be an agonist or an antagonist. A full agonist produces complete receptor activation leading to a maximal response; a partial agonist produces a submaximal response and possible blockage of agonist activation. An antagonist does not produce a physiological response but is able to block the response of an endogenous ligand or exogenous agonist.

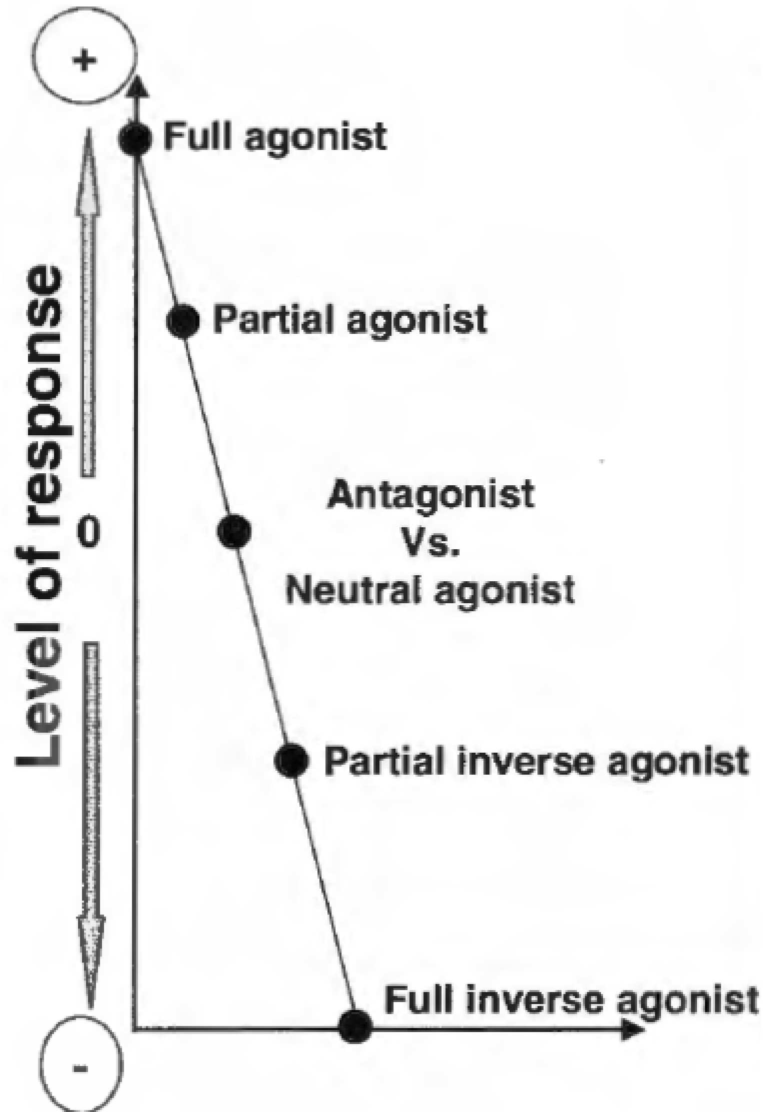


Table 1

Abbreviation	**Term**	**Definition**	**Formula, if applicable**
AUC	Area under the curve	The area under a curve generated by the plotting of data collection points of drug plasma concentration vs time. This represents the total drug exposure to the body over time.	A/a + B/b…
CL	Clearance		CL = Vd* Kel or Dose/AUC
C_{max}	Cmax	The maximum plasma concentration that a drug reaches in the body after a drug has been administered but before the next dose.	measured value
C_0	Dose at time 0	The plasma drug concentration immediately after i.v. administration of a drug	
D	Dose	The amount of drug to administered at a given time	D = Vd * Co or Target Co*Vd/F
T	Dosing interval	The time between drug dose administrations	Determined by target concentrations and clearance of a given drug
F	Bioavailability	Amount of drug that reaches systemic circulation after administration. This is the drug available to exert a biological effect	Fabs = (AUC_{noniv}/AUC_{iv}) * $(Dose_{iv}/Dose_{non\text{-}iv})$
F_{abs}	Absolute bioavailability	The bioavailability of a drug when administered via a non i.v. route compared to the bioavailability of the same drug administered i.v.	Fabs = (AUC_{noniv}/AUC_{iv}) * $(Dose_{iv}/Dose_{non\text{-}iv})$
F_{rel}	Relative bioavailability	A comparison of the bioavailability between two different dosage forms administered via different routes.	Frel = (AUC doseA/AUC dose B) x Doseb/DoseA)

Table 1 (continued)

K_{el}	Elimination rate Constant	Fraction of a volume of drug eliminated per unit time)	K_{el} = Ln (C1/C2]/t2-t1
MRT_{iv}	Mean residence time after iv administration	A representation of the average time a molecule stays bound to a target or it remains active in the body after i.v. administration	MRTiv = AUMCiv/AUC or Vd_{ss}/Cl
MRT	Mean residence time	A representation of the average time a molecule stays bound to a target or the time it remains active in the body	MRT = AUMC/AUC or 0.693CL/ Vd(ss)
R_{ac}	Accumulation Ratio	Ratio of accumulation of a drug going from a single dose to steady state with repeated administration	1/1-e-kel*t
t1/2	Elimination Half Life or half life	Time it takes for the plasma concentration of a drug to decrease by half	t1/2 = 0.693/K el
T_{max}		The time at which C_{max} is observed	Measured value
Vd	Volume of Distribution	The volume in which a drug is distributed in the body	Vd = total amount of drug in the body/plasma drug concentration or D/C_0
Vd_{ss}	Volume of distribution steady state	The volume in which a drug is distributed in the body when the drug reaches plasma steady state.	Vd_{ss} = Cl/MRT

Chapter 2

Pain, Inflammation, and Pain Management in Zoo Animals

2.1 Mechanisms of Pain

Pain is defined as an "unpleasant sensory and emotional experience associated with actual or potential tissue damage".[121] While this definition was originally developed for humans, the definition also applies to animals.[122] The sensation of pain is a complex physical, neurological, and social phenomenon that arises from the interaction of multiple physiological processes and is likely experienced differently from species to species depending upon their intrinsic cognitive and neuroanatomical structures.[123-125] While it has been argued that only humans feel pain, because they are the only species that can "verbally report the sensation of pain", it cannot be ignored that animals also communicate obvious signs of discomfort based upon movement, body position, tail and ear position, facial expression, and other nonverbal cues.[124,126] This concept of only humans feeling pain is a philosophical argument more than reality considering the nonverbal communication that animals have in contrast to human's dependence on spoken words.[124,127,128] Even in human patients, an inability to communicate verbally does not negate the possibility that an individual is experiencing pain and is in need of appropriate pain management. The lack of verbal communication of pain should not be an excuse for not providing effective and adequate analgesia to all patients.[121,126,129,130]

It is a universally held belief that animal pain and suffering are recognized as clinically important conditions that can negatively affect an animal's quality of life and it is a fundamental obligation of veterinarians to manage and attempt to control the perception of pain in all of our

patients.[124,127,128,131] This means that specific details such as drugs administered, techniques, or husbandry methods tailored to an individual animals species, breed, age, procedure performed, degree of tissue trauma, individual behavioral characteristics, and patient health status have become a routine part of veterinary care[129,131] This specific tailoring of analgesics to the nuances of an individual patient combined with adequate pain assessment based upon the species being treated should ultimately result in superior analgesia vs simply giving an arbitrary dose of a drug in a "one size" fits all modality.[129] his becomes especially important with zoological species, because the nuances of drug metabolism have not been investigated in many species.[4]

Because of the pain as a "human only" debate, the term nociception has gained favor in zoological species veterinary literature[4] Nociception, in contrast to pain, is defined as the response to external or internal noxious stimuli, and is a more precise term than just pain for animals because of human's deficiencies in overcoming verbal requirements and communicating fully with multiple species[123,126,131-135] The nonverbal communication of animals when exposed to unpleasant stimuli cannot be denied, therefore the term nociception will be used in this dissertation to refer to the specific anatomical structures and physiological processes associated with noxious stimuli and the term pain will be utilized as a general descriptive term for the sensations the patient is experiencing when exposed to such noxious stimuli. Pain scales and facial pain scores have been developed to provide an objective assessment of the perception of pain based upon body language and behaviors for domestic animals, but outside of exotic companion mammals, there are many species in which such objective aids for pain assessment are needed.[127,132,133,136]

The fundamental anatomical structures and physiological processes that allow for the sensation of pain is ubiquitous in vertebrates, but the specific details and a thorough understand

of specific mechanisms still has only been elucidated and thoroughly described in humans.[123,134,137,138] Nociception is the process by which information of tissue damage or the potential for tissue damage as the result of noxious stimuli is assessed and processed by the central nervous system and the brain.[123,130,135,137,139] Nociception, is an inclusive term, in that as the as perception of a noxious stimulus, nociception includes the processes of nerve signal transduction, conduction, transmission, and perception of the noxious stimulus.[139,140] This has been described as both a conscious and a nonconscious process.[124,127,139] Sufficient mechanical, noxious chemicals, or warming/heat stimuli will activate the nociceptor receptors.[123,130,135,137,140] Nociceptive receptor stimulation triggers action potentials within afferent unmyelinated C-neurofibers or weakly myelinated Aδ-fibers with cell bodies located in the dorsal root ganglia of the spinal cord (Figure 5).[123,130,135,137,139-141] Excitatory glutamate receptors and inhibitory GABAergic and glycinergic receptors are considered the primary dorsal root ganglia receptors of the spinal cord.[130,135,137,140] The central pain signaling pathways of the vertebral cord conduct signals to the brain where the perception and associated thought process responses to the stimuli occur.[123,130,135,137] The initial response an animal has to a noxious stimuli may act to modulate that stimuli in a positive or negative manner.[130,135,141,142] This results in a variability that can be unpredictable in that a stimuli which may elicit an extreme response in one patient, may not have as severe of a response in another due to individual variation in nociceptive nerve patterns, the central processing of afferent input, summation of inputs and inhibitory interactions within the nervous system.[141,143] This is ultimately why some species, and individuals within a specific species, may appear to have a higher "pain tolerance" than others. The descending neural pathways are generally considered inhibitory and are often specific targets for modulation by endogenous opioids and exogenous opioids, cholecystokinin, neurotensin, acetylcholine,

endogenous and exogenous cannabinoids, α-adrenergic agonists, and serotonin, thus altering signals from the brain to the peripheral tissues.[123,130,135,137]

The sensation of pain can be described based upon the chronicity of the painful stimuli as either acute or chronic.[122,141] Acute pain follows a specific bodily injury, may be severe in nature, and may result in rapid and extreme physiological and psychological response from affected individuals.[122,137,141] In contrast, chronic pain, or long term periods of exposure to painful stimuli can induce a systematic stress response associated with elevations in endogenous corticosteroids, antidiuretic hormone (ADH), catecholamines, aldosterone renin, angiotensin II, and glucose, along with decreases in insulin and testosterone.[122] These whole-body changes as a result of chronic pain can result in a generalized catabolic state with muscle protein catabolism and lipolysis, in addition to retention of water and sodium and excretion of potassium, and other significant changes in essential physiological processes.[122] Prolonged painful sensations can decrease the rate of healing, in addition to having adverse effects on multiple organ systems, including the cardiovascular system, renal function and fluid retention, and gastrointestinal tract functions such as nutrient and fluid absorption[122,130,144]

The specific neuropathways involved with pain sensation provide a means of classifying pain based upon neurobiologic status. Nociceptive pain can be considered an early warning, physiological protective mechanism detecting and minimizing contact with noxious stimuli.[139] This protective role depends upon and results in an immediate action/reaction to the stimuli, including reflexive actions such as the withdrawal reflex.[139] The second neurobiological classification of pain is both adaptive and protective, in that this type of pain, known as inflammatory pain, occurring after injury results in the body decreasing physical activity and contact to the affected area.[139] This type of pain is associated with the hallmark features of

inflammation, including redness, swelling, and loss of function associated with activation and production of an organisms intrinsic inflammatory mediators.[139] Local effects at the site of injury can lead to upregulation of inflammatory mediators affecting blood flow and neuropeptides altering sensation to that area.[123,135,137] Because of this the control of inflammation may be a key component of providing analgesia to patients. The alleviation of pain is also important to limit secondary responses in patients which can have both negative physiological and psychological outcomes.

The final neurobiologic classification can be considered maladaptive, in that it often results secondary to abnormal function of the nervous system.[139] This type of dysfunctional pain can occur as a result from damage to the nervous system (ie neuropathic pain) or in conditions when no damage or inflammatory condition is identifiable.[139] Allodynia is a specific type of neuropathic pain where stimuli normally not perceived as painful under normal conditions evokes a pain response due to abnormal nerve function resulting in neuropathic pain.[132] This can lead to negative stereotypical behaviors such as pacing, swaying or licking commonly seen in zoological species. This type of pain is perhaps the most difficult to treat because the exact nature of the neurological dysfunction can be challenging to localize and pinpoint.[132]

Pain has also been described as somatic, visceral, neuropathic, or mixed based upon the specific body systems and anatomic locations affected.[130,132,145] The neurobiological classifications of pain are often limited to humans because of deficits in complete anatomic and neurobiological descriptions of zoological species. anatomical classifications of pain provide good descriptive evaluations of pain sensations applicable across multiple species, allowing for intraspecies variation in anatomy and physiology. Somatic pain involves painful stimuli affecting the peripheral structures of the body, including skin, synovium, connective tissues,

muscles, and periosteum.[132,145] Somatic pain can be associated with conditions such as muscle strains/injuries, joint injuries, osteoarthritis, fractures, and bone metastasis.[133,145] Visceral pain involves the internal organs or the viscera, including structures of the abdomen, pelvis and thorax.[132,145] This type of pain is most often associated with pain associated with gastrointestinal disease such as colic, organ metastasis, or inflammatory intestinal disease.[132,145] Visceral nerve sensors are not activated by cutting or heat stimuli, as somatic nociceptors are.[132] Neuropathic pain occurs due to lesions of or dysfunction in the central and peripheral nervous system.[145] This type of pain may result from injury, inflammation, or chronic diseases causing dysfunction of the nerves, spinal cord, or brain.[130,132,145]

2.2 Pharmacological Management of Pain ....

Veterinary medicine has advanced significantly over the last 25 years in terms of attempts at managing animal pain. In a study conducted in the 1990's involving domestic dogs and cats hospitalized for invasive surgical procedures, ranging from limb amputations, limb-sparing bone cancer resection, thoracotomy, cervical vertebral instability repair, to humeral fracture, repair only 1% of the cats and 40% of the dogs received any form of pain management, pharmaceutical or otherwise, while hospitalized.[146] In current times, there is a concern for polypharmacy in zoological medicine because of the lack of through understanding of the unique physiology that affects the pharmacokinetics and pharmacodynamics of drugs in zoo species.[25] In addition there are no studies that have evaluated the long term effects of administration of any drugs in zoo species.[25] Unfortunately these knowledge deficits result in the assumption that pharmaceutical compounds evaluated for single dose pharmacokinetics will be safe when administered chronically, and that drugs safely administered chronically to domestic animals will

subsequently be safe when administered in a similar manner to non-domestic species These limitations in species-specific understanding of the basic metabolism and fundamentals of drug pharmacokinetic parameters in non-domestic species limits not only individual drug usage, but also leads to an increased potential for negative effects from polypharmacy in zoological medicine.[25] With more drugs commercially available, zoo veterinarians may be more likely to administer multiple drugs for the treatment of complex clinical conditions, such as long term pain management in a geriatric animal with chronic arthritis.[25]

This lack of information in zoological medicine, combined with the advances in domestic animal pain management, creates a conundrum that must be addressed. The complex nature of the pain pathways in the body dictate that pain management may be best accomplished with a multifaceted approach. There is no single drug that provides analgesia for all types of pain, no matter what classification system that is used to describe that pain. Multimodal analgesia as a form of polypharmacy is the combination of multiple drugs and/or nonpharmaceutical techniques to target different points of the pain pathway.[147] This approach takes into account the additive or synergistic effects of drugs providing analgesia.[96,147] For example, combining the anti-inflammatory effects of an NSAID with the central nervous system effects of an opioid will provide better analgesia to a surgical patient's post-traumatic fracture repair than either class of drugs administered individually. The second advantage of multimodal analgesia is that the synergistic effects of different medications may result in lower overall doses administered of each individual drug, thus reducing the potential for the development of negative/undesirable side effects associated with administration of a given drug.[25,147] Nonpharmaceutical methods of multimodal analgesia, such as physical therapy, acupuncture or other methods can also minimize undesirable effects of drugs by decreasing drug dosages.[139,148]

In 2012, an online multispecies survey investigating analgesics in mega-vertebrates was discussed and links to a survey uploaded on Surveymonkey®.com was posted on the American Association of Zoo Veterinarians (AAZV) list serve (Appendix 1). This provided survey access to all AAZV members throughout the world. The results of this survey were subsequently published in two separate articles in the Journal of Zoo and Wildlife Medicine. This survey was an effort to elucidate and understand the types of drugs administered, methodologies employed for pain management, document successful and unsuccessful anecdotal doses administered, and anecdotal nonpharmacological treatment methods employed, and any observed side-effects of pain management modalities used for captive mega-vertebrates. This survey also served as a starting point for determination of drugs that needed study in mega-vertebates because they were commonly administered, but lacked published pharmacokinetic, pharmacodynamic, and long-term administration studies.

Mega-vertebrate is a collective term used to describe a very large vertebrate. Commonly housed zoo species classified as mega-vertebrate include 3 recognized species of elephant (African bush elephant: *Loxodanta africana*, , African forest elephant: *Loxodonta cyclotis*) and the Asian elephant: (*Elephas maximus*), 3 species of rhinoceros (Southern white rhinoceros: *Cerathotherium simum*, Black rhinoceros: *Diceros bicornis*, and Indian rhinoceros: *Rhinocerus unicornis*), 3 species of giraffe (Northern giraffe: *Giraffa camopardalis*, Reticulated giraffe: *Giraffa reticulata*, and Southern giraffe: *Giraffa giraffa*), and the common hippopotamus (*Hippopotamus amphibious*).[149-152] The specific details and presence of individual subspecies of giraffe and hippopotamus continue to be points of scientific debate. There have been few published pharmacokinetic studies in mega-vertebrates, with published references often only being single dose studies, not comprehensive, single dose, multiple dose, and IV studies (see

Kottwitz et al, and Boothe et al, 2016- Appendix 2 and 3).[153,154] The limitations of published scientific literature, the popular nature of these animals in zoos, and the unstable wild populations make them ideal candidates for pharmacological studies because scientific data attained has benefitted both captive and wild populations.

2.3 Critique of the Mega-vertebrate Analgesia Survey

Surveys and questionnaires are a key component of evaluating trends in human medicine and social sciences.[155] An ideal survey controls for error by ensuring that each member of a population has an equal chance of being included in the sample, that samples members are randomly selected in large enough numbers to ensure an accurate representation of the study population, and that the chosen sample responds to the questions asked.[155] Surveys seem to have lagged with use in evaluating drug trends of domestic animals, likely because many times medications administered to domestic animals are either targeted for that species or there is published data released at the time of or shortly after a drug is commercially available that can guide clinicians in drug and treatment choices. Despite real and perceived deficiencies, surveys and questionnaires are gaining in frequency.[156] Surveys provide a valuable resource for clinicians in describing disease stages and exploring treatment options.[157,158] Surveys also can serve as a beginning point to guide research choice for future studies based upon clinician perception of important drugs or disease conditions requiring further investigation.[156]

A key component to an effective questionnaire is asking the correct questions in a manner that gives responses that fit that questionnaire's research goals.[156,159] The mega-vertebrate analgesia survey had two major shortcomings that have been realized after publication of the data. The first was length of the survey (See Appendix 1 Mega-vertebrate Analgesia Survey

Questions). Communication with fellow zoo veterinarians after the articles were published often had the same feedback (Unpublished data): Despite the success and ease of use with the online survey, it was just too long to be practical. A shorter survey, perhaps one that contained the same questions, but only focused on a single group of animals, such as elephants only or rhino only, would have had a higher response rate. Nonresponse bias from questions not being answered has been proven to have the potential to alter outcomes of survey data.[159,160]

The second shortcoming of this survey that was realized after publication that was that despite stating that the survey investigated institutional use patterns, not data associated with individual animals, readers misunderstood and assumed data presented in the resultant publication was for individual animals. While the survey detailed 59 different institutions, it contained information about every mega-vertebrate housed at that respective institution, including historic populations. This means the actual data of the survey was that for hundreds of animals, not just the current captive population, but extracting specific animal data was not possible because of how the questions were written, unless a participating institution chose to write additional information about that animal. Necropsy results were reported within the survey in some instances with comments about perceived negative effects of drugs administered. Unfortunately, not every institution provided an equal degree of detail. This type of question bias is not uncommon in surveys, and may not become apparent until after the survey is complete.[160] It may have been alleviated with multiple single species group surveys and specific emphasis on providing information about unusual case responses or necropsy findings. Ultimately this survey has provided essential information on current analgesia modalities for these species and information about drugs that require further investigation. This information played a role in designing the study funded by Morris Animal Foundation (grant D15ZO-007)

for the investigation pharmacokinetics and pharmacodynamics of firocoxib and flunixin meglumine in Asian and African elephants.

2.4 Inflammation and Pain

Inflammation in lower invertebrates was described as a localized process, with sponges and jellyfish responding with primitive mesodermal cells (amoebocytes) capable of phagocytosis being mobilized to the specific point of injury.[161] Healing is accomplished via collagen fibril proliferation and ectodermal hyperplasia.[161] While there have been advances in the understanding of higher organisms, these original descriptions remain valid today. Inflammation in vertebrate species is characterized by a systemic process resulting in the 5 cardinal signs of heat, redness, swelling, pain and loss of function.[162] Microcirculation effects, including arteriolar dilation and increased permeability of capillary venules, leading to formation of an inflammatory exudate rich in leukocytes are associated with 3 of these cardinal signs.[161,162] The infiltrate is first composed largely of polymorphonuclear cells, then later mononuclear cells, which transform to macrophages in the interstitial space.[161,162] Inflammation is driven by multiple mediators synthesized or released from cell storage sites in a timed manner in both the early and later phases of acute inflammation and also with chronic inflammation.[161,162] Mediators can also interact with each other and other mediators by addition, antagonism, or synergism.[162]

2.5 Anti-inflammatory Drugs

The use of various compounds by humans to control inflammation dates back centuries. Stone tablets from the Sumerian period confirm the Assyrians used the extract of willow leaves

for musculoskeletal pain and that the Egyptians used a mixture of myrtle and willow leaves for joint pain and for the relief of pain and inflammation associated with wounds.[163] In ancient Greece, Hippocrates first prescribed an extract from willow bark and leaves that contained salicin, a form of the active ingredient in aspirin for the treatment of pain.[163,164] St. Kevin of Glendalough is reported to have used an extract of willow bark to treat painful musculoskeletal conditions in southern Ireland in the 7th century.[163] In 1785, the Reverend Edmund Stone of Chipping Norton described in a letter to the Royal Society of Medicine the antipyretic properties of the willow tree and willow bark.[94,163]

Salicin itself was not actually described in willow bark as the active ingredient until the 17th century.[163,164] The first veterinary use of a synthetic drug, the sodium salt of salicyclic acid, was reported in 1875.[94] Acetylsalacyclic acid, what we know today as aspirin, was introduced as a pain reliever into the German market by Bayer in 1899.[163,165] Bayer also began to aggressively promote aspirin to thousands of human physicians, creating the first known example of mass marketing of a pharmaceutical product.[165] Despite this widespread marketing and subsequent patient use, the German patent office refused to patent aspirin, stating that the industrial process for production was insufficiently novel to warrant a patent.[163] Ibuprofen, the first of the non-aspirin NSAIDs for human use, was identified by the Boots Company in 1951, and by the 1970's it was being widely prescribed for the treatment of painful musculoskeletal conditions.[163]

The discovery of the veterinary NSAID phenylbutazone paralleled that of ibuprofen with discovery in 1949 and marketing to humans beginning in 1952.[166] Veterinarians in the early 1950s began prescribing phenylbutazone to multiple species, particularly horses, for the management of lameness.[166] Despite early controversy as to its effectiveness, phenylbutazone was prescribed heavily to humans and animals in the 1950's and 60s, but ultimately was

removed entirely from the human market by the mid 1980's due to concerns over adverse side effects.[166] Although there have been profound adverse events noted in humans and some domestic species, phenylbutazone persists as a commonly prescribed NSAID to domestic horses for musculoskeletal pain, lameness, and other pain related conditions.[166]

The term "nonsteroidal anti-inflammatory drug" was first coined by Merck and Company's laboratories in 1963, with the intent to distance this type of drug from corticosteroids due to rising evidence of risks of corticosteroid administration in human patients.[166] To understand the drive of a major pharmaceutical company to do this, one must first understand the physiological effects of these drugs. Glucocorticoids are among the most widely used, and unfortunately often misused, class of drugs in veterinary medicine today with excellent anti-inflammatory properties that are in some situations superior to those of NSAIDs.[167] The depth of information on optimal glucocorticoid dosing regimens and intervals in many species is still quite variable due to species differences in drug metabolism and specific drug action.[167] Therapeutic protocols for non-domestic species and zoo animals are often the product of clinical experience, claimed common sense, and information extrapolated from human medicine or from another related species.[167] The results of administration of this class of drugs can be nonspecific, in that they have concurrent profound metabolic effects regardless of the initial disease process.[167] These adverse metabolic affects from corticosteroids can be difficult to separate pharmacologically from the therapeutic benefits, making glucocorticoids a potent therapy, yet potentially dangerous, especially after long term treatment.[167-171]

Pharmacological doses of glucocorticoids for anti-inflammatory purposes can have a substantial negative feedback effect on endogenous glucocorticoid regulation, suppressing both hypothalamic and pituitary hormone secretion (Figure 6).[167-170] Both exogenous corticosteroids

and endogenous cortisol inhibits adrenocorticotropin hormone (ACTH) secretion and corticotropin-releasing hormone (CRH) secretion through negative feedback at both hypothalamic and pituitary levels.[167] This is important when considering recovery of the hypothalamic-pituitary-adrenal axis (HPAA) from exogenous glucocorticoid administration, even if those drugs are administered at therapeutic doses.[167] Glucocorticoid drugs with anti-inflammatory effects, but no effects on the HPAA, have not yet been identified.[167] The extent of this negative feedback is species specific and is dependent on the dose, potency, half-life, and duration of the specific glucocorticoid drug administered.[167] These negative effects in a body from corticosteroid administration are due to drug effects mimicking excessive endogenous cortisol. [138,139,141-143] They can include fluid retention, hyperglycemia, dyslipidemia, arteriosclerosis, muscle wasting, hair loss, osteoporosis, immunosuppression, and behavior changes including apparent euphoria/hyperactivity and/or changes in cognition.[167,168,170-172] The similarities between long term corticosteroid administration and hyperadrenocorticsm (Cushing's syndrome), or endogenous hypercortisolemia, were noted almost immediately after wide spread use began in humans, with similar findings occurring in animals.[167,173-175] Long term administration of supraphysiological doses may also lead to adrenocortical atrophy and decreased adrenal secretory reserve.[167]

The anti-inflammatory effects of corticosteroids occur through deactivation of genes associated with encoding cytokines, chemokines, adhesion molecules, inflammatory enzymes, receptors and protein molecules associated with inflammation.[168,170] Cellular effects are most often associated with protein synthesis.[168] These functions are the result of 4 basic mechanisms of action: 1) the classical genomic mechanism of action caused by the cytosol glucocorticoid receptor (cGR); 2) secondary nongenomic effects also initiated by the cGR; 3) membrane bound

glucocorticoid receptor (mGR) mediated nongenomic effects and 4); nonspecific nongenomic effects caused by interaction with cellular membranes.[167,176] This gene activation accounts for some of the adverse events seen from corticosteroid use, but others, such as changes in psyco-behavioral activities, occur due to mechanisms that have not yet been explained.[171,176] The genes affected by corticosteroids include immunosuppressive genes, but corticosteroids also activate genes associated with adverse metabolic events, including disruption of blood glucose metabolism, fat metabolism, bone healing, and bone growth.[167,170,176-178] This risk of adverse events, despite the obvious positives of corticosteroid administration, justified Merck's actions to try to brand a new and distinct class of drugs distanced from the negative effects of corticosteroids.

Despite this long history of being prescribed to alleviate pain and discomfort, the mechanism of action of nonsteroidal anti-inflammatory drugs, was not described until the 1970's.[179-182] NSAIDs produce both analgesic and negative side effects by inhibiting specific enzymes in the arachidonic acid (AA) pathway, impairing the production of prostaglandins.[183] These effects are considered much more specific than the more global effects of corticosteroids, but nonetheless still may have significant effects.

2.6 Cyclooxygenase

Arachidonic acid (AA) is a 20 carbon unsaturated fatty acid that plays a pivotal role in inflammation as the precursor of eicosanoid inflammatory mediators.[162,165,179-182] AA is an esterified cell membrane phospholipid, released from damaged tissue following the action of lipocortins, endogenous peptides which activate phospholipase A_2.[162,184] AA serves as the base substrate for prostaglandin (PG)H synthase, more commonly known as cyclooxygenase (COX),

and several lipoxygenases (LO), including 5-LO, 12-LO, and 15-LO.[162] Each enzyme is a component of an individual enzymatic cascade where action of enzymes further down the cascade leads to the formation of eicosanoids.[162] These substances are also described as autacoids, as they act locally at the site of synthesis.[162] They have a short half-life, so that continued effect depends stimuli resulting in a maintained synthesis and release.[162,183,185] Early in acute inflammation, several PGs are synthesized from AA by different downstream enzymes.[162,184] These prostanoids are integral for the inflammatory pathway and subsequent pain, as well as perform essential physiologic functions on target tissues.[183-185] NSAIDs exert both analgesic and anti-inflammatory effects by inhibiting PG synthesis through blocking the rate-limiting step in this pathway at the site of cyclooxygenase (COX).[162,183,184,186] COX isoenzymes facilitate the conversion of AA to prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2) via oxidation and reduction reactions upstream from specific bioactive prostaglandins.[162,179,181,184-187]

The COX enzyme has two distinct active sites, one called the cyclooxygenase active site and the other the peroxidase active site.[186] The cyclooxygenase site cyclizes AA and adds a hydroperoxyl group to carbon 15 to form prostaglandin G_2 (PGG_2).[186,188-190] The separate peroxidase site of the same COX enzyme then reduces this hydroperoxy group to the hydroxy group to form PGH_2.[186,188-190] NSAIDs specifically inhibit the cyclooxygenase active site of COX, but have no effect on the peroxidase active site.[186,188-190] Obviously if there is decreased inflammation, there will subsequently be decreased inflammation associated pain and discomfort, so there is an intimate association between these effects and the effects of NSAIDs on COX isoenzymes.

The first two COX isoenzymes COX-1 and COX-2 were described in the 1990's in humans and human models.[187-194] A third isoenzyme, COX-3, which appears to exert its effects almost exclusively in the central nervous system and heart, was not described until 2002.[195] There is little detail of the specific structure or isoenzymes outside of human or human model studies. While it is assumed that these isoenzymes are conserved across species, that has yet to be proven.[196] There are assumptions that have been made that the basic isoenzyme structures and functions of all COX isoenzymes are conserved across species lines. A study in alpaca (*Vicugna pacos*) investigating COX isoenzyme activity suggests that there may be species differences in COX-1 function, with whole blood circulating TxB_2 levels were determined to be very low (See Appendix 4, Kottwitz et al., 2018).[197] These low circulating levels of TxB_2 are indicative of decreased constitutive COX-1 isoenzyme activity. Alpaca and other camelids are species where glandular stomach (C-3) ulceration has been well documented.[198-200] It is possible that decreased circulating TxB_2 levels in healthy adult alpacas may indicate a predisposing factor of decreased COX-1 isoenzyme activity if associated gastric protective prostaglandins produced by COX-1 are also low. Low constitutively produced COX-1 also indicate a higher risk for adverse effects occurring in these species if COX-1 selective inhibitory drugs are administered, even at therapeutic doses. Further investigation into species specific isoenzyme structure, tissue location, and function in alpaca and other camelids are needed to prove any definite differences and risk.[197]

COX-1 is thought to occur as a membrane-bound enzyme present in most cells of the body in humans.[162,191,192,201] COX-1 and COX-2 have essentially identical enzymatic actions for the conversion of AA ultimately to PGH_2 in the species investigated.[162,186,192,201] Depending on the tissue where this metabolism happens and the specific COX isoenzyme present, PGH_2 can be

converted to prostaglandins PGD_2, PGE_2, PGF2a, and PGI_2 (prostacyclin), and to thromboxane (TX) (Figure 7).[186,191,192]

COX-1 is expressed constitutively in many tissues in the body of mammals, and is relatively stable, with only small fluctuations in concentrations occurring in response to stimulation by hormones or other mediators.[162,191,192,201] There is direct involvement by COX-1 in generalized "housekeeping" functions in the body, ranging from blood clotting, regulation of vascular homeostasis, renal blood flow and protection, protection from gastric acid secretions, and coordination of the actions of circulating hormones.[162]

COX-2 on the other hand exists in humans and human models in both an inducible and constitutive isoform.[201,202] Pro-inflammatory cytokines, growth factors, lipopolysaccharide (LPS), and mitogens may all serve as stimulators of COX-2 synthesis.[162,187] There are mixed results in the literature as to the specific degree of involvement of COX-1 and COX-2, however it is now generally accepted that COX-2 is the isoform that facilitates inflammatory PG's at sites of inflammation.[162,203,204]

COX-3 is a variant of COX-1, derived from the same gene, but it retains Intron-1 after transcription and translates into a COX enzyme with additional amino acids in its structure.[205] [195,206] The mechanism of COX-3 is not fully understood in humans or domestic animals.[195,205-207] It appears that some acetaminophen and NSAIDs such as diclofenac and ibuprofen are potent inhibitors of COX-3 expressed in cell culture, but these NSAIDS are highly polar, and as a result are unlikely to cross the blood brain barrier and reach therapeutic concentrations in the brain to affect this enzyme.[195,205,207] It is possible there are additional COX isoenzymes, including a COX-4 which is thought to be a variation of COX-2.[205,208] This particular isoenzyme was identified experimentally in the cytosol where LPS-induced COX-2 was identified as a

membrane-bound enzyme and COX-2 induced with the NSAID diclofenac existed only in the cytoplasm of culture cells.[205] It was also noted in studies of experimentally induced carrageenan pleurisy in the rat, a late-induced COX-2 appeared during the resolution phase of the experimentally induced inflammatory response.[209]

There are commercially available assays utilized for measuring some PG substrate concentration as a means of determining COX isoenzyme activity, however the rapid degradation of some PG their utility as a quantifiable substrate for may experimental protocols. In addition, there have been complications identified with reproducibility and subsequent verification of experimental protocols for quantifying both COX-1 and COX-2 activity.[210] ThromboxaneB_2 and PGE_2 (and metabolites) are two PGs often evaluated in *in vitro* and *ex vivo* COX-isoenzyme experiments, specifically those utilized to measure NSAID inhibition of COX isoenzymes.[201,210-215] Thromboxane A_2 which is initially produced by thromboxane degradation has a half-life of about 30 seconds after it is synthesized from PGH_2.[184] It is non-enzymatically degraded into biologically inactive TXB_2, which is more stable and provides for an experimentally measurable substrate.[184] ThromboxaneA_2 is predominantly derived from platelet COX-1 in the body, but can also be produced by other cell types, including macrophage COX-2 after enzyme induction.[216-218]

Experimental studies have shown that a deficiency of COX-2 produced by knockout gene manipulation reduces the level of PGE_2 production by roughly 75%, while the deficiency of COX-1 reduces the PGE_2 level by 25% initially.[219] In this model, a COX-2 inhibitor (NS-398) was administered after inflammation was induced with carrageenan.[219] The PG produced in wild type mice fell to levels seen in COX-2 knockout mice.[219] Interestingly though, by day 7 following carrageenan treatment, higher numbers of inflammatory cells were detected in mice

deficient in COX-2 and little resolution of inflammation was apparent compared to wild-type or COX-1-knockout mice.[219] PGE_2 plays an important role in inflammation as it is involved in all processes leading to the leading to three of the classic recognized signs of inflammation: redness, swelling and pain.[216,220] It must be understood that while pain is associated with, it is not just a product of inflammation. Instead pain is multifaceted entity dependent upon the specific stimuli, the location of the stimuli, the species involved, and other factors of which limiting PG with NSAIDs via COX inhibition is just one component.[96,130,147,221-223] It is possible to have a stimuli induce pain before activation of inflammatory mediators completing their activity and it is possible to have an inflammatory response with negligible or very minor pain associated with it. In the physiological processes associated with the effects of PGE_2, redness and edema result from increased blood flow into the affected tissue through PGE_2-mediated augmentation of arterial blood flow, vasodilation, and increased microvascular permeability.[216] A mechanism of pain activation that can be controlled by NSAID administration is pain associated with the action of PGE_2 on peripheral sensory neurons and on central sites within the spinal cord and the brain.[216] PGE_2 is one of the most abundant PG's produced in the human body and has been characterized as exhibiting versatile biologic activities, however it also rapidly degrades, necessitating the measurement of metabolites to accurately quantify levels.[184] PGE_2 is considered an inducible isoenzyme, typically induced experimentally with cellular exposure to lipopolysaccharide or some other inflammatory stimulating substrate.[201,210-215] This multiple facet involvement with both pain and inflammation makes PGE_2 valuable when investigating COX-2 isoenzyme activity in all species.

When considering NSAID effects in zoological species, it is important to understand that COX inhibition by these drugs is dependent upon the interactions of COX isoenzymes and the

specific NSAID drug that is administered to that species. Because these drugs focus specifically on the COX pathways, with limited effects on other pathways in the body associated with nociception, other sensitizers such as bradykinin and nerve growth factor, in the case of neurogenic pain can still alter nociception and pain signaling significantly during tissue injury and inflammation.[147] This also means that these drugs may have a "ceiling effect" depending upon the species as COX isoenzymes are inhibited, but other mechanisms of pain are not, which can ultimately result in a perceived lack of effectiveness in pain management of NSAIDs in some species.[147] To date it is thought that COX isoenzymes are conserved across all species, however that has not yet been proven, so it is possible that an identified lack of effect may also be due to COX isoenzyme structural differences preventing the binding of an NSAID administered. The studies published specific to the structure of COX-1 or COX-2 are largely human studies and it is assumed that this isoenzyme is the same for animals too.[224]

Most, conventional non-selective NSAIDs enter the specific NSAID binding sites of both COX-1 and COX-2 isoenzymes, thus acting as reversible competitive inhibitors of both enzymes.[186,225] There is variation in these drugs though, because aspirin acetylates the active cyclooxygenase site, forming a covalent bond, and thus acting as an irreversible inhibitor of both COX-1 and COX-2 isoenzymes.[186,226] COX-2 specific inhibitors such as firocoxib, celecoxib and rofecoxib have a larger structure than conventional NSAIDs.[186] In humans, the COX active site of both isoforms is nearly identical and is created by a long hydrophobic channel of the enzyme with a tyrosine (at position 385) and a serine (at position 530) at the apex of active site.[188,226]

COX-1 is inhibited by NSAIDs by binding of the drug to arginine at position 120.[188,226] COX-2 specific inhibitors have a conformation that preclude them from readily fitting into the NSAID binding site of COX-1, but allows them to readily bind in the so-called "side pocket" of

the NSAID binding site of COX-2.[186,225,227] COX-2 in humans has a similar molecular structure as to COX-1, with similar active sites for the attachment of AA to NSAIDs.[226,227] The COX-2 active site has a slightly larger volume due to a larger central channel, which creates a wider entrance for drug molecules.[226-228] This means that that the specific channel of COX-2 can accommodate larger drugs than COX-1.[226,228] This channel size difference determines some of the COX-2 selectivity of certain NSAIDs.[186,224,225,228,229]

This active site difference is the result of a single amino acid change, where an isoleucine at position 523 in COX-1 is substituted with a valine at 523 in COX-2 resulting in a change in enzyme conformation.[204,224,226,229] That valine is smaller by a single methyl group, creating a gap in the wall of the channel, which provides access to the side pocket that is the binding site for COX-2 selective inhibitors.[224,226,228,229] The larger isoleucine at position 523 in COX-1 blocks access to the side pocket, and thus prevents COX-2 specific inhibitors from converting AA.[226,228] This point has been described in humans but has not been identified in any zoo species. As the changes in binding site of appear to be the result of a single amino acid difference, this ultimately means that as species lines are crossed, it is possible that other mutations in COX isoenzymes may result in changes in activity of specific NSAIDs, or prevent any activity at all. Until specific differences in COX isoenzyme activity are identified and subsequent sequencing of the COX-isoenzymes determines mutations, it is generally accepted that these enzymes are conserved across species lines, however that has not yet been proven.

Kinetic studies have established two distinct mechanisms in humans by which COX-2-specific NSAIDs inhibit COX-1 and COX-2. Specific COX-2 inhibitors exhibit a time-dependent reversible inhibition of COX-2.[186,225,230] When this happens, the COX-2 specific inhibitor appears to alter the active cyclooxygenase site following binding.[186,225,230] At very high doses,

however, these same compounds act as time-independent reversible inhibitors of COX-1, with the degree of inhibition of COX-1 dependent on arachidonic acid concentration, drug concentration, and affinity for the active site.[186,225,230]

Aspirin is a unique NSAID in that it inhibits COX isoenzymes by acetylation of the hydroxyl group of the serine at position 530, preventing AA to have access to the tyrosine at position 385, and thus preventing AA from being metabolized to PG via steric hindrance.[231] This covalent bond results in an irreversible inhibition of COX-1 isoenzymes which is very different from the reversible inhibitory function of other NSAIDs. Cyclooxygenase-2 retains serine at its active site, which is now in position 516 due to the change in structure with the side pocket formation.[228] Aspirin acetylates the serine at position 516 in COX-2 as drug concentration increases, but due to the larger size of the catalytic channel, AA is able to bypass the acetylated structure and be metabolized to PG.[232] Because of these differences in binding, aspirin can be considered to have irreversible inhibition of COX-1 even at low doses, and partial, limited, and rapidly reversable inhibition of COX-2 at high drug concentrations.[233-235]

2.7 Adverse Effects of Nonsteroidal Anti-inflammatory Drug Administration

NSAIDs have a more focused mode of action than the broader reaching anti-inflammatory effects of corticosteroids, due to the specific inhibition of the cyclooxygenase isoenzymes. However, there have been important negative effects documented from NSAID administration thought to be directly associated with cyclooxygenase inhibition that must be considered for all species. Gastrointestinal side effects are cited as the most common adverse event in humans receiving NSAIDs.[186,201,226] Prostaglandins have a specific role in protecting against gastric ulceration by reducing gastric acid production, stimulating gastric fluid secretion,

increasing mucous production of the stomach lining, secretion of bicarbonate in the duodenum to neutralize the acidic pH of the stomach, and increasing gastric vasodilation in monogastric omnivores like humans.[236] The anatomical distinction between digestive systems must be considered when prescribing NSAIDS to zoological species because of the variation in physiological functions of the stomach in ruminants, pseudoruminants, foligivores, insectivores, or other others. It also must be noted that experimental studies have failed to show an increased risk of gastric ulceration post NSAID administration in some zoological species. This is despite glandular stomach ulceration being a commonly identified sequellae in some species and evidence of low circulating COX-1 levels, suggesting increased risk of gastric ulceration secondary to COX-1 inhibiting drug administration.[197,199,200,237] Because COX isozenyzmes are tissue specific, and circulating levels only give a representation of tissue concentrations, further investigation with species specific studies are needed to fully understand this apparent discrepancy.

The association of potential negative effects from alterations in PG levels due to COX inhibition and the disruption of normal physiological functions is an important concern in wildlife and zoo species. One example is the massive die off, and now threat of extinction, of three species of Gyps vultures as a result of environmental exposure to the COX-2 inhibitor diclofenac induced renal failure.[26,27] Ketoprofen administration has been experimentally shown to demonstrate toxic effects at low doses in captive Gyps vultures, paralleling the toxicity seen in wild vultures.[238] In contrast, other studies shave determined that diclofenac in pied crows (*Corvus albus)* and turkey vultures (*Cathartes aura*), and the COX-2 inhibitor meloxicam in American kestrels (*Falco sparveriu*s) did not result in the same acute, fulminant renal failure and death, as diclofenac in Gyps vultures, but is toxic in Steppe Eagles (*Aquila nipalensis*), thus

indicating species specific adverse events do occur after exposure to NSAIDS.[27,239-241] Carprofen, flunixin meglumine, and phenylbutazone have also been found to have the potential for toxicity in cape vultures (*Gyps coprotheres*).[242] While the die-off of Gyps vultures to the brink of extinction due to exposure to NSAIDs has driven concern and recent experimental studies involving NSAID toxicity in avian species as validated those concerns with some species, such studies and published information are severely lacking in other zoological species.

In humans, prostaglandins are involved in 3 key aspects of normal renal function: 1) control of renin release, 2) regulation of vascular tone and 3) control of tubular function.[186,243] These functions are tied specifically to the renal tubules and macula densa, with COX-1 being found in the cells of the medullary collecting ducts and COX-2 being identified in the macula densa of the juxtaglomerular apparatus and the adjacent cells of the cortical ascending limb of the loop of Henle in rats, rabbits, and dogs.[243] Mice that lack the gene for COX-1 production do not show significant signs of kidney disease, yet those that lack a COX-2 gene have kidneys that fail to develop, resulting in early death.[244] However, the full function of COX-2 is not fully understood in human renal physiology, much less any zoological species.[226] It is thought that renal injury can specifically occur in humans and animals as a result of alterations in renal perfusion that then leads to ischemia and ultimately kidney failure.[186,245]

It has been determined that the brain contains high concentrations of prostaglandins.[246] Cyclooxygenase-1 is distributed throughout the brain, but has the highest concentration in the forebrain.[247] High levels of COX-2 are found in the brain also, especially in the hippocampus, neocortex, and amygdala.[247] Cyclooxygenase-2 expression increases in the spinal cord of domestic dogs post injury.[248] There are few reports of neurological signs associated with NSAID administration in non-domestic animals, other than the ferret (*Mustella puturo furos*), where a

large percentage of those ferrets exposed to the NSAID ibuprofen.[249] Neurological signs including disorientation, collapse, and seizwere the most commonly reported clinical sign of acute toxicity.[249]

Prostaglandins are integral in reproduction, and the capacity for fetal tissues to produce PG's is increased during pregnancy and parturition.[250 251] Constitutive COX-1 in the amnion may be important for maintenance of a healthy pregnancy.[250 251] Prostaglandins synthesized from COX-2 are thought to be important for initiating uterine contractions during parturition, with COX-2 synthesis increasing substantially immediately before the start of and during parturition.[250 251] The concentrations of PG's in blood and amniotic fluid also increases during labor in humans.[250 251] It has also been determined experimentally that mice lacking COX-2 are infertile due to decreased ovulation.[252] The implications pharmacological manipulation of PG in regard to endangered species conservation are enormous in zoological institutions. While there is no research to date investigating infertility associated with NSAID administration in any zoological species, the possibility should be considered by veterinarians prescribing COX inhibiting drugs to reproductively active animals.

The other tissue specific effects of COX isoenzymes identified in humans, including effects on the CNS, liver function, white blood cell function, endothelial cell function, vascular smooth muscle, and other cardiovascular system effects, should also not be ignored, but do not appear to have the same potential to cause clinical adverse effects in zoological species as do the gastric, renal, or reproductive effects of these drugs.[186,226] Further study is needed in all zoo species, and the potential for adverse effects should never be ignored or denied with NSAIDs in any species.

The differences in COX-1 vs COX-2 isoenzyme activities exhibited by different NSAIDs, specific selectivity of COX-2 versus COX-1, can be expressed as the $^{COX\text{-}1}/_{COX\text{-}2}$ inhibitory ratio.[95,201] This ratio is derived from an in vivo or in vitro study in which the inhibitory effect, usually expressed as the inhibitory concentration to inhibit 50% of activity (IC_{50}), is measured from stimulating cells that are capable of expressing products of these enzymes.[94,95] This percentage of inhibition has also been expressed in veterinary literature as a different percentage, typically 80% or 95% range of inhibition.[94] In the whole blood assay, the source for COX-1 products (thromboxane A_2 or its metabolite thromboxane B_2) is platelets, and the source of COX-2 products (e.g. PGE_2) is leukocytes.[186] The ratio is therefore expressed as $^{COX\text{-}1\ [IC50]}/_{COX\text{-}2\ [IC50]}$, or simply $^{COX\text{-}1}/_{COX\text{-}2}$. The higher the value is above 1.0, the more specific the drug is for COX-1 compared with COX-2. The lower the value is below 1, the more specific a drug is for COX-2 compared to COX-1.[94] A value of 1 means equal selectivity with a drug capable of binding and inhibiting both isoenzymes. There is subjective value placed on the magnitude of the ratio to consider the drug as ‘‘sparing”, “specific”, or “preferential” for each respective isoenzyme. These terms are based on the inhibitory concentration ratios as measured in a given species at a labeled drug dose. A COX-2 specific drug will inhibit COX-2, but not COX-1 at label drug doses in a specific species. A COX-2 preferential drug will inhibit COX-2 at a lower drug concentration than COX-1, but there will also be some inhibition at the labeled drug dose for a species. COX-1 sparing is the same as COX-2 selective, in that at the labeled drug dose, it will inhibit COX-2 but not COX-1 in a given species. A COX-2 specific drug is the same as a COX-2 selective drug. A COX-nonspecific drug will inhibit both COX-1 and COX-2 at labeled doses. It is important to understand that while these ratios are reported, the

validation, confirmation, and interpretation of the data may be problematic because the published literature may report differing ratios, even for the same drug in the same species.[94,162]

Figure 5

Single painful stimuli evoke two successive and qualitatively distinct sensations referred to as “first” and “second” pain sensation. Under normal conditions, innocuous sensations of low intensity, such as touch or vibration, are transmitted from the periphery to laminae III and IV of the dorsal horn via Aβ fibers. The signal is then relayed to the brain through the dorsal column somatosensory pathway. Noxious thermal or mechanical input (transduction), the protective nociceptive ‘‘first pain’’ experience, will activate the Aδ fibers, which have small receptive fields. These fibers function as a warning system and are protective to the animal. With increased intensity of the stimulus, C-fibers also conduct impulses along with Aδ fibers. C-fibers have a larger receptive field compared to Aδ fibers. They are responsible for the ‘‘second pain’’ experience. The Aδ and C-fibers enter the dorsal horn of the spinal cord, wherein Aδ fibers solely and C-fibers predominantly terminate in laminae I and II. The signal is then relayed to the brain by way of the spinothalamic tract. Image and description originally published in Mathews, 2008.[130]

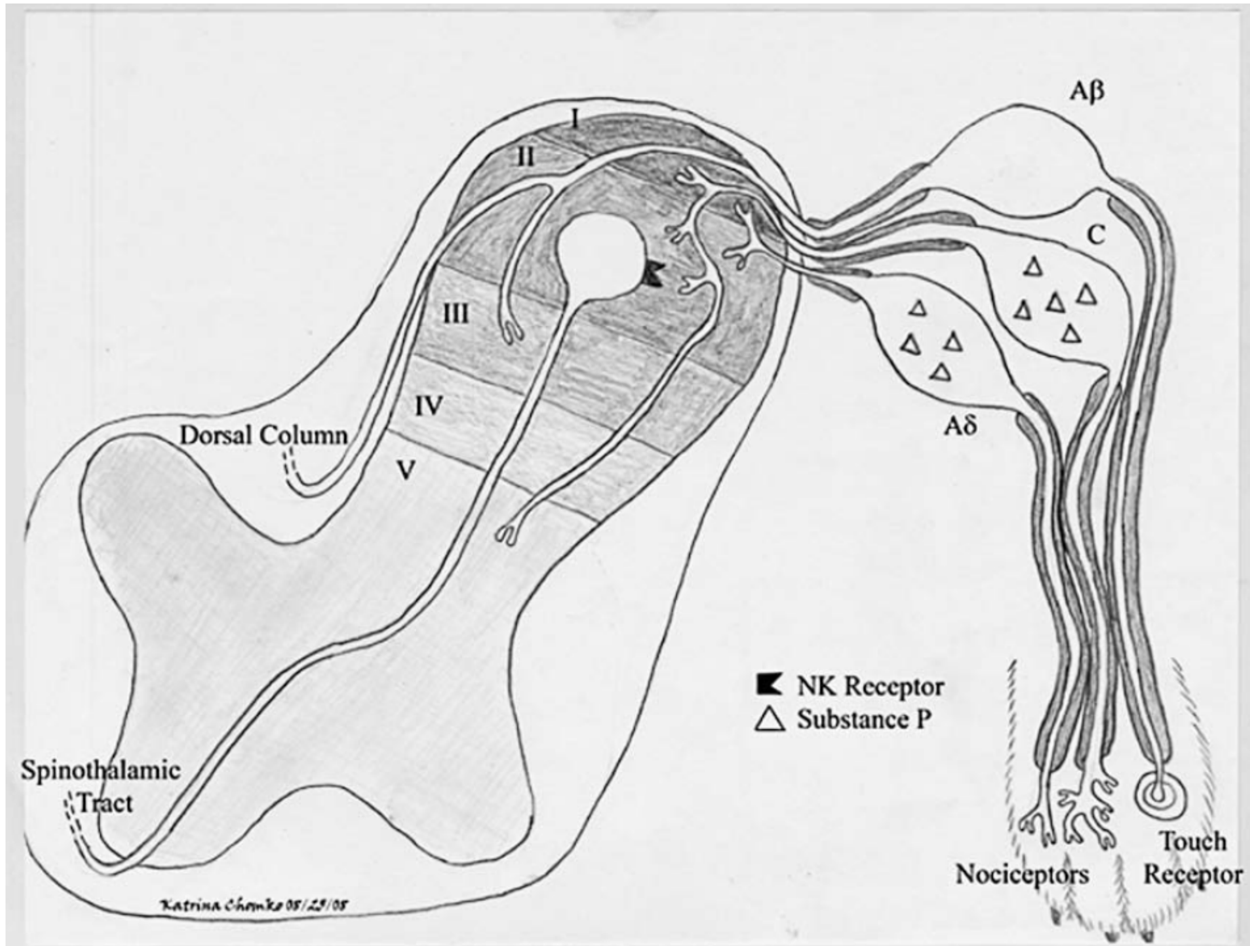


Figure 6

Exogenous glucocorticoids act in a similar way to endogenous cortisol, inhibiting both the hypothalamus to secrete corticotrophin releasing hormone (CRH) and the pituitary gland to secrete adrenocorticotrophic hormone (ACTH) thus decreasing endogenous production of cortisol.

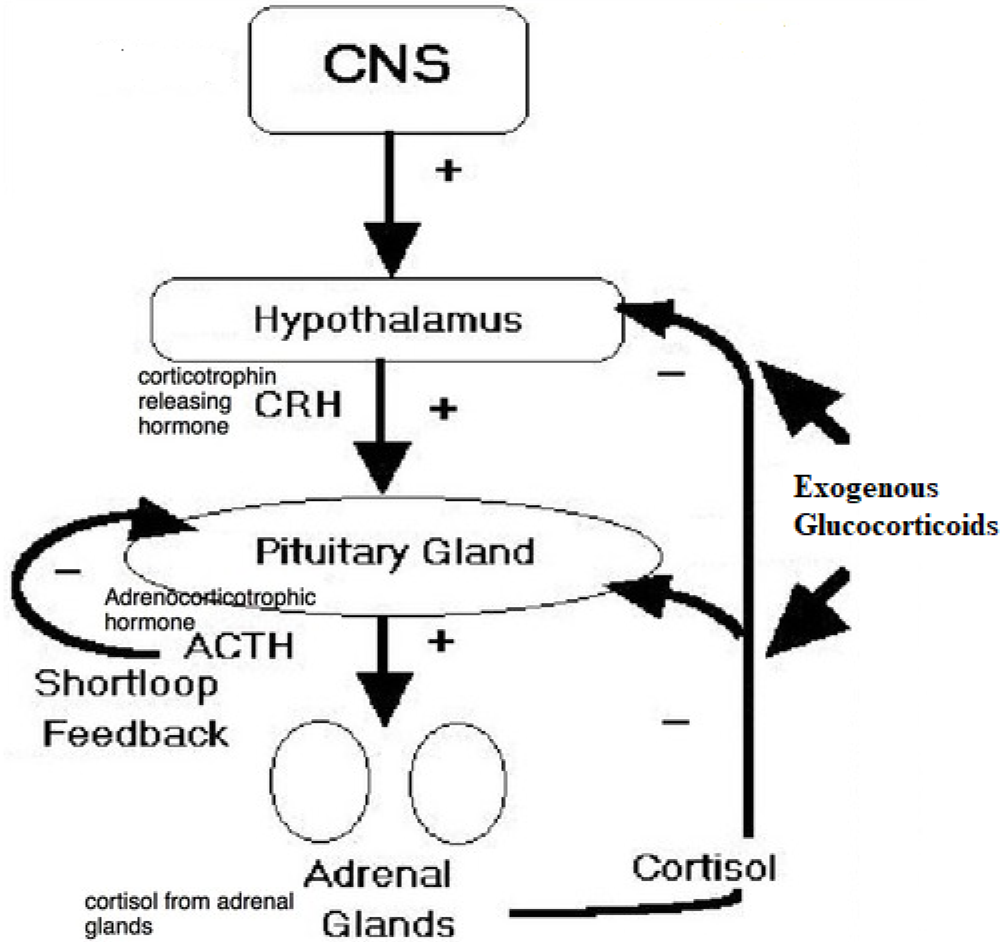
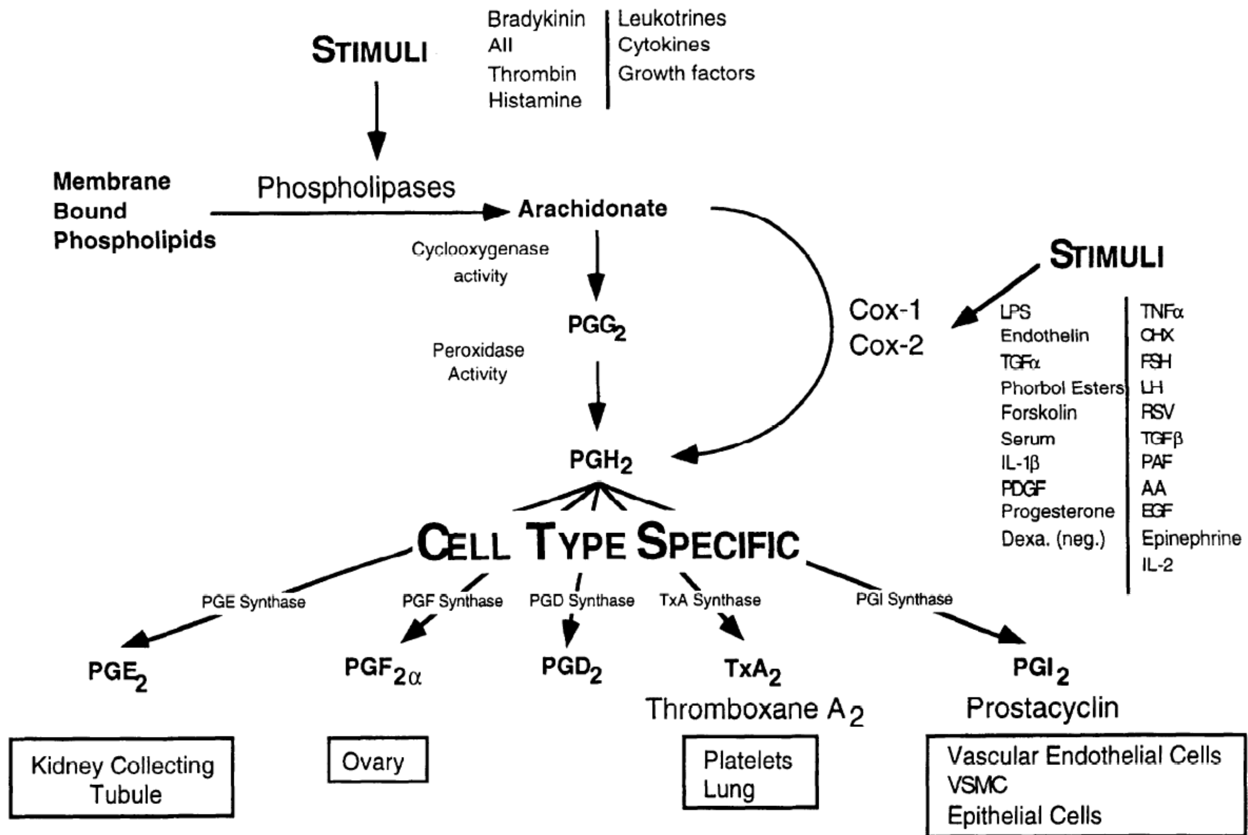


Figure 7

Arachidonic acid metabolism via cyclooxygenase pathway as described in humans. The regulation of arachidonic acid metabolism to PGH2 and specific, cellular specific types of PG is dependent upon both COX-1 and COX-2. Determination of the biological role of each COX isoform and the specific organs affected by that enzyme is still ongoing. Determination of the specific role of each cyclooxygenase isoenzyme in different organisms is ongoing and may vary between species. Figure originally published in Williams, 1996.[224]



Chapter 3

Assessment of *in vitro* and *ex vivo* Cyclo-oxygenase Inhibition in Asian Elephants

3.1 Flunixin Meglumine and Firocoxib Drug Descriptions

Based upon the data collected in the Mega-vertebrate Analgesia Survey, the drug flunixin meglumine was the 2nd most common administered NSAID to captive elephants, while firocoxib was the 5th most common.[153] While pharmacokinetic studies have been performed for other NSAIDs, there are no published pharmacokinetic or pharmacodynamic studies for elephants with either flunixin meglumine or firocoxib.[25,79,113,253,254] The frequency of administration of these NSAID drugs indicates a need for both pharmacokinetic and pharmacodynamic studies, especially considering the differences in effects and specific functions noted in other species.

Flunixin meglumine has an ambiguous designation in the veterinary literature as it is both described as a "non-selective" NSAID, exhibiting equal effects on both COX-1 and COX-2 isoenzymes in domestic animals, or as a COX-1 selective drug, exhibiting the largest effect on COX-1.[213,255,256] Conversely, firocoxib is characterized as a COX-2 selective drug because it tends to inhibit COX-2 while exerting a sparing effect on COX-1.[181,220,221] Cox-2 selectivity, in theory, minimizes the negative physiological effects associated with either COX-1 exclusive or predominately COX-1 inhibiting drugs.[213] That is to not say that a COX-2 predominate NSAID is without the potential for negative side effects. These drugs may still have some inhibitory effect on COX-1 despite being described as COX-2 selective.[181,220,221] The degree of inhibition of COX-1 and/or COX-2 can vary substantially between species.[257] This means while these drugs may have a specific function and selectivity in domestic animals, it is quite possible for

there to be a very different selectivity for COX isoenzymes when administered to non-domestics, especially zoo animals that may have unique species specific drug metabolism. This can also result in substantial fatalities in wildlife due to these differences in species specific COX isoenzyme inhibition, for example, the increased COX-2 selectivity of diclofenac, and subsequent post ingestion inhibition of renal blood flow leading to ischemic renal necrosis and ultimately renal failure that has decimated populations of some Asian vulture species.[258-260]

3.2.1. *In Vitro* and *Ex Vivo* Evaluation of Cyclo-oxygenase Isoenzyme Inhibition by Aspirin, Flunixin Meglumine and Firocoxib in Asian Elephants. To be submitted to ..... Pharmacological Research ....

3.2.2 Abstract

Background/Purpose: to evaluate the inhibition *in vitro* and *ex vivo* of firocoxib, flunixin meglumine and aspirin in Asian elephants (*Elaphas maximus*).

Animals: 6 healthy adult Asia elephants for *in vitro* evaluations. 6 different healthy adult elephants for *ex vivo* determinations following 8 consecutive doses of firocoxib. 3 healthy adult Asian elephants for *ex vivo* determinations following 3 consecutive doses of flunixin meglumine. 5 Asian elephants receiving firocoxib therapeutically for a minimum of 3 consecutive months.

Methods: Blood was collected from 6 elephants and subjected to *in vitro* analysis of COX-1 or COX-2 isoenzyme inhibition by firocoxib, flunixin meglumine, and aspirin. Blood was collected at the beginning prior to drug administration and after 8 consecutive doses (0.1 mg/kg by month every 24 hours) of firocoxib. Blood was collected from 3 elephants following administration of 3 consecutive doses of flunixin meglumine (1.1 mg/kg, by mouth every 24

hours) and blood was collected from 5 adult elephants receiving firocoxib therapeutically. All samples were processed the same, with TxB_2 being determined in whole blood as an indication of COX-1 inhibition and PGE+M being determined in plasma after induction with LPS as an indicator of COX-2 inhibition.

Results: Firocoxib had an *in vitro* IC_{50} COX-1:COX-2 ratio of 60.7, Flunixin meglumine had an IC_{50} COX-1:COX-2 ratio of 0.003 *in vitro*. Aspirin exhibited an IC_{50} COX-1:COX-2 ratio of 0.01 in vitro. *Ex vivo* TxB_2 concentrations decreased in all elephants receiving multiple doses of firocoxib, from 21329 +/- 5473 pg/ml to 16063 +/- 3883 pg/ml (avg: -21 +/- 26.2%. The long-term treatment elephants had TxB_2 levels of 15826 +/- 585 pg/ml. *Ex vivo* PGE_2+M levels increased from a mean of 17.1 +/- 5.09 pg/ml to a mean of 26.1+/- 13.5 pg/ml. Long-term administration resulted in mean PGE_2 +M concentrations of 11.3 +/- 2.62 pg/ml. *Ex vivo* TxB_2 decreased from 24,721 +/-5257 pg/ml to 170 +/- 48.8 pg/ml. PGE_2+M levels determined *ex vivo* were 16.9 +/-1.32 pg/ml prior to the elephant receiving 3 doses of flunixin meglumine and were 16.4 +/- 1.29 pg/ml post. The mean decrease in PGE2+M levels was – 3.20 +/- 4.62 pg/ml..

Conclusions: Firocoxib demonstrated preferential inhibition of COX-2 while both flunixin meglumine and aspirin were preferential for COX-1. A paradoxical increase in ex vivo PGE2+M levels post firocoxib administration was identified. More than a 10 fold decrease in circulating TxB_2 levels was identified.

3.2.3 Introduction

Appropriate pain management is the function of a balance between risks of side effects of the drugs administered and the benefits of pain modulation for a patient taking those drugs.[126]

All drugs function by altering or facilitating specific processes within the body. These affects can be individual and unique, with differences within the same species and between different species.[67,261] Understanding the pharmacodynamics of how a drug functions within a given species provides an opportunity to tailor treatment, such as pain management, in a precise and specific manner for a patient.[261]

Nonsteroidal anti-inflammatory drugs (NSAIDs) are the most commonly administered pain medication given to elephants for control of somatic and visceral pain.[153] These drugs function by inhibiting cyclooxygenase isoenzymes in the body and play a role in decreasing peripheral pain sensation by modulating or decreasing inflammation.[96,126,147] In addition, because of the presence of cyclooxygenase-2 (COX-2) expression in the CNS, COX-2–specific NSAIDS may have a specific central nervous system effects on the perception of pain.[96,126,262] Cyclooxygenase (COX) is the obligate, rate-limiting enzyme for the conversion of arachidonic acid into prostaglandins, ultimately affecting multiple systems within the body, not just inflammation (Figure 8).[262,263] While there are multiple COX isoenzymes, the COX-1 and COX-2 isoenzymes are generally thought of as the specific forms most commonly inhibited by NSAID administration (Figure 8).[210,225,264]

COX-1 is required for essential physiological functions, and so it is constitutively produced by multiple cells in the body,[191,194,245,265-267] Intrinsic protective mechanisms of renal, gastrointestinal, or platelet function are controlled by prostanoids produced by COX-1 which act as local signaling mediators.[180,181,187,229,268,269] COX-2 is produced following induction in response to inflammatory stimuli such as injury or trauma.[187,264,268] Production of COX-1 is associated with the control of cellular division, angiogenesis and cardiovascular functions, as well as having a role in influencing the water and electrolyte balance controlled by the

kidneys.[179,180,187,191,268-272] COX-1 is thought to play a role in tissue blood flow during inflammatory states, while the uncontrolled action of COX-2 may initiate the undesirable effects associated with the inflammatory process.[180,187,211,219,262] For example, COX-2 is considered more efficient than COX-1 in oxygenating eicosapentaenoic, gamma-linolenic, alpha-linolenic and linolenic acids, the key fatty acids involved in the inflammatory cascade.[192,229,268] Because the function of both COX-1 and 2 are physiologically important, understanding the impact of specific NSAIDs on their function influences predictions of drug safety and efficacy.

Determination of COX-1/COX-2 isoenzyme selectivity both *in vitro* and *ex vivo* in whole blood has become standard for evaluating the pharmacodynamics of NSAIDs in humans and domestic animals, including dogs, cats, and horses.[164,212-214,255,256,273-276] These isoenzyme studies are limited in zoological species and wildlife, with no *ex vivo* studies known to have been performed, to date.[196] To the best of the authors' knowledge, this study describes the first evaluation of COX inhibition *in vitro* and *ex vivo* in Asian (*Elephas maximus*) and African elephants (*Loxodanta africana* and *Loxodanta cyclotis*).

3.2.4. Materials and Methods

This study was approved by the Institutional Animal Care and Use Committee of Auburn University, Auburn, Alabama (Protocols: 2015-2609 and 2018-3224) and the Animal Care and Use Committee and Research Committees of each participating institution housing the elephants. A commercially available ELISA kit (Thromboxane B_2 EIA, Cayman Chemical Co., Ann Arbor, Michigan, USA) was used to determine Thromboxane B_2 levels in serum as an indicator of whole blood COX-1 isoenzyme activity. A second commercially available assay was utilized to determine Prostaglandin E_2 and its metabolites (Prostaglandin E_2 and Metabolites EIA, Cayman

Chemical Co., Ann Arbor, Michigan, USA) as an indicator of whole blood COX-1 isoenzyme activity.

Blood samples were collected from 4 healthy adult female Asian elephants for *in vitro* assays to assess the effects of firocoxib, flunixin meglumine and aspirin on COX-1 and Cox-2 activity. Samples for *ex vivo* analysis were also collected from 4 healthy adult female and 2 male Asian elephants participating in a multiple oral dose firocoxib pharmacokinetic study, with samples obtained prior to drug administration and after administration of 8 oral doses of 0.1 mg/kg of commercially available firocoxib (Previcox® tablets, Merial Inc, Duluth, GA 30096-4640 USA). In addition, blood was collected from 2 healthy, adult female Asian elephants and one male participating in a multiple oral dose flunixin meglumine study. These 3 elephants received 3 consecutive oral doses of 1.1 mg/kg flunixin meglumine, and samples were obtained prior to drug administration and after 3 consecutive oral doses of 1.1 mg/kg of the drug. Finally, blood was collected from 5 female Asian elephants that had been receiving firocoxib orally at a range of 0.58 to 0.1 mg/kg for a minimum of 90 days for comparison to the other studies levels. There were no elephants identified receiving multiple doses of flunixin meglumine beyond the pharmacokinetic study elephants. No elephants were identified as receiving aspirin therapeutically or as part of a pharmacokinetic trial at the time of this study.

Participating elephants ranged in age from 23 to 63 years of age. All elephants, except those in the long-term administration study, were determined to be healthy based upon physical examination, and routine blood analysis (CBC and serum chemistry profile) prior to the beginning of the study. The long-term administration study participants were being treated for chronic orthopedic conditions, but otherwise were deemed healthy based upon physical examination and routine bloodwork. Blood collection for all samples followed the same

methodology. Blood (no more than 30 ml at one time) was collected aseptically from an ear vein into glass blood collection tubes, using a 21 gauge 0.75 inch (19 mm) butterfly catheter (BD Vacutainer Safety-Lok, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA, for tablets or for paste Sureflo winged infusion set, Terumo Medical Products, Somerset, NJ 08873, USA). All samples were processed immediately after collection, with serum and plasma stored at 20°C for repeat analysis.

In vitro determination of serum TxB_2 concentrations (as an indicator of *in vitro* COX-1 inhibition), was performed by collecting 3 ml of blood into six plain glass blood collection tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA) that had been preloaded with drug (firocoxib, flunixin meglumine, or aspirin) at the following concentrations per individual tube: 0, 0.01, µMol, 0.1 µMol, 1 µMol, 10 µMol, 100 µMol, and 1000 µMol. *Ex vivo* determination of COX-1 activity in animals receiving drug was also determined via serum TxB_2, by collecting 10 ml of blood into plain glass blood collection tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA).

All blood samples for serum TxB_2 determinations were held at 20°C for a minimum of 2 hours to allow a clot to form, then centrifuged twice at 2000 x g for 10 minutes.[211-213] The supernatant serum was harvested and divided into 250 µl aliquots and stored at -20C prior to analysis. Immediately prior to analysis, samples were purified with ethanol to precipitate proteins and filtered through a disposable extraction column (Bakerbond SPE Otadecyl, C-18 extraction columns, Mallinckrodt Baker, Inc, Phillipsburg, New Jersey 08865, USA). This methodology provided a manufacturer's specified detection limit of 5 pg/ml for undiluted samples.

Determinations of serum PGE_2 +M as an indicator of *in vitro* COX-2 inhibition, was performed by collecting 3 ml of blood into six lithium heparin glass blood collection tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA) that had been preloaded with drug (firocoxib, flunixin meglumine, or aspirin) at the following concentrations per individual tube: 0, 0.01 µMol 0.1 µMol, 1 µMol, 10 µMol, 100 µMol, and 1000 µMol . Tubes were also pretreated with aspirin to inhibit platelet and monocyte COX-1 production of TxB_2[214,277]. *Ex vivo* determination of COX-2 activity was also determined via serum PGE_2 +M, by collecting 10 ml of blood lithium heparin containing glass blood collection tubes (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA) preloaded with 100 µg acetylsalicylic acid to inhibit COX-1 isoenzyme activity.[211,212] Fifty µg E. coli LPS, serotype 026:B6 (Sigma Aldrich Co., 3050 Spruce Street, St Louis, Missouri 63103) per ml of blood was added to induce COX-2.[212,214] Samples were incubated at 37°C for 24 hours in an incubator with an orbital shaker set at 30 rotations/minute, then centrifuged twice at 2000 × g for 10 minutes. Plasma was harvested, re-centrifuged at 2000 × g, then divided into 250 µl aliquots and stored at -20C prior to analysis.[211-213,274] All samples analyzed were first purified by chilled acetone precipitation for removal of proteins. The supernatant was removed and dried under nitrogen. This methodology provided a detection limit of 2 pg/ml per the manufacturer's specifications. Purified samples for each PGE_2+M assay were diluted at 1:1 and 1:4 with commercially available buffer (PGE EIA Assay Buffer, Cayman Chemical Co., Ann Arbor, Michigan, USA).

The standard curves for each assay were derived from the manufacturer's standard provided with each assay kit, prepared per the manufacturer's specifications and validated for that specific assay. The concentration of TxB_2 or PGE_2+M and metabolites in each sample were

calculated from the standard curve for each dilution, per the manufacturer's instructions and as described in previously published studies.[211,212,257] All assay plates were read at a wavelength of 415 nm and all results are reported as mean ± 1 standard deviation as calculated using commercially available software (Microsoft Excel, Microsoft Redmond campus, Redmond, Washington, USA).

The sigmoidal dose-response curves were analyzed using GraphPad Prism v8 (GraphPad Software, San Diego, CA 92108 USA). Data was analyzed using a nonlinear curve fitting model in the four parameter logistic equation: $\%\text{inhibition} = I_0 + (I_{max}-I_0)/\{1+10^{[(LogIC50C)*nH]}\}$ where % inhibition is the inhibition of eicosanoid (TxB_2, PGE_2 + M) production expressed as a percentage of the baseline value; C is the logarithmic value of the test compound concentration; IC_{50} is the specific drug concentration that resulted in 50% of maximal inhibition of COX-1 (IC_{50} COX-1) or COX-2 (IC_{50} COX-2) and serves as a determination of drug potency; Imax is the maximum inhibition or a determination of drug efficacy; I_0 is the baseline inhibition; nH is the Hill coefficient equal to the slope of the concentration–response curve, which also serves as a determination of drug affinity. Selectivity for either COX-1 or COX-2 was determined by calculating the COX-1:COX:2 ratio using the equation COX-1 IC_{50}/ COX-2 IC_{50}. Additionally IC_{20} and IC_{80} concentrations were determined.

3.2.5 Results

Firocoxib had an *in vitro* IC_{50} COX-1:COX-2 ratio of 60.7, The IC_{20} COX-1:COX-2 inhibition ratio for firocoxib was calculated using the Hill coefficients to be 2.7, while the IC_{80} inhibition ratio was calculated to be 1366. (Table 2, Figures 9,10). Flunixin meglumine exhibited a COX-1:COX-2 ratio of 0.0030 *in vitro*. The IC_{20} COX-1:COX-2 inhibition ratio for

flunixin meglumine was calculated using the Hill coefficients to be 0.0029, while the IC_{80} inhibition ratio was calculated to be 0.003. (Table 2, Figures 9,10). Aspirin exhibited a COX-1:COX-2 ratio of 0.01 *in vitro*. The IC_{20} COX-1:COX-2 inhibition ratio for aspirin was calculated using the Hill coefficients to be 0.01, while the IC_{80} inhibition ratio was calculated to be 0.013. (Table 2, Figure 8,9).

Ex vivo TxB_2 concentrations, decreased in all elephants receiving multiple doses of firocoxib, from a mean of 21329 +/- 5474 pg/ml to 16063+/- 3883 pg/ml for an average percentage decrease of 21.1 +/- 26.8 percent. The elephants that received firocoxib long term had mean COX-1 levels of 15826 +/- 585 pg/ml. *Ex vivo* PGE+M levels increased in elephants receiving multiple doses of firocoxib from a mean of 17.1 +/- 5.09 pg/ml to a mean of 26.1 +/- 13.5 pg/ml post 8 doses. The elephants that had been receiving firocoxib long term (>3 months) inducible circulating PGE +M concentrations, with a mean of 11.3 +/- 2.62 pg/ml.

Ex vivo analysis of TxB_2 n in elephants receiving multiple doses of flunixin meglumine revealed a decrease in mean thromboxane levels from 24,721 +/-5257 pg/ml to 170 +/- 48.8 pg/ml. PGE+M levels determined *ex vivo* were 16.9 +/-1.32 pg/ml prior to the elephant receiving 3 doses of flunixin meglumine and were 16.4 +/- 1.29 pg/ml post. The mean decrease in PGE+M levels was – 3.21 +/- 4.62 pg/ml. *Ex vivo* analysis results are summarized in Table 3.

3.2.6 Discussion

This study was conducted to correlate the *in vitro* and *ex vivo* effects of firocoxib and flunixin meglumine in Asian elephants. Because of the species status as an International Union for the Conservation of Nature (IUCN) endangered species, access to both healthy adult animals and clinical patients is limited.[278] It is impossible to perform any study specifically inducing

pain to measure specific anti-nociceptive effects of these drugs due to the elephants IUCN designation. Instead pain response must be evaluated in Asian elephants and in many cases other zoo animals utilizing subjective or perceived pain scores and other described noninvasive methods of determining alleviation of pain and painful responses.[279 280-282] No clinical patients receiving flunixin meglumine in North American zoological or conservation institutions at the time of this study were identified to allow evaluation of this drug beyond *in vitro* and those 3 participating in the pharmacokinetic study. That is despite this drug having been identified as a commonly administered NSAID in previous surveys.[153] It is uncertain why this occurred, considering the reported frequency of administration of this drug. It is possible it is only administered for single or a very limited number of doses.

COX isoenzyme selectivity has been utilized as a means of determining NSAID COX potency and selectivity.[207,211,212,214,245,269,277,283,284] A IC_{50} COX-1/IC_{50} COX-2 ratio across the entire therapeutic dose range that is less than one signifies a drug that is selective for COX-1.[201] Conversely a IC_{50} COX-1/IC_{50} COX-2 ratio greater than one across a dose range indicates a drug is selective for COX-2. [201] If the ratio is one across a therapeutic dose range, that indicates a drug that is nonselective drug. [201] Unfortunately there can be a high degree of variability in the results of studies of the relative selectivity of an NSAID depending upon the tissue utilized, the assay system, assay methodology, and the species from which test cell lines are developed.[277,284] The results from *in vitro* cell determinations of cyclooxygenase inhibition are dependent on the specific cell type, with tumor cells, cloned cells or other types of cell sources resulting in differences in COX selectivity determined.[196,284] These systems are generally acceptable for screening of relative selectivity of a drug for the isoenzymes, but variability in methods can prevent them from being physiologically appropriate. Because of this, the use of whole blood

assays involving the target species has become recognized as the gold standard for determining COX selectivity[245,277] This form of *in vitro* assay is minimally invasive and can be utilized as a means of screening not just for drug potency and efficacy but also as a screening for potential toxicity of an NSAID prior to administration to an endangered or threatened species from which tissue samples or cell cultures.[284] The recent die off of vultures and now recognized apparent sensitivity of those species to NSAID drugs has driven a search for methods such as whole blood assays as safe methods of evaluating physiological effects of drugs.[26] ....

Species variations are possible with blood isoenzyme activity analysis in the respective assay utilized to evaluate thromboxane and PGE-M. These AA metabolism products have been evaluated in other species with different assays, including individual laboratory developed assays, commercially available ELISA's and HPLC.[207,211,212,214,245,269,277,283,284] There is potential for different assays to provide different results. The determination of COX-isoenzyme selectivity should be recognized as only one method of determining drug safety, as drug safety may be influenced by other parameters, including drug metabolites and method of metabolism, the degree of acidity of any prodrug, the plasma half-life of the drug in question in the specific species being studied, any nuances to liver metabolism and the degree of enterohepatic recycling that may occur in a species, nuances in renal filtering ability including renal blood flow, and the potential for polymorphisms in both drug metabolism and COX isoenyzmes.[197,284]

Typically the COX-1/COX-2 inhibition ratios are based on the IC_{50} values of each isoenzyme.[277] [285] The IC_{50} indicates that concentration of drug is needed to inhibit 50% of an enzymes activity.[277,284,285] While the IC_{50} provides a general reference point, it has been suggested that peak anti-inflammatory and analgesic activity may only occur with NSAIDs at concentrations above the COX-2 IC_{80} level[284,285] Conversely, protection from potential negative

adverse effects associated with COX-1 inhibition may only occur at or below the COX-1 IC_{20} level. [284,285] These values should not be over interpreted, because adverse events do not specifically occur below the IC_{20} and analgesia does not specifically only occur above the IC_{80} of a given drug in a given species.[277] Also, an IC_{80} COX-1:COX-2 ratio does not necessarily indicate the degree of inhibition of COX-1 attained with therapeutically efficacious concentrations. [277,285] This interpretation is further complicated as adverse effects are recognized as being associated with both COX-1 and COX-2 specific functions.[260,286,287]

Not all compounds have similar slopes to their respective inhibition curves. [284] This curve may vary dramatically when comparing different species due to species specific physiology and differences in constitutive COX-1 function and induction capacity of COX-2 activity[197,284,287] The drug and species respective Hill coefficient (From the Hill-Langmuir equation) becomes important when comparing the inhibition of enzymes as it is a way to quantify the degree of interaction between drug and drug binding site.[288] Care must be taken to not over interpret the Hill coefficient for a given inhibition curve as it is a specific descriptor to that individual inhibition curve for the drug and species being evaluated. [288] Point data, such as IC_{50}, allows a calculation of ratios but it does not give an indication of slope of the inhibition curve.[284] A drug with a steep inhibition curve has a narrow range when going from no enzyme inhibition to maximal inhibition. [288] A drug with a flatter inhibition curve indicates that a change in enzyme inhibition requires a relatively greater change in drug concentration. [284,288] So when evaluating COX-inhibition, on must consider not only the specific inhibition ratios, but also the slope and shape of the inhibition curve. [288] [284,285]

Firocoxib showed a COX-2 selectivity *in vitro* with COX-1/COX-2 ratios being greater than 1 for IC_{20}, IC_{50}, and IC_{80} inhibition levels Not only that but the IC_{50} level rose steeply with

a Hill equation coefficient of 2.348 for COX-2, suggesting a rapid rise in inhibition and strong potency to inhibit COX-2. This preference for COX-2 inhibition has been demonstrated with studies in other species.[257,273,289] Flunixin meglumine was the opposite, showing IC_{20}, IC_{50}, and IC_{80} COX-1/COX-2 inhibition ratios well below 1 *in vitro*, which suggests that this drug prefers COX-1 inhibition relative to COX-2 in elephant serum (Table 2, Figure 9,10). Flunixin also demonstrated similar Hill coefficients of 1.415 for COX-1 and 1.437 for COX-2. There was no detectable COX-2 inhibition at drug concentrations below 10 µM, however as drug concentrations increased, the percentage of COX-2 inhibition also rose. Aspirin exhibited IC_{20}, IC_{50}, and IC_{80} COX-1/COX-2 inhibition ratios below 1 *in vitro*, suggesting that this drug favors COX-1 inhibition relative to COX-2 (Table 2, Figure 9,10). The Hill coefficient for both COX-1 and COX-2 inhibition were both less than one (COX-1: 0.622, COX-2:0.719) which appears compatible with a concentration inhibition slope that indicates irreversible binding of aspirin to COX-1 as previously described.[189,231] As drug concentrations increased, there was an increase in COX-2 inhibition, suggesting some inhibitory effects at high concentrations. These assays also validate and confirm that the potent inhibition of COX-1 by aspirin will result in no interference with COX-2 from the addition of very small concentrations (100 µg/ml of blood) of aspirin as a means of decreasing COX-1 interferences with PGE_2-M assays. This coincides with previously described COX inhibition studies of other species.[277]

Ex vivo studies using whole blood assays provide a more physiologically relevant determination of a respective NSAID's effects on the species studied.[211,277,283,287,290,291] These studies involve the use of clinical administration of the drugs and provide with a means of evaluating in-body changes to isoenzyme function that may occur in a patient post drug exposure. [211,277,283,287,290,291] In the case of the elephants in this study, only baseline, prior to

drug administration and after the required doses were received were collected. The collection of multiple samples as drug concentrations rise to steady state and then undergo peak and troughs are necessary to accurately determine a Hill coefficient in *in vivo* studies.[211,277,283,287,290,291]

Ex vivo TxB_2 levels, as an indicator of circulating COX-1 activity decreased in all elephants receiving multiple doses of firocoxib, from a mean of 21,329 +/- 5,473 pg/ml to 16,063 +/- 3,883 pg/ml for an average percentage decrease of 21.1 +/- 26.8 percent. The elephants that received firocoxib long term had mean COX-1 levels of 15,826 +/- 585 pg/ml. This indicates that the decrease in COX-1 levels appears to stabilize once the drug reaches steady state. Unfortunately, there is no reference to describe what "normal" circulating TxB_2 levels are in Asian elephants, so it is not possible to draw conclusions beyond there is a slight decrease then stabilization of circulating levels of TxB_2. Reference standards need to be established to further interpret these results.

Ex vivo PGE_2+M levels, as an indicator of COX-2 isoenzyme activity increased in elephants receiving 8 doses of firocoxib from a mean of 17.1 +/- 5.09 pg/ml to a mean of 26.1 +/- 13.5 pg/ml after 8 doses. This paradoxical increase cannot be explained. It is the opposite of the decreased levels of induced PGE_2+M that was observed in domestic horses receiving 7 consecutive doses of the same formulation of firocoxib.[273] It is possible that this change is a result of induction of the isoenzyme within these study elephants. Multiple blood collections occurred during the study period, which may have caused an undetected inflammatory response, resulting in more isoenzyme production, and as a result, PGE+M production after exposure to the same amount of LPS. The corresponding serum concentration of firocoxib at the time of sample collection, which was a trough level, was 46.71 ng/ml (range: 29.9 - 68.1 ng/ml) (see chapter 5). This is comparable to drug concentrations measured in domestic horses 35.4 ng/mL (range, 21.6

to 40.7 ng/mL) in COX inhibition/drug efficacy studies.[211] Further determinations of serum COX isoenzyme inhibitions will need to be coupled with serum drug concentrations to determine specifics of this relationship.

The elephants that had been receiving firocoxib long term (>3 months) had lower inducible circulating PGE_2 +M levels, with a mean of 11.3 +/- 2.62 pg/ml. This decrease is expected as firocoxib is thought to preferentially inhibit COX-2. These therapeutic monitoring elephants also had serum drug concentrations comparable to the multiple dose study elephants with a mean of 41.22 ng/ml (range: 28.8 - 53.6 ng/ml). This decrease, combined with perceived relief in pain/discomfort of these elephants observed by keepers and attending veterinarians can be associated with a positive therapeutic response at a dose of ~0.1 mg/kg.

It must be noted that one of the elephants that provided samples for the long-term evaluation of firocoxib died from causes not related to the study after their portion of the study was completed. As reported by the attending veterinarian and review of medical records, there were no indications of adverse events associated with firocoxib administration noted at necropsy or during histopathologic evaluation of all tissues per AZA guidelines of this elephant. One is too small of a number of elephants to suggest safety of this drug. That said, because mortality studies are not possible, the only alternative is to monitor cases such as this one and retrospectively compare necropsy results to determine if there are specific identifiable tissue changes.

Ex vivo analysis of thromboxane levels as a reflection of COX-1 inhibition in elephants receiving multiple doses of flunixin meglumine as part of the pharmacokinetic study revealed a decrease in mean thromboxane levels from 24,721 +/-5,257 pg/ml to 170 +/- 48.8 pg/ml. This resulted in a mean change of -99.3 +/- 0.32 percent of circulating TxB_2 levels for these elephants.

A similar decrease following multiple consecutive doses was also observed in domestic horses.[213] This substantial decrease in circulating TxB_2, and associated COX-1 isoenzyme inhibition is concerning, considering the constitutively expressed nature of COX-1 and its essential functions within the body. The possibility of assay dysfunction falsely causing this drop were considered, however no specific assay breakdown could be identified, and these samples showed repeated results with other samples processed on the same assay plate not having unexpected results. This is considered a real result. Further investigation, including the repeated dose evaluation of COX-1 inhibition by flunixin meglumine in therapeutic cases, evaluation of blood clotting times as a physiological decrease in function, and evaluation of elephants receiving this drug therapeutically is indicated. TxB_2 is involved with blood clotting, however, no bleeding dyscrasias, such as hematoma formation during blood collection, were noted in any of the elephants in this study. The study population was too small (N=3) to make any type of a general statement about this decrease, other than further study is indicated.

PGE+M levels determined *ex vivo* were 16.9 +/-1.32 pg/ml prior to the elephant receiving 3 doses of flunixin meglumine and were 16.4 +/- 1.29 pg/ml post. The results for each individual elephant were positive and negative both, but the mean decrease in PGE+M levels was – 3.21 +/- 4.62 pg/ml. *Ex vivo* analysis results are summarized in Table 3. The corresponding serum concentration of flunixin meglumine at the time of sample collection, which was a trough level, was 0.75 ug/ml (range, 0.35-1.13 ng/ml. This is less than drug concentrations measured in domestic horses 1.29 ug/mL (range, 0.18 to 8.74 μg/mL) in COX inhibition/drug efficacy studies.[211]

No adverse events were identified in elephants in that study that received flunixin meglumine. There were no elephants identified receiving multiple doses of flunixin meglumine

beyond the pharmacokinetic study elephants. This is despite flunixin meglumine being previously reported as a commonly administered drug.[153] It is possible that flunixin meglumine is not used as a long-term care drug as firocoxib is, thus limiting the number of available patients. Aspirin was not evaluated *ex vivo* due to the inability to identify subjects receiving the drug either in pharmacokinetic study or therapeutically for multiple doses.

In conclusion, COX isoenzyme evaluations *in vitro* and *ex vivo* serve as a tool for evaluating efficacy and drug safety. The information gathered can be valuable, especially in animals that are ICUN classified as endangered or otherwise have limited ability for sample collection. They should be combined with other methods of evaluation, including pharmacokinetic studies and if possible clinical and necropsy records of patients receiving the drug therapeutically in order to construct a thorough drug profile for use in the species investigated.

Table 2

Inhibition ratios of COX-1 and COX-2 at IC_{20}, IC_{50}, and IC_{80} as determined by nonlinear regression analysis and log transformation of data to best fit. Values calculated utilizing the Hill equation as determined by simultaneously fitting percent inhibition values from drug concentrations of 0, 0.01, 0.1, 1, 10, 100, 100 µM of each respective drug. IC50 is the specific drug concentration that results in 50% of maximal inhibition of COX-1 (IC50 COX-1) or COX-2 (IC50 COX-2) and can serve as a determination of drug preference for specific COX isoenzyme. If the ratio is above 1, that drug favors COX-2. If the ratio is below 1 that drug favors COX-1. Firocoxib showed favor of COX-2 with all ratios above 1. Flunixin meglumine and aspirin were both preferentially selective for COX-1.

Drug	**Inhibitory Concentration**	**Cox-1**	**Hill coefficient**	**Cox-2**	**Hill coefficient**	**Cox 1:Cox 2 inhibition ratio**
	IC_{20}	0.091		0.034		2.7
Firocoxib	IC_{50}	3.68	0.374	0.061	2.348	60.7
	IC_{80}	150		0.1095		1366
	IC_{20}	0.023		7.95		0.0029
Flunixin Meglumine	IC_{50}	0.062	1.415	20.860	1.437	0.003
	IC_{80}	0.165		54.737		0.003
	IC_{20}	0.0002		0.0301		0.0066
Aspirin	IC_{50}	0.0020	0.622	0.207	0.719	0.0097
	IC_{80}	0.0183		1.422		0.0129

Table 3

Determination of concentration of TxB_2 and PGE_2+M as an indicator of change in COX-1 and COX-2 isoenzyme activity respectively in 6 Asian elephants that were part of a firocoxib pharmacokinetic trial, 3 Asian elephants that were part of a flunixin meglumine pharmacokinetic trial, and 5 Asian elephants (therapeutic monitoring column) that received firocoxib for a minimum of 3 months as part of a therapeutic protocol for management of osteoarthritis, foot disease, or other chronic painful conditions.

		Pre multiday (pg/ml)	**Post multiday (pg/ml)**	**Percent change**	**Therapeutic monitoring (pg/ml)**
Firocoxib	**Mean**	21329	16063	-24.7	15826
TXB2	**SD**	+/-5473	+/-3883	+/-26.8	+/-585
Firocoxib	**Mean**	17.1	26.1	49.6	11.3
PGE2+M	**SD**	+/-5.09	+/-13.5	+/-42.6	+/-2.62
Flunixin Meglumine	**Mean**	24721	170	-99.3	
TXB2	**SD**	+/-5257	48.8	+/-0.32	
Flunixin Meglumine	**Mean**	16.9	16.4	-3.21	
PGE2+M	**SD**	+/-1.32	1.29	+/-4.62	

Figure 8

Aracadonic acid (AA) metabolism. Cyclooxygenase plays a key role in metabolizing AA to cyclic endoperoxides which have specific actions on specific organs in the body. These actions are not all inclusive and it appears that there may be overlap of action/organs affected that require further study. Note PGI_2, PGD_2 and PGE_2's effects on vasodilation as an example of this overlap. It is unknown if these effects are conserved across all species.

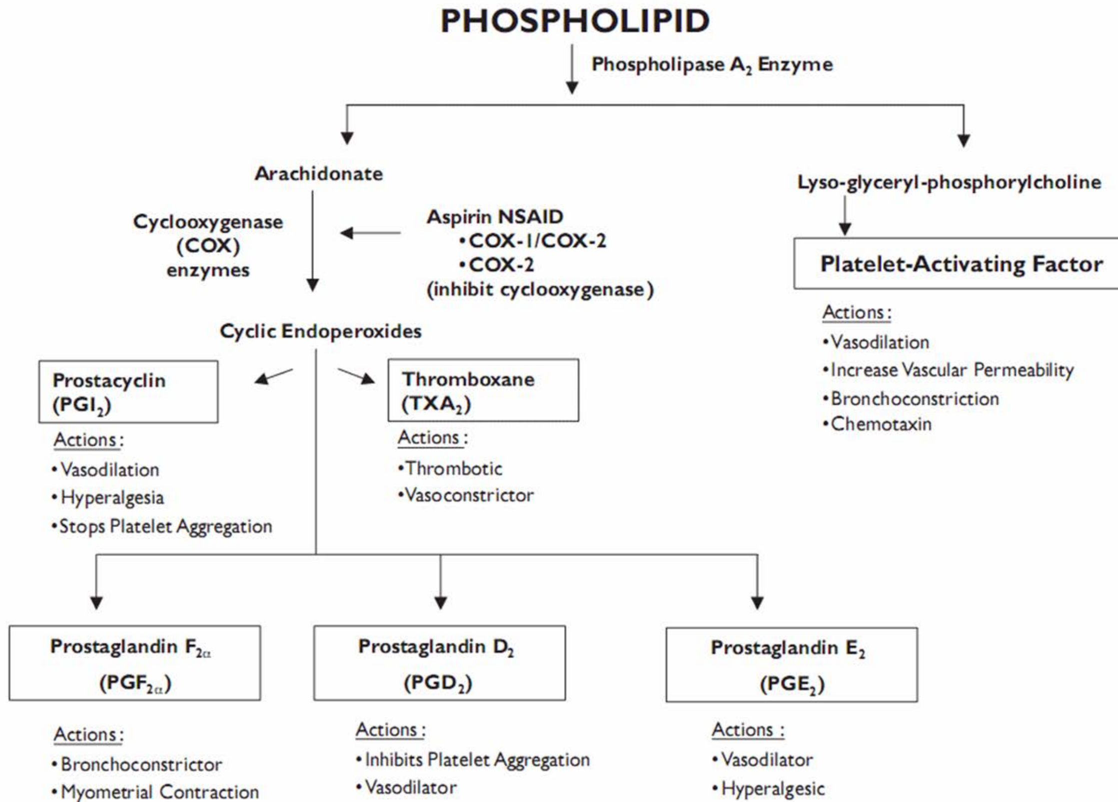


Figure 9

Dose response curves based on *in vitro* analysis of COX-1 inhibition by firocoxib, flunixin meglumine, and aspirin at various micromolar concentrations in Asian elephant (N=6) whole blood. The graph represents IC_{50} inhibition expressed as a percentage of baseline value analyzed with GraphPad Prism v8 statistical analysis software utilizing a 4 parameter logistical equation to determine a nonlinear best fit curve for percentage of inhibition at increasing drug concentrations. Whole blood platelets served as the source of TxB_2 as an indicator of COX-1 isoenzyme activity. COX-1 inhibition was not detectable below 0.01 µMolar drug concentrations. Both aspirin and flunixin meglumine showed high COX-1 inhibition relative to Firocoxib.

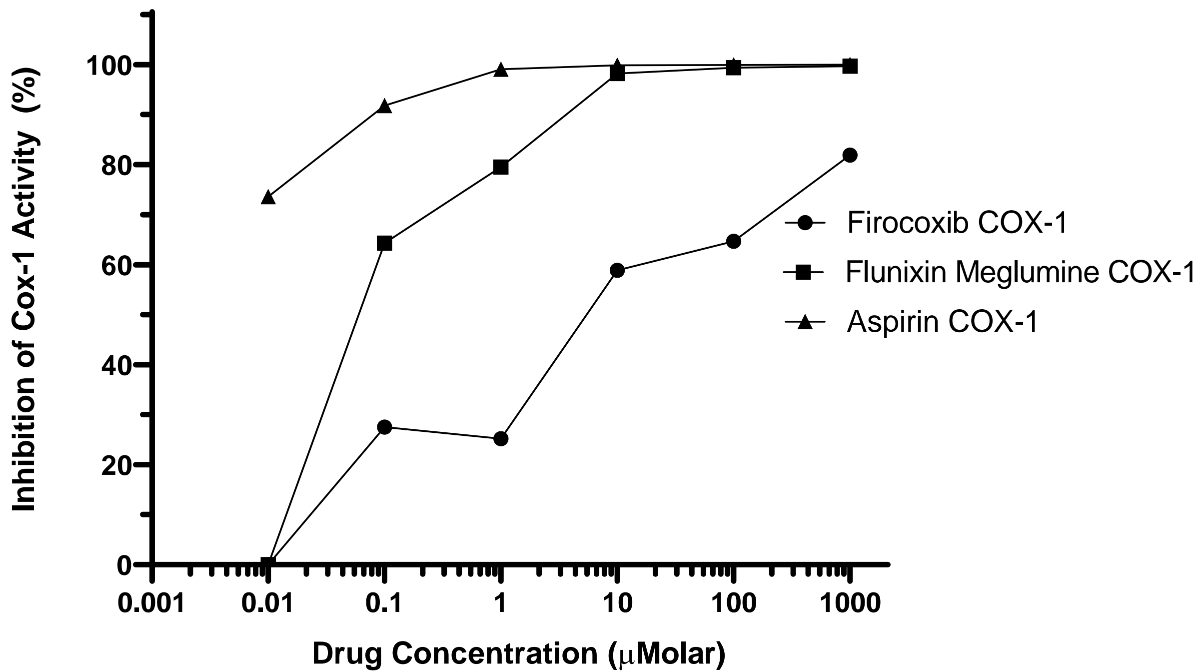
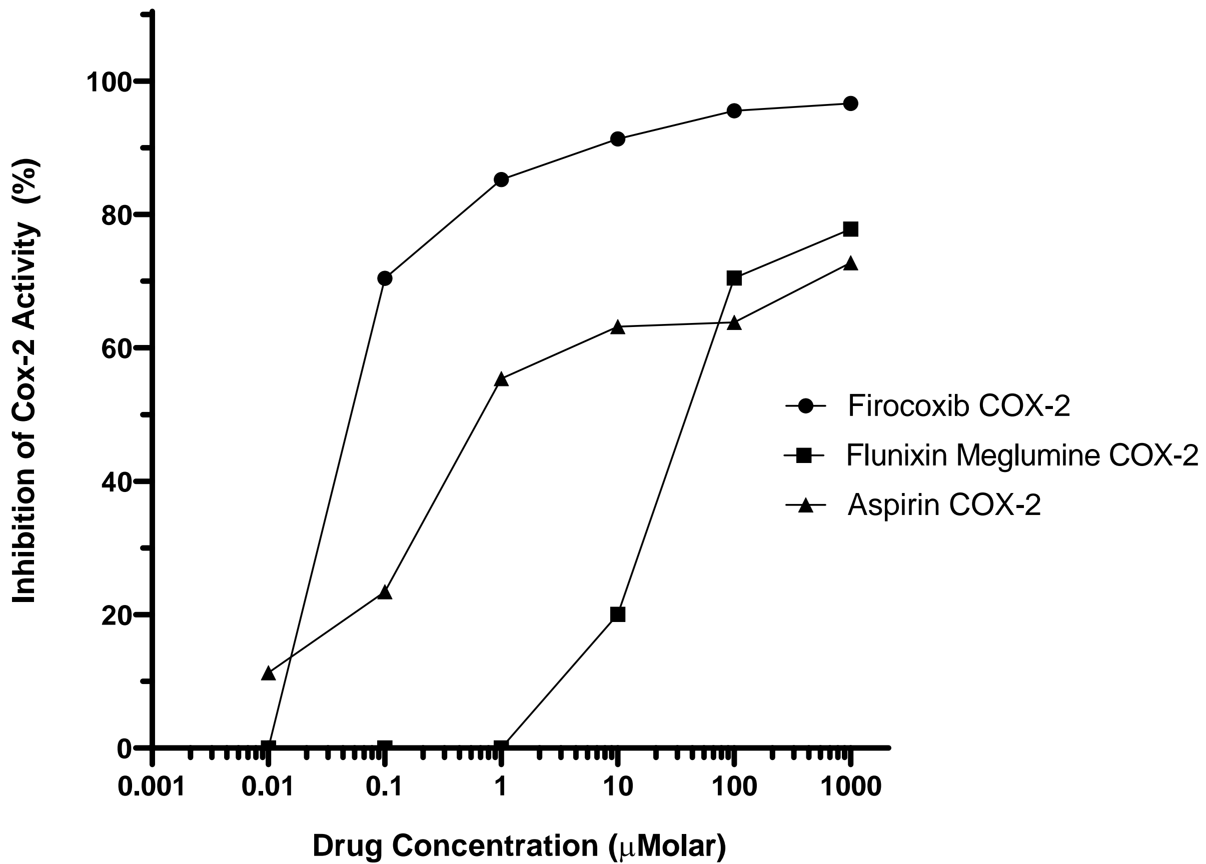


Figure 10

Dose-response curves based on *in vitro* analysis of COX-2 inhibition by firocoxib, flunixin meglumine, and aspirin at various micromolar concentrations in Asian elephant (N=6) whole blood. The graph represents IC_{50} inhibition expressed as a percentage of baseline value analyzed with GraphPad Prism v8 statistical analysis software utilizing a 4 parameter logistical equation to determine a nonlinear best fit curve for percentage of inhibition at increasing drug concentrations. Lipopolysaccharide was utilized to induce whole blood production of PGE_2 plus metabolites as an indicator of activity of COX-2. COX-2 inhibition was not detectable below 0.01 μMolar drug concentrations. Firocoxib showed preferential COX-2 selectivity relative to both flunixin meglumine and aspirin.



Chapter 4

Pharmacokinetics of Oral Single Dose Firocoxib in African Elephants (*Loxodanta africana*)

4.1 Background Information about zoo pharmacokinetic studies

Pharmacokinetic studies of serum or plasma drug concentration data for the treatment of diseases, especially chronic diseases, in zoo animals is limited.[25,109] Differences between and within species can affect the pharmacokinetics of all drugs, including NSAIDs.[61,113] The indications, pharmacokinetics, pharmacodynamics, and toxicology of pharmaceuticals are well established through strict regulatory mechanisms in humans and labeled species, which are typically only domestic animals.[25] Physiological variation between species creates a unique individualized environment which may alter drug absorption, distribution, metabolism and excretion.[25,292 293] These differences have been identified in elephants in a study that identified multiple clinically relevant differences in the pharmacokinetics of ketoprofen compared to the pharmacokinetics of that drug reported in horses.[113] In that specific study it was identified that absorption, mean half-life, area under the concentration-time curve (AUC), mean residence time, apparent volume of distribution (VD), plasma clearance (CL), and maximum plasma concentration (Cmax) values were significantly different between the S and R enantiomer of ketoprofen for both i.v. and oral administration.[113]

There have also been differences in NSAID pharmacokinetics reported between Asian and African elephants.[79,113] A study evaluating pharmacokinetics of phenylbutazone determined that statistically significant differences ($P < 0.0001$) in clearance (CL), terminal half-life $T_{1/2}$, and

mean residence time (MRT) occurred between African and Asian elephants administered a 2-mg/kg oral dose.[79]. This suggests that different treatment regimens for Asian and African elephants should be used with phenylbutazone.[79] While these are only two studies, the evaluation of these drugs implies that care should be taken when administering NSAIDs to elephants, and what is an appropriate drug dose for an Asian elephant may not be appropriate for an African elephant.

As described in Chapter 2 of this dissertation, accurate determination of a drug's bioavailability, oral absorption, and clearance require an i.v. drug administration study. These are key in determination of appropriate drug doses and dosing intervals.[291] These parameters can be estimated mathematically by comparing administration via different routes with less of a degree of precision. Vd for a given drug can only be determined accurately after i.v administration because i.v. administration is the only route of administration that guarantees 100% of an administered drug reaches systemic circulation.[294] Some drug studies will report VDss/F or VD/F (volume of distribution/dose) as the value from that study. [294] These are of little clinical or scientific relevance unless the concurrent bioavailability of the drug is also known for the specific route being reported in a study. [294] If the bioavailability is 100%, as is seen with an i.v. study, then Vd=Vd/F. If bioavailability or F is less than 100%, then the Vd will increase proportionately.[294] This mathematical difference can result in inaccuracies in calculations of drug dose and loading dose. It is not possible to perform lethal dose (LD50) drug studies on zoo animals, so an appropriate therapeutic index cannot be calculated. This is a cause of concern with NSAIDs, considering the associated risk for adverse events in those drugs.

For a drug dose to be effective, it must be sufficient to reach serum concentrations that fall within its therapeutic range. Drugs do not always reach serum or plasma steady state during

a single dose study. Steady state of a drug is defined as the time point where the rate of drug entering the blood stream is equal to the rate of clearance from the body.[86] The drug dosing interval and elimination half-life determine a given drugs time to steady state[294] Typically it takes repeated doses of the drug for a time lasting from 3 to 5 half-lives for a given drug to reach steady-state (Figure 11).[86] Steady state also results in serum concentrations that are consistently in the therapeutic range of the drug, with the trough, or lowest drug concentration, ideally not falling out of this range. Typical administration of NSAID's are not a single dose, instead therapeutic regimens typically are repeated doses over a period of time, because of this single dose drug studies alone miss important parameters necessary for implementing NSAID use for controlling pain and inflammation. If a drug is administered at a dosing interval greater than 5 half-lifes, there is little concern for drug accumulation so long as the dose is appropriate for that species.[86] Obviously a toxic dose is possible with a single dose if the species the drug is administered as a single dose that exceeds the effective range for that drug[295]. The differences in physiology make predicting specific nuances of a drugs effective range difficult in zoo animals.[295] The ultimate goal of any determining any NSAID pain management protocol is to dose a patient at a dose that keeps plasma levels within the therapeutic or effective range of plasma drug concentrations, without dosing at a level that falls below the therapeutic range and staying below toxic levels[86]

The pharmacokinetic studies described in this dissertation began with the goal to thoroughly evaluate the NSAID firocoxib in both Asian and African elephants, including i.v., single dose oral, multiple dose, oral, and therapeutic monitoring of long-term administration patients. However, the final outcome did not achieve all of those goals as initially intended, instead providing a single dose study, with limited data on multiple dose and therapeutic

monitoring for African elephants, and a much more thorough study of this drug in Asian elephants. Those two studies can serve as examples to compare the traditional single dose studies done in zoological medicine with a more complete study providing more complete information for an NSAID. This single dose study will be submitted to the Journal of Zoo and Wildlife Medicine for publication.

4,2 Pharmacokinetics of Single Dose Oral Administration of Firocoxib to African Elephants (*Loxodanta africana*) to be submitted to the Journal of Zoo and Wildlife Medicine ....

4.2.1 Abstract ....

Background/Purpose: To provide a recommended dosing regimen for firocoxib in African elephants (*Loxodanta africana*) based on pharmacokinetics determined after single oral dose of 0.01 mg/kg or 0.1 mg.kg administered as a paste or tablet.

Animals: 6 healthy adult African elephants

Methods: 4 elephants were administered firocoxib tablets at dose of 0.01 mg/kg and then, following a minimum 30 day washout period, 0.1 mg/kg. Two elephants were administered firocoxib paste at a dose of 0.01 mg/kg, and then following a 30 day minimum washout period, a dose of 0.1 mg/kg. Serum samples were collected incrementally post drug administration and analyzed via HPLC.

Results: AUC_{inf} was determined to be 1588+/-362 h*mg/ml, clearance, not corrected for bioavailability was 88.1 +/-2.17 ml/min/kg, (C_{max}) was 31.3 +/- 6.6 ng/ml, T_{max} was 6.36+/- 1.79 hr, the apparent volume of distribution (Vz/F) was 10.93 +/-7.25 L/kg, disappearance harmonic mean half-life was 66.4 hr with a pseudostandard deviation of 59.2 hr. and a rate constant of

0.008 1/hr. for tablets. AUC_{inf} was determined to be 814.4 h*mg/ml, clearance, not corrected for bioavailability was 132 ml/min/kg, (C_{max}) was 44 +/- 12.5 ng/ml, T_{max} was 7.0 +/-1.41 hr, the apparent volume of distribution (Vz/F) was 6.935 mL/kg, harmonic mean half-life disappearance of 36.4 hours with a disappearance rate constant of 0.019 1/hr. for tablets

Conclusions: Firocoxib administered at a dose of 0.01 mg/kg is not an appropriate dose for African elephants. Firocoxib administered at a dose of 0.1 mg/kg to African elephants results in serum levels comparable to domestic horses, including comparable peak serum concentrations and long half-life. While study numbers were low, there is sufficient evidence to suggest that 0.1 mg/kg is an appropriate dose for firocoxib in African elephants.

4.2.2 Introduction..

African elephants are long lived, hindgut fermenters with a digestive tract consisting of a single stomach with both glandular and nonglandular portions and a well-developed cecum.[22] Because of these anatomical similarities to the digestive tract equine pharmacokinetic and pharmacodynamic data have traditionally been used to extrapolate oral drug doses for elephants.[13,296] The lack of literature on the physiology of African elephants, combined with their enormous size, complicates assessment of dosing requirements utilizing dose extrapolation and estimation methods such as allometric scaling.[30]

Arthritis, foot disease, and other medical conditions thought to cause both pain and discomfort are well documented in captive elephants.[174,297-303] A 2006 survey of 78 zoological institutions accredited by the Association of Zoos and Aquariums (AZA), reported that 33% of the responding institutions participating at least once case of elephant foot pathology thought to cause pain and discomfort annually with[301] This same study reported that 7% of all of the

African elephants surveyed had a concurrent diagnosis of arthritis in addition to foot pathology.[301] A more recent study reported an even higher incidence of lameness/stiffness (62%:136/220) and foot lesions (59%:129/220) identified through the evaluation of individual medical records of Asian and African elephants housed in AZA accredited zoos in the United States.[297] Lameness associated with arthritis appears to be directly associated with age, with arthritis being significantly more common in older elephants.[297,299,304]

Attempts have been made to reduce foot pathologies, arthritis, and other orthopedic pathologies in institutions housing elephants by controlling individual animal body condition and weight, regular and routine foot and nail care, adjustments of exercise plans to ensure adequate activity, and changes in flooring substrate.[297,301,302,305] As a preventative measure against these pathologies and to alleviate the potential discomfort due to concrete substrates, some zoological institutions have renovated elephant housing to increase the amount of natural or shock-absorbent substrates.[302] Despite a recent study demonstrating potentially conflicting results, the generally accepted premise in elephant management is that when provided with natural substrates in captivity, elephants appear to benefit from improved foot and joint health thought to be due to increased tissue blood flow along along with associated filing of the nails and foot pad, and increased movement of foot muscles, tendons and joints.[297,301,305,306]

Despite the changes in husbandry, arthritis, foot problems and other painful conditions still occur in captive elephants.[297] One of the first steps for effectively medically managing discomfort in patients of any species is administration of drugs at species-appropriate doses and dosing intervals to control both pain and inflammation.[153,295] There is a limited number of scientific studies performed on the pharmacokinetics of analgesics in elephants.[153] In addition, published doses in both formularies or case reports for elephants are not always based on actual

pharmacokinetic studies, but instead may be extrapolated from doses administered in domestic horses.[153] The long life of captive elephants, combined with chronic disease conditions create a need for scientifically determined analgesic and anti-inflammatory drug choices to appropriately manage chronic medical conditions such as osteoarthritis. A 2016 survey identified nonsteroidal anti-inflammatory drugs (NSAIDs) as the most commonly administered analgesics to captive elephants, however there was substantial variability identified in both in dosing regimens and reported efficacy between and within facilities reported in that survey.[153]

The analgesic and anti-inflammatory effects of NSAIDs are considered to be due to the drugs inhibition of cyclooxygenase (COX) isoenzymes, specifically COX-1 or COX-2.[212,213] The COX-1 isoform is constitutively expressed and is considered responsible for normal homeostatic functions, including platelet function, renal blood flow and protection of the intestinal lining.[212,213] The COX-2 isoform is primarily induced in response to lipopolysaccharide and cytokine stimulation and is considered mainly responsible for the synthesis of proinflammatory prostanoids.[212,213,229,307,308] Firocoxib is a coxib type drug, generally characterized as a COX-2 selective NSAID because it tends to inhibit COX-2 while exerting sparing effects on COX-1.[213,255,308] It is important to understand that despite this designation of COX-2 selectivity, it is possible to see adverse side effects associated with COX-1 inhibition after administration of COX-2 inhibitory drugs as the inhibition of both isoenzymes is dependent on the degree of COX-selectivity of the drug in the specific species being evaluated .[213] In addition, it is known that COX-2 isoenzymes are expressed constitutively in the duodenum of some species and also contribute to renal blood flow and normal kidney function.[202,309] This study was approved by the Institutional Animal Care and Use Committee of Auburn University, Auburn, Alabama (Protocols: 2015-2609 and 2018-3224) and the Animal

Care and Use Committee and Research Committees of each participating institution housing the elephants. The goal of this study was to evaluate the pharmacokinetics of a single dose of firocoxib administered orally to captive African elephants.

4.2.3 Materials and Methods

4.2.3.1 Animals

Elephants for this study were chosen based on the criteria of being at least 10 years old, having no signs of illness based on clinical history, physical examination, and routine health screening (CBC, serum chemistry profile and if possible urinalysis|), and having not received any other medications for at least a 30 day period before the study began. Blood work (CBC, serum chemistry profile and if possible, urinalysis|) was repeated within 1 month of completion of the study to assess post drug health status. These selection criteria resulted in a total of 6 healthy females from institutions housing captive African elephants in the U.S. Ages ranged from 33 to 43 years and weights ranged in weight from 2086 to 4075 kilograms.

4.2.3.2 Drug Administration and Sample Collection

Elephants were chosen randomly to receive paste or tablets. Due to circumstances not related to this study, two of the elephants chosen to receive firocoxib paste withdrew before sample collection occurred. Four elephants were administered firocoxib orally in tablet form (Previcox® tablets, Merial Inc, Duluth, GA 30096-4640 USA) at a dose of 0.01 mg/kg and then following a minimum 30 day washout period a single dose of 0.1 mg/kg. The canine tablet formulation was utilized because the equine tablet formulation had not yet been released to the U.S. market when the study began. The equine tablet formulation of firocoxib tablets was not

evaluated. Two elephants received firocoxib paste (Equioxx® paste Merial Inc, Duluth, GA 30096-4640 USA) at a single dose of 0.01 mg/kg and tehn following a minimum 30 day washout period a single dose of 0.1 mg/kg orally. All drugs were administered in the morning, prior to feeding the A.M. diet. Firocoxib was administered with a food item (for example: apple, bread, peanut butter, sweet feed) preferred by each participating elephant. After drug administration, the elephant was offered and all consumed all of their normal morning diet without restriction.

Ten ml of blood was collected from an ear vein using a 21-gauge butterfly catheter (BD Vacutainer Safety-Lok, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA, or Sureflo winged infusion set, Terumo Medical Products, Somerset, NJ 08873, USA). at -5 minutes, 15, 30, 45, and 60 minutes, 1.5 2,4,6,8, 12, 24, 48, 72, 96, and 120 hours post drug administration. These times were specifically chosen to allow for collection times that coincided with the elephant care schedules of participating facilities. Samples were placed into glass tubes and centrifuged for 10 min at 2500 rpm. Because firocoxib can bind to plastic of storage tubes, serum was decanted into glass vials and kept frozen at -20C. until time of analysis.[310]

4.2.3.3 Drug Analysis

Elephant serum was analyzed for firocoxib concentrations by high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection based upon previously described methodologies.[311-313] The HPLC system consisted of a Waters 600 Controller (pump), a Waters 717 Autosampler, and a 2487 UV-Visible detector (Waters Corporation™, Milford, MA, USA). Separation was achieved with a Luna PFP (2), 5 µm, 150 x 4.6 mm column (Phenomenex®,

Torrance, CA, USA) maintained at 40 °C. The mobile phase consisted of 45:55:0.025 acetonitrile/water/trifluoroacetic acid (VWR®, Radnor, PA, USA) with the flow rate set to 1 mL/min. The standard curve was generated with a range from 2 to 100 ng/mL by fortifying African elephant serum with known amounts of firocoxib (Toronto Research Chemicals Inc. (TRC), Toronto, Ontario, Canada) reference standard and accepted if the coefficient of determination (r^2) was at least 0.999 and the predicted concentrations were within 10% of the actual concentrations. Firocoxib was extracted from elephant serum with solid phase extraction (SPE) cartridges (Waters Oasis® HLB 3 cc, 60 mg (Waters Corporation™, Milford, MA, USA). Briefly, previously frozen serum samples were thawed and vortexed, then mixed with water that contains 5% acetic acid and vortex. The SPE cartridges were conditioned with acetonitrile and then water. The aqueous serum samples were loaded and allowed to elute by gravity. The cartridges were rinsed with water that contains 5% acetic acid, and then with water: methanol (75:25 v/v) solution. Vacuum of ~10 in of Hg was used to remove the residual solvent. Firocoxib was eluted with acetonitrile which was then evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 40: 60 acetonitrile: water that contains 5 % acetic acid (v/v), vortex/mixing, and then the solution was centrifuged at 16000 x g at room temperature. The injection volume was 100 µL. The retention time for firocoxib was 9.8 min and UV absorbance was monitored at 290 nm.

The linear correlation coefficient for firocoxib in African elephant serum was 0.998. The limit of detection (LOD) was 1 ng/mL and the lower limits of quantification (LLOQ) was 2 ng/mL. The Precision (RSD%) for firocoxib in African elephant serum at concentrations of 2, 20, 50, and 100 ng/mL were 16.57%, 2.43%, 0.81%, 0.71% respectively. The Accuracy for

firocoxib in African serum 2, 20, 50, and 100 ng/mL were 99.78%, 97.49%, 101.60%, and 99.22% respectively.

4.2.3.4 Pharmacokinetic Analysis

The serum drug concentrations determined from 4 elephants receiving tablets and 2 receiving paste at a 0.01 mg/kg dose were not consistently above the LOD of the assay to allow for statistical analysis, including pharmacokinetic modeling. As a result of this initial analysis, further trials with the dose of 0.01 mg/kg were suspended.

Serum fircoxib concentration versus time data was subjected to non-compartmental analysis using computer software (Phoenix WinNonLin V7, Pharsight, Cetara, Pharsight Corp., Princeton, New Jersey 08540, USA)). Area under the curve to infinity (AUC-inf)was determined using the log-linear trapezoidal method. The actual maximum concentration (Cmax) occurring at time to maximum concentration (Tmax) were recorded. Concentrations at 12 hours (C12) and at the last time point collected (Cmin) were also recorded. The slope of the terminal component of the drug-elimination time curve was based on non-linear regression. Because firocoxib was only administered to African elephants orally, and was not administered intravenously, the terminal component could not be confirmed to be elimination and thus both the elimination rate constant and half-life were reported as disappearance rate constant and disappearance half-life. Disappearance half-life was reported as harmonic mean +/- pseudostandard deviation. Neither clearance (CL) nor volume of distribution (Vd) could be determined and are reported uncorrected for bioavailability (Vd/F or CL/F). Other parameters included addition mean residence time (MRT) and the percent of the AUC that was extrapolated from the terminal component of the curve. The relative bioavailability (percent) of firocoxib was calculated based on the ratio of the AUCs using the formula $(AUC_{paste}/Dose_{paste})x(Dose_{tab}/AUC_{tab})$.

Pharmacokinetic parameters were reported as mean and standard deviation with 95% confidence intervals. Statistical analysis was performed using Systat v13.2 (Systat Software, Inc. San Jose, CA 95131 USA). Normality was assessed using a Shapiro-Wilk's test or a modified Anderson-Darling test to estimate normality in the event of low sample numbers. The student's paired t-test was used to assess significant differences (P=0.05) between T1/2, Cmax, Tmax, and AUCinf between the single dose oral paste and oral tablet formulations.

4.2.4 Results

No abnormalities for any of the participants were noted in the CBC, serum chemistry profile and urinalyses post study. Drug concentration vs time for paste is depicted in Figure 12 for paste and Figure 13 for tablets. Pharmacokinetic results are summarized in Table 4. AUC-inf was determined to be 1588+/-362 h*mg/ml for tablets and 814 h*mg/ml for paste. Statistical significance of the differences in these two values could not be determined. The clearance, not corrected for bioavailability was 88.1 +/-2.17 ml/min/kg for tablets and 132 m.l/min/kg for paste. The maximum detected concentration (C_{max}) observed was 31.3 +/- 6.6 ng/ml for firocoxib tablets and 44 +/- 12.5 ng/ml for paste. This occurred at a T_{max} of 6.36+/- 1.79 hr for tablets and 7.0 +/-1.41 hr for paste. Both of these parameters were determined to not have statistically significant differences (C_{max} p value: 0.823, T_{max} p value 0.967). The harmonic mean disappearance half-life was 66.4 hours with a pseudostandard deviation of 59.19 hr. and a rate constant of 0008 1/hr. for tablets. Conversely firocoxib paste had a harmonic mean half-life disappearance of 36.4 hours with a disappearance rate constant of 0.019 1/hr. These parameters did not have statistically significant differences (p value: 0.206). MRT was 7.22 +/- 25.7 hr. for tablets and 28.9 hr for paste. The apparent volume of distribution (Vz/F) was 10925 +/-7254 for

tablets and 6925 mL/kg for paste. The relative bioavailability for paste compared to tablets was 50%.

4.2.5 Discussion

There is a pronounced lack of pharmacokinetic studies in captive elephants, especially for drugs used to manage pain and inflammation.[4] Dosage regimens in elephants have traditionally been based upon single dose studies or extrapolated from the assumption that pharmaceuticals administered to elephants undergo the same absorption, distribution, metabolism and excretion as the domestic horse.[13,296,314] This association in presumed similarity is flawed because, while elephants are hind gut fermenting herbivores, they have a fundamentally different form of liver metabolism compared to horses in that elephants produce bile alcohols while horses produce bile acids as a major metabolite of cholesterol metabolism.[76,77] The specifics of these differences and how they may affect drug metabolism are not fully understood, but do demonstrate a need for single dose i.v. oral, and long-term administration studies of drugs administered to elephants, especially NSAIDs which have the potential to be administered frequently and sometimes for long periods of time.[174,297-303] [153]

Firocoxib has been evaluated extensively in horses and domestic dogs with pharmacokinetic, pharmacodynamic, COX-inhibition, and efficacy studies for specific disease conditions.[275,313,315-321] The reported adverse effects in all species appear to be minimal.[322-326] The only complication noted with the study was an apparent aversion to the firocoxib paste formulation by both elephants receiving the paste as perceived by those elephants individual keepers. There were insufficient elephants in this study to determine if that aversion was due to taste or consistency of the paste, however, the assessment of the elephant care staff of those

elephants was that that it may have been consistency of the paste, with observations of slow chewing and moving of the food item around in the elephants mouth as if to investigate the texture when the drug was administered. All 4 elephants receiving tablets readily consumed the tablet with a food item such as apple, peanut butter sandwich, or banana with no observed aversion to the tablet. This study represents the first attempt to evaluate pharmacokinetics in African elephants. Unfortunately, this is an incomplete drug study, lacking sufficient numbers to prevent estimation of drug parameters and lacking an i.v component to allow accurate determination of both clearance, bioavailability, and volume of distribution. Because of that, the information presented here should be considered pilot data only providing suggestions for drug administration, however that may change with information gathered from a larger study.

The variation identified between both formulations, with tablets having nearly twice the AUC (1588+/-362 vs 814 h*ng/ml for tablets vs paste), higher relative half-life (66.4 +/-59.2 hr for tablets vs 36.4 hr for paste), higher MRT (72.2 +/- 25.7 hr vs 28.86 hr) and differences in apparent volume of distribution (200% vs 50%) could be related to the perceived aversion of the paste formulation and decreased total drug intake. With only two elephants in that portion of the study it was not possible to determine reliable standard deviations or to gather more information as to if those drug differences are real or not.[327] The differences in study number and variation in parameters evaluated makes a comparison of tablet form to paste form unreliable with this data.[328,329] A larger study with more elephants receiving both drug forms is needed to accurately compare and contrast the pharmacokinetics of the tablet form vs the paste form.[328,329]

Care should also be taken when attempting to extrapolate information and compare pharmacokinetics of the same drug between species as there may be subtle physiological differences that can create large variation in drug metabolism. The pharmacokinetic variables in

this study were lower than those determined in a similar domestic equine study[275] While elephants are a hind gut fermenter like a horse, they have relatively short intestines for body size and rapid gut transit times, which lead to lower digestibility coefficients and a much higher amount of undigested food passing through compared with those in domestic hoofstock[22] This higher percentage of undigested food could negatively affect drug absorption from the gastrointestinal tract. Intravenous studies are needed to further explore that as a possible reason for lower serum concentrations.

Despite shortcomings, there is clinically relevant information that can be gathered from this study. First is that the firocoxib tablets were readily consumed by the elephants. Palatability for medications is very important in zoological medicine.[4,25] A drug has little clinical value if it cannot be successfully administered to the patient consuming it. Second firocoxib appears to have a long half-life. This means once a day dosing, or even every other day dosing is possible, depending upon the dose and clinical presentation of a patient. This can be advantageous with elephants that have an aversion to taking medications. Further evaluation is needed to determine parameters for specific dosing intervals and dosing rates beyond what is in this study. Both the tablet and the paste formulation has a higher T_{max} than a domestic horse does, with the tablet having 6.36+/-1.79 hr and paste having 7.0 +/-1.41 hr versus respective referenced domestic horse values of 3.2 +/- 0.9 hr for tablets 1.17 +/- 0.41 hr for paste in domestic horses.[275] This longer time to T_{max} indicates that firocoxib administered orally may not be the best way to administer this drug to elephants with acute, painful injuries. The lower threshold of drug effect is not known in this species, however that longer time indicates that administration of firocoxib utilizing the iv formulation may be more appropriate in the event of acute perceived painful

injury requiring therapy. Further studies must be done to fully evaluate the i.v. formulation in addition to the tablet and paste oral formulations.

4.3 Assessment and Conclusions of this study

This study represents the shortcomings of pharmacokinetic studies performed in zoological medicine including low sample size, with 4 or fewer participants, and resultant possible limitation on parameters or variation in parameters that cannot be explained with the data gathered.[4,25,330-332] Only having 2 participants receiving firocoxib paste can result in sampling error if those two are outliers to the larger African elephant population. Not being able to collect i.v. data in this study limited important pharmacokinetic parameters, like elimination half-life which will have an effect on determination of both dose and dosing regimen when attempting to determine a safe and efficacious dose of this drug to administer to African elephants.

That said, this study also can serve as preliminary data to better shape studies to further characterize the pharmacokinetics and pharmacodynamics of firocoxib in African elephants. Knowing the relatively long disappearance half-life, the slower time than domestic horses to T_{max}, and plasma concentrations can serve to adjust dosing times and rates with the next study as that information is correlated with an i.v. study. It must be noted that there was a distinct aversion to i.v. administration of any drug and an aversion to multiple blood collection times in a 24 hour period with the African elephants during this study. The concern over ear sloughing due to accidental extravascular administration of a drug was cited by 3 institutions approached to participate in this study. This aversion was despite the firocoxib formulation intended to be used was a drug approved for i.v. administration in domestic horses (Equioxx® injectable, Merial

Inc, Duluth, GA 30096-4640 USA) However, in the concurrent Asian elephant study, blood was collected from rear leg veins on multiple participants. The median saphenous vein can also serve as a vein i.v. access that apparently was not recognized by the African elephant keepers or attending veterinarians.   Suggesting the use of a rear leg vein may provide an alternate access point allowing a more thorough follow up study.

The apparent paste aversion noted in two of the elephants in this study is concerning. Not only did it potentially affect the pharmacokinetic parameters evaluated, it also paralleled the apparent aversion to paste identified in a concurrent flunixin meglumine paste pharmacokinetic study. There appears to be sufficient evidence based on these two studies to consider a more thorough evaluation of elephant's response to taste and/or texture of items offered for oral consumption. If a true aversion to a paste texture is identified that will limit other drugs formulated for domestic horses that are available in paste form (such as phenylbutazone).

Figure 11

Graphic depiction of drug concentration after repeated doses at a drug dosing interval that is shorter than the time it would take to repeatedly eliminate the drug from the orgamism. As doses are repeated serum or plasma drug concentration will increase as the drug accumulates in the system until it reaches the steady state point, or the point where overall drug intake is at equilibrium with drug elimination. Note while it takes 3-5 half lifes for a drug to reach steady state, it will also take 3-5 half-life periods after the last dose for a drug to clear an organism's system. Depending upon the individual NSAID, this steady state level is the goal for therapeutic management of patients with chronic diseases. Ideally the peak should remain below a toxic level and the trough should remain or only spend a minimal amount of time below therapeutic concentrations.

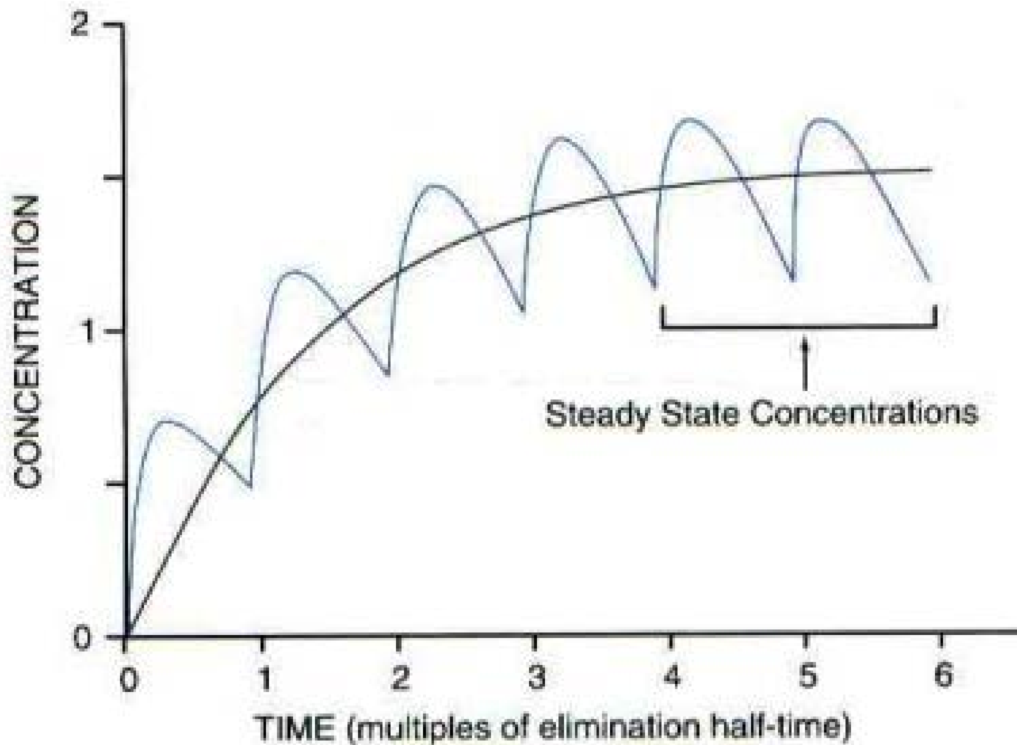


Table 4

Pharamacokinetic parameters for firocoxib after single oral dosing of firocoxib (0.1 mg/kg) tablets (n=4) or paste (n=2) in African Elephants . The elimination rate constant (λ) and half-life were reported as disappearance rate constant and disappearance half-life. Disappearance half-life was reported as harmonic mean + pseudostandard deviation. Both clearance (CL) and volume of distribution (Vd) are reported uncorrected for bioavailability (F). Other parameters included addition mean residence time (MRT) and the percent of the AUC that was extrapolated from the terminal component of the curve. The relative bioavailability (percent) of firocoxib was calculated based on the ratio of the AUCs using the formula $(AUC_{paste}/Dose_{paste})x(Dose_{tab}/AUC_{tab})$.

	Dose Form: Tablets N=4			**Dose Form: Paste N=2**	
Parameter	**Mean**	**Standard Deviation**		**Mean**	**Standard Deviation**
AUC%extrap (%)	23.58	+/-14.86		4.60	n
AUCinf (h*ng/mL)	1588	+/-362		814	n
Clss/F (ml/min/kg)	88.1	+/-2.17		132	n
Cmax (ng/mL)	31.3	+/-6.60		44	+/-12.5
Cmin (ng/ml)	3.23	+/-1.98		1.79	+/-1.40
Disappearance Half-life (hours)	66.4	+/-59.2		36.4	n
Dissappearance λ (1/hour)	0.008	+/-0.007		0.019	n
MRT (hours)	72.2	+/-25.7		28.9	n
Tmax (Hour)	6.36	+/-1.79		7.00	+/-1.41
Vd/F (mL/kg)	10926	+/-7254		6925	n
$F_{relative}$ (%) Paste:Tablets				50%	

Figure 12

Drug serum concentration versus time graph for a single dose of (0.1 mg/kg) of firocoxib paste administered to 2 African elephants. The maximum concentration detected (C_{max}) was 44+/-12.48 ng/ml detected at time (Tmax) 7.00+/- 1.41 hours. The last detectable serum concentration identified at 48 hours post drug administration was 2.67 ng/ml.

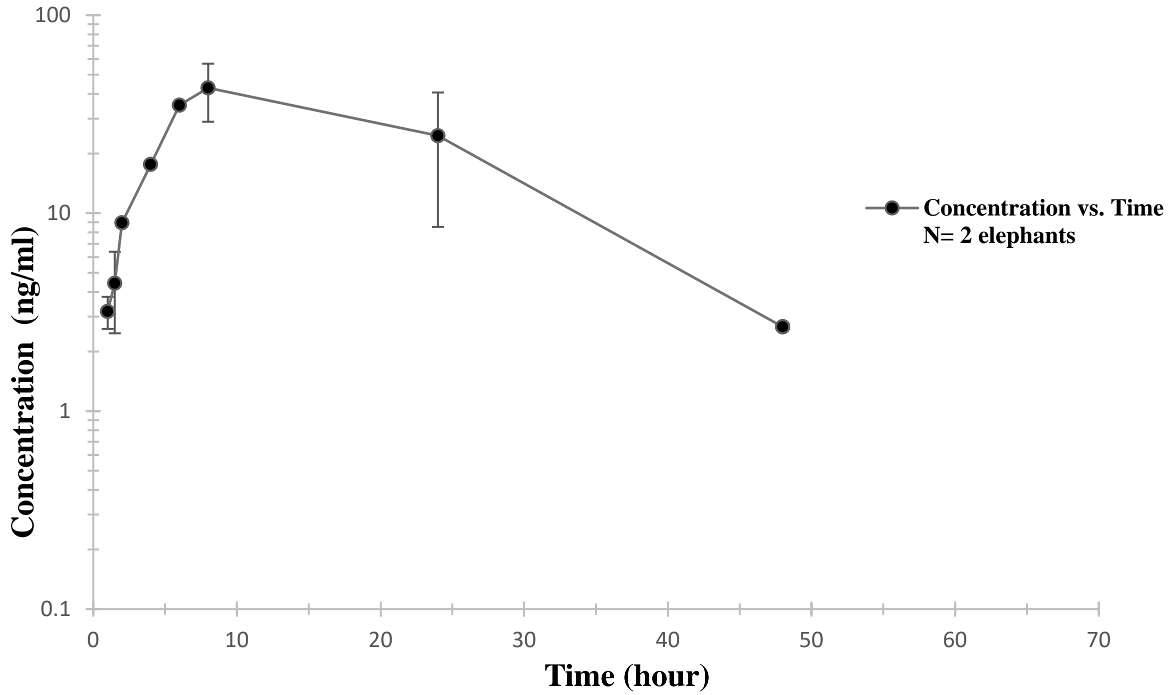
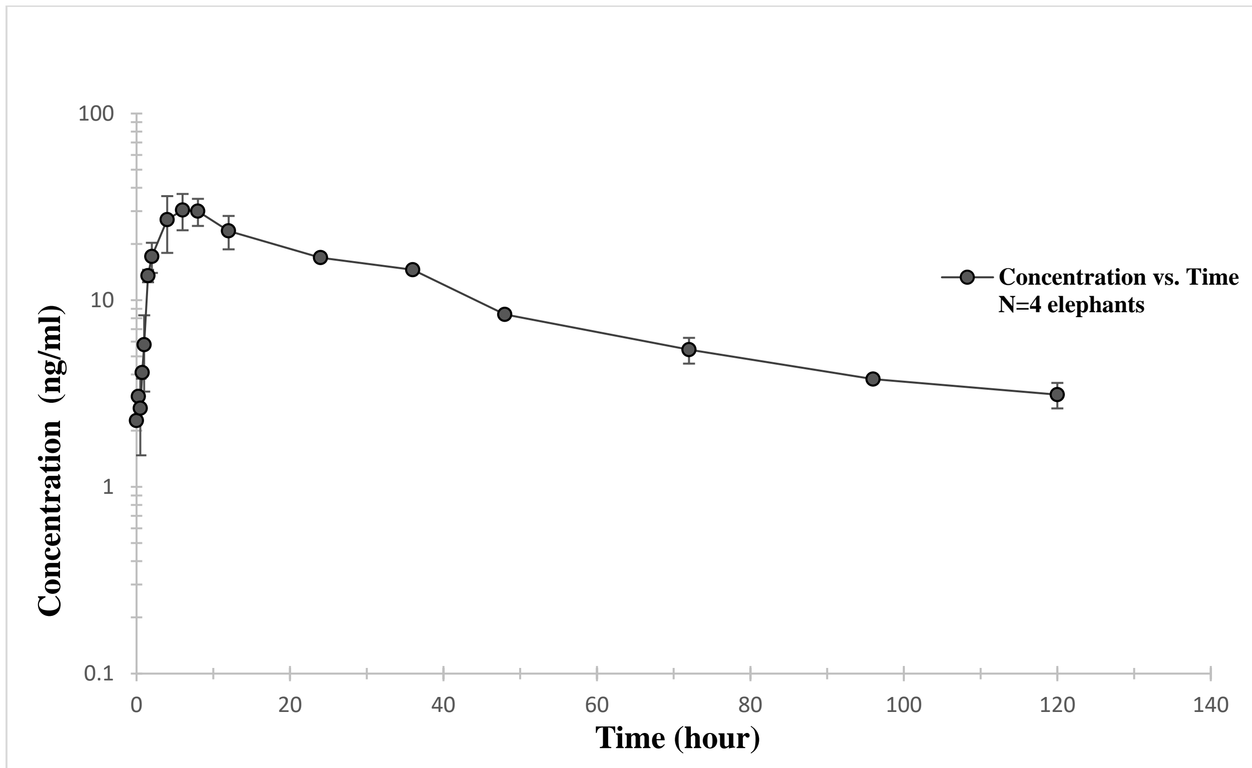


Figure 13

Drug serum concentration versus time graph for a single dose (0.1 mg/kg) of firocoxib tablets administered to 4 African elephants. The maximum concentration detected (C_{max}) was 31.3+/-6.6 ng/ml detected at time (T_{max}) 6.36+/- 1.79 hours. The serum concentration at 120 hours post drug administration was 3.12+/-0.49 ng/ml.



Chapter 5

Pharmacokinetics of Single Oral and IV Dose and Multiple Oral Dosing of Firocoxib in Asian Elephants (*Elephas maximus*)

Background information: parallels of humans, domestic animals and zoos

Drugs are a fundamental means of managing disease, pain, and other medical conditions that contribute to the quality of life of all animals. We are progressing beyond the single i.m. dart delivered or single oral dose pharmacokinetic study as the only form of drug study done in zoo animals.[25] Drug development in humans and animals alike undergo a series of clearly defined steps from initial theory to FDA drug approval. .[333,334] The initial development phase is performed to characterize the drug molecule.[334] As a suitable drug candidate advances, more extensive and costly registration phase studies are then conducted. [333,334] All animal health drugs (excluding medical devices) are required to be approved by an appropriate regulatory agency prior to marketing.[334] In the United States, this authority is the US FDA Center for Veterinary Medicine. The goal is to ensure quality, safety, and effectiveness.[333,334] These stages are composted of toxicology studies, following the FDA Center for Veterinary Medicine and the Veterinary International Conference on Harmonization guidelines, including studies on target animal safety, genetic toxicology studies, 90-day chronic rodent studies, 90-day chronic nonrodent studies, two-generation reproduction studies in rats, and a teratology study.[334] These studies must be completed and evaluated before a drug can be approved for a specific species. [333,334] The total time may take 5 to 15 years or more and cost more than $100 million US dollars to advance an animal health drug from initial concept to market. [334]

This process is problematic in zoo animals due to limits on both animal and species availability, funding, and the lack of a positive economic outcome relative to the economic investment by a company pursuing drug approval. The economics alone, combined with the time investment, means that drugs specifically approved for zoo animals are not likely to happen. Luckily, drugs are available for zoo veterinarians to prescribe patients in the US since clinicians currently may utilize drugs approved for both humans and animals under the Animal Medicinal Drug Use Clarification Act (AMDUCA), and extrapolate their use and indications from domestic animals and humans to non-approved species.[110] This availability of drugs to prescribe does not mean that drug studies should not be done. We have entered a world of polypharmacy in zoos as captive populations are living longer, capture of wild animals is being limited, attempts to prolong life for conservation are occurring and unique species-specific concerns are identified.[25,128] The elucidation of drug toxicity seen in nondomestic species and associated difficulties with attempting to extrapolate doses between species is another driving force to not randomly prescribe drugs that have not been evaluated in some way in the species being treated and to carefully consider downstream effects of drugs in the environment as those could directly impact zoo animals administered a drug therapeutically.[325 335,336] More thorough evaluations of drugs, including i.v., single dose administration, multiple dose administration, and information developed from careful therapeutic monitoring along with evaluation of perceived efficacy, *in vitro* and *ex vivo* pharmacodynamic studies can all be utilized to develop safer and more effective treatment regimens. The elephant studies in this chapter will be submitted to the Journal of Zoo and Wildlife Medicine for publication.

5.2 Pharmacokinetics and pharmacodynamics of IV, Single Dose, Multiple Dose and Therapeutic Monitoring of Firocoxib to Asian Elephants to be submitted to the Journal of Zoo and Wildlife Medicine

5.2.1 Abstract

Background/Purpose: To provide a recommended dosing regimen for firocoxib in Asian elephants (*Elephas maximus*) based on pharmacokinetics determined after single dose iv, single dose oral multiple doses (8 consecutive) with paste or tablet formulations and to determine efficacy based on therapeutic monitoring in clinical patients.

Animals: All elephants were healthy adults for the following trials: i.v: 3 elephants, 4 elephants single dose oral paste, 6 elephants single oral dose tablets, 4 elephants for multiple dose paste, 6 elephants for multiple dose tablets. Therapeutic monitoring elephants were 5 adults that had been receiving firocoxib for a minimum of 90 days consecutively

Methods: Doses administered were as follows: i.v.- 0.1 mg/kg, oral single dose: 0.01 mg/kg and 0.1 mg/kg. multiple dose trial consisted of 8 consecutive 0.1 mg/kg doses administered 24 hours apart. Therapeutic drug monitoring consisted of repeated evaluation of peak and trough levels for elephants receiving 0.85 to 0.1 mg/kg tablets orally.

Results: Firocoxib administered at 0.01 mg/kg had serum levels at or below the LOD of the assay. Firocoxib administered at 0.1 mg/kg had a long half-life with all dosing regimens examined. Paste appeared to be absorbed more quickly and reach higher serum concentrations after both single and repeated dosing. Therapeutic monitoring serum levels were comparable to the serum levels of the multiple dose trial.

Conclusions: Firocoxib administered at a dose of 0.01 mg/kg is not an appropriate dose for African elephants. Firocoxib administered at a dose of 0.1 mg/kg to African elephants results in serum levels comparable to domestic horses, including comparable peak serum concentrations and long half-life. While study numbers were low, there is sufficient evidence to suggest that 0.1 mg/kg is an appropriate dose for firocoxib in Asian elephants.

5.2.2 Introduction... X

Arthritis and foot diseases are well documented in captive elephants.[297-303] There are published reports citing incidence as high as 70% of Asian and African elephants in captivity in North America as being affected by musculoskeletal disorders, including trauma, arthritis, and foot lesions. A 2019 study reported a high incidence of lameness/stiffness (62%:136/220) and foot lesions (59%:129/220) identified through the evaluation of individual medical records of Asian and African elephants housed in AZA accredited zoos in the United States.[297] In 2006, a survey of 78 zoological institutions accredited by the Association of Zoos and Aquariums (AZA), 33% of the responding institutions reported having at least once case of elephant foot pathology annually.[301] Not only that, but 18% of the Asian elephants in the AZA survey had been diagnosed with arthritis.[301] This condition appears to be directly associated with age, with arthritis being significantly more common in older elephants.[297,299,304]

Attempts have been made to reduce foot pathologies, arthritis, and other orthopedic pathologies by facilities housing elephants by focusing on regular, routine foot and nail care, adjustments of exercise plans, and changes in flooring substrate.[297,301,302,305,306] Obesity also appears to be an important factor when assessing the development of arthritis in captive elephants housed in native environments.[297,302,305] As a preventative measure against these

pathologies and to alleviate the potential discomfort due to concrete substrates, some zoological institutions have renovated elephant housing to increase the amount of natural or shock-absorbent substrates.[305] When provided with natural substrates in captivity, elephants appear to benefit from improved foot and joint health thought to be due to increased blood flow, along with associated filing of the nails and foot pad, and increased movement of foot muscles, tendons and joints.[301,305]

Despite the changes in husbandry, arthritis, foot problems and other painful conditions still occur in captive elephants. One of the first steps for effectively managing discomfort with medications in patients of any species is administration of drugs at species-appropriate doses and dosing intervals to control both pain and inflammation.[153] While online and published formularies exist, there is an extremely limited number of scientific studies performed on the pharmacokinetics of analgesics in elephants.[153] In addition, many published doses in both formularies or case reports for elephants are not based on actual pharmacokinetic studies but instead are extrapolated from doses administered in domestic horses.[153] A 2016 survey identified Nonsteroidal Anti-inflammatory Drugs (NSAIDs) as the most commonly administered pharmaceuticals for analgesia in elephants, however there was substantial variability in dosing regimens and reported efficacy between and within facilities participating in that survey.[153] .....

The analgesic and anti-inflammatory effects of NSAIDs are considered to be due to the drugs inhibition of specific cyclooxygenase (COX) isoenzymes. These enzymes act to inhibit the cyclooxygenase (COX) isoenzymes COX-1 and COX-2.[212,213] The COX-1 isoform is constitutively expressed in the body and is considered responsible for normal homeostasis, including platelet function, gastrointestinal mucosal integrity and platelet activation. [180,181] The COX-2 isoform is primarily induced in response to lipopolysaccharide and cytokine stimulation

and is considered mainly responsible for the synthesis of proinflammatory prostanoids.[180,181,129,229,307] Firocoxib is generally characterized as a COX-2 selective NSAID because it tends to inhibit COX-2 while exerting a sparing effect on COX-1.[213,255] Cox-2 selectivity is thought to minimize negative physiological effects, including renal perfusion, gastrointestinal mucosal integrity and platelet activation associated with either COX-1 exclusive or predominately COX-1 inhibiting drugs.[213] Firocoxib appears to have a COX-2 preference in Asian elephants (See Chapter 3). It is important to understand that despite this selectivity, it is possible that adverse side effects associated with COX-1 inhibition may still be observed with highly selective COX-2 inhibitory drugs as the degree of inhibition of both isoenzymes is dependent on the degree of specific drug COX-selectivity.[213] In addition to that, it is known that COX-2 isoenzymes are expressed constitutively in the duodenum of some species and also contribute to renal blood flow and function, so their inhibition may also contribute to adverse events observed post drug administration.[202,309] .... This study was approved by the Institutional Animal Care and Use Committee of Auburn University, Auburn, Alabama (Protocols: 2015-2609 and 2018-3224) and the Animal Care and Use Committee and Research Committees of each participating institution housing the elephants. The goal of this study was to determine a recommended dosing regimen for firocoxib based on pharmacokinetic studies in captive Asian elephants through individual single dose i.v. administration, single dose oral administration and multiple dose oral administration pharmacokinetic studies. These results were compared with therapeutic drug monitoring (TDM) evaluations of elephants having received firocoxib long term (>3 months) for management of orthopedic or other sources of chronic pain as an attempt to determine real world perceived efficacy and viability of use of this drug for pain management in captive Asian elephants.

5.2.3 Materials and Methods

5.2.3.1 Animals

All Asian elephants (n=15) in these studies were from North American institutions housing captive Asian elephants. Elephants (n=10, 4 males and 6 females) for the pharmacokinetic studies (i.v., single dose, and multiple dose) were 12 to 43 years in age (median 28 years). They were chosen based on the criteria of being at least 10 years old, having no signs of illness based on clinical history, physical examination, and routine CBC and serum chemistry profiles, and having not received any other medications for at least a 30 day period prior to the start of each phase of the study. Elephants (n=5, all female) that were part of the long-term TDM portion of this study ranged in age from 31 to 65 years of age (median 42 yrs). These animals were receiving firocoxib, and no other drug, for at least 90 days prior to study for treatment of chronic conditions associated with osteoarthritis or other joint conditions.

5.2.3.2 Drug Administration and Sample Collection

For the pharmacokinetic studies, elephants were chosen randomly to receive paste or tablets for single dose studies. The dose form received (ie paste or tablets) for the single dose study remained as the same dose form for the respective multiple dose studies. Six elephants (2 males and 4 females) were administered firocoxib orally in tablet form (Previcox® tablets, Merial Inc, Duluth, GA 30096-4640 USA) at a dose of 0.01 mg/kg and then following a minimum 30 day washout, 0.1 mg/kg. One elephant received Firocoxib paste (Equioxx® paste, Merial Inc, Duluth, GA 30096-4640 USA) orally at a dose of 0.01 mg/kg. Four elephants (2 males and 2 females) received firocoxib paste at a dose of 0.1 mg/kg orally for single and

multiple dose studies. The three elephants (2 males and 1 female) in the i.v. portion of the study received the commercially available equine injectable formulation (Equioxx® injectable, Merial Inc, Duluth, GA 30096-4640 USA). After a minimum washout period of 90 days multiple dosing occurred at a dose of 0.1 mg/kg. The canine tablet formulation (Previcox®) was utilized for single and multiple dose studies because the equine tablet formulation had not yet been released to the U.S. market at the beginning of the study. For the TDM study, all 5 elephants received firocoxib (Previcox® tablets Merial Inc, Duluth, GA 30096-4640 USA) in tablet form at doses ranging from 0.58 mg/kg to 0.1 mg/kg.

All oral doses of firocoxib were administered in the morning, prior to feeding the A.M. diet. Oral doses were administered with a food item (e.g, apple, bread, peanut butter, sweet feed) preferred by each participating elephant. Elephants were not specifically fasted prior to drug administration, although at least 10 hours had lapsed since fresh hay for overnight consumption was last provided. After drug administration, all elephants were offered their normal morning diet without restriction. For i.v. dosing, a 21-gauge butterfly catheter (Sureflo winged infusion set, Terumo Medical Products, Somerset, NJ 08873, USA). was placed in an ear vein and the drug was administered undiluted. A minimum of 10 mls sterile saline was used to flush the catheter post drug administration prior to catheter removal.

Ten ml of blood was collected from an ear vein or the median saphenous vein at each sample collection time point using a 21-gauge butterfly catheter (BD Vacutainer Safety-Lok, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA, or Sureflo winged infusion set, Terumo Medical Products, Somerset, NJ 08873, USA). Samples were placed into glass tubes and centrifuged for 10 min at 2500 rpm. Because firocoxib can bind to plastic of storage tubes, serum was decanted into glass vials and kept frozen at -20C. until time of

analysis.[310] Blood collection occurred at the following times for i.v and single dose studies: -5 minutes, 15, 30, and 60 minutes, 1.5 2,4,6,8, 12, 24, 48, 72, 96, and 120 hours post drug administration.

At least 90 days lapsed between the final single oral dose study and the multiple dose study. For the multiple dose study, firocoxib was administered every 24 hrs for 8 consecutive doses. Blood was collected at the following times: -5 minutes and 9, 24, 33, 48, 57, 72, 96, 120,144, 240, 246, 252, 264, 273, 288, 312, 336, 360, 385, and 408 hours post the first dose. This resulted in collection times to 168 hours post administration of the 8th and final dose. These times were specifically chosen to allow for sample collection times that coincided with elephant care-takers schedules within participating facilities. The samples collected at 72, 96, 120, 144, and 168 hours post the first dose represent trough times.

Blood, and if possible, urine was collected for complete blood count (CBC), serum chemistry analysis, and urinalysis as by routine procedures for each participating institution before and after each drug administration trial.

5.2.3.3 Drug Analysis

Elephant serum was analyzed for firocoxib concentrations by high pressure liquid chromatography (HPLC) with ultraviolet (UV) detection based on previously described methodologies.[311-313] The HPLC system consisted of a Waters 600 Controller (pump), a Waters 717 Autosampler, and a 2487 UV-Visible detector (Waters Corporation™, Milford, MA, USA). Separation was achieved with a Luna PFP (2), 5 µm, 150 x 4.6 mm column (Phenomenex®, Torrance, CA, USA) maintained at 40 °C. The mobile phase consisted of 45:55:0.025 acetonitrile/water/trifluoroacetic acid (VWR®, Radnor, PA, USA) with the flow rate set to 1

mL/min. The standard curve was generated ranging from 2 to 100 ng/mL by fortifying Asian elephant serum with known amounts of firocoxib (Toronto Research Chemicals Inc. (TRC), Toronto, Ontario, Canada) reference standard and accepted if the coefficient of determination (r^2) was at least 0.999 and the predicted concentrations were within 10% of the actual concentrations. Firocoxib was extracted from elephant serum with solid phase extraction (SPE) cartridges (Waters Oasis® HLB 3 cc, 60 mg (Waters Corporation™, Milford, MA, USA). Briefly, previously frozen serum samples were thawed and vortexed, then mixed with water that contained 5% acetic acid and was subsequently vortex. The SPE cartridges were conditioned with acetonitrile followed by water. The aqueous serum samples were loaded and allowed to elute by gravity. The cartridges were rinsed with water that contained 5% acetic acid, and then with a water: methanol (75:25 v/v) solution. Vacuum of ~10 in of Hg was used to remove the residual solvent. Firocoxib was eluted with acetonitrile which was then evaporated to dryness under a stream of nitrogen. The residue was reconstituted with 40: 60 acetonitrile: water that contains 5 % acetic acid (v/v), vortex/mixing, and then the solution was centrifuged at 16000 x g at room temperature. The injection volume was 100 µL. The retention time for Firocoxib was 9.8 min and UV absorbance was monitored at 290 nm.

The linear correlation coefficient for firocoxib in Asian elephant serum was 0.998. The limit of detection (LOD) was 1 ng/mL and the lower limits of quantification (LLOQ) was 2 ng/mL. The Precision (RSD%) for firocoxib in Asian elephant serum at concentrations of 2, 20, 50, and 100 ng/mL were 16.69%, 4.89%, 5.88%, and 3.04% respectively. The Accuracy for firocoxib in Asian elephant serum at concentrations of 2, 20, 50, and 100 ng/mL were 113.59%, 99.07%, 99.09%, and 101.38% respectively.

5.2.3.4 Pharmacokinetic Analysis

Serum firocoxib concentration versus time data was subjected to noncompartmental analysis using computer software (Phoenix WinNonLin V7, Pharsight, Cetara). Area under the curve to infinity (AUC_{inf}) and were determined using the log-linear trapezoidal method. The extrapolated maximum dose (C_0) after i.v. administration was based on non-linear regression and for oral administration, the maximum concentration (C_{max}) occurring at time to maximum concentration (T_{max}) were determined directly from serum concentrations after oral dosing. The lowest concentration at the last time point collected (C_{last}) were also recorded. The slope of the terminal component of the drug-elimination time curve was based on non-linear regression. For the i.v. administration, the elimination rate constant (λz) and half-life T1/2 λz, clearance at steady state (CLss) and volume of distribution (Vd) were reported. For oral administration, the ratio of volume of distribution or clearance to oral bioavailability were also reported (Vdss/F and CLss/F). For animals receiving drug i.v. the absolute bioavailability was calculated using the formula Fabs = 100 (AUCpo*Div/AUCiv*Dpo) where D is the dose following oral (po) or i.v. administration. The relative bioavailability for tablets versus paste was calculated using the formula Frel= (AUC Da *Db/AUC Db*Da). Other parameters included addition mean residence time (MRT) and the percent of the AUC that was extrapolated from the terminal component of the curve were also recorded.

Multiple dose pharmacokinetic analysis was divided into a part "A" which involved samples taken during drug administration to a point 168 hours post first dose administration. Part "B" consisted of analysis of samples collected after the 8th dose was administered to a point 336 hours after the first dose was administered. The AUC_{last} or the area under the curve to the last collection point at administration of the last dose, and AUC_{inf} for part B were determined

using the were determined using the log-linear trapezoidal method. The maximum concentration (C_{max}) occurring at time to maximum concentration (T_{max}), and the average serum concentration observed (C_{av}) were determined directly from serum concentrations after oral dosing for both phase A and B. The elimination rate constant (λz) and the ratio of volume of distribution or clearance to oral bioavailability were also reported (Vd/F and CLss/F). The terminal half-life (T1/2 λz) was reported for part B, after the administration of the last dose. The relative bioavailability (F) was determined for both drug formulations in both parts A and B. Other parameters including mean residence time (MRT) and the percent of the AUC that was extrapolated from the terminal component of the curve were also recorded for part B

Mean peak and trough +/- standard deviation values were determined for the TDM elephants. Attending veterinarians and elephant keepers were asked to rate perceived efficacy of effect in the elephants treated with the following scale: 0 (no effect) 1 (minimal noticeable alleviation perceived discomfort), 2 (moderate noticeable alleviation of pain/discomfort observed) 3 (good alleviation of pain/discomfort observed) or 4 (excellent effect, all signs of pain resolved).

Pharmacokinetic parameters were reported out as mean and standard deviation with 95% confidence intervals. Statistical analysis was performed using Systat v13.2 (Systat Software, Inc. San Jose, CA 95131 USA) Normality was assessed using a Shapiro-Wilk's test. The student's paired t-test was used to assess significant differences (P=0.05) between T1/2, Cmax, Tmax, and AUCinf between the single dose oral paste and oral tablet formulations.

5.2.4 Results

The serum drug concentrations determined from 6 elephants receiving tablets and one receiving paste at a 0.01 mg/kg dose were not consistently above the LOD of the assay to allow for statistical analysis, including pharmacokinetic modeling. As a result of this initial analysis, further trials with the dose of 0.01 mg/kg were suspended.

Two elephants originally planned to receive the paste form of firocoxib at the 0.1 mg/kg dose withdrew from the study due to circumstances unrelated to the study. This reduced the number of animals receiving the paste at 0.1 mg/kg to 4. No elephant refused sample collection for any study. Drug concentration vs time for single dose i.v. administration are depicted in Figure 14, single dose paste administration are depicted in Figure 15, and for single dose tablet administration are in Figure 16. Time 0 results for all elephants for all single dose drug formulations was below the limits of detection of the study (2 ng/ml). Following i.v. administration of 0.1 mg/kg, firocoxib had an AUC_{inf} of 1444 +/- 639 h*ng/ml and a C_0 of 65 +/- 10.2 ng/ml. Firocoxib exhibited an eliminations half-life of 35.5+/-28.0 hour, a Vd of 4.57 +/-4.07 L/kg and Cl of 97.7+/-0.90 ml/h/kg after i.v. administration. Single dose oral tablet administration of a dose of 0.1 mg/kg had an AUC_{inf} of 1332 +/-878 h*ng/ml with a Cmax of 49.1 +/-3.27 ng/ml that occurred at a T_{max} of 5.71 +/- 3.91 hours. Half-life was 34.3 +/- 30.3 hours for oral tablet administration. Single dose oral administration of firocoxib paste at dose of 0.1 mg/kg had an AUC_{inf} of 1455+/-634 h*ng/ml with a C_{max} of 61.8 +/-14.7 ng/ml that occurred at a T_{max} of 4.08+/- 1.62 hours. Half-life was 19.9 +/- 12.7 hours for oral single dose paste administration. Relative bioavailability of tablets:paste was determined to be 92.3% This value was the same for absolute bioavailability of tablets. The absolute bioavailability of paste was 100%

Drug concentration vs time for multiple dose tablet administration are depicted in Figure 17, and for multiple dose paste administration are depicted in Figure 18. The AUC_{all} for part A was determined to be 6727+/- 2290 h*ng/ml for the tablet formulation and 7741+/-2696 h*ng/ml for the paste formulation. C_{max} for part A was 34.8+/-5.92 ng/ml at a T_{max} of 11.5+/-5.92 hours and 51.5+/-6.42 ng/ml at a T_{max} of 21.00+/-24 hours for paste. The C_{ave} for tablets during part A was 23.3+/-10.47 ng/ml while it was 42.3+/-8.71 ng/ml for paste. VD/F for part A for tablets was 214+/- 52.9 L/kg while VD/F for paste was 25.5+/-21.1 L/kg.

The AUC_{inf} for part B was determined to be 6341+/- 3003 for the tablet formulation and 5613+/-2262 h*ng/ml for the paste formulation. C_{max} for part B was 75.8+/-15.5 ng/ml at a Tmax of 176+/-2.86 hours after the first dose was administered and 95.5+/-29.3 ng/ml at a T_{max} of 177+/-3.33 hours for paste. The terminal half-life (terminal T1/2 λ) was 84+/-32.2 hours for tablets and 62.9+/- 2.25 hours for paste in part B. VD/F for part B for tablets was 8.45+/- 2.13 L/kg while VD/F for paste was 2,007+/-721 L/kg. CLss/F was 102+/-23 for tablets and 76+/-71 ml/h/kg for paste in part B. MRT_{inf} was determined to be 89+/-31 for tablets and 76+/-71 hours for paste in part B. Relative bioavailability (F) for tablets was determined to be 88.52% and for paste was 112.97%

The mean peak concentration 8.5 hours after drug administration for TDM elephants was 61 +/-12.5 ng/ml. Mean trough immediately before the daily dose was administered were 40+/-7.64 ng/ml. The mean perceived efficacy of effect was 3.6 with a range of 3-4 for all elephants. No perceived efficacy was lower than a 3.

5.2.5 Discussion ....

There is a well documented lack of pharmacokinetic studies in captive elephants.[4,109] Many published studies have focused on antimicrobials, with those of NSAIDs having been single dose only studies.[4,109] Dosage regimens in elephants have traditionally been based upon single dose studies or extrapolated from the assumption that pharmaceuticals administered to elephants undergo the same absorption, distribution, metabolism and excretion as the domestic horse.[13,296,314] This association is presumed flawed because elephants have a fundamentally different form of liver metabolism compared to horses in that elephants produce bile alcohols while horses produce bile acids as the predominate means of cholesterol metabolism.[76,77] The specifics of these differences and how they may affect drug metabolism are not fully understood, but do demonstrate the need for single dose and long-term studies of drugs administered to elephants.

Following i.v. administration, firocoxib exhibited a large Vd (4.57+/-4.01 L/kg), slow clearance (97.7 +/- 1.90 ml/h/kg) and long elimination half-life (35.5+/-28 hours). The Vd and clearance were substantially different from those reported in domestic horses, however terminal half-life appeared comparable.[275,313,321] Firocoxib tablets administered as a single dose of 0.1 mg/kg exhibited a similar Vd/F (4.52+/-3.46 L/kg) and long elimination half-life (34.3+/-30.3 hours) as identified following i.v. administration. Interestingly firocoxib paste administered at 0.1 mg/kg had a much lower Vd/F than tablets of 2.06 +/-0.84 L/kg) and an elimination half-life almost half of that of both i.v. and firocoxib tablets.(19.9+/-12.8 hours). However, this difference in half-life was not statistically significant (p value 0.164). AUC_{inf} was comparable for a single dose of 0.1 mg/kg for i.v., (1444+/-639 h*ng/ml), tablets (1332+/-878 h*ng/ml) and paste (1455+/-634 h*ng/ml) . The oral paste achieved a C_{max} near that of the C_0 of i.v. administration (65.0+/_10.2 for i.v. vs 61.8+/-14.8 ng/ml for paste), which were both higher than tablets (49.1

+/-3.27), however this difference was not statistically significant (p=0.122). These values were slightly lower than those reported in domestic horses, however horses also exhibited the difference in C_{max} with paste having a higher concentration than tablets. [275] In domestic horses this difference was contributed to a bioavailability (F) which was also seen in the elephants in this study with firocoxib past having an F of 100.8% and tablets an F of 92.3%. The T_{max} for both single dose oral tablets and paste were comparable (5.71+/-3.91 vs. 4.08+/-1.62 hours) and this slight difference was not statistically significant (p=0.823)

Analysis of the multiple dose trials had to be divided into a Part A, which was the time during which sufficient doses (0.1 mg/kg every 24 hours) were submitted for firocoxib to reach a steady state level, and then Part B the time from last dose until the drug was eliminated from the body. Ideally the last sample should be near the LOD of the assay used for evaluation to minimize extrapolated information statistical analysis. The parameters reported for the multiple dose study differ from the single dose study due to accumulation and the drug reaching steady state. This is also reflected by the higher C_{max} (51.5+/- 6.42 ng/ml for tablets, 95.5 +/-29.3 ng/ml for paste) from Phase B. It needs to be noted that while samples were collected to a point of 336 hours, or 168 hours (7 days) following the last dose there was still detectable serum levels (13.43 ng/ml for tablets and 3.5 ng/ml for paste) at the time of last sample collection. This is a reflection of the long half-life of this drug, The long half-life is something that must be considered with therapeutic administration of this drug, as it provides for convenient once per day dosing, but it will likely not have an immediately observable effect, instead taking multiple doses or a loading dose to attain steady serum levels. Conversely in the event of an adverse event or some other reason to stop the drug, serum levels will persist for several days. The multiple dose study had a higher terminal half-life (84.4+/- 32.2 hours) for the tablet formulation than the

paste (26.8+/-2.25 hours), or the half-lifes observed in the single dose studies. Firocoxib undergoes hepatic metabolism and primarily fecal excretion with some associated renal excretion.[313] Asian elephants may engage in some level of enterohepatic recycling of firocoxib tablets, as has been previously reported for phenylbutazone. It seems unusual to see this only in the tablet formulation though, so additional research is needed to determine if there is something with that specific formulation that is not in the paste formulation that may contribute to enterohepatic recycling.

The C_{max} reported of firocoxib for both paste and tablets for Part A as the drug rose to steady state was lower than that for the single dose study, however that is likely not due to differences in drug dose, instead it is likely due to skew in reported values because the samples taken at times 68, 96, 120, 144, and 168 hours post initial dose administration were trough times. This was done to accommodate staffing of the participating elephant institutions and ensure minimum levels were stabilized at steady state.. The C_{avg} also observed is most likely skewed lower because of the collection of those trough samples without corresponding peaks. The C_{max} reported for Part B (75.8 +/-15.5 ng/ml for tablets and 95.5+/-29.3 ng/ml for paste), is a representation of C_{max} at steady state as that sample was taken after the administration of the 8th dose. Steady state was achieved by 96 hours (5 doses) for both doses based upon stabilization and lack of variation in trough doses observed. This corresponds with the long half-life observed with this drug. This C_{max} and steady state trough levels are also comparable to the mean peak (61 +/-12.5 ng/ml) and mean trough (40+/- 7.64 ng/ml) values identified in the TDM elephants. It is impossible to do pain/antinociception studies in animals like elephants, so assessment of pain drug perceived efficacy falls upon the attending veterinarian and elephant staff.[153,280] Unfortunately there has not yet to be a standardized pain score scale adapted for either African or

Asian elephants as there has been in domestic animals.[127,132,133,137,337] Instead the attending veterinarians and/or elephant care staff were asked to subjectively rank the perceived efficacy of firocoxib. The mean perceived efficacy of effect was 3.6 with a range of 3-4 for all elephants. No perceived efficacy was lower than a 3 signifying that firocoxib has a high perceived efficacy by veterinarians administering it to Asian elephants for conditions causing chronic pain such as osteoarthritis or pododermatitis.

No adverse events were reported in any of the elephants receiving firocoxib in this study. Routine laboratory tests (CBC, serum chemistry profile, and urinalysis) evaluated after each phase of this study had no identified abnormalities. It must be noted that one of the TDM elephants that participated in that portion of this study was euthanized over a year after samples were collected for reasons not related to the study. A complete necropsy was done per the elephant TAG/SSP guidelines with no abnormalities that could be associated with long term (>than 1 year) firocoxib administration were noted.

In conclusion of these studies, firocoxib has a very long half-life, with a relatively slow rise to steady state, which means multiple doses or possibly a loading dose is required to reach serum levels associated with therapeutic efficacy. This study has described the pharmacokinetics of single i.v., oral paste, oral tablets, multiple dose oral tablets, and oral paste, and TDM of animals receiving firocoxib, a drug that appears both safe and efficacious for long term management of painful chronic conditions in captive Asian elephants such as pododermatitis or osteoarthritis.

5.3 Conclusions and Assessment of Study

This study represents a thorough pharmacokinetic study, composed of i.v. single dose, multiple dose, and therapeutic monitoring of clinical patients. The study had two shortcomings,

one being its length to be this thorough spanned nearly 3 years. As a result, participating facilities were not always able to contribute throughout the entire study time. To perform this type of a study in the future at a participating zoo, This time frame must be made clear from the beginning to establish parameters for participating institutions. Factors such as season/time of year, staff changes, seasonal variation within the or elephant facility, or other factors need to be monitored and it must be realized that they can detrimentally harm a long term study ..

The other shortcoming of this study is the relatively low number of elephant participants. Some of the parameters evaluated, such as C_{max} for the multiple dose study, have a large SD which is likely a result of outliers in the small population (N=4). The inclusion of more animals should minimize outliers and normalize data sufficiently to decrease that variation between samples. Despite the low numbers, this study was fortunate that a complete and thorough necropsy was performed on the TDM elephant that was euthanized. The necropsy evaluation of animals in zoos receiving drugs as part of a drug study is not routine.

Table 5

Noncompartmental pharmacokinetic parameters for firocoxib in Asian elephants following single dose intravenous (N=3) administration and single dose oral administration of either tablet (N=6) or paste (N=4) formulation. Data is presented as Mean +/- Standard Deviation (SD).

	Single Dose (0.1 mg/kg) i.v. N=3		Single Dose (0.1 mg/kg) tablets N=6		Single Dose (0.1 mg/kg) paste N=4	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
AUCinf (h*ng/ml)	1444	+/-639	1332	+/-878	1455	+/-634
AUC % Extrap (%)	17.1	+/-10.15	13.8	+/-11.9	10.14	+/-8.33
Tmax (hours)	-	-	5.71	+/-3.91	4.08	+/-1.62
Cmax (ng/ml)	-	-	49.1	+/-3.27	61.8	+/-14.8
Co (ng/ml)	65.0	+/-10.2	-	-	-	-
λ (1/hours)	0.052	+/-0.06	0.035	+/-0.02	0.051	+/-0.04
T1/2 λ (hours)	35.5	+/-28.0	34.3	+/-30.3	19.9	+/-12.8
Vd (L/kg)	4.57	+/-4.07	-	-	-	-
Vd/F (L/kg)	-	-	4.515	+/-3.46	2.058	+/-0.84
Cl (ml/h/kg)	97.7	+/-1.90	-	-	-	-
MRT (h)	35.5	+/-28.0	72.222	+/-25.7	28.813	+/-14.1
relative F (%)		-	92.3%	-	100.8%	-

Table 6

Noncompartmental pharmacokinetic parameters for firocoxib in Asian elephants following multiple dose oral administration of either 8 consecutive doses of firocoxib tablets (N=6 elephants) or paste at 0.1 mg/kg (N=4 elephants) every 24 hours. Note A is pharmacokinetics up through the point of the last dose, or when firocoxib reached steady state, while B is the analysis of parameters from the last dose to the point of serum levels falling below detection limits of the assay or the last timepoint measured. Data is presented as Mean +/- Standard Deviation (SD).

	A		B		A		B	
	Multiple dose oral tablet N=6		**Multiple dose oral tablet N=6**		**Multiple dose oral paste N=4**		**Multiple dose oral paste N=4**	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
AUCall (h*ng/ml)	6727	+/-2290	-	-	7741	+/-2696	-	-
AUinf (h*ng/ml)	-	-	6341	+/-3003	-	-	5613	+/-2262
AUC % Extrap (%)	-	-	22.0	+/-11.2	-	-	3.39	+/-2.43
Tmax (hours)	11.5	+/-5.92	176	+/-2.86	21.0	+/-24	177	+/-3.33
Cmax (ng/ml)	34.8	+/-14.8	75.8	+/-15.5	51.5	+/-6.42	95.5	+/-29.3
Cavg (ng/ml)	23.3	+/-10.47	-	-	42.3	+/-8.71	-	-
λ (1/hours)	0.002	+/-0.001	0.009	+/-0.004	0.006	+/-0.004	0.026	+/-0.002
terminal $T_{1/2}$ λ (hours)	-	-	84.4	+/-32.2	-	-	62.9	+/-2.25
Vd/F (L/kg)	214	+/-52.9	8.45	+/-2.13	25.5	+/-21.1	2.01	+/-0.72
CLss/F (ml/h/kg)	-	-	102	+/-23	-	-	51.0	+/-20.6
MRTinf (h)	-	-	89.1	+/-30.7	-	-	75.7	+/-71
relative F (%)	-	-	-	112.97%	-	-	-	88.52%

Figure 14

Concentration versus time curve for 3 Asian elephants administered a single dose of 0.1 mg./kg firocoxib i.v..

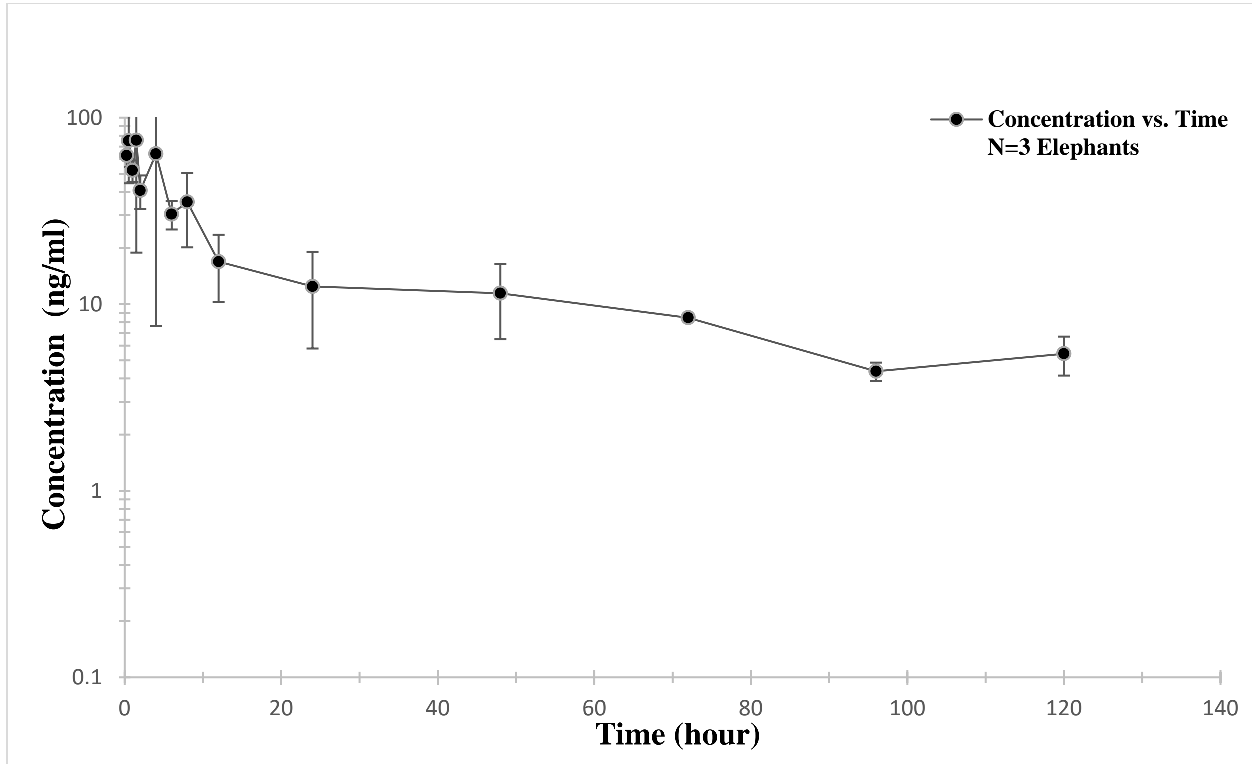


Figure 15

Concentration versus time curve for 6 Asian elephants administered a single dose of 0.1 mg./kg firocoxib tablets administered orally.

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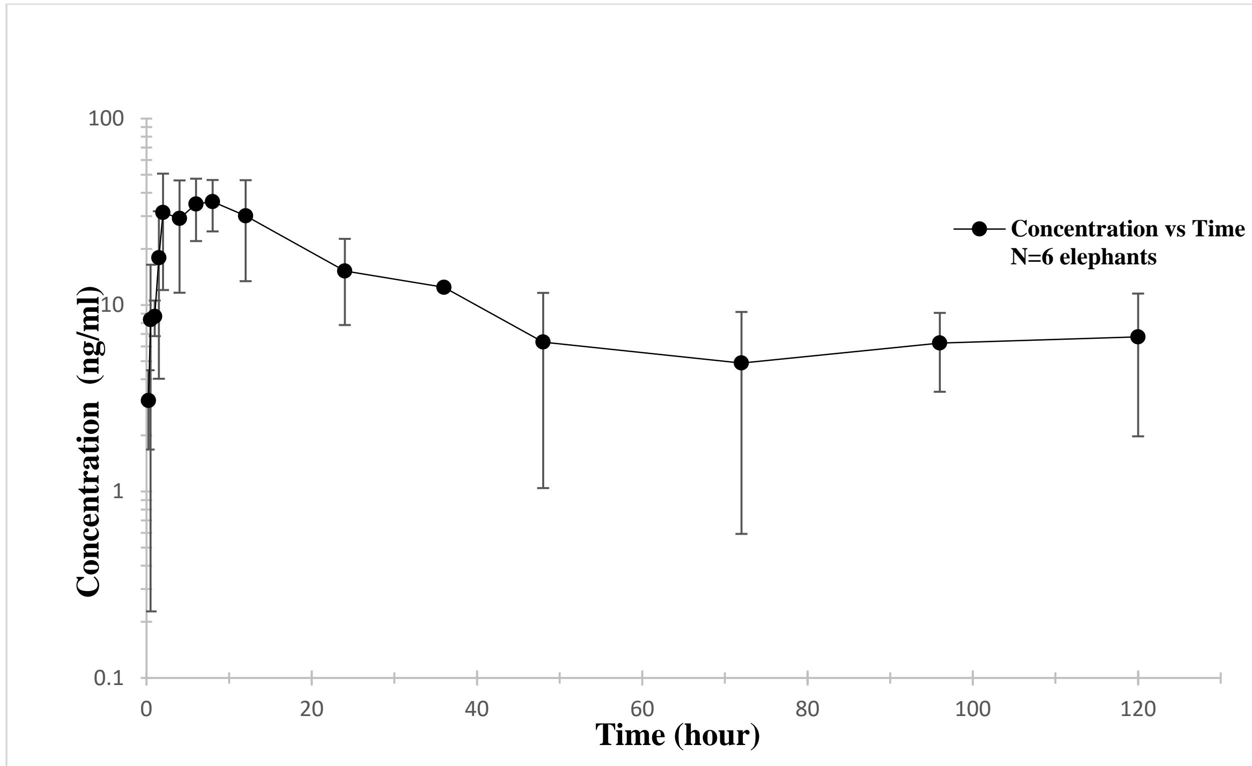


Figure 16

Concentration versus time curve for 4 Asian elephants administered 0.1 mg./kg firocoxib paste administered orally.

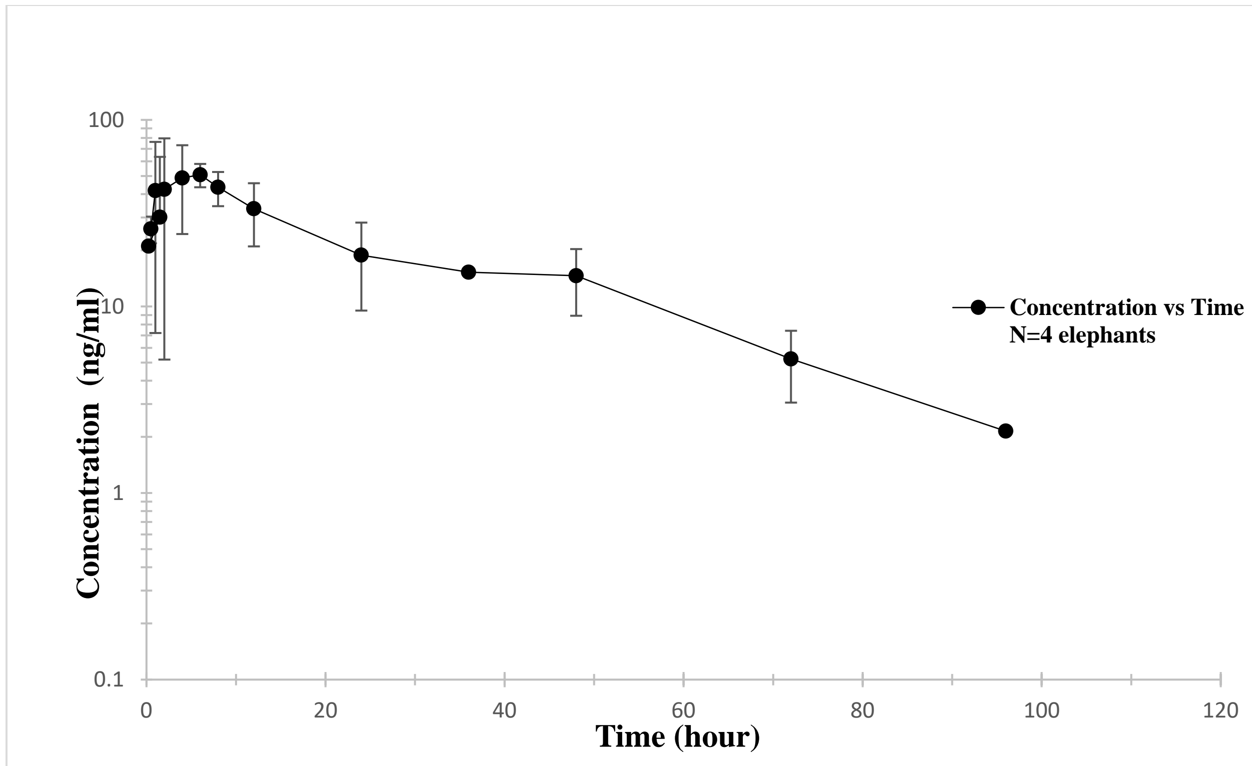


Figure 17

Plot of concentration versus time for 6 Asian elephants administered 0.1 mg/kg firocoxib tablets orally every 24 hours for 8 consecutive doses. The first sample represented was collected 9 hours post first dose administration. Times from 72 to 168 hours represent trough times, immediately before AM firocoxib dose was administered. The mean serum concentrations at these times ranged from 38.4 +/- 10.3 to 46.7+/- 13.8 ng/ml. Peak concentration achieved after the 8th doses (174 hours) was 70.2 +/- 14.3 ng/ml. The serum drug concentration at the last measured sample was 13.4 +/- 5.9 ng/ml

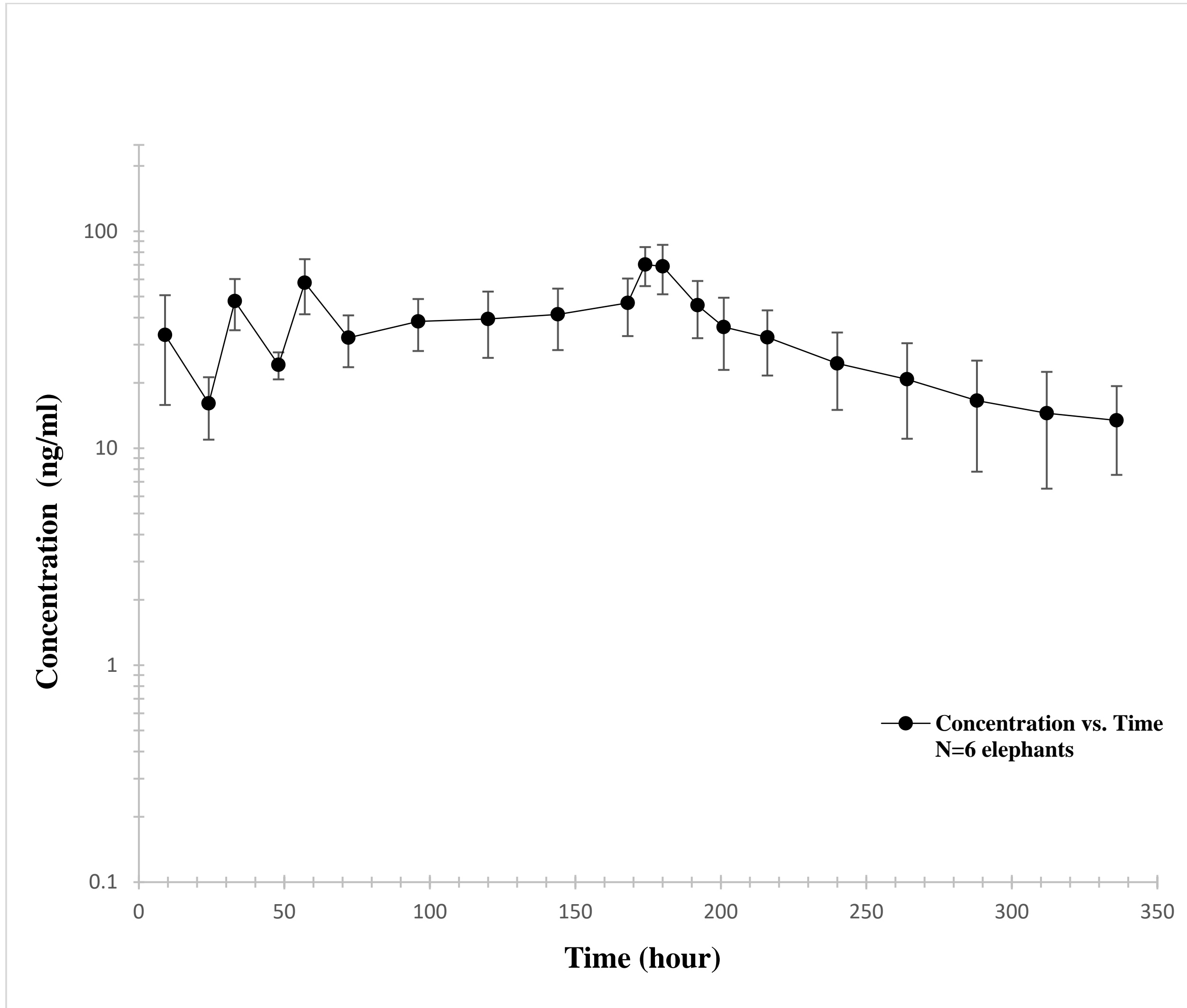
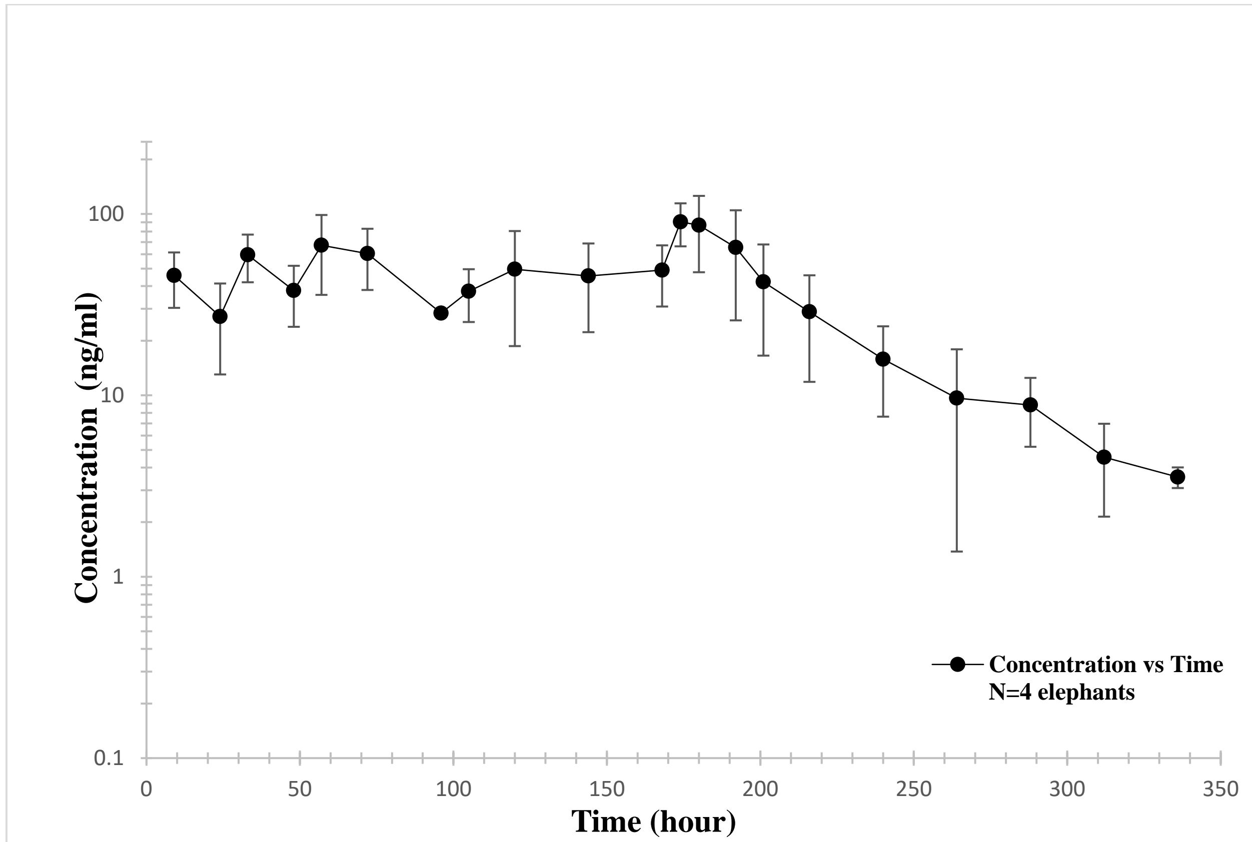


Figure 18

Plot of concentration versus time for 4 Asian elephants administered 0.1 mg/kg firocoxib paste orally every 24 hours for 8 consecutive doses. The first sample represented was collected 9 hours post first dose administration. Times from 72 to 168 hours represent trough times, immediately before AM firocoxib dose was administered. The mean serum concentrations at these times ranged from 28.4 ng/ml to 49 +/- 18.1 ng/ml. Peak concentration achieved after the 8th doses (174 hours) was 90+/- 24.1 ng/ml. The serum drug concentration at the last measured sample was 3.54 +/- 0.46 ng/ml



Chapter 6

Conclusions and Future Research

6.1 What is an ideal NSAID?

It has been proposed in human medicine that an "ideal analgesic" should have a rapid onset of action, act over an extended period of time, reduce awareness of pain quickly, minimize interruption of daily activity due to pain, be well tolerated with minimal adverse effects, and produce analgesia for a variety of conditions in different patient populations.[338]. This definition can be further expanded for long term management of painful conditions such as arthritis. It has been stated in human medicine that an ideal NSAID should be COX-2 preferential, thus substantially decreasing the risk of gastrointestinal system toxicity, should have limited or no cardiovascular or renal toxicity that occurs post administration, and is generally as well tolerated (and palatable).[339] To date there have not been broad recommendations for the prescription of NSAIDs in veterinary medicine. The variety of species in zoological medicine may complicate formulation of a single set of criteria, however it does not mean that some of these criteria cannot be applied to the use of NSAIDs in zoological patients. It is logical to think that an ideal NSAID for zoo animals is one that is palatable and easy to administer, minimizing stress to both patient and veterinarian. An ideal NSAID should have a rapid onset with a prolonged time of action, thus minimizing dosing frequency. This ideal NSAID should be potent and efficacious enough to minimize interruption of daily activity due to pain while also producing analgesia over a wide range of pain types in different patient populations. Finally, an ideal NSAID for zoo animals should be well tolerated with no to minimal adverse gastrointestinal, cardiac, or renal effects.

This may mean COX-2 selectivity is indicated in an ideal drug, however because of the renal specific adverse events documented in some vulture species, further study is needed confirm the safety of COX-2 selective drugs in nonmammalian species.

Chapter 3 of this dissertation detailed specifics of the differences in the COX inhibition of three NSAIDs administered to elephants: firocoxib, flunixin meglumine and aspirin (the latter as a control). This dissertation reports the pharmacokinetics of firocoxib. The pharmacokinetics of flunixin meglumine have been simultaneously studied in both Asian and African elephants as a partner drug study to the firocoxib studies reported here. The studies of these two different drugs were funded as one by the Morris Animal Foundation (grant- D15ZO-007) with the intent to determine which, if either, would be more appropriate for treatment of pain in elephants. The pharmacokinetics of flunixin meglumine have been reported by collaborating colleagues at Oregon State University (J.M Christiansen) and University of Pennsylvania (U. Bechert). The results of their collaborative work with flunixin meglumine are summarized in Table 8, allowing for a comparison of the potential use of either of the two drugs, based on the criteria above, for treatment of pain in elephants.

6.2 Considerations of firocoxib vs flunixin meglumine

6.2.1 COX- isoenzyme preference

Drug potency and efficacy of COX isoenzyme inhibition were described for both of these drugs in Asian elephants in Chapter 3 as determined by the COX-isoenzyme inhibition studies. Firocoxib demonstrated both potency and preference for the COX-2 isoenzyme. While the paradoxical increase in COX-2 (PGE+M) seen during the *ex vivo* evaluation of the multiple dose study is not clearly explainable, the TDM *ex vivo* evaluation provided an indication of safety of

long-term administration of firocoxib. This safety is further indicated when considered in light of no apparent changes in serum blood profiles and the negative necropsy results obtained from the one TDM elephant. That said, more information such as additional necropsy results, are needed to confirm the safety of firocoxib in Asian elephants. Conversely, the results of the *ex vivo* study of flunixin meglumine in Asian elephants demonstrated a precipitous drop (-99.31 +/-0.32%) in circulating thromboxane after multiple oral doses of flunixin meglumine. This causes concern, because of TxB_2's and associated COX-1's essential functions in the body. No abnormalities were noted in the study elephants, but parameters like blood clotting times were not evaluated and should be with future study.

As such, in vitro and ex vivo pharmacodynamics studies support firocoxib preference to flunixin meglumine in terms of both potential efficacy and safety.

6.2.2. Ease of administration

As stated in Chapter 4 of this dissertation, many drugs can only be administered orally to zoo patients, making palatability very important in zoological medicine.[4,25] A oral formulation of a drug has little clinical value if it cannot be successfully administered to the patient consuming it. In the flunixin meglumine studies, 6 of 20 (30%) Asian elephants refused or spit out flunixin meglumine paste. There was even higher refusal of this drug formulation in African elephants with 8 of 17 (47%) of the participating elephants refusing or tasting then immediately spitting out the drug. This taste aversion was so severe that it was noted at one institution that it appeared as if one Asian elephant watched another elephant in an adjoining paddock refuse the drug, and she subsequently refused all food items offered by keepers for a time period after that. This refusal to take flunixin meglumine paste negatively impacted both drug studies because

institutions housing elephants were randomly assigned which drug was administered first. For those institutions for which flunixin meglumine was assigned as the first trial, several subsequently withdrew from the entire study. While additional Asian elephants were successfully recruited to complete the concurrent firocoxib study, additional Africans could not be recruited. Conversely, no elephant refused or was reluctant to receive firocoxib tablets. It is not clear if it was the taste, or the consistency of the flunixin meglumine paste that led to the refusal, but based on this finding alone, firocoxib is preferred to flunixin meglumine in terms of palatability and ease of administration.

6.2.3. Pharmacokinetics and the dosing regimen

The goal of an ideal NSAID having a rapid onset of activity with a prolonged dosing period that minimizes dosing frequency was evaluated by the pharmacokinetic studies performed as detailed in Chapter 4 and 5 of this dissertation, which focused on drug naïve, apparently healthy animals. The pharmacokinetics of flunixin meglumine are detailed in Table 7. Note that firocoxib was also evaluated at two doses (0.01 mg/kg and 0.1 mg/kg) in both Asian and African elephants. At the 0.01 mg/kg oral dose, serum firocoxib concentrations were not sufficiently detectable to allow for statistical reporting or modeling and as such, 0.01 mg/kg was determined not acceptable for both Asian and African elephants. At the higher dose of 0.1 mg/kg, comparisons between the two species is limited to single oral dose administration. In general, while the disposition of the drugs did not dramatically differ, based on these studies, between Africans and Asians, comparison of the disposition between the two drugs did reveal some differences. When considering onset and duration of activity as a criteria for an ideal NSAID, the AUC, C_{max}, T_{max}, Clearance, and half-life (which determines duration of exposure and thus

dosing interval, and time to steady-state and thus time to evaluate safety or efficacy) are the critical parameters of interest. Flunixin meglumine at a 1.5 mg/kg (the higher dose evaluated) had a T_{max} of 1.5+/-2.01 hours for Asian elephants and 2.0+/-1.57 hours for African elephants. This was comparable to firocoxib in African (6.3 +/-1.79 hours for tablets, 7.00+/-1.4 hours for paste) and Asian (6.3 +/-1.79 hours for tablets, 7.00+/-1.4 hours for paste) elephants. The half-life of 10.+/-3.35 hours for Asian and 6.2+/-2.86 hours for African elephants means that twice a day dosing is indicated for flunixin meglumine to maintain therapeutic concentrations, This is compared to half-lives greater than 30 hours for both firocoxib tablets and paste in both species, which indicate that once a day dosing and possibly even every other day (based upon the multiple dose study detailed Chapter 5) may be indicated in some patients with this drug. Ultimately there are mixed outcomes comparing firocoxib to flunixin meglumine, in that flunixin meglumine has a desired rapid time to maximum serum concentrations, however the short half - life means multiple times per day dosing may be necessary when treating a chronic condition like pododermatitis or osteoarthritis. Multiple day dosing of flunixin meglumine becomes problematic when considering the difficulties in drug administration encountered with flunixin meglumine. Based on pharmacokinetics, firocoxib is the preferred drug. The time to steady-state with firocoxib may be as long as 3 days, thus a loading dose (0.2 to 0.3 mg/kg) may be indicated if a rapid response is desired.

6.2.4 The evaluation of TDM elephants

Chapter 5 gives an indication of efficacy of firocoxib. Based on the subjective evaluation of perceived efficacy ratings, firocoxib provides good to excellent pain relief to Asian elephants being treated for pododermatitis or osteoarthritis with once a day administration at doses

comparable to 0.1 mg/kg. The serum levels determined in both TDM and multiple dose elephants were lower than those identified in domestic horses after 12 consecutive oral doses.[340] There were no long-term administration TDM elephants or necropsy results identified for flunixin meglumine. Further study of flunixin meglumine is needed to determine if there are associated adverse physiological changes associated with administration of this drug, and to evaluate perceived efficacy in therapeutic patients.

In addition, Chapter 5 provides information regarding the firocoxib concentrations monitored therapeutically in clinical patients. This latter information is important because pain and efficacy studies generally are not possible in zoo animals unless performed observing suffering from spontaneous pain diseases associated with disease. The development of an objective, minimally invasive methodology for evaluating efficacy and the potential of adverse events of NSAID therapy would be ideal, but that does not currently exist. Instead this remains a future research endeavor that holds promise with the current advances in pharmacogenomics in humans and domestic animals that may be applied to zoological medicine[85,221]

In summary, the studies detailed in this dissertation indicate that more thorough pharmacokentic, pharmacodynamic, and TDM studies are possible in captive elephants than traditional single dose studies. Firocoxib appears clearly superior to flunixin meglumine as an NSAID to choose for management of chronic pain in Asian elephants. At this time there is no indication that this information cannot also be considered in African elephants, however additional studies, specifically of i.v. multiple consecutive doses, and TDM with perceived efficacy evaluation of firocoxib is indicated in African elephants for confirmation.

Table 7

Pharmacokinetic parameters obtained from single dose (0.8 mg/kg and 1.5 mg/kg) and multiple dose (1.1 mg/kg x 3 consecutive doses 24 hours a part) oral administration of flunixin meglumine to Asian and African elephants. This study represented a tandem study implemented by collaborators.

Pharmacokinetic parameter	0.8 mg/kg		1.5 mg/kg		1.1 mg/kg	
	African (n=8)	Asian (n=8)	African (n=6)	Asian (n=7)	African (n=4)	Asian (n=6)
C_{max} (µg/ml)	2.5 ± 0.65	2.1 ± 0.76	4.4 ± 0.67	7.2 ± 1.47	4.0 ± 1.42	2.7 ± 1.34
T_{max} (hr)	2.80 ± 1.98	1.3 ± 0.51	2.0 ± 1.57	1.5 ± 2.01	3.5 ± 1.00	2.3 ± 0.82
T½ (hr)	6.7 ± 6.91	5.5 ± 4.79	6.2 ± 2.86	10.2 ± 3.35	6.1 ± 0.11	12.6 ± 5.06
Kel (hr-1)	1.1 ± 0.07	0.1 ± 0.07	0.1 ± 0.05	0.07 ± 0.02	0.11 ± 0.10	0.06 ± 0.19
AUC(0-∞) (hr x µg/ml)	20.5 ± 10.65	29.4 ± 11.60	33.1 ± 5.25	48.6 ± 16.07	139 ± 66.5	163.2 ± 79.5
AUMC(0-∞) (hr x µg2/ml)	397 ± 464	285 ± 264	289 ± 93.3	694 ± 293	1497 ± 960	2079 ± 661
MRT (hr)	11.0 ± 4.68	11.2 ± 6.85	9.9 ± 2.21	13.2 ± 4.55	10.3 ± 2.13	12.6 ± 5.06

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Appendix 1

Megavertebrate Analgesia Survey

Demographics

This survey is being conducted by Scott B. Citino, DVM, Dipl. ACZM and Jeffrey Zuba, DVM to collect information about what AAZV members are using for analgesia in megavertebrates (Elephant, Rhinoceros, Hippopotamus, and Giraffe). Please have only one veterinarian from your institution complete this survey. Try to have all of your analgesic data for giraffe, elephants, rhinoceros, and hippos available before you complete the survey. Try to fill out as much as you can. Collated data from this survey will be presented during the Analgesia Session of the 2012 AAZV conference in Oakland, CA. and/or be posted on the AAZV website or published in the Journal of Zoo and Wildlife Medicine

*** 1. Please fill in the demographic information below**

Name:

Institution:

Address 1:

Address 2:

City/Town:

State/Province:

ZIP/Postal Code:

Country:

Email Address:

Phone Number:

Megavertebrate Analgesia Survey

Use of Non-Steroidal Anti-inflammatories in Giraffe

*** 2. Have you used non-steroidal anti-inflammatory agents (NSAIDS) in Giraffe for analgesia?**

- Yes
- No

Megavertebrate Analgesia Survey

Use of Non-Steroidal Anti-inflammatory agents in Megavertebrates

Use of Non-Steroidal Anti-inflammatories in Giraffe

***2. Have you used non-steroidal anti-inflammatory agents (NSAIDS) in Giraffe for analgesia?**

- Yes
- No

Megavertebrate Analgesia Survey

Use of Non-Steroidal Anti-inflammatories in Giraffe, cont.

***3. What NSAIDS have you used in giraffe?**

- [ ] Flunixin Meglumine
- [ ] Phenylbutazone
- [ ] Ketoprofen
- [ ] Etodolac
- [ ] Carprofen
- [ ] Meloxicam
- [ ] Ibuprofen
- [ ] Other (please specify)

4. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each NSAID you have safely used in giraffe: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance.

***5. What is the average duration of treatment for each NSAID you have safely used in giraffe?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Flunixin Meglumine	☐	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***6. What is the longest duration of treatment for each NSAID you have safely used in giraffe?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***7. For what type of pain have you used each NSAID in giraffe?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Flunixin Meglumine	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***8. For what type of pain have you used each NSAID in giraffe?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

*9. What adverse effects have you seen with the use of each NSAID in giraffe?

*10. Rate the following NSAIDs as to their overall analgesic efficacy in giraffe?

	Excellent	Good	Fair	Poor or no effect	N/A
Flunixin Meglumine	○	○	○	○	○
Phenylbutazone	○	○	○	○	○
Ketoprofen	○	○	○	○	○
Etodolac	○	○	○	○	○
Carprofen	○	○	○	○	○
Meloxicam	○	○	○	○	○
Ibuprofen	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

11. Any other comments about using NSAIDS in giraffe?

Use of Non-steroidal Anti-inflammatory Agents in Elephants

***12. Have you used non-steroidal anti-inflammatory agents (NSAIDS) in Elephants for analgesia?**

- Yes
- No

Use of Non-Steroidal Anti-inflammatories in Elephants, cont.

***13. What NSAIDS have you used in elephants?**

- [ ] Flunixin Meglumine
- [ ] Phenylbutazone
- [ ] Ketoprofen
- [ ] Etodolac
- [ ] Carprofen
- [ ] Meloxicam
- [ ] Ibuprofen
- [ ] Other (please specify)

14. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each NSAID you have safely used in elephants: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. Any differences between Asian and African elephants?

*** 15. What is the average duration of treatment for each NSAID you have safely used in elephants?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Flunixin Meglumine	☐	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

*** 16. What is the longest duration of treatment for each NSAID you have safely used in elephants?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***17. For what type of pain have you used each NSAID in elephants?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Flunixin Meglumine	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***18. For what type of pain have you used each NSAID in elephants?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***19. What adverse effects have you seen with the use of each NSAID in elephants?**

***20. Rate the following NSAIDs as to their overall analgesic efficacy in elephants?**

	Excellent	Good	Fair	Poor or no effect	N/A
Flunixin Meglumine	○	○	○	○	○
Phenylbutazone	○	○	○	○	○
Ketoprofen	○	○	○	○	○
Etodolac	○	○	○	○	○
Carprofen	○	○	○	○	○
Meloxicam	○	○	○	○	○
Ibuprofen	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

21. Any other comments about using NSAIDS in elephants?

Use of Non-steroidal Anti-inflammatory Agents in Rhinoceroses

***22. Have you used non-steroidal anti-inflammatory agents (NSAIDS) in Rhinoceroses for analgesia?**

- Yes
- No

Use of Non-Steroidal Anti-inflammatories in Rhinoceroses, cont.

***23. What NSAIDS have you used in rhinoceroses?**

- ☐ Flunixin Meglumine
- ☐ Phenylbutazone
- ☐ Ketoprofen
- ☐ Etodolac
- ☐ Carprofen
- ☐ Meloxicam
- ☐ Ibuprofen
- ☐ Other (please specify)

24. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each NSAID you have safely used in rhinoceroses: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. Any differences between rhinoceros species?

***25. What is the average duration of treatment for each NSAID you have safely used in rhinoceroses?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Flunixin Meglumine	☐	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***26. What is the longest duration of treatment for each NSAID you have safely used in rhinoceroses?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***27. For what type of pain have you used each NSAID in rhinoceroses?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Flunixin Meglumine	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***28. For what type of pain have you used each NSAID in rhinoceroses?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***29. What adverse effects have you seen with the use of each NSAID in rhinoceroses?**

***30. Rate the following NSAIDs as to their overall analgesic efficacy in rhinoceroses?**

	Excellent	Good	Fair	Poor or no effect	N/A
Flunixin Meglumine					
Phenylbutazone					
Ketoprofen					
Etodolac					
Carprofen					
Meloxicam					
Ibuprofen					
Other					

Other (please specify)

31. Any other comments about using NSAIDS in rhinoceroses?

Use of Non-steroidal Anti-inflammatory Agents in Hippopotamus

***32. Have you used non-steroidal anti-inflammatory agents (NSAIDS) in Hippopotamus for analgesia?**

- Yes
- No

Use of Non-Steroidal Anti-inflammatories in Hippopotamus, cont.

***33. What NSAIDS have you used in hippopotamus?**

- ☐ Flunixin Meglumine
- ☐ Phenylbutazone
- ☐ Ketoprofen
- ☐ Etodolac
- ☐ Carprofen
- ☐ Meloxicam
- ☐ Ibuprofen
- ☐ Other (please specify)

34. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each NSAID you have safely used in hippopotamus: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance.

***35. What is the average duration of treatment for each NSAID you have safely used in hippopotamus?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Flunixin Meglumine	☐	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***36. What is the longest duration of treatment for each NSAID you have safely used in hippopotamus?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***37. For what type of pain have you used each NSAID in hippopotamus?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Flunixin Meglumine	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***38. For what type of pain have you used each NSAID in hippopotamus?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Flunixin Meglumine	☐	☐	☐	☐	☐
Phenylbutazone	☐	☐	☐	☐	☐
Ketoprofen	☐	☐	☐	☐	☐
Etodolac	☐	☐	☐	☐	☐
Carfprofen	☐	☐	☐	☐	☐
Meloxicam	☐	☐	☐	☐	☐
Ibuprofen	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

*39. What adverse effects have you seen with the use of each NSAID in hippopotamus?

*40. Rate the following NSAIDs as to their overall analgesic efficacy in hippopotamus?

	Excellent	Good	Fair	Poor or no effect	N/A
Flunixin Meglumine	○	○	○	○	○
Phenylbutazone	○	○	○	○	○
Ketoprofen	○	○	○	○	○
Etodolac	○	○	○	○	○
Carprofen	○	○	○	○	○
Meloxicam	○	○	○	○	○
Ibuprofen	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

41. Any other comments about using NSAIDS in hippopotamus?

Megavertebrate Analgesia Survey

Use of Narcotic Analgesic agents in Megavertebrates

Megavertebrate Analgesia Survey

Use of Narcotic Analgesics in Giraffe

***42. Have you used narcotics in Giraffe for analgesia?**

- Yes
- No

Megavertebrate Analgesia Survey

Use of Narcotic Analgesics in Giraffe, cont.

***43. What narcotic analgesics have you used in giraffe?**

- [ ] Butorphanol
- [ ] Fentanyl
- [ ] Morphine
- [ ] Buprenorphine
- [ ] Tramadol
- [ ] Other (please specify)

44. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each narcotic analgesic you have safely used in giraffe: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance.

***45. What is the average duration of treatment for each narcotic analgesic you have safely used in giraffe?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Other	☐	☐	☐	☐	☐	☐
Butorphanol	☐	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐	☐

Other (please specify)

***46. What is the longest duration of treatment for each narcotic analgesic you have safely used in giraffe?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***47. For what type of pain have you used each narcotic analgesic in giraffe?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Butorphanol	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐
Morphine	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***48. For what type of pain have you used each narcotic analgesic in giraffe?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***49. What adverse effects have you seen with the use of each narcotic analgesics in giraffe?**

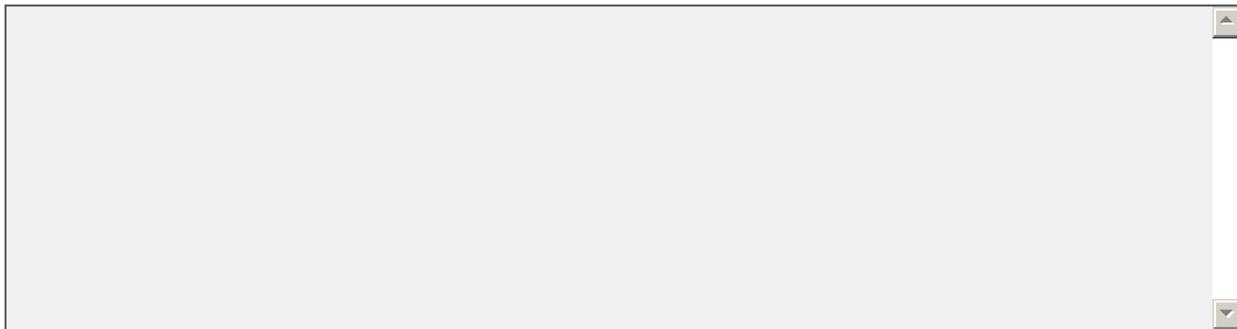
***50. Rate the following narcotic analgesics as to their overall analgesic efficacy in giraffe?**

	Excellent	Good	Fair	Poor or no effect	N/A
Butorphanol	○	○	○	○	○
Fentanyl	○	○	○	○	○
Morphine	○	○	○	○	○
Buprenorphine	○	○	○	○	○
Tramadol	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

Megavertebrate Analgesia Survey

51. Any other comments about using narcotic analgesics in giraffe?



Use of Narcotic Analgesics in Elephants

***52. Have you used narcotics in elephants for analgesia?**

- Yes
- No

Use of Narcotic Analgesics in Elephants, cont.

*53. What narcotic analgesics have you used in elephants?

- [ ] Butorphanol
- [ ] Fentanyl
- [ ] Morphine
- [ ] Buprenorphine
- [ ] Tramadol
- [ ] Other (please specify)

54. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each narcotic analgesic you have safely used in elephants: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. Differences between Asian and African Elephants?

***55. What is the average duration of treatment for each narcotic analgesic you have safely used in elephants?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Butorphanol	☐	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***56. What is the longest duration of treatment for each narcotic analgesic you have safely used in elephants?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***57. For what type of pain have you used each narcotic analgesic in elephants?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Butorphanol	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐
Morphine	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

Megavertebrate Analgesia Survey

***58. For what type of pain have you used each narcotic analgesic in elephants? Chronic pain is pain lasting > 3 months; Check all that apply**

	Acute	Chronic	Mild	Moderate	Severe
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***59. What adverse effects have you seen with the use of each narcotic analgesics in elephants?**

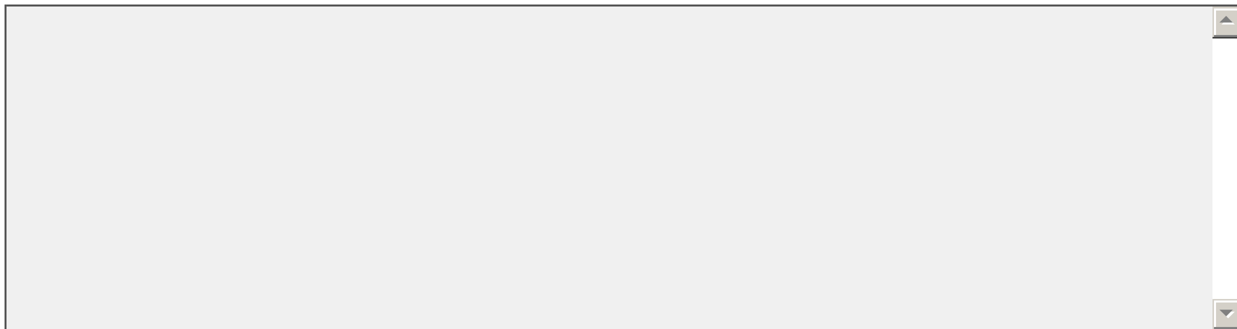
***60. Rate the following narcotic analgesics as to their overall analgesic efficacy in elephants?**

	Excellent	Good	Fair	Poor or no effect	N/A
Butorphanol	○	○	○	○	○
Fentanyl	○	○	○	○	○
Morphine	○	○	○	○	○
Buprenorphine	○	○	○	○	○
Tramadol	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

Megavertebrate Analgesia Survey

61. Any other comments about using narcotic analgesics in elephants?



Use of Narcotic Analgesics in Rhinoceroses

***62. Have you used narcotics in rhinoceroses for analgesia?**

- Yes
- No

Use of Narcotic Analgesics in Rhinoceroses, cont.

***63. What narcotic analgesics have you used in rhinoceroses?**

- [ ] Butorphanol
- [ ] Fentanyl
- [ ] Morphine
- [ ] Buprenorphine
- [ ] Tramadol
- [ ] Other (please specify)

64. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each narcotic analgesic you have safely used in rhinoceroses: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. Differences between rhinoceros species?

***65. What is the average duration of treatment for each narcotic analgesic you have safely used in rhinoceroses?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Butorphanol	☐	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***66. What is the longest duration of treatment for each narcotic analgesic you have safely used in rhinoceroses?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***67. For what type of pain have you used each narcotic analgesic in rhinoceroses?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Butorphanol	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐
Morphine	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

Megavertebrate Analgesia Survey

***68. For what type of pain have you used each narcotic analgesic in rhinoceroses? Chronic pain is pain lasting > 3 months; Check all that apply**

	Acute	Chronic	Mild	Moderate	Severe
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

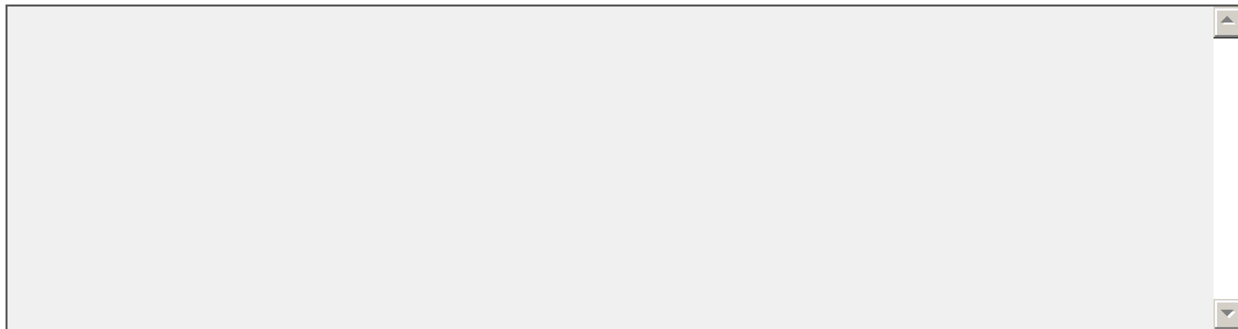
***69. What adverse effects have you seen with the use of each narcotic analgesic in rhinoceroses?**

***70. Rate the following narcotic analgesics as to their overall analgesic efficacy in rhinoceroses?**

	Excellent	Good	Fair	Poor or no effect	N/A
Butorphanol	○	○	○	○	○
Fentanyl	○	○	○	○	○
Morphine	○	○	○	○	○
Buprenorphine	○	○	○	○	○
Tramadol	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

71. Any other comments about using narcotic analgesics in rhinoceroses?



Megavertebrate Analgesia Survey

Use of Narcotic Analgesics in Hippopotamus

***72. Have you used narcotics in hippopotamus for analgesia?**

- Yes
- No

Use of Narcotic Analgesics in Hippopotamus, cont.

*73. What narcotic analgesics have you used in hippopotamus?

- [ ] Butorphanol
- [ ] Fentanyl
- [ ] Morphine
- [ ] Buprenorphine
- [ ] Tramadol
- [ ] Other (please specify)

74. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each narcotic analgesic you have safely used in hippopotamus: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance.

***75. What is the average duration of treatment for each narcotic analgesic you have safely used in hippopotamus?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Butorphanol	☐	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***76. What is the longest duration of treatment for each narcotic analgesic you have safely used in hippopotamus?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***77. For what type of pain have you used each narcotic analgesic in hippopotamus?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Butorphanol	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐
Morphine	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***78. For what type of pain have you used each narcotic analgesic in hippopotamus? Chronic pain is pain lasting > 3 months; Check all that apply**

	Acute	Chronic	Mild	Moderate	Severe
Butorphanol	☐	☐	☐	☐	☐
Fentanyl	☐	☐	☐	☐	☐
Morphine	☐	☐	☐	☐	☐
Buprenorphine	☐	☐	☐	☐	☐
Tramadol	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***79. What adverse effects have you seen with the use of each narcotic analgesic in hippopotamus?**

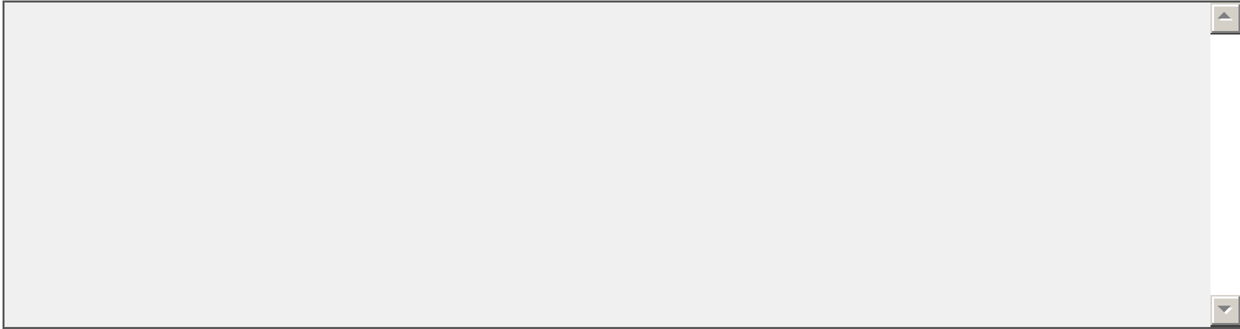
***80. Rate the following narcotic analgesics as to their overall analgesic efficacy in hippopotamus?**

	Excellent	Good	Fair	Poor or no effect	N/A
Butorphanol	○	○	○	○	○
Fentanyl	○	○	○	○	○
Morphine	○	○	○	○	○
Buprenorphine	○	○	○	○	○
Tramadol	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

Megavertebrate Analgesia Survey

81. Any other comments about using narcotic analgesics in hippopotamus?



Use of Other Drugs and Methods for Analgesia in Megavertebrates

Use of Other Drugs and Methods for Analgesia in Giraffe

Other drugs and methods include gabapentin, amantadine, local anesthetics, alpha-2-adrenergic agonists, corticosteroids, glucosamine/chondroitin, polysulfated glycosaminoglycan, omega 3 & 6 fatty acids, acupuncture, etc.

***82. Have you used other drugs or methods for analgesia in giraffe?**

- Yes
- No

Use of Other Drugs and Methods for Analgesia in Giraffe, cont.

***83. What other drugs and methods have you used for analgesia in giraffe?**

- [ ] Gabapentin
- [ ] Amantadine
- [ ] Local Anesthetics (i.e., Lidocaine, etc)
- [ ] Corticosteroids (i.e., Prednisone, Dexamethasone, etc)
- [ ] Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)
- [ ] Polysulfated Glycoaminoglycan/Hyaluronic Acid
- [ ] Glucosamine/Chondroitin
- [ ] Omega 3&6 Fatty Acids
- [ ] Acupuncture
- [ ] Low Level Laser Therapy
- [ ] Other (please specify)

84. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each other drug and method have safely used for analgesia in giraffe: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. For methods, provide the type of equipment used, method of application, etc.

Megavertebrate Analgesia Survey

***85. What is the average duration of treatment for each other drug or method you have safely used for analgesia in giraffe?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Gabapentin	☐	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

[ ]

Megavertebrate Analgesia Survey

***86. What is the longest duration of treatment for each other drug and method you have safely used for analgesia in giraffe?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

[ ]

***87. For what type of pain have you used each other drug and method for analgesia in giraffe?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Gabapentin	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

Megavertebrate Analgesia Survey

***88. For what type of pain have you used each other drug and method for analgesia in giraffe?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***89. What adverse effects have you seen with the use of each other analgesic drug and method in giraffe?**

***90. Rate the following other drugs and methods as to their overall analgesic efficacy in giraffe?**

	Excellent	Good	Fair	Poor or no effect	N/A
Gabapentin	○	○	○	○	○
Amantadine	○	○	○	○	○
Local Anesthetics (i.e., Lidocaine, etc)	○	○	○	○	○
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	○	○	○	○	○
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	○	○	○	○	○
Polysulfated Glycoaminoglycan/Hyaluronic Acid	○	○	○	○	○
Glucosamine/Chondroitin	○	○	○	○	○
Omega 3&6 Fatty Acids	○	○	○	○	○
Acupuncture	○	○	○	○	○
Low Level Laser Therapy	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

91. Any other comments about using other drugs and methods for analgesia in giraffe?

Use of Other Drugs and Methods for Analgesia in Elephants

Other drugs and methods include gabapentin, amantadine, local anesthetics, alpha-2-adrenergic agonists, corticosteroids, glucosamine/chondroitin, polysulfated glycosaminoglycan, omega 3 & 6 fatty acids, acupuncture, etc.

***92. Have you used other drugs or methods for analgesia in elephants?**

- Yes
- No

Use of Other Drugs and Methods for Analgesia in Elephants, cont.

***93. What other drugs and methods have you used for analgesia in elephants?**

- ☐ Gabapentin
- ☐ Amantadine
- ☐ Local Anesthetics (i.e., Lidocaine, etc)
- ☐ Corticosteroids (i.e., Prednisone, Dexamethasone, etc)
- ☐ Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)
- ☐ Polysulfated Glycoaminoglycan/Hyaluronic Acid
- ☐ Glucosamine/Chondroitin
- ☐ Omega 3&6 Fatty Acids
- ☐ Acupuncture
- ☐ Low Level Laser Therapy
- ☐ Other (please specify)

94. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each other drug and method have safely used for analgesia in elephants: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. For methods, provide the type of equipment used, method of application, etc. Differences between Asian and African elephants?

***95. What is the average duration of treatment for each other drug or method you have safely used for analgesia in elephants?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Gabapentin	☐	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

Megavertebrate Analgesia Survey

***96. What is the longest duration of treatment for each other drug and method you have safely used for analgesia in elephants?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

Megavertebrate Analgesia Survey

***97. For what type of pain have you used each other drug and method for analgesia in elephants?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Gabapentin	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

[ ]

***98. For what type of pain have you used each other drug and method for analgesia in elephants?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***99. What adverse effects have you seen with the use of each other analgesic drug and method in elephants?**

***100. Rate the following other drugs and methods as to their overall analgesic efficacy in elephants?**

	Excellent	Good	Fair	Poor or no effect	N/A
Gabapentin	○	○	○	○	○
Amantadine	○	○	○	○	○
Local Anesthetics (i.e., Lidocaine, etc)	○	○	○	○	○
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	○	○	○	○	○
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	○	○	○	○	○
Polysulfated Glycoaminoglycan/Hyaluronic Acid	○	○	○	○	○
Glucosamine/Chondroitin	○	○	○	○	○
Omega 3&6 Fatty Acids	○	○	○	○	○
Acupuncture	○	○	○	○	○
Low Level Laser Therapy	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

101. Any other comments about using other drugs and methods for analgesia in elephants?

Use of Other Drugs and Methods for Analgesia in Rhinoceroses

Other drugs and methods include gabapentin, amantadine, local anesthetics, alpha-2-adrenergic agonists, corticosteroids, glucosamine/chondroitin, polysulfated glycosaminoglycan, omega 3 & 6 fatty acids, acupuncture, etc.

* 102. Have you used other drugs or methods for analgesia in rhinoceroses?

- Yes
- No

Megavertebrate Analgesia Survey

Use of Other Drugs and Methods for Analgesia in Rhinoceroses, cont.

***103. What other drugs and methods have you used for analgesia in rhinoceroses?**

- ☐ Gabapentin
- ☐ Amantadine
- ☐ Local Anesthetics (i.e., Lidocaine, etc)
- ☐ Corticosteroids (i.e., Prednisone, Dexamethasone, etc)
- ☐ Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)
- ☐ Polysulfated Glycoaminoglycan/Hyaluronic Acid
- ☐ Glucosamine/Chondroitin
- ☐ Omega 3&6 Fatty Acids
- ☐ Acupuncture
- ☐ Low Level Laser Therapy
- ☐ Other (please specify)

104. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each other drug and method have safely used for analgesia in rhinoceroses: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. For methods, provide the type of equipment used, method of application, etc. Differences between species?

***105. What is the average duration of treatment for each other drug or method you have safely used for analgesia in rhinoceroses?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Gabapentin	☐	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***106. What is the longest duration of treatment for each other drug and method you have safely used for analgesia in rhinoceroses?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***107. For what type of pain have you used each other drug and method for analgesia in rhinoceroses?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Gabapentin	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

***108. For what type of pain have you used each other drug and method for analgesia in rhinoceroses?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***109. What adverse effects have you seen with the use of each other analgesic drug and method in rhinoceroses?**

***110. Rate the following other drugs and methods as to their overall analgesic efficacy in rhinoceroses?**

	Excellent	Good	Fair	Poor or no effect	N/A
Gabapentin	○	○	○	○	○
Amantadine	○	○	○	○	○
Local Anesthetics (i.e., Lidocaine, etc)	○	○	○	○	○
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	○	○	○	○	○
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	○	○	○	○	○
Polysulfated Glycoaminoglycan/Hyaluronic Acid	○	○	○	○	○
Glucosamine/Chondroitin	○	○	○	○	○
Omega 3&6 Fatty Acids	○	○	○	○	○
Acupuncture	○	○	○	○	○
Low Level Laser Therapy	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

111. Any other comments about using other drugs and methods for analgesia in rhinoceroses?

Use of Other Drugs and Methods for Analgesia in Hippopotamus

Other drugs and methods include gabapentin, amantadine, local anesthetics, alpha-2-adrenergic agonists, corticosteroids, glucosamine/chondroitin, polysulfated glycosaminoglycan, omega 3 & 6 fatty acids, acupuncture, etc.

***112. Have you used other drugs or methods for analgesia in hippopotamus?**

- Yes
- No

Use of Other Drugs and Methods for Analgesia in a Hippopotamus, cont.

***113. What other drugs and methods have you used for analgesia in hippopotamus?**

- [ ] Gabapentin
- [ ] Amantadine
- [ ] Local Anesthetics (i.e., Lidocaine, etc)
- [ ] Corticosteroids (i.e., Prednisone, Dexamethasone, etc)
- [ ] Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)
- [ ] Polysulfated Glycoaminoglycan/Hyaluronic Acid
- [ ] Glucosamine/Chondroitin
- [ ] Omega 3&6 Fatty Acids
- [ ] Acupuncture
- [ ] Low Level Laser Therapy
- [ ] Other (please specify)

114. Please provide the dose (mg/kg), route (PO, IM, etc.), dosing interval (SID, BID, etc.), and a qualitative evaluation of efficacy for each other drug and method have safely used for analgesia in hippopotamus: For doses please use actual weights or the best possible estimated weights. Please provide any "tricks" you use for administration or to improve patient compliance. For methods, provide the type of equipment used, method of application, etc.

***115. What is the average duration of treatment for each other drug or method you have safely used for analgesia in hippopotamus?**

	1 day	1 day to 1 week	2 weeks	3 weeks	1 month	> 1 month
Gabapentin	☐	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐	☐

Other (please specify)

***116. What is the longest duration of treatment for each other drug and method you have safely used for analgesia in hippopotamus?**

	< 1 week	1 week to 2 weeks	1 month to 6 months	6 months to 1 year	1 year or greater
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***117. For what type of pain have you used each other drug and method for analgesia in hippopotamus?**
Somatic=activation of pain receptors in either the body surface or musculoskeletal tissues;
Visceral=pain when internal organs are damaged or injured;
Neuropathic=pain is caused by injury or malfunction to the spinal cord and peripheral nerves;
Check all that apply.

	Somatic	Visceral	Neuropathic	Mixed
Gabapentin	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐
Other	☐	☐	☐	☐

Other (please specify)

[ ]

Megavertebrate Analgesia Survey

***118. For what type of pain have you used each other drug and method for analgesia in hippopotamus?**
Chronic pain is pain lasting > 3 months;
Check all that apply

	Acute	Chronic	Mild	Moderate	Severe
Gabapentin	☐	☐	☐	☐	☐
Amantadine	☐	☐	☐	☐	☐
Local Anesthetics (i.e., Lidocaine, etc)	☐	☐	☐	☐	☐
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	☐	☐	☐	☐	☐
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	☐	☐	☐	☐	☐
Polysulfated Glycoaminoglycan/Hyaluronic Acid	☐	☐	☐	☐	☐
Glucosamine/Chondroitin	☐	☐	☐	☐	☐
Omega 3&6 Fatty Acids	☐	☐	☐	☐	☐
Acupuncture	☐	☐	☐	☐	☐
Low Level Laser Therapy	☐	☐	☐	☐	☐
Other	☐	☐	☐	☐	☐

Other (please specify)

***119. What adverse effects have you seen with the use of each other analgesic drug and method in hippopotamus?**

Megavertebrate Analgesia Survey

*120. Rate the following other drugs and methods as to their overall analgesic efficacy in hippopotamus?

	Excellent	Good	Fair	Poor or no effect	N/A
Gabapentin	○	○	○	○	○
Amantadine	○	○	○	○	○
Local Anesthetics (i.e., Lidocaine, etc)	○	○	○	○	○
Corticosteroids (i.e., Prednisone, Dexamethasone, etc)	○	○	○	○	○
Alpha-2-Adrenergic Agonists (i.e., Xylazine, etc)	○	○	○	○	○
Polysulfated Glycoaminoglycan/Hyaluronic Acid	○	○	○	○	○
Glucosamine/Chondroitin	○	○	○	○	○
Omega 3&6 Fatty Acids	○	○	○	○	○
Acupuncture	○	○	○	○	○
Low Level Laser Therapy	○	○	○	○	○
Other	○	○	○	○	○

Other (please specify)

121. Any other comments about using other drugs and methods for analgesia in hippopotamus?

Megavertebrate Analgesia Survey

Megavertebrate Analgesia - Final Questions

122. What is the first drug or method you reach for when analgesia is needed in a giraffe?

123. What is the first drug or method you reach for when analgesia is needed in a elephant?

124. What is the first drug or method you reach for when analgesia is needed in a rhinoceros?

125. What is the first drug or method you reach for when analgesia is needed in a hippopotamus?

The End

Thanks for taking the time to complete this survey. Results from this survey will either be presented at the 2012 AAZV conference or posted on the AAZV website.

Appendix 2

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RESULTS OF THE MEGAVERTEBRATE ANALGESIA SURVEY: ELEPHANTS AND RHINO

Jack Kottwitz, D.V.M., Matthew Boothe, D.V.M., Roy Harmon, B.S., Scott B. Citino, D.V.M., Dipl. A.C.Z.M., Jeffery R. Zuba, D.V.M., and Dawn M. Boothe, D.V.M., Ph.D., Dipl. A.C.V.I.M., Dipl. A.C.V.C.P.

Abstract: An online survey utilizing Survey Monkey linked through the American Association of Zoo Veterinarians listserve examined current practices in megavertebrate analgesia. Data collected included drugs administered, dosing regimens, ease of administration, efficacy, and adverse events. Fifty-nine facilities (38 housing elephants, 33 housing rhinoceroses) responded. All facilities administered nonsteroidal anti-inflammatory drugs (NSAIDs), with phenylbutazone (0.25–10 mg/kg) and flunixin meglumine (0.2–4 mg/kg) being most common. Efficacy was reported as "good" to "excellent" for these medications. Opioids were administered to elephants (11 of 38) and rhinoceroses (7 of 33), with tramadol (0.5–3.0 mg/kg) and butorphanol (0.05–1.0 mg/kg) being most common. Tramadol efficacy scores were highly variable in both elephants and rhinoceroses. While drug choices were similar among institutions, substantial variability in dosing regimens and reported efficacy between and within facilities indicates the need for pharmacokinetic studies and standardized methods of analyzing response to treatment to establish dosing regimens and clinical trials to establish efficacy and safety.
Key words: Analgesic, elephant, NSAID, opioid, pain management, rhinoceros.

BRIEF COMMUNICATION

Pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" by the International Association for the Study of Pain.[6] The inability of a human or an animal to communicate verbally with a caregiver does not negate the possibility that that individual is experiencing pain or is in need of appropriate pain-relieving medications.[6] It is well recognized that painful stimuli and chronic pain can have a direct impact on behavior, attitude, and responsiveness to otherwise normal stimuli, in addition to affecting normal physiologic functions such as sleep, appetite, and digestion.[6,11]

One of the first steps for effectively managing discomfort with medications in any patient is administration of drugs at species-appropriate doses and dosing intervals to control both pain and inflammation. While online and published formularies exist, there is an extremely limited number of scientific studies performed on the pharmacokinetics of analgesics in elephants.[1,2,5,9,10,15,16] To date, there are no specific pharmacokinetic studies available in any species of rhinoceros to support dosing regimens for drugs used for pain management. Published studies[8,9,12,13] of opioids administered to rhinoceros having focused on administration for sedation and general anesthesia, not analgesia. In addition, many published doses in both formularies and case reports for these species are not based on actual pharmacokinetic studies but instead are extrapolated from doses administered in domestic horses.[4,5,9,10,12–15]

To better characterize medications, usage patterns, and methods of providing analgesia to megavertebrates, a link to an online survey (using www.surveymonkey.com) was posted on the American Association of Zoo Veterinarians listserve from March 2012 through September 2013. Response to this survey was worldwide, with 59 zoological institutions from North America, Europe, Africa, and Australia participating. Of the 59 responding institutions, 35 institutions housed elephants and 33 housed at least one species of rhinoceros. Species housed by reporting institutions included Asian elephants (*Elaphas maximus*), African elephants (*Loxodanta* spp.), southern white rhinoceros (*Ceratotherium simum simum*), Indian or greater one-horned rhinoceros (*Rhinoceros unicornis*), and Sumatran rhinoceros (*Dicerorhinus sumatrensis*). For the purposes of this survey, African elephant species, including both

From the Department of Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama 36849, USA (Kottwitz, M. Boothe, Harmon, D. M. Boothe); White Oak Conservation Center, Yulee, Florida 32097, USA (Citino); and the San Diego Zoo Safari Park, Department of Veterinary Services, Escondido, California 92109, USA (Zuba). Correspondence should be directed to Dr. Kottwitz (jack_kottwitz@hotmail.com).

Table 1. Summary of nonsteroidal anti-inflammatory drug (NSAID), opioid, and other methods of analgesia provided to captive elephants. Caution should be taken with higher dosing levels with the data in this chart. Of these analgesics, only phenylbutazone (3 mg/kg per 48 hr for Asian; 2 mg/kg per 24 hr for African), ibuprofen (6 mg/kg per 12 hr for Asian; 7 mg/kg per 12 hr for African), ketoprofen (1 mg/kg every 48 hr to 2 mg/kg every 24 hr, p.o. or i.v. for Asian), and butorphanol (single dose 0.015 mg/kg i.m. or i.v.) have drug doses and dosing schedules supported by pharmacokinetic evaluation of these drugs in elephants.[1,2,5,15] Pharmacodynamic studies are not available. Many of the drug doses listed are derived from domestic equine doses.

Drug	Institutions reporting use of drug in elephants ($n = 38$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
NSAIDs											
Phenylbutazone	27	Asian elephant, African elephant	0.25–6	p.o.	s.i.d. to b.i.d.	6	18	1	0	2	1 institution reported oral ulceration
Flunixin meglumine	25	Asian elephant, African elephant	0.28–1.1	p.o.	s.i.d. to b.i.d.	6	15	1	1	3	2 institutions reported loss of appetite, gastrointestinal upset, gas distension, mild colic
	2	African elephant	0.4–1.0	i.v.	s.i.d. to b.i.d.						1 institution reported sloughing of ear post i.v. injection
	4	Asian elephant, African elephant	0.28–1.1	i.m.	s.i.d. to b.i.d.						—
Ibuprofen	20	Asian elephant, African elephant	1–6 mg/kg for Asian 1–7 mg/kg for African	p.o.	s.i.d. to b.i.d.	6	16	1	2	2	Questionable efficacy for more severe pain management
	2	Asian elephant	1–7	Rectally	s.i.d. to b.i.d.						—

Table 1. Continued.

Drug	Institutions reporting use of drug in elephants ($n = 38$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
Ketoprofen	8	Asian elephant, African elephant	1 mg/kg every 48 hr to 2 mg/kg every 24 hr	p.o.	s.i.d. to b.i.d.	3	4	0	0	0	—
	1	Asian elephant	1.0–2.0	i.v.	s.i.d. to b.i.d.						1 institution reported sloughing of ear post i.v. injection
	1	African elephant	0.963	i.m.	s.i.d.						—
Firocoxib	4	African elephant	0.1	p.o.	—	2	2	0	0	0	—
Carprofen	3	African elephant, Asian elephant	0.2–0.3	p.o.	s.i.d.	0	1	1	1	0	2 institutions reported gastrointestinal upset, moderate colic postadministration
Meloxicam	3	African elephant	0.2	p.o.	s.i.d.	1	1	1	0	0	—
Acetaminophen	3	African elephant	0.5–1.5	p.o.	s.i.d. to b.i.d.	0	0	1	2	0	—
Vedaprofen	2	Asian elephant, African elephant	0.45–0.7	p.o.	s.i.d. to b.i.d.	0	0	1	0	1	—
	1	Asian elephant	0.5–1	Rectally	b.i.d.						—
Etodolac	1	Asian elephant	2–2.7	p.o.	s.i.d.	0	1	0	0	0	—
Aspirin	1	Asian elephant	62.5 mg total (weight not given)	p.o.	b.i.d.	0	0	0	1	0	Questionable efficacy, no analgesic effect observed

Table 1. Continued.

Drug	Institutions reporting use of drug in elephants ($n = 38$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
Opioids											
Tramadol	4	Asian elephant, African elephant	0.5–3	p.o.	b.i.d.	1	1	1	2	0	Questionable efficacy with doses of 1.0 mg/kg or less; decreased responsiveness and depression noted at higher doses
	1	Asian elephant	1	Rectally	Not reported						—
Butorphanol	4	Asian elephant	0.01–0.08	i.m.	s.i.d.	0	3	1	0	0	Mild sedation with higher doses
Fentanyl	2	Asian elephant, African elephant	0.078 mcg/kg	Transdermal patch	Not reported	0	2	0	0	0	Mild sedation with higher doses
Other modes of analgesia											
Glucosamine and chondroitin sulfate	14	Asian elephant, African elephant	Extrapolated from horse dose or 1.1 to 1.5	p.o.	s.i.d. or b.i.d.	0	5	4	3	2	Multiple compounded products have been utilized
Gabapentin	6	Asian elephant, African elephant	0.4–6	p.o.	s.i.d. or b.i.d.	1	2	2	0	1	1 institution reported difficulty administering due to taste aversion
	1	Asian elephant	1	Rectally	s.i.d.	0	1	0	0	0	None noted

Table 1. Continued.

Drug	Institutions reporting use of drug in elephants ($n = 38$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
Corticosteroids	5	African elephant	Prednisolone: up to 1	p.o.	s.i.d.	1	3	0	1	0	—
	1	African elephant	Dexamethasone: 0.05	p.o.	s.i.d.						—
	1	African elephant	0.05	i.v.	Single dose						—
Local anesthesia infusion	3	Asian elephant, African elephant	To effect (10–30 ml 1% Lidocaine)	s.q.	s.i.d.	0	2	1	0	0	Limited time of effect reported by all institutions
Acupuncture	2	Asian elephant	N.A.	—	1 to 2 times per week	0	0	2	0	0	—
Polysulfated glycosamino glycans	2	African elephant	Dose extrapolated from horse dose	i.m.	Every 3 days for 5 doses	Appeared to have mild positive effect					—
Cold laser therapy	2	Asian elephant	80 joules across 4–8 points	—	Reducing frequency: 3 times per week for 3 wk, then once a week, then every other week	0	0	2	0	0	—
Pentosan polysuflate	1	Asian elephant	3	i.m.	Every 7 days	No effect noted posttherapy					Administered to treat long-standing gait abnormality, with no change in gait noted
Omega fatty acids	1	African elephant	No dose reported	p.o.	s.i.d.	No efficacy reported					—
Amantadine	1	—	No dose reported	p.o.	—	No efficacy reported					—
α-2 agonists	1	Asian elephant	No dose reported	i.m.	s.i.d.	No efficacy reported					Administered for sedation purposes

[a] s.i.d. = once per day; b.i.d. = twice per day or every 12 hr.

African bush elephants (*Loxodonta africana*) and African forest elephants (*Loxodonta cyclotis*), were grouped together under their common genus.

Data collected about drugs utilized and modality of analgesia provided were compiled using Microsoft Access into the following categories: facility information; signalment (age, genus, and species); drug or modality, including name and specific drug formulation utilized; and dosing regimen, including route, drug vehicle, dose in "mg/kg," dosing interval, and duration. Patient information regarding pain treated was also collected. Pain duration was defined as "acute" if of less than 3 mo and as "chronic" if of more than 3 mo. Types of pain treated included somatic (activation of pain receptors in either the body surface or musculoskeletal tissues), visceral (activation of pain sensors when internal organs are damaged or injured), neuropathic (pain caused by injury or malfunction to the spinal cord and peripheral nerves), or mixed (pain involving two or more of the first three categories). Perceived efficacy of analgesia provided, defined as the perception by associated humans of resolution of the painful condition warranting administration of an analgesic drug or other form of pain relief, was ranked subjectively on a scale of 1 (poor) to 4 (excellent). Ease of use and complications associated with using each method of analgesia were described as comments from the respondent. Adverse events were characterized as reported by responding institutions. Mild adverse events described included mild colic and loss of appetite, while severe adverse events described included complete loss of appetite, severe colic, or evidence of physiologic changes, such as elevated renal values. All institutions reported administration of oral medications in both elephants and rhino using a variety of food items, including fruit, such as cored apples or watermelon, bread products, or peanut butter. All modalities utilized, number of institutions utilizing each, dosing-treatment schedules, perceived efficacies, and reported side effects are outlined in Table 1 for elephants and in Table 2 for rhino.

Analgesics were divided into three drug categories: nonsteroidal anti-inflammatory drugs (NSAIDs), opioids, and other non-NSAID–non-opioid drugs. Nonpharmacologic analgesic interventions also were recorded. NSAIDs administered to both elephants and rhinoceroses included phenylbutazone, flunixin meglumine, ibuprofen, ketoprofen, firocoxib, carprofen, and meloxicam. NSAIDs that were administered to elephants, but not to rhinoceroses, included acetaminophen, vedaprofen, etodolac, and aspirin. Opioid drug administration was much less commonly reported than was NSAID use for both elephants and rhinoceroses. Opioids administered included tramadol, butorphanol, and fentanyl. Other products administered to both elephants and rhinoceroses with the intent of controlling pain included gabapentin, corticosteroids, α-2 agonists, local anesthesia infusion, pentosan polysulfate, glucosamine and chondroitin sulfate, and omega 3/6 fatty acids. Amantadine was administered to elephants only. Other nonpharmaceutical methods of analgesia reported in elephants exclusively included acupuncture and cold laser therapy.

NSAIDs were administered for all four types of pain to both elephants and rhinoceros, with somatic pain being the most common indication, and neuropathic pain the least. As with NSAIDs, opioid drugs were administered most commonly for somatic pain followed by visceral, mixed, and, lastly, neuropathic pain. Interestingly, combinations of analgesics were used more commonly for neuropathic pain than for visceral or mixed pain, presumably in an attempt to deliver multimodal analgesia in the face of an insufficient response to previous therapy. The perceived efficacy of NSAIDs was variable, with acetaminophen, carprofen, and aspirin all reported as the least effective for analgesia in elephants (Table 1). Carprofen also had a low reported perceived efficacy score in rhinos. NSAIDs generally demonstrated higher perceived efficacy scores in all rhinoceros species compared to elephants (Table 2).

Adverse effects associated with NSAIDs in elephants were largely gastrointestinal, including loss of appetite and mild to moderate colic. Difficulties in oral administration of some of the drugs were noted in multiple institutions, which was attributed to elephants avoiding the taste of compounded and commercially available oral preparations. Data were not sufficient to allow identification of any one NSAID associated with taste aversion in elephants. A single incidence of sloughing of the ear after intravenous injection in an ear vein was described for both flunixin meglumine and ketoprofen in elephants. The only adverse event associated with NSAIDs reported in rhinoceroses was taste aversion, particularly for both flunixin meglumine and phenylbutazone.

Opioid drugs, specifically tramadol, butorphanol, and fentanyl, were less commonly administered, with only 11 institutions housing elephants and seven housing rhinoceroses reporting their use. Tramadol appeared to have the lowest perceived efficacy of these three drugs, with question-

Table 2. Summary of nonsteroidal anti-inflammatory drug (NSAID), opioid, and other methods of analgesia provided to captive rhinoceroses. Caution should be taken when considering higher dosing levels with the data in this chart. To date there have been no experimental pharmacokinetic or pharmacodynamic studies performed for any of these drugs or scientific evaluations of these methods of providing analgesia in rhino. Many of the drug doses listed are derived from domestic equine doses.

Drug	Institutions reporting use of drug in rhinoceros ($n = 33$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
NSAIDs											
Phenylbutazone	25	White rhino, Black rhino, Indian rhino	3–10	p.o.	s.i.d. to b.i.d.	5	16	4	0	0	Higher doses (>4 mg/kg not given more than 3 days); taste aversion noted with some animals
	1	White rhino	4	i.v.	One-time dose						—
	4	White rhino, Indian rhino	1.1	i.m.	s.i.d.						—
Flunixin meglumine	24	White rhino, Black rhino, Indian rhino, Sumatran rhino	0.20–1.6	p.o.	s.i.d. or e.o.d.	6	14	4	0	0	Taste aversion noted with some animals
	4	White rhino, Black rhino	0.5–1	i.v.	s.i.d. or b.i.d						—
	5	Black rhino, Indian rhino	0.50–1.1	i.m.	s.i.d.						—
Ibuprofen	1	White rhino	1	p.o.	s.i.d.	—	—	—	—	—	—
Ketoprofen	3	White rhino, Black rhino	0.5	p.o.	s.i.d. to b.i.d.	1	1	0	0	1	—
	1	Black rhino	0.5	i.v.	s.i.d. to b.i.d.						
Firocoxib	4	White rhino	0.088–0.1	p.o.	s.i.d.	2	2	0	0	0	No adverse events reported
Carprofen	2	Black rhino	0.78–2.0	p.o.	s.i.d.	0	0	2	0	0	—
Meloxicam	2	Indian rhino	0.2	p.o.	s.i.d.	0	0	2	0	0	—
Suxibuzone	1	White rhino	6	p.o.	b.i.d.	0	1	0	0	0	—

Table 2. Continued.

Drug	Institutions reporting use of drug in rhinoceros ($n = 33$)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
Opioids											
Tramadol	4	White rhino, Black rhino	0.8–3.0	p.o.	b.i.d.	1	2	0	0	1	Mild to moderate sedation noted
Butorphanol	3	White rhino, Black rhino	0.05–1.0	p.o.	b.i.d.	2	1	0	0	0	Sedation, anorexia, decreased appetite noted on 1.0 mg/kg dose
Fentanyl	1	White rhino	0.09 mcg/kg per hr	Transdermal patch	—	0	0	0	1	0	Sedation noted, but no apparent analgesia observed
Other modes of analgesia											
Glucosamine and chondroitin sulfate	7	White rhino, Indian rhino	1.1–4.0	p.o.	s.i.d. or b.i.d.	0	2	2	1	2	Questionable efficacy reported by 2 institutions
	2	White rhino	1–22.5	i.m.	Once per week to once per month						
Gabapentin[b]	4	Black rhino	2.5–5.0	p.o.	s.i.d.	0	1	1	0	3	—
	1	White rhino	1	Rectally	s.i.d.						
Corticosteroids	1	White rhino	Prednisolone: 0.2	i.m.	One-time administration	0	0	0	0	1	Site of dart injection became infected
	1	White rhino	Dexamethasone: 10	p.o.	s.i.d.	0	0	0	0	1	
Local anesthesia infusion	1	Indian rhino	Topical Lidocaine–Applied to skin	Topically	s.i.d.	0	0	0	1	0	No effect noted after 5 days of therapy

Table 2. Continued.

Drug	Institutions reporting use of drug in rhinoceros (n = 33)	Species treated	Dose administered (mg/kg)	Route	Dosing frequency[a]	No. of institutions reporting perceived efficacy					Adverse effects noted
						Excellent	Good	Fair	Poor	No response	
Pentosan polysuflate	1	Black rhino	3	i.m.	Every 7 days	No effect noted post therapy					—
Omega fatty acids	1	Black rhino	No dose reported	p.o.	s.i.d.	0	1	0	0	0	—

[a] s.i.d. = once per day; b.i.d. = twice per day or every 12 hr.
[b] Note: Gabapentin often administered in conjunction with NSAID or opioid drugs, including phenylbutazone and tramadol.

able perceived efficacy, if any, reported at lower doses. Fentanyl was administered to elephants at two institutions in the form of a transdermal patch, with good perceived efficacy reported by both institutions. This is in contrast to one institution utilizing a transdermal fentanyl patch for rhinoceros analgesia, which reported poor perceived efficacy. Adverse events reported for opioid drugs in elephants were limited to tramadol, which included sedation, depression, and decreased responsiveness after tramadol administration. Questionable perceived efficacy, if any effect for tramadol at low doses, was also reported by three institutions. Anorexia and decreased appetite were noted at doses greater than 0.7 mg/kg of butorphanol administered orally to rhinoceroses. There were reports of mild to moderate to severe sedation and decreased responsiveness in rhinoceroses with all opioid drugs.

Non-NSAID–nonopioid methods of analgesia had variable perceived efficacy, with perceived efficacy ranging from no effect noted for glucosamine and chondroitin sulfate, pentosan polysulfate, and amantadine to fair for low-level laser therapy (Tables 1, 2). The most significant adverse events reported with nonpharmaceutical methods of analgesia, such as low-level laser therapy or acupuncture, were lack of cooperation from the patient being treated or no noted effect from treatment.

An important limitation to this study is that survey results reflected institutional rather than individual animal responses. Institutions reporting also included historical information for animals that were no longer alive or no longer at that individual institution. Unfortunately, not all institutions reported the individual information as historic or current collection data, so it is not possible to accurately calculate individual population numbers for statistical analysis of perceived efficacy or incidence of side effects. Data can only be reported as institutional data. Despite the limitations of calculating exact numbers of animals as responses, the results of this survey clearly show that NSAIDs are the most common form of analgesia administered to captive elephants and rhinoceroses, with phenylbutazone and flunixin meglumine being the most common of the NSAIDs utilized. Interestingly, there were no adverse events associated with NSAID administration reported in rhinoceroses. A previous case report[14] describes bullous skin lesions in a southern white rhinoceros associated with oral administration of firocoxib. However, no lesions of this type were reported by any institution

administering firocoxib to either elephants or rhinoceroses in this survey.

Sedation associated with opioid drug administration to both elephants and rhinoceroses is an expected side effect, especially considering the known sensitivities to these drugs and their use as anesthesia drugs in these species.[7–10,12,13,15,16] Improved information from pharmacokinetic trials of these drugs will likely alleviate these negative dose-related effects.

Based upon institutional responses, nonpharmaceutical methods of analgesia were often utilized in conjunction with pharmaceutical methods or were utilized in an attempt to minimize total drug doses out of concern for variable perceived efficacy or possible negative side effects.

The variability reported in dosing regimens for NSAIDs and opioid drugs is of concern. This survey identified variability in doses that varied as much as 20-fold between institutions or within individual institutions. The commonly accepted narrow safety margin of many NSAIDs is of concern in megavertebrates, in part because of the lack of scientific drug trials upon which dosing regimens are based.[3,4] While the information presented here may be utilized as general guidelines, it is not to be used as a specific reference for drug doses or method of analgesia. Further studies in the specific pharmacokinetics of the more commonly utilized methods of analgesia, including NSAID and opioid drugs, are needed to fully analyze the safety and efficacy of these medications in megavertebrates.

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Appendix 3

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RESULTS OF THE MEGAVEREBRATE ANALGESIA SURVEY: GIRAFFE AND HIPPOPOTAMUS

Matthew Boothe, D.V.M., Jack Kottwitz, D.V.M., Roy Harmon, B.S., Scott B. Citino, D.V.M., Dipl. A.C.Z.M., Jeffery R. Zuba, D.V.M., and Dawn M. Boothe, D.V.M. Ph.D., Dipl. A.C.V.I.M., Dipl. A.C.V.C.P.

Abstract: Results of an online survey posted on the American Association of Zoo Veterinarians listserv examined the patterns of analgesic medication and pain management modalities used for captive giraffe and hippopotami. Compiled data included signalment, drugs administered, dosing regimens, subjective efficacy scores, ease of administration, and adverse events. Nineteen institutions exhibiting hippopotami (*Hippopotamus amphibious*) and pygmy hippopotami (*Choeropsis liberiensis*) and 45 exhibiting giraffe (*Giraffa camelopardalis* spp.) responded. Phenylbutazone was the most-commonly administered nonsteroidal anti-inflammatory drug (NSAID), followed by flunixin meglumine, but doses varied widely. Eight institutions reported adverse events from NSAID administration. Tramadol was the most-commonly administered opioid followed by butorphanol. Only one adverse event was reported for opioids. Twenty-three of 45 institutions exhibiting giraffe utilized alternative analgesia methods including gabapentin, glucosamine-chondroitin, local anesthetics, and low level laser therapy. Six of 19 institutions exhibiting hippopotami administered omega 3-6 fatty acids, gabapentin, glucosamine-chondroitin, and α-2 adrenergics to provide analgesia. While all reporting zoological institutions administered similar drugs, there was substantial variation and diversity in both dosing regimens and frequencies, indicating the need for both preclinical and clinical studies supporting dosing regimens.
Key words: Analgesia, giraffe, hippopotamus, NSAID, opioid, pain management.

BRIEF COMMUNICATION

Pain management in domestic and nondomestic animals is a ubiquitious goal for practicing veterinarians. Acute injury or chronic pain from arthritis can have a direct impact on the behavior and attitude of animals of any species. Physiologic functions may also be affected by pain, potentially resulting in multiple adverse events including depression, tachycardia, anorexia, weight loss, immunosuppression, and delayed wound healing.[7] The initial step for effective management of pain in species such as giraffes and hippopotami is the administration of proper pain management medications or other pain management modalities at the appropriate dose and interval for that species. Implementation of suitable analgesia plans is complicated by the general lack of pharmacokinetic and pharmacodynamic data for these species. To date there are no published pharmacokinetic or pharmacodynamics studies for any species of giraffe or hippopotamus. In addition, confounding factors such as patient mortality have arisen throughout the history of administration of drugs administered for analgesia and anesthesia in exotic mammals, further necessitating accurate treatment and dosing information.[8,13]

Current published pharmacologic data focus on the effectiveness of various agents for immobilization and anesthesia in giraffe and hippopotami.[4,5,10,12] While studies exist for other megavertebrates, such as elephants, there are currently no published references analyzing the pharmacokinetics or pharmacodynamics of analgesics in any species or subspecies of giraffe or hippopotamus.[1,2] An online survey (using www.surveymonkey.com) was posted on the American Association of Zoo Veterinarians listserv from 2012 through September 2013 that collected information to characterize the current administration of drugs and other methods of providing analgesia in the aforementioned genera. A total of 59 institutions from North America, Europe, Africa, Australia, and Asia responded to the survey. Of the 59 responding institutions, 45 responding institutions housed at least one sub-

From the Department of Anatomy, Physiology, and Pharmacology, College of Veterinary Medicine, Auburn University, Auburn, Alabama 36849, USA. (M. Boothe, Kottwitz, Harmon, D. Boothe); the White Oak Conservation Center, Yulee, Florida 32097, USA (Citino); and the San Diego Zoo Safari Park, Department of Veterinary Services, Escondido, California 92109, USA (Zuba). Present address (Boothe): Pediatric Cardiology, University of Florida, Gainesville, FL 32610, USA. Present address (Kottwitz): P.O. Box 37, Notasulga, AL 36866 USA. Correspondence should be directed to Dr. Kottwitz (jack_kottwitz@hotmail.com)

species of giraffe and 19 institutions housed at least one species of hippopotamus.

Data collected from this survey was compiled using Microsoft Access (Microsoft Corporation, Redmond, Washington 98052, USA) into the following categories: 1) signalment, including genus, species, and facility; 2) drug or modality, including name and specific formulation utilized; and 3) dosage regimen, including route, drug vehicle, dose in mg/kg, dosage interval, and duration of treatment. Pain duration was defined as acute if lasting less than 3 mo and chronic if lasting more than 3 mos. The survey attempted to further categorize pain as somatic, visceral, neuropathic, or mixed. Somatic pain was defined as activation of pain receptors on either the body surface or in musculoskeletal tissues. Visceral pain was defined as pain when internal organs were damaged or injured. Neuropathic pain was defined as pain caused by injury or malfunction to the spinal cord or peripheral nerves. Mixed pain was defined as pain involving two or more of the first three categories. Unfortunately, despite this attempt at correlating the reasons specific drugs were administered to a specific type of pain, the survey data did not provide clearly defined answers, with some drugs being used for multiple types of pain while others were not specifically classified by respondents. Additional information was derived from comments from responding institutions that allowed for subjective scoring of perceived efficacy of analgesia provided, which were ranked 1 through 4, where: 1 = poor, 2 = fair, 3 = good, and 4 = excellent. Perceived efficacy of analgesia provided can be defined as the perception by associated humans of resolution of the painful condition warranting administration of an analgesic drug or other form of pain relief to a veterinary patient. It needs to be noted that perceived efficacy should not be interpreted as actual efficacy due to an intrinsic bias for positive results; however, perceived efficacy does provide some guidelines for future research to determine actual efficacy. Ease of use and complications associated with each method of analgesia were described based upon comments from the respondent. Information about adverse events was provided by responding institutions as well. These were scored subjectively by the authors based on survey comments as: 1 = mild, 2 = moderate, or 3 = severe. Minor adverse events identified included mild gastrointestinal discomfort, softer stools, and mild diarrhea while severe adverse events included gastric ulceration, vasculitis-dermatitis, and evidence of physiologic changes such as elevated renal values.

Pharmaceutical methods of providing analgesia were further divided into three groups: NSAIDs, opioid drugs, and others including non-NSAID-nonopioid drugs, nutraceuticals, or other methods of analgesia. The NSAID drugs that were administered to both hippopotami and giraffe included phenylbutazone, flunixin meglumine, ibuprofen, ketoprofen, firocoxib, carprofen, and meloxicam (Tables 1, 2). The NSAIDs that were administered only to giraffe included acetaminophen, aspirin, carprofen, suxibuzone, and vedaprofen (Table 1). No drugs were identified that were administered to hippos but not to giraffes. Opioid drug administration to both genera was much less commonly reported than was NSAID use. Opioids administered included tramadol and butorphanol, as well as one institution using "other" opioids in giraffe, with the drug name not provided in the survey. Non-NSAID-nonopioid methods of analgesia reported in both genera included glucosamine and chondroitin, gabapentin, corticosteroids, local anesthetics, and $\alpha 2$ agonists. Omega 3-6 fatty acids were exclusively reported in hippopotami. Low-level laser therapy and local anesthetics were exclusively reported in giraffe.

The perceived efficacy scores of NSAIDs were highly variable, with the overall efficacy reported as fair to excellent for the majority of NSAIDs administered to either giraffe or hippo. Flunixin meglumine and phenylbutazone were the most-commonly administered NSAIDS for both genera, but each had two reported perceived efficacy scores of "poor or no effect." Adverse effects from NSAIDs administered to giraffe were largely associated with the gastrointestinal system including soft stools, gastrointestinal discomfort, and diarrhea. Two of eight total reported adverse events for NSAIDs administered to giraffe were reported following ketoprofen administration. These two adverse events involved the renal system and included mild hematuria. Four adverse events were reported in the hippo. Three of these adverse events involved the gastrointestinal system: hematochezia following administration of phenylbutazone; a postmortem diagnosis of severe ulcerative gastritis following administration of phenylbutazone; and postmortem gastritis-abomasitis following administration of ibuprofen. Neither animal with postmortem changes was reported as demonstrating abnormalities while alive. The NSAID doses administered were also highly variable. Phenylbutazone had an extreme

variation in doses ranging from 0.75–20 mg/kg in giraffe (Table 1) and from 2.0–6.8 mg/kg in the hippo (Table 2). The other NSAID doses also varied but none to the extent of phenylbutazone.

Opioid drugs were less-commonly administered than were NSAIDs for analgesia in both giraffe and hippo. Tramadol and butorphanol were the only drugs utilized in both genera, with a report of one "other" opioid that was not identified within the survey. Perceived efficacy was again variable, with butorphanol ranging from fair to excellent. Tramadol was perceived as having poor to no effect in two giraffe. There were two reported adverse events in the giraffe consisting of sedation or drowsiness (or both) and one report of decreased fecal production. There were no reported adverse events after tramadol administration to hippo.

Non-NSAID-nonopioid methods of analgesia had variable perceived efficacy, ranging from poor for gabapentin and glucosamine-chondroitin to excellent for local anesthetics. Responses to the survey for non-NSAID-nonopioid methods of analgesia did not provide easily extractable dosage amount, route, and frequency information. As such, only name, number of institutions using, and perceived efficacy were analyzed for non-NSAID-nonopioid methods of analgesia. Specific statements of 'no effect observed' were noted. Corticosteroids and glucosamine-chondroitin were the most common of the non-NSAID-nonopioid treatments administered. There was a single report of mild sedation in a giraffe receiving gabapentin.

A significant limitation to this study was the inability to describe individual animal responses; instead, the survey reflects institutional responses which may be for single or multiple animals. Additionally, some but not all institutions reported historical information for animals that were either no longer present at that institution or that were no longer living. This resulted in broad-reaching data, reflecting more animals than what is accounted for by the number of responding institutions. As a result, readers must note it is not possible to accurately calculate individual species population numbers for statistical analysis of efficacy or incidence of side effects for the given drugs. Data can only be reported as institutional data, except for adverse events, which were reported as individual instances. There were no positive or negative control groups for the data presented in this study. All information provided is based upon institutional reports, which may vary widely depending upon institutional records, the accuracy of records, and the proficiency of data reporting by each institution.

Despite the limitations, this survey has demonstrated that NSAID drugs are the most common form of analgesia administered to captive giraffe and hippopotami, with phenylbutazone and flunixin meglumine being the most common NSAID drugs utilized (Tables 1, 2). Side effects reported for NSAIDs in giraffe involved the gastrointestinal system including gastrointestinal discomfort and diarrhea, severe gastric ulceration postmortem with no clinical signs, and the urinary system including mild hematuria. Side effects for NSAIDs in hippopotami were gastrointestinal but, more disconcertingly, included a report of vasculitis-dermatitis. A case report describing vasculitis-dermatitis has been previously reported in a Nile hippopotamus, but this was not linked to the administration of firocoxib.[11] There is only one report of a presumed adverse event from NSAID administration in the veterinary literature involving megavertebrates, and which resolved at the cessation of NSAID administration: a case of bullous skin lesions in a white rhino associated with oral administration of firocoxib.[13] This type of associated lesion was not noted by any institution administering firocoxib to giraffe.

It is important to note that oral administration was the most common method of administering analgesics to giraffe and hippo. Both species have fermenting foregut digestive systems, with the giraffe having a true rumen while the hippotamus has a three-chambered pseudo-rumen. Previous studies have determined that the unique physiology of the rumen may significantly alter drug absorption and solubility in ruminants versus monogastric species.[9] Dilution due to the increased volume of the rumen, binding to plant material in rumen contents, deactivation by ruminal microbes, rumen pH, and temperature may all be factors that affect oral drug absorption.[9] While the digestive system may affect drug absorption, care needs to be taken with some of the higher doses observed in this survey. Analgesic doses should not be based explicitly on the digestive system of a species. The differences in oral absorption due to gastrointestinal physiology justify individual pharmacokinetic studies for all medications if at all possible.

This survey primarily identified the substantial variability in NSAIDs dosages both between and within institutions, demonstrating the need for compilation and organization of formal formularies for these species. The NSAIDs were the most-

Table 1. Giraffe: Reported NSAID, opioid, and other non-NSAID-nonopioid use in giraffe for all institutions. Caution should be taken with the higher dosing levels expressed in the data in this chart. Many dosages were derived from dosages used in domestic large animal species, including domestic horses and cattle, as there have not been any pharmacokinetic or pharmacodynamics studies of these drugs performed in giraffe to date. Data reported in this chart represent institutional data, except for adverse events, which represent individual specimen data. — Represents data that was not recorded.

	Drug	Institutions reporting use of drug in giraffe	Dose administered (mg/kg)	Route	Dosing frequency	Number of institutions reporting perceived efficacy					Adverse events reported
						Excellent	Good	Fair	Poor	No response	
NSAIDs	Phenylbutazone	39	0.75–20	p.o.	q.o.d., b.i.d.	9	25	7	0	3	Softer stools; possible GI discomfort; taste aversion noted at many institutions
		2	0.73–0.85	i.m.	q24h	0	0	0	1	0	—
		1	4	i.v.	once	0	1	0	0	0	—
	Flunixin meglumine	28	0.23–1.9	p.o.,	q24h, b.i.d.	6	15	3	1	6	Possible elevation of renal values; taste aversion noted at many institutions
		13	0.5 - 1.1	i.m.	q24h,b.i.d.	1	5	2	2	0	Pain reported at injection site by two institutions
		2	1.1	i.v.	once	0	2	0	0	0	—
	Ketoprofen	9	0.5–3	p.o.	q24h	0	4	5	0	2	Mild hematuria noted at two institutions
		2	2–3	i.m.	q24h	0	0	0	2	0	—
		1	2	i.v.	q24h	0	1	0	0	0	—
	Firocoxib	10	0.045–0.3	p.o.	q24	3	2	4	0	1	—
		1	0.13	i.m.	once	0	0	0	0	1	—
	Meloxicam	7	0.05–0.6	p.o.	q.o.d., q24h	2	4	0	0	1	—
		2	0.02 (neonates) 0.2 (adults)	i.m.	q24h	0	2	0	0	0	—
	Etodolac	3	5–10	p.o.	q24h	0	2	1	0	0	—
	Ibuprofen	2	4	p.o.	q24h	0	1	1	0	0	—
	Carprofen	1	2.2	p.o.	Not specified	0	1	0	0	0	—
	Suxibuzone	1	3	p.o.	b.i.d.	0	1	0	0	0	—
	Vedaprofen	1	1.5	p.o.	q24h	0	0	0	1	0	—
	Celecoxib	2	2.2	p.o.	q24h	0	0	1	1	0	—
	Acetaminophen	1	No dose provided	p.o.	Not specified	0	0	1	0	0	—
	Aspirin	1	30	p.o.	q24h	0	1	0	0	0	—

Table 1. Continued.

	Drug	Institutions reporting use of drug in giraffe	Dose administered (mg/kg)	Route	Dosing frequency	Number of institutions reporting perceived efficacy: Excellent	Good	Fair	Poor	No response	Adverse events reported
Opioids	Tramadol	5	0.48–3	p.o.	q24, b.i.d.	1	1	0	2	1	Drowsiness at higher doses. Possible decreased fecal production at higher doses.
	Butorphanol	1	0.02	p.o.	q24h	0	1	1	0	1	—
		2	0.1–0.3	i.m.	q24h	0	1	0	0	0	—
		1	0.17	i.v.	once	0	0	0	0	1	Difficult to assess response, was administered while patient was anesthetized for surgical procedure
Other modes of analgesia	Glucosamine-chondroitin	15	4–21.6	p.o.	q24h, b.i.d.	0	3	7	2	3	Extreme taste aversion noted with some apple-flavored equine products
	Gabapentin	8	0.2–16	p.o.	b.i.d.	2	2	1	3	0	Possible lethargy at high doses
	Corticosteroids	5	Prednisone: 0.5–1 dexamethasone powder: one 5 or 10-g packet	p.o.	Once, q24h	1	2	0	1	1	—
		2	Dex-SP: 0.337		0	0	0	0	0	2	—
		1	Dexamethasone: 0.1	i.m.	q24h	0	0	0	0	1	—
	Local anesthetics	3	Variable doses		once	2	0	1	0	0	Noted as utilized only for local analgesia
	α2 adrenergics	2	Medetomidine (+ ketamine) : 0.04	i.m.	once	0	2	0	0	0	—
	Low level laser therapy	1				0	0	0	0	1	—
	Haluronic acid	2	150–300 mg total dose administered concurrently with NSAIDs	p.o.	q24h	0	1	0	0	0	—
	Polysulfated glycosaminoglycans	3	1.0–1.5	i.m.	q14–21 days	1	1	0	0	0	—

Table 2. Hippopotamus: Reported NSAID, opioid, and other non-NSAID-nonopioid use in hippopotami for all institutions. Caution should be taken with the higher dosing levels expressed in the data in this chart. Many dosages were derived from dosages used in domestic large animal species, including domestic horses, as there have not been any pharmacokinetic or pharmacodynamic studies performed to date in any species of hippopotamus. Data reported in this chart represent institutional data, except for adverse events, which represent individual specimen data. — Represents data that was not recorded.

	Drug	Institutions reporting use of drug in Hippopotamus	Dose administered (mg/kg)	Route	Dosing frequency	Number of institutions reporting perceived efficacy					Adverse events reported
						Excellent	Good	Fair	Poor	No response	
NSAIDs	Phenylbutazone	12	2–6.8	p.o.	q.o.d–b.i.d.	2	8	1	1	0	Ulcerative gastritis-abomasitis (identified postmortem) in one animal; intermittent fecal occult blood testing while receiving phenylbutazone reported in one animal
	Flunixin	9	0.3–1.3	p.o.	q24h, b.i.d.	2	4	1	1	1	—
	meglumine	2	1.1	i.m.	q24h	0	1	0	0	1	Response difficult to evaluate in some animals
		1	1.1	i.v.	q24h	0	1				—
	Ketoprofen	2	0.8–2.0	p.o.	q24h	0	1	0	0	1	—
		1	2.17	i.m.	q24h	0	1	0	0	0	—
	Etodolac	3	5–10	p.o.	q24h	0	2	1	0	0	—
	Meloxicam	2	0.1–0.15	p.o.	q24h	1	0	0	0	1	—
		1	0.1	i.m.	q24h	1	—	—	—	—	—
		1	0.1	s.q..	q24h	1	—	—	—	—	—
	Ibuprofen	1	800–8,000 mg per day	p.o.	q24h, b.i.d.	0	0	0	1	0	Ulcerative gastritis-abomasitis (identified postmortem) in one animal
	Celexicob	1	2.2	p.o.	q24h	—	—	—	—	—	No perceived efficacy given
	Firocoxib	2	0.09–0.11	p.o.	q24h	0	1	0	0	0	1 animal developed vasculitis-dermatitis while receiving drug

Table 2. Continued.

	Drug	Institutions reporting use of drug in Hippopotamus	Dose administered (mg/kg)	Route	Dosing frequency	Number of institutions reporting perceived efficacy					Adverse events reported
						Excellent	Good	Fair	Poor	No response	
Opioids	Tramadol	1	3	p.o.	q24h	1	0	0	0	0	—
	Butorphanol	1	0.02	p.o.	q24h	0	1	0	0	0	—
Other modes of analgesia	Glucosamine-chondroitin	4	0.1–7 mg/kg	p.o.	b.i.d.	0	2	0	1	1	—
	α2 agonists	1	Medetomidine (+ ketamine and butorphanol): 0.04 mg/kg	i.m.	Once	0	1	0	0	0	For chemical restraint
	Corticosteroids	1	Dexamethazone (Azium powder) 5–10 mg packets	p.o.	q24h	0	1	0	0	0	—
	Gabapentin	1	1.5	p.o.	b.i.d.	0	0	1	0	0	Administered concurrently with phenylbutazone
	Omega 3-6	1	Dose not specified	p.o.	Not specified	0	1	0	0	0	Noted as being administered specifically for skin

utilized analgesic medication, and dosages varied more than 20-fold. The variable efficacy, possible narrow safety margins, and potential for adverse events associated with NSAIDs is of concern with the doses identified.[3,5,6] Hence, the purpose of this study is to serve as evidence to support future studies in establishing reliable dosage modalities of drugs used for pain management in the giraffe and the hippopotamus. Pharmacokinetic and pharmacodynamics studies are necessary to establish safe and efficacious treatment plans that aid in pain management of these genera.

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Appendix 4

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DETERMINATION OF WHOLE BLOOD LEVELS OF CYCLOOXYGENASE-1 AND -2 ISOENZYME ACTIVITY IN ALPACA (*Vicugna pacos*)

J.J. Kottwitz, M.A. Edmondson[1] and D.M. Boothe
Department of Anatomy, Physiology and Pharmacology
The Department of Clinical Sciences[1], College of Veterinary Medicine, Auburn University, AL, 36849, USA

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ABSTRACT

This study determined concentrations of thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) and metabolites (PGEM) in alpacas utilising commercially available assays. Twenty healthy adult alpaca (11 castrated males and 9 females) participated in this study. Four ELISAs were utilised: two to quantitate TxB_2 in serum as an indicator of *in vitro* cyclooxygenase-1 (COX-1) activity, one quantitated PGE_2 and a fourth assay quantitated prostaglandin E_2 and metabolites (PGEM) in plasma as indicators of *in vitro* cyclooxygenase-2 (COX-2) isoenzyme activity after leukocyte exposure to lipopolysaccharide. Known standards were utilised for confirmation of assay results. Alpaca serum TxB_2 concentrations were substantially lower than reported in other species and those quantitated in four clinically normal horses using identical methods (alpaca=616±294, 95% CI [47, 753] pg/ml; horse=6087± 855, 95% CI [3964, 8210] pg/ml). Alpaca plasma mean PGEM concentration was 50±28, 95% CI [21, 111] pg/ml. Whole blood determination of TxB_2 and PGE_2 have been utilised in multiple species to evaluate COX isoenzymes for evaluating nonsteroidal anti-inflammatory drug (NSAID) COX inhibition. This study demonstrates that TxB_2 as measured using these methods may not be an acceptable method for determination of COX-1 response to NSAIDs in alpaca. The low whole blood TxB_2 levels identified by these assays may also indicate an intrinsic sensitivity to these drugs that warrants further investigation.

Key words: Alpaca, COX-1, COX-2, cyclooxygenase, prostaglandin E_2, thromboxane

Cyclooxygenase-1 (COX-1) is constitutively produced as it is required for essential physiological functions (Kujubu *et al,* 1991; Morita *et al,* 1995; O'Banion *et al,* 1991; Papich, 2008; Ren *et al,* 1995; Xie *et al,* 1991). The prostanoids produced by COX-1 may act as local signaling mediators and hormones mitigating control of intrinsic protective mechanisms of renal, gastrointestinal, or platelet function (Kawahara *et al,* 2015; Smith *et al,* 1994; Vane *et al,* 1998; Wilson *et al,* 2004). COX-2 is generally thought to be induced in response to injury, trauma and other inflammatory stimuli, with associations with the control of cellular division, angiogenesis and cardiovascular functions, as well as a role in influencing water and electrolyte balance controlled by the kidneys (Kawahara *et al,* 2015; Morita *et al,* 1995; Ren *et al,* 1995; Streicher and Wang, 2008; Timmers *et al,* 2007; Vane *et al,* 1998; Wilson *et al,* 2004). COX-1 is thought to play a role in tissue blood flow during inflammatory states, while the uncontrolled action of COX-2 may initiate the undesirable effects of the inflammatory process (Duz *et al,* 2015; Vane *et al,* 1998). COX-2 is considered more efficient than COX-1 in oxygenating eicosapentaenoic, gamma-linolenic, alpha-linolenic and linolenic acids, the key fatty acids involved in the inflammatory cascade (Kawahara *et al,* 2015; Otto and Smith, 1995). Because the function of both COX-1 and 2 are physiologically important, understanding the impact of therapeutic drugs on their function influences predictions of drug safety and efficacy.

Accordingly, understanding species differences in the expression of these two isoforms might facilitate predicting safety and efficacy of cyclooxygenase inhibitors. Many of the adversities associated with NSAID administration may be considered the sequelae of COX-1 inhibition (Amagase *et al,* 2014; Rodrigues *et al,* 2010; Sinha *et al,* 2013; Jones and Budsberg *et al,* 2000). Drugs that target COX-2 preferentially may be regarded as safer; however, species differences in enzyme catalytic activity could mitigate the safety of these drugs. The purpose of this study was to determine concentrations of TxB_2 as an indicator of COX-1 isoenzyme activity and concentrations of PEGM as an indicator of the

SEND REPRINT REQUEST TO J.J. KOTTWITZ email: jack_kottwitz@hotmail.com

inducibility of COX-2 isoenzymes in alpacas utilising commonly utilised, commercially available assays as a prelude to investigating response to drugs that may alter cyclooxygenase isoenzyme levels.

Materials and Methods

Two commercially available ELISA kits were used to determine Thromboxane (TxB_2) levels in serum as an indicator of COX-1 activity: Assay 1a (Thromboxane B_2 Express EIA) followed by Assay 1b (Thromboxane B_2 EIA, Cayman Chemical Co., Ann Arbour, Michigan, USA). Prostaglandin E_2 (PGE_2) was measured in plasma as an indicator of COX-2 activity using 2 commercial ELISA kits: Assay 2a (Prostaglandin E_2 Express EIA kit) and Assay 2b (Prostaglandin E_2 and Metabolites EIA, Cayman Chemical Co., Ann Arbor, Michigan, USA).

This study was approved by the Institutional Animal Care and Use Committee of Auburn University, Alabama (Protocols: 2013-2039 and 2014-2549). All animals in this study underwent a minimum 6 month washout period prior to sample collection. Blood for pilot data analysis was obtained from 7 adult alpacas (4 castrated males and 3 intact females) randomly selected from the Auburn University Small Ruminant Herd. Because TxB_2 was undetectable using Assay 1a, the full study using blood collected from a total of 20 randomly selected alpacas (11 castrated males and 9 intact females) was analysed using only Assay 1b. All animals were housed in an outdoor paddock on the veterinary campus.Three adult male castrated horses (two Thoroughbreds and one American Quarter Horse) and one adult female Percheron, privately owned by one of the authors, served as positive controls. These animals were maintained solely on free range costal Bermuda grass pasture. Blood was collected on premises from these animals.

Collection and analysis of both alpaca and horse blood utilised the same methodology. Blood (10 ml) was collected aseptically from the jugular vein, using a 12 ml syringe and an 18 gauge, 1.5 inch (38 mm) needle. All samples were processed and analysed in triplicate immediately after collection with processed serum and plasma stored at -20°C for repeat analysis. All assays (Assay 1a, 1b, 2a, 2b) were repeated to verify results.

Determination of TxB_2 as an indicator of COX-1, was performed by collecting 10ml of blood and immediately transferring the blood to a plain glass blood collection tube (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA). Blood was incubated at 20°C for 2 hours, then centrifuged twice at 2000 x g for 10 minutes (Beretta *et al,* 2005). The supernatant serum was harvested and divided into 250 µl aliquots prior to analysis. Pilot serum samples were analysed with Assay 1a according to the manufacturer's instructions, using the purification option to remove contaminants that may interfere with the assay. This methodology provided a manufacturer's specified detection limit of 45 pg/ml for undiluted samples. Samples were purified with ethanol to precipitate proteins and filtered through a disposable extraction column (Bakerbond SPE Otadecyl, C-18 extraction columns, Mallinckrodt Baker, Inc, Phillipsburg, New Jersey 08865, USA). For Assay 1b, serum samples from the group of 20 alpaca were processed utilising the same methodology as above, but without the purification step as per the manufacturer's instructions for that specific assay. This methodology provided a manufacturer's specified detection limit of 5 pg/ml for undiluted samples. Samples for both Assay 1a and 1b were diluted 1:10, 1:25, 1:50 and 1:100 with EIA buffer after purification. Known dilutions of assay standard were aliquoted for measurement with each plate for quality control purposes.

For determination of PGE_2, 10 ml of blood was collected as for COX-1 (via TxB_2) determination, then immediately transferred to a 10 ml lithium heparin blood collection tube (Vacutainer, Becton, Dickinson and Company, Franklin Lakes, New Jersey 07417, USA) to which 50 µg *E. coli* LPS, serotype 026:B6 (Sigma Aldrich Co., 3050 Spruce Street, St Louis, Missouri 63103) to induce COX-2 and 100 µg acetylsalicylic acid to inhibit COX-1 (Beretta *et al,* 2005) were added. Samples were transferred to 10 ml glass tubes, incubated at 37°C for 24 hours, then centrifuged twice at 2000 × g. Plasma was harvested, re-centrifuged at 2000 × g, then divided into 250 µl aliquots (Beretta *et al,* 2005). Samples analysed with Assay 2a were purified per the manufacturer's instructions which was as with Assay 1a to remove possible contaminants. Samples analysed with Assay 2b were purified by acetone precipitation for removal of proteins. This difference in purification allowed for the detection of PGEM. This methodology provided a detection limit of 36 pg/ml for Assay 2a and 2 pg/ml for Assay 2b. The supernatant was removed and dried under nitrogen. Purified samples for each PGE_2 assay were diluted at 1:10, 1:25, 1:50 and 1:100 with commercially available buffer (PGE EIA Assay Buffer, Cayman Chemical Co., Ann Arbor, Michigan, USA).

The standard curves for each assay were derived from the manufacturer's standard provided with

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each assay kit, prepared per the manufacturer's specifications. The concentration of TxB_2 or PGE_2 and metabolites in each sample were calculated from the standard curve for each dilution per the manufacturer's instructions and as described in previously published studies (Beretta *et al,* 2005; Brideau *et al,* 2001). All assay plates were read at a wavelength of 415 nm and all results are reported as mean ± 1 standard deviation as calculated using commercially available software (Microsoft Excel, Microsoft Redmond campus, Redmond, Washington, USA).

Results

Results of all assays performed are summarised in Table 1. Thromboxane concentrations were not detectable within the linear range of the standard curve in alpaca serum at any dilution utilising assay 1a in the serum of the 7 pilot alpacas, whereas equine serum TxB_2 concentrations were 4146 ± 229 pg/ml, 95% CI [3829, 4463].

The Thromboxane Express (TxB_2 Express) assay did not show measurable results in Alpaca. The Thromboxane Assay (TxB_2) assay showed measurable results for alpaca, but those results were 1/10 of the results for horses utilising the same assay.These results indicated a substantially lower level of Thromboxane activity as an indicator of intrinsic Cycloxygenase-1 activity in alpacas compared to horses. This low COX-1 level will complicate thorough evaluation of drugs affecting COX-1 levels and may be a predisposing factor for well documented health problems in alpaca, such as C3 ulceration. Prostaglandin E_2 and metabolites levels were comparable between horses and alpaca, suggesting that inducibility of COX-2 is comparable between these two species.

Serum TxB_2 concentrations in the full study based on Assay 1b were 616 ± 294, 95% CI [478, 753] pg/ml at the 1:10 dilution. All other dilutions were below the detection limits of the assay.Equine mean serum TxB_2 levels were 6087 ± 855, 95% CI [3964, 8210] pg/ml at the 1:10 dilution using the same methodology.

PGE_2 was detectable in both horses and alpaca with assay 2a. In alpaca, the mean concentration was 34 ± 10, 95% CI [27, 41] pg/ml and for horses, mean concentrations were 34 ± 6, 95% CI [25, 43] pg/ml. For PGEM, concentrations were detectable in both horses and alpaca at the 1:10 dilution with assay 2a. In alpaca, the mean concentration was 50 ± 28, 95% CI [21, 111] pg/ml and for horses, mean concentrations were 34 ± 6, 95% CI [26, 43] pg/ml.

Discussion

The results determined in this study indicate that these commercially available ELISA kits are not appropriate bioanalytical assays to evaluate COX activity in alpaca. Evaluation of COX activity as a means of *in vitro* evaluation of NSAID drugs was first published in human medical literature (Patrignani *et al,* 1994). It is important to distinguish between the presence of COX isoenzymes and the activity of these enzymes. The specific inhibition of the activity of these isoenzymes is the target of NSAID drug therapy. Because of this, quantification of enzyme activity is an important component of understanding how NSAIDs function in different animals and describing how different drugs target both COX-1 and COX-2. The original human study utilised a combination of radioimmunoassay and Western blot to determine whole blood PGE_2 and TxB_2 concentrations via radioimmunoassay detection of H3 labeled [3H] PGE_2 or [3H] TxB_2 and specific rabbit anti-PGE_2 or anti-TxB_2 sera (Patrignani *et al,* 1994). Thromboxane A2 (TxA2) is produced from arachidonic acid metabolism via COX-1 activity by

Table 1. Summary of Assays performed at a 1:10 dilution for both Alpaca and Horse.

Dilution 1:10							
Assay	**Name of assay**	**Species**	**Number of Animals**	**Mean (pg/ml)**	**Standard Deviation**	**Low Range (pg/ml)**	**High Range (pg/ml)**
1a	TxB_2 Express	Alpaca	7	not detectable	---	---	---
1a	TXB_2 Express	Horse	4	4146	± 229	3984	4308
1b	TxB_2	Alpaca	20	616	± 294	329	1487
1b	TxB_2	Horse	4	6087	± 855	5483	6692
2a	PGE_2 Express	Alpaca	7	34	±10	27	41
2a	PGE_2 Express	Horse	4	34	± 6	25	43
2b	PGE_2 + metabolites	Alpaca	20	50	± 28	21	111
2b	PGE_2 + metabolites	Horse	4	34	± 6	30	39

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multiple cells within the body, but is considered to not be a measurable circulating hormone as it is rapidly hydrolysed to TxB_2, which is then metabolised slower and eventually eliminated from the body by the kidneys (Patrignani *et al,* 1994; Patrono *et al,* 1986).

Previous studies have demonstrated variability in intrinsic TxB_2 levels in domestic animals, with horses, pigs and cats having much lower concentrations than dogs or cattle (Patrono *et al,* 1986; Reinke and Brune, 1988 and Van Hoogmoed *et al,* 2004). Horses were utilised as a comparative control for this study because of previously published studies having documented this intrinsically lower level. The mean TxB_2 level determined in the alpaca in this study is lower than the lowest reported in the literature in domestic animals and is approximately 10% of that of the mean of horses evaluated by the same assays. Conversely, the levels of TxB_2 and PGE_2 and PGEM detected for horses in this study were comparable to previously published results, which indicates that the results are repeatable and that the camelid-specific species difference is likely real using these tests (Patrono *et al,* 1986).

Both species evaluated in this study had similar inducible COX-2 levels, measured by PGE_2 and metabolite analysis after induction with LPS (Patrono *et al,* 1986; Reinke and Brune, 1988 and Van Hoogmoed *et al,* 2004). PGE_2 is constitutively produced as a primary product of arachidonic acid metabolism via COX-2 enzymatic activity in multiple cells in the body as a result of external stimuli and activation of that pathway (Hamburg and Samuelsson, 1971). In whole blood assays, the source for COX-1 products (TxB_2) is platelets and the source of COX-2 products (PGE_2 and metabolites) is leukocytes. This methodology utilising whole blood has been generally accepted as the gold standard for determining COX-1/COX-2 enzyme activity specificity because it incorporates the proteins, cells, platelets and circulating enzymes into the assay that normally occur in circulating blood (Papich, 2008). These components are not present in isolated cells or enzyme systems.

In veterinary drug studies, commercially available ELISAs have largely replaced the more expensive and cumbersome radioimmunoassays and as a result have become the accepted standard for determining TxB_2 and PGE_2 (and metabolites) to ultimately evaluate COX-1 and COX-2 inhibition (Beretta *et al,* 2005; Brideau *et al,* 2001; Cunibeti *et al,* 2012; Davis *et al,* 2015; Duz *et al,* 2015; Minter *et al,* 2011). It needs to be noted that use of liquid chromatography–mass spectrometry (LC-MS) has been described for determining both TxB_2 and PGE_2 concentrations in serum, but these methodologies have largely been regulated to human studies only with few published studies in animals to date (Cao *et al,* 2011; Knych *et al*, 2015). The authors have an ongoing study developing LC-MS methodology for comparison with the results of this study. While the prostaglandin (PGE_2 and PGEM) levels were comparable to other domestic species, what was surprising with this study was the serum TxB_2 concentrations in alpaca that were not measurable with Assay 1a and were substantially lower than that of horses when measured with Assay 1b, indicating that the use of these specific bioanalytical assays to evaluate COX activity in this species is not appropriate.

The assays utilised to determine TxB_2, PGE_2 and PGEM in this study have been utilised in multiple animal species including domestic dogs, cats and horses indicating cross-species efficacy for these assays (Beretta *et al,* 2005; Brideau *et al,* 2001; Cunibeti, *et al,* 2012; Duz, *et al,* 2015). The fact that levels were detectable in horses and control values were as expected based on known standards indicate that there was no assay or human malfunction that resulted in the unusually low levels that were detected. Despite the history of use of ELISA in multiple species and validation through known standards, one variable with the multiple assays used in this study is the antibody bound to the wells of each 96 well plate. Assay 1a utilises a goat polyclonal anti-mouse IgG antibody bound to the individual well, while the Assay 1b utilises a mouse, monoclonal anti-rabbit IgG antibody. These both bind preferentially to a monoclonal rabbit antibody specific for TxB_2. These assays function based on the competition between TxB_2 and a TxB_2 acetylcholinesterase (AChE) conjugate for those limited well bound antibody sites. The AChE Tracer + TxB_2 conjugate is maintained at a constant level while the concentration of free TxB_2 varies. This allows for an inverse quantitation of serum TxB_2 levels. It is possible that there may be decreased binding of the free TxB_2 or AChE + TxB_2 conjugate to the TxB_2 specific antibody because of differences in the alpaca TxB_2 protein. While it may be possible, it was beyond the scope of this study to determine and quantitate any structural differences in alpaca TxB_2 Monoclonal anti-thromboxane antibodies, as was utilised in Assay 1b, have traditionally shown high specificity for TxB_2 with limited detectable cross reactivity (Reinke and Brune, 1988). Further studies are indicated, including specific mapping of the structure.

This is the first study of COX isoenzyme activity in alpaca the veterinary literature. The clinical implications in alpaca of low TxB_2 indicating low intrinsic COX-1 levels determined by these ELISAs are substantial. The low TxB_2 and subsequent COX-1 levels detected in this study imply that this assay may not be useful to assess NSAID COX isoenzyme inhibition in this species. In addition, if these results are real, the low levels of TxB_2 as an indicator of COX-1 activity may indicate a substantially higher risk of toxicity in alpaca for medications that inhibit these enzymes. NSAIDS favoring COX-1 inhibition, such as phenylbutazone and flunixin meglumine, are commonly administered and recommended for camelids with clinical disease (Drew *et al*. 1992; Navarre *et al,* 2001a; Navarre *et al,* 2001b). Further study is needed to determine the severity of this increased risk in alpaca. Pending further investigations, including verification of low TxB_2 levels using different analytical methods, such as LC-MS, practitioners may be advised to use caution when prescribing these medications, or others that affect COX isoenzymes.

The association between decreased COX-1 activity and gastric ulceration is well established in both humans and animals (Amagase *et al,* 2014; Rodriguez *et al,* 2010; Sinha *et al*, 2013; Jones and Budsberg *et al,* 2000). Gastric ulceration or ulceration of the glandular compartment of the stomach in the absence of administration of NSAIDs has been documented in camelids including alpacas (Cebra *et al,* 2003; Smith *et al,* 1994). To date, a definitive etiological agent has not yet been determined and there are few publications examining the effects of NSAIDS in alpacas or similar species. A study describing the association of prostaglandins with intestinal motility in llamas showed no response to NSAID drug exposure, but did not specifically investigate association of COX isoenzymes and the protective layer of the glandular gastric third compartment (C3) (Van Hoogmoed *et al,* 2004). The presence of these enzymes does not necessarily describe their specific activity. The association of constitutively expressed COX-1 with the protective prostaglandin layer of the glandular stomach leads to the conclusion that an animal with intrinsically low COX-1 levels is likely to be prone to gastric ulceration. This is concerning for all South American camelids, not just alpaca, because low levels of TxB_2 as an indicator of COX-1 observed in this study may be a potential predisposing factor for the C3 ulceration problems observed in these species (Cebra *et al,* 2003; Smith *et al*, 1994).

Further studies are indicated, including evaluation of gastric mucosal COX-1 levels to expand our knowledge base regarding COX enzymes in alpaca. This data is compelling in its implication that the unique physiology of the alpaca in respect to COX isoenzymes could affect NSAID metabolism and it may be a contributing or predisposing factor for gastric ulceration secondary to administration of these drugs. Further study of this same nature is also indicated in other camel and camelid species.

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