ABSTRACT

DAVIS, JENNIFER LYNN. Pharmacokinetics of antimicrobial drugs in the horse.
(Under the direction of Mark G. Papich.)

Oral antimicrobial drug therapy in horses is limited by a lack of approved formulations, cost, poor bioavailability and adverse effects. Additionally, little is known about the relationship between plasma concentration and tissue concentrations in horses. Therefore, the purpose of this research was to determine the factors that affect oral absorption and tissue distribution of antimicrobial drugs in the horse. In order to examine this, we performed pharmacokinetic studies using drugs that represent a broad range of physicochemical characteristics as well as drugs that represent different classes based on the Biopharmaceutics Classification System (BCS), a system developed to screen drugs and predict oral drug absorption in humans based on the solubility and permeability characteristics.

Experiments were also performed to study drug concentrations in the tissue – the site of drug action. Interstitial fluid was used to represent unrestricted drug diffusion between capillaries and tissue. The aqueous humor of the eye was chosen to represent areas with physiologically restricted drug penetration. In vitro experiments were performed to determine plasma protein binding, lipophilicity and mucosal permeability of commonly used drugs in the horse.

Four pharmacokinetic studies were completed using voriconazole and orbifloxacin (BCS Class I), cephalexin (BCS Class III) and doxycycline (BCS Class IV). An additional study was done comparing itraconazole capsules (BCS Class II) and itraconazole solution (BCS Class I) to determine the effect of increasing the solubility of a drug on oral absorption.
As predicted based on the BCS, voriconazole and orbifloxacin had high bioavailability (135% and 68%, respectively), while cephalixin and doxycycline had low bioavailability (5% and 2.8%, respectively). Itraconazole capsules had a low bioavailability (12%) while the solution had a high bioavailability (64%). These studies confirm our hypothesis that oral drug absorption can be predicted based on the solubility and permeability characteristics of the drug.

Drug concentrations in the interstitial fluid were determined and were found to be highly correlated with the plasma protein binding of the drug. In contrast, drug concentrations in the aqueous humor were found to correlate well with the lipophilicity of the drug.

Our studies next examined methods to confirm the BCS classification based on effective permeability ($P_{eff}$) of the equine small intestine using an *in vitro* Ussing chamber model. Fluconazole, metronidazole, marbofloxacin and cephalixin were used in this study because these drugs exhibit high solubility, but they differ in their lipophilicity, molecular weight and oral bioavailability in horses. Good correlations were found between permeability and bioavailability and between permeability and lipophilicity. A strong correlation was also found between the permeability and molecular weight, which may be caused by paracellular transport of drugs with low molecular weight.

In summary, this series of studies established, for the first time, that *in vitro* methods can be used to successfully predict oral drug absorption and tissue distribution in horses. The results of these studies have important implications. They will help horses and horse owners by providing a greater selection of effective drugs for treating diseases in horses. They will assist veterinarians by providing them with greater choices of drugs and more information.
about the potential effectiveness of drugs they prescribe. These studies will also assist the pharmaceutical industry by helping to efficiently screen potential drug candidates for development. Lastly, these studies can be used by regulatory agencies to provide information that may be used as a guide to evaluate oral absorption studies in horses and the effects of formulation on oral drug absorption.
PHARMACOKINETICS OF ANTIMICROBIAL DRUGS IN THE HORSE

by

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DEDICATION

I would like to dedicate this dissertation to my parents, Charles and Nancy Davis, for their unconditional love, support and patience during the last 16 years of my higher education. I also dedicate this to Chris for 19 years of love and friendship. Without him, I would never have become a veterinarian.
BIOGRAPHY

Jennifer Lynn Davis was born in Heidelberg, Germany on December 14, 1972. She received her Bachelor of Science degree in Animal Science and her Doctor of Veterinary Medicine from VA Tech. She then did an internship at Mississippi State University in Equine Medicine and Surgery. From there, she completed a residency in Equine Internal Medicine at North Carolina State University, during which time she received a Master’s degree in Specialized Veterinary Medicine and achieved board certification as a specialist in Large Animal Internal Medicine. Following this, she began a PhD program and residency in Clinical Pharmacology, which she completed in May 2006. She also received board certification as a specialist in Veterinary Clinical Pharmacology in 2005.
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I would like to begin by thanking the members of my PhD committee. Dr. Mark Papich has been an excellent mentor and role model for me over the past four years. I respect and admire him for his dedication, professionalism and intellect. I hope to one day become as talented and accomplished as he is. Drs. Sam Jones, Brian Gilger and Sarah Gardner have been my mentors and friends for the past 7 years. They have played an integral role in my development as a veterinarian, a researcher, and a person. I truly feel privileged and honored to have worked with these four people and I hope to continue to work with them throughout the remainder of my career. To all of you, I thank you from the bottom of my heart.

I also need to thank Beth Salmon for all of her help with the pharmacokinetic studies, as well as for being a wonderful person and friend during the good and bad times. Beth is one of the best people I know, and I will always be in her debt for all of her support.

This research would also not have been possible without the help of Dr. Butch Kukanich, Dr. Tara Bidgood and Delta Dise. They provided me with technical and mathematical assistance. More importantly they made me laugh, they supported me unconditionally and they are (and hopefully always will be) my friends as well as my colleagues.

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1. INTRODUCTION

Antimicrobial drug therapy with oral medications in horses is limited by a lack of approved formulations, cost, poor bioavailability, as well as an unacceptable risk of side effects, particularly those that involve the gastrointestinal tract. According to the USDA-FARAD, there are currently only 6 oral antimicrobial formulations marketed for use in the horse, four of which are trimethoprim/sulfadiazine combinations, one is a combination of pyrimethamine and sulfadiazine, and the other is the antifungal drug griseofulvin. Because of this, horses are usually treated with drugs formulated for humans or small animals, which requires manipulation of the dosage form through compounding, administration of a large number of low dose strength tablets and capsules or large volumes of solutions or suspensions. These formulations are also very expensive, particularly when used to treat such a large animal on a mg/kg basis.

Another concern in the use of oral antimicrobial drugs in horses is the poor oral bioavailability of compounds that are well absorbed in other species. Examples of this include ketoconazole (Prades et al, 1989), amoxicillin (Wilson et al, 1988) and cefadroxil (Duffee et al, 1997). Low oral bioavailability of antibiotics can cause changes in the gastrointestinal microflora, produce diarrhea, and increase the risk of life-threatening colitis. The unique gastrointestinal anatomy and physiology of the horse may be responsible for these differences, but this has not been thoroughly investigated.

A better tool is needed to evaluate oral drug absorption for horses. By developing a system to study the in vitro characteristics of a drug and make predictions regarding oral absorption based on these characteristics, drugs can be screened prior to administering the drug to animals. Such a system is already in use in human medicine.
and is being proposed for use in dogs to predict oral drug absorption and therefore gain bioequivalence waivers for generic or altered drug formulations. More efficient in vitro evaluation of formulation candidates will save drug sponsors time and money and improve drug availability for horses. Evaluation of drug candidates for people has been conducted with the Biopharmaceutics Classification System (BCS). The BCS classifies drugs based on their solubility and permeability characteristics (Amidon et al, 1995). The development of a similar system for horses would have obvious benefits, the most important of which may be more efficient evaluation of drug candidates for horses and eventually approval and marketing of more drugs specifically formulated for this species.

Once the drug is absorbed, its distribution in the body, and particularly its penetration to the site of the infection, will determine the outcome of therapy. The majority of infections occur in the extracellular (interstitial) fluid, therefore the concentration of protein unbound drug at this site will be associated with therapeutic success. When infections occur in other areas, such as the eye, brain and prostate, which are protected by functional or anatomic barriers, therapeutic agents are prevented from reaching these sites to treat infections (Barza, 1993). By studying the in vitro physicochemical properties of drug candidates for horses and then relating them to the ability of the drug to reach therapeutic concentrations at its intended site of action, we can more accurately predict which drugs will be successful for therapy in these animals.

The purpose of this research was therefore twofold. First, the physicochemical factors affecting oral drug absorption in the horse were assessed in order to begin the development of a BCS for horses. Second, these physicochemical factors were further investigated to determine which characteristics would predict drug penetration into the
interstitial fluid as well as the aqueous humor, which was used to represent those areas protected by specialized anatomical barriers. Chapters 2 and 3 will review the literature regarding oral absorption and tissue distribution of drugs in the horse to lay the scientific foundation for these studies. Chapters 4, 5, 6, 7 and 8 detail pharmacokinetic studies performed to begin to assess the feasibility of using in vitro/in vivo correlations in the horse. Chapter 9 is a report on an in vitro method developed to study the mucosal permeability of drugs in the horse, one of the most important factors in oral absorption. Chapters 10 and 11 will summarize our findings and detail plans for future research in this area that will ultimately lead to our goal of developing a BCS for horses.
1.1 References


2. LITERATURE REVIEW: ORAL ABSORPTION

2.1 THE BIOPHARMACEUTICS CLASSIFICATION SYSTEM

Oral absorption of pharmaceuticals is determined by drug factors and animal (species) factors. Each of these is equally important in achieving good oral absorption, and they are often interrelated. These relationships have been extensively studied and defined in humans, resulting in a classification system that can predict the oral absorption of immediate release dosage formulations of drugs on the basis of certain physicochemical characteristics. This system is called the Biopharmaceutics Classification System (BCS) and will be described in detail below.

The BCS is used by the United States Food and Drug Administration (FDA) as a scientific approach to identify drugs that may be eligible for waivers of in vivo bioavailability and/or bioequivalence studies for immediate release solid dosage forms (FDA Guidance for Industry, 2000). These waivers are designed to improve the efficiency of drug development and the drug review process, thereby saving pharmaceutical companies time and money during the development of generic or altered drug formulations. Additionally, this system is frequently used by pharmaceutical companies early in drug development to identify the best drug candidates for further development. Recent efforts have also focused on using the characteristics identified by the BCS to predict not only drug absorption, but also in vivo drug disposition, including drug elimination and efflux transporter-enzyme interactions (Wu and Benet, 2005).

In order to identify appropriate drug candidates, the BCS specifies four different categories based on the solubility and permeability characteristics of the drug (Amidon et al, 1995). Class I drugs are characterized by high solubility and high permeability,
therefore oral absorption is high and minimally affected by the formulation. For these
drugs, gastric emptying time is the rate limiting step for oral absorption of rapidly
dissolving dose forms. Class II drugs have low solubility and high permeability. Drug
dissolution is the rate limiting step for absorption of these compounds. These drugs often
have a prolonged absorption time, and absorption may be highly variable based on the
formulation and *in vivo* variables. Class III drugs have high solubility, but low
permeability. Permeability across the intestinal membranes thus becomes the rate
limiting step for absorption of these drugs. Similar to Class I drugs, formulation for
immediate release dosing forms has little influence on absorption. Class IV drugs have
both low solubility and low permeability, and therefore oral absorption is often poor.

Class I drugs are currently the only drugs eligible for bioequivalence waivers,
providing they also meet the following criteria: 85% dissolution within 30 minutes *in
vitro*, stability in the gastrointestinal tract (GIT), no significant effects of excipients on
rate or extent of oral dosing, a wide therapeutic index, and absorption in the intestine,
rather than the buccal cavity (FDA Guidance for Industry, 2000). More recently,
extensions of biowaiver eligibility have been proposed for Class II and Class III drugs
(Yu et al, 2002). Investigations of the potential use of a BCS in veterinary species have
also been performed, however the determination of interspecies differences still requires
further study and bioequivalence waivers have not yet been granted for any veterinary
drugs (Martinez et al, 2002a,b). In order to extend the BCS to include horses, we must
first examine the definitions of drug solubility and permeability and adapt them to this
species, based on the methods of drug administration and the physiologic characteristics
specific to the horse. In addition, one must show that a measure of permeability
(measured by LogP in your studies), correlates with another accepted measure of permeability, the $P_{\text{eff}}$ (effective permeability).

2.2 **Drug Solubility**

The aqueous solubility is directly proportional to the number of hydrogen bonds that can be formed with water molecules (Stenberg et al, 1999), and drugs must go into solution prior to being absorbed across the intestinal membranes. Therefore, the aqueous solubility has a direct effect on the fraction of the drug available for absorption. Drugs must not only be able to form a solution in aqueous media, but they must also be able to go into solution in the gastrointestinal tract before they pass to the distal intestine or are eliminated in the feces. The majority of drugs dissolve in the stomach, so that they are already in solution and available for absorption in the small intestine. Therefore, dissolution must occur at a faster rate than gastric emptying to ensure optimum absorption. The following discussion focuses on the methods for determining drug solubility and dissolution rate, as well as the properties of a drug that will affect its ability to form a solution.

2.2.1 **Solubility Classification**

In order for a drug to be classified as highly soluble (BCS Class I and III), the highest dose strength must be soluble in $\leq 250$ mL of water at a temperature of $37 \pm 1^\circ$C, over a pH range of 1-7.5. These conditions were defined based on the typical human bioequivalence studies in which the drug is administered orally with 250 mL (approximately 8 oz or 1 cup) of water (Yu et al, 2002). An equation was developed to determine the solubility classification as described by the dose number (Do):
Do = \frac{M_o}{V_o}/C_s

where \( M_o \) is the maximum dose strength of the drug formulation, \( V_o \) is the volume of fluid administered (250 mL), and \( C_s \) is the saturation solubility of the compound (Table 2-1). According to the BCS, drugs with a \( D_o < 1 \) are classified as having high solubility. For poorly soluble drugs, the \( C_s \) is usually \( < 0.1 \text{ mg/mL} \) (Horter and Dressman, 2001).

The rate at which a drug goes into solution is also a factor in determining drug absorption. In order for a drug to be considered for a bioequivalence waiver, no less than 85% must dissolve within 30 minutes (FDA Guidance for Industry, 2000). Dissolution testing is performed using specialized equipment that simulates intestinal mixing. The dissolution must be examined in a United States Pharmacopeia (USP) Apparatus I at 100 rpm or Apparatus II at 50 rpm in a volume of 900 mL of media. The dissolution testing is also performed using three different types of media including 0.1 N HCl or Simulated Gastric Fluid USP without enzymes, buffer with a pH of 4.5, and buffer with pH 6.8 or Simulated Intestinal Fluid USP without enzymes. At least 12 dosage units must be tested, with recommended sampling times of 10, 15, 20, and 30 minutes. For bioequivalence waivers, the dissolution of the new drug formulation is then compared to a reference product and a similarity factor \( (f_2) \) is calculated which is a measure of the percent of dissolution between the two product curves. Dissolution profiles are considered similar when the \( f_2 \) is \( \geq 50 \).

2.2.2 Factors Affecting Drug Solubility

The important factors of drug dissolution are summarized in the modified Noyes-Whitney equation (Dressman et al, 1998; Horter and Dressman, 2001):

\[
DR = (A^*D/h) * [C_s - (X_\theta/V)]
\]
Where DR is the dissolution rate, A is the surface area, D is the diffusion coefficient, h is the thickness of the boundary layer adjacent to the dissolving surface, $X_d$ is the amount of drug already dissolved, and V is the volume of the dissolution media. The physicochemical factors that can influence this relationship are summarized below.

The saturation solubility ($C_s$) is one of the main determinants of the concentration gradient across the boundary layer, which in turn, is the driving force for drug dissolution. The $C_s$ can be affected by drug polymorphisms, that is, the ability of a drug to exist in more than one crystalline structure with different physicochemical properties, including solubility and dissolution rate (Doelker, 2002). It can also be affected by the drug’s ability to be solubilized in surfactants or ingested foodstuffs. Bile can increase the solubilization capacity of poorly soluble drugs. The relationship between the lipophilicity of a drug and its ability to become solubilized in bile was described by Collett and Koo (1975). Subsequently, a model was developed to predict the ability of a compound to become solubilized based on its Log P value and aqueous solubility (Mithani et al, 1996). Other factors, such as molecular weight and drug-micelle interactions caused by repulsive forces in charged molecules may also affect solubilization capacity (Horter and Dressman, 2001).

The pH of the dissolution media, including gastrointestinal lumen contents, can affect both the extent and rate of aqueous solubility by modifying the proportion of ionized to unionized drug. This proportion is described by the modified Henderson-Hasselbeck equation (Seydel and Schaper, 1981):

For weak acids:  $\%\text{unionized} = \frac{100}{1 + \text{antilog}(\text{pH}-\text{pKa})}$

For weak bases:  $\%\text{unionized} = \frac{100}{1 + \text{antilog}(\text{pKa}-\text{pH})}$
In general, drugs in the ionized state have higher total solubility than those in the unionized state (Martinez and Amidon, 2002). For weak acids, solubility will increase linearly with increases in pH at values greater than \( \text{pKa} + 1 \), because a larger proportion of the drug exists in the ionized form (Horter and Dressman, 2001). The inverse relationship is true for weak bases. The rate at which dissolution occurs is also affected in a similar manner. For weak acids, dissolution is faster at a higher pH when the drug is predominantly ionized. In contrast, for weak bases, dissolution is faster at a lower pH.

The dissolution rate of a drug is strongly related to its particle size. Small particle size increases the exposed surface area, which in turn causes a faster dissolution rate (Dressman et al, 1998; Horter and Dressman, 2001; Martinez and Amidon, 2002). Therefore, micronization of drug particles to 3-5 \( \mu \text{m} \) can enhance drug dissolution and the subsequent oral bioavailability. Exceptions occur when the dissolution medium has little ability to wet the surface of the particle. This can lead to drug agglomeration and a decrease in effective surface area (Solvang and Finholt, 1970).

Both the volume and viscosity of fluids in the gastrointestinal tract affect the solubility of a drug. Increasing the volume of fluids available for drug dissolution will increase the overall solubility of that compound. The dose:solubility ratio is commonly used to determine the amount of fluid necessary to dissolve a given amount of drug. For example, a tablet with dose strength of 500 mg and a solubility of 15 \( \mu \text{g/mL} \) will require 33 liters of fluid for complete dissolution, as is the case with griseofulvin (Dressman et al, 1998). This is often a problem for poorly soluble BCS Class II and IV drugs, since there is rarely enough fluid present in the gastrointestinal lumen for complete drug dissolution. Higher viscosity fluids will decrease the dissolution rate of a drug, even
though they may increase the overall solubility, by causing a decrease in the diffusivity of the compound in the dissolution media (Braun and Parrott, 1972).

Feeding can affect the oral absorption of compounds in several different ways: It can change the pH of the gastric contents, alter gastric emptying and gastrointestinal motility, and stimulate the secretion of bile components. Feeding usually causes an increase in gastric pH, a decrease in intestinal pH and a delay in gastric emptying. This can enhance or hinder drug solubility, based on the physicochemical properties specific to each drug. Feeding of lipids causes an increase in the secretion of bile acids and phospholipids (Persson et al, 2005). This has been shown to improve drug bioavailability, presumably by increasing the solubilization of lipophilic drugs, such as griseofulvin (Crounse, 1961). Another mechanism by which this may increase drug absorption is through direct uptake of the drug-lipid micellar complexes into the intestinal lymphatic system, which would allow the drug to bypass the first-pass metabolism effects of the liver (Porter and Charman, 2001). Complexation of some drugs with protein constituents of food may also increase solubility. The classic example of this is dicoumarol, which forms complexes with the milk protein, casein and increases bioavailability (Macheris and Koupparis, 1986).

2.3 Drug Permeability

Once a drug is solubilized, it must be transported across the intestinal membrane to reach the systemic circulation. This can occur via two different routes: between the cells (paracellular) or through the cells (transcellular).
2.3.1 Permeability Classification

A drug is considered to be highly permeable based on the BCS if it meets the criteria of > 90% absorption in humans after oral administration. This calculation must be based on pharmacokinetic studies in which the oral bioavailability is determined via mass-balance measurements or in comparison to an intravenous reference dose (FDA Guidance for Industry, 2000). Direct measures of \textit{in vivo} or \textit{in vitro} permeability are also allowed, and these will be discussed in section 2.3.4.

2.3.2 Paracellular Drug Permeability

Drug movement by the paracellular route is less common and limited by the tight junctions formed between the cells of the intestinal membrane. Therefore, this is usually considered a minor route of drug absorption, except for small, hydrophilic compounds. Paracellular transport occurs by diffusion, or through convective volume flow through the intercellular pores. The number of paracellular spaces available for drug absorption limits paracellular absorption because it is dependent on intestinal surface area. Pore size also is important, which in humans is approximately 3-10 Å (Lennernas, 1995). This does not appear to be consistent across species, however. Pore size is similar to humans in the rat, but larger in the dog (He et al, 1998). The small pore size for humans limits paracellular permeability to drugs with a molecular weight of <200 Da (Lennernas, 1995). In dogs, this limit may be closer to 600 Da, which would increase the number of drugs available for this route of absorption (He et al, 1998). There is no data available on pore size or molecular weight limits for the paracellular route in the horse.
2.3.3 Transcellular Permeability

Transcellular permeability is the most common route of drug transport across the intestinal membrane. This is a highly complex process that requires movement through a lipid bilayer with four distinct regions with differing water and lipid content (Tieleman et al, 1997). The outermost layer has a high proportion of water molecules and is responsible for interactions with the surrounding area. The second region has the highest molecular density and very few water molecules. This layer represents the greatest barrier to drug diffusion across the membrane. The third region is composed mostly of nonpolar tails and limits drug penetration that is caused by molecular size and shape. The fourth region is the most hydrophobic and limits drug penetration based on the hydrophilicity of the compound.

The majority of drugs that cross the membrane transcellularly do so via passive diffusion. Passive diffusion is governed by the molecular weight of the compound, drug lipophilicity, polar surface area, and molecular flexibility (Palm et al, 1996). Molecular size is negatively correlated with drug permeability. Lipophilicity, often measured as the logarithm of the octanol:water partition coefficient at pH 7.4 (Log P) or at pH 5.5-7.5 (Log D), is positively correlated with drug permeability. Using a mathematically derived Log P (Log $P_0$) optimal drug permeability would theoretically occur at a value between 2 and 7 (Seydel and Schaper, 1981). However, this may not be applicable to drug absorption in vivo, as many drugs with a Log P of greater than 5 are not absorbed, due to partitioning and trapping within the lipid membrane. More recent experiments have found an even stronger correlation between the polar surface area and the permeability of the molecule (Palm et al, 1998). The polar surface area reflects the sum of the surfaces of
the polar atoms present in the molecule. Usually, this includes all oxygen, nitrogen and attached hydrogen molecules. There is an inverse correlation between polar surface area and intestinal permeability. Molecular flexibility is a function of a compound’s ability to undergo conformational changes that will eventually affect the polar surface area of the molecule.

Gastric and intestinal mucous layers also have a significant effect on permeability. These layers are made up primarily of lipids and proteins, with smaller amounts of mucin and DNA. The lipid components may significantly decrease the diffusion of small lipophilic molecules, possibly by trapping the drug in the mucus layer (Larhed et al, 1998). Small hydrophilic molecules are unaffected.

Transcellular drug transport is not always passive. Active transport mechanisms exist that allow for the uptake of some drugs that may have limited permeability otherwise. The classic example of this is the oligopeptide transporters and β-lactam antibiotics (Terada and Inui, 2004). Drugs that cross the intestinal membrane via these transporters often do not correlate well with the previously mentioned parameters.

2.3.4 Models for Determining Drug Permeability

Given the importance of permeability in a drug’s oral absorption, several models for studying permeability have been developed. The BCS currently recognizes permeability based on in vivo intestinal perfusion studies in humans, in vivo or in situ intestinal perfusion studies in animals, in vitro permeation experiments across epithelial cell monolayers (Caco-2 cell model), or excised human or animal intestinal tissue (Ussing chamber studies).
Human In Vivo Intestinal Perfusion Models

Intestinal perfusion models rely on measurements of drug disappearance from an isolated intestinal lumen following oral administration. The human perfusion model uses specialized instruments placed in the proximal jejunum of fasted, healthy volunteers using fluoroscopic or endoscopic techniques (Lennernas, 1997). Open, semiopen, and closed perfusion techniques have been developed. The open model uses a triple lumen tube that has a mixing segment which allows for mixing of gastrointestinal contents and the perfusate containing the drug. Samples are taken from the aboral end of the segment, approximately 20-30 cm distal to the mixing segment (Gramatte et al, 1994). A major disadvantage of this method is that the composition of the perfusate can change along the length of the mixing and sampling segment, thus making absorption conditions difficult to define (Lennernas, 1998). The semiclosed model uses an occluding balloon proximal to the testing segment which prevents contamination from the proximal intestine and creates equilibrium of the luminal composition (Ewe and Summerskill, 1965). However, this system, as well as the open system, has low recovery of the nonabsorbable volume marker PEG 4000, and they both require perfusion flow rates 2-20 times higher than in the physiologic state (Lennernas, 1998).

Because of these disadvantages, a closed perfusion model was developed that uses 2 balloons to isolate a 10 cm segment of intestine with minimal proximal or distal contamination (Knutson et al, 1989). This system also had >95% recovery of PEG 4000, lower flow rates, and the controlled absorption conditions allowed for an accurate study of transport and metabolism mechanisms in the intestine. Specialized catheters have been created for this method, one for the proximal jejunum, and one for the colon. The
main disadvantage of the closed method is that it cannot be used in the distal small intestinal segments, as the open and semiopen systems can.

A good correlation between the effective permeability calculated using this technique and the extent of oral absorption in humans has been reported, and the absorption half-life estimated using this method accurately predicts the time to maximum absorption in pharmacokinetic studies (Lennernas et al, 1997). This model is well correlated with other techniques used to measure permeability but it is better suited to determine drug absorbed by active transport mechanisms, and may be useful to study drug efflux patterns and intestinal metabolism (Lennernas et al, 1997). This method is not considered useful in horses at this time, due to the difficulty of accurate placement of a catheter in the unanesthetized horse, and the lack of a suitable catheter system for use in the equine intestine which has a larger diameter than the human intestine.

Animal In Situ Intestinal Perfusion Models

The in situ perfusion technique is most commonly performed in rats and is similar to the human in vivo model previously discussed. The main difference is that the rats are anesthetized during sampling, and the flow rate of the perfusate is much lower in rats than in humans (Fagerholm et al, 1996). An excellent correlation between rat in situ permeability and human in vivo permeability was found, although the effective permeability is on average 3.6 times higher in humans than in rats for passively absorbed compounds (Fagerholm et al, 1996). Based on this, a scaling factor has been determined for comparing human and rat data.

As with the in vivo human perfusion studies, this method would be difficult to adapt for use in the horse due to the difficulty and expense of prolonged anesthesia, the
risk factors associated with open abdominal surgery, and the need for the development of specialized catheterization equipment adapted to the size and scope of the equine small intestine.

*Caco-2 Cell Monolayers*

Caco-2 cells are an immortal cell line derived from human colonic adenocarcinoma cells. They are the most common method used for determining drug permeability in human medicine due to ease and availability. The cells differentiate spontaneously under normal culture conditions and form continuous monolayers. Because these cells were derived from the colon total permeability is lower in the Caco-2 system when compared to human in vivo perfusion studies (Artursson, 1991). However, the cell monolayers may have absorptive capacities similar to small intestinal cells (Hidalgo et al., 1989). The primary drawback to Caco-2 cell monolayers is the variable expression of active transporter and efflux systems, as well as intestinal metabolizing enzymes, including the major intestinal metabolizing enzyme, CYP 3A4. Induction of upregulation of these variables, or transfection of the cell cultures with the cDNA necessary for expression of these variables, has been used to account for this deficiency (Cummins et al., 2001; Crespi et al., 1996; Chu et al., 2001). Other inherent limitations of this system include lack of a mucus layer and the homogeneity of the cell cultures. These cells do not normally produce the mucus layer that is present in the intestine and which can impede the absorption of some compounds, particularly those that are rapidly permeable (Lennernas, 1997). To overcome this limitation, co-cultures with other mucus producing cells have been developed (Hilgendorf et al., 2000). These co-cultures also increase the cellular heterogeneity, as would be more consistent with live intestinal tissues. Despite
the alterations that have been developed to more closely mimic *in vivo* conditions, the Caco-2 cell line cannot be used to study regional differences in intestinal permeability, therefore limiting its overall effectiveness (Ingels and Augustijns, 2003).

Despite all these limitations, good correlations have been found between Caco-2 permeability and relative oral absorption in humans, when methodological and culture conditions are standardized (Ingels and Augustijns, 2003). Because Caco-2 cell systems are so widely used, guidelines have been developed to relate Caco-2 permeability to human in vivo permeability. If the calculated permeability in the Caco-2 cells reaches 13.3 to 18.1 x 10^-6 cm/s, it predicted *in vivo* permeability would be 2 x 10^-4 cm/s (Sun et al, 2004). This correlates with an absorbed fraction of >90%, qualifying drugs that meet this crieteria to be classified as highly permeable according to BCS standards.

Unfortunately, there are no similar cell cultures that have been developed for horses. Given the obvious differences between human and equine intestinal absorption, metabolizing capacity and transporters system, the data obtained from human Caco-2 cell cultures cannot be extrapolated for use in the horse.

*Ussing Chamber Studies*

The Ussing chamber is a specially designed apparatus that consists of two compartments separated by layer of intestinal mucosa (Figure 2.1). The integrity of the tissue is maintained by bathing the segment in a balanced electrolyte solution supplemented with 95%/5% oxygen and carbon dioxide. Measurement of transepithelial resistance ensures that the integrity is intact, as the TER will decrease once the tissue becomes nonviable or the intestinal permeability increases due to damage to the membrane or leakiness of the intercellular junctions (Blikslager et al, 2002). For *in vitro*
permeability experiments, the compartment attached to the mucosal surface of the intestine is exposed to a solution containing the drug, and the appearance of the drug on the opposite ‘serosal’ side of the intestine is measured. From this, the effective permeability is calculated using the equation

\[ P_{\text{eff}} = \frac{(dC/dt)}{A*1/C_0} \]

where \( dC/dt \) is the slope of the concentration versus time curve for the individual drug from 30-120 minutes; \( A \) is the area of the mucosal segment in the Ussing chamber, and \( C_0 \) is the initial concentration of drug in the mucosal chamber.

This method has several advantages over the Caco-2 cell monolayer. Because it uses actual intestinal mucosa, the tissue has all the characteristics of that segment of intestine, and can be used to study drug permeability along different segments of the intestine. Cell heterogeneity and the intercellular junctions are similar to \textit{in vivo} conditions (Ingels and Augustijns, 2003). Additionally, whereas drug must be in solution with the Caco-2 monolayer system, some authors suggest drug suspensions can be used, which may be useful for the study of more lipophilic compounds (Gotoh et al, 2005). There is also some evidence that drug-drug interactions, active transport systems and the role of p-glycoprotein on drug absorption can be studied using this method (Gotoh et al, 2005).

Comparison of \textit{in vivo} human intestinal perfusion data with Ussing chamber data obtained using rat jejunal mucosa has shown a good correlation between calculated permeability and oral absorption, although permeability is approximately 4-5 times lower in the Ussing chamber model (Lennernas et al, 1997). Lower permeability is attributed to species differences in the intestinal surface area and lipid composition, and a lack of
blood flow to the tissue, active transport mechanism co-factors, mucus layer, and gastrointestinal motility. Despite this, as with the Caco-2 monolayer system, the relative ranking of permeability of studied compounds between humans and rats was highly correlated.

The Ussing chamber technique is the most adaptable for species of veterinary interest because tissues of practically any animal can be placed in the chamber and validated. The advantage for our studies was that this method has already been validated as a method of study in the horse. Numerous studies have been performed using the Ussing chamber method to study the effects of various anti-inflammatory drugs on epithelial resistance and recovery following ischemia (Tomlinson and Blikslager, 2005; Tomlinson et al, 2004; Campbell et al, 2002). The effectiveness of this model as a tool for studying the permeability of antimicrobials in the horse is discussed in greater detail in chapter 9.

2.4 **INTERSPECIES DIFFERENCES AFFECTING DRUG SOLUBILITY AND PERMEABILITY**

Application of the current BCS to the horse, and other veterinary species is limited until investigators fully characterize interspecies differences that may influence drug solubility, permeability and, ultimately, bioavailability. Variations in physiologic factors that differ among species require further study, to develop *in vitro in vivo* correlations (IVIVC) to describe the impact on oral absorption to develop a BCS for veterinary species. These factors are discussed below, with particular reference to differences between horses and humans.
2.4.1 Gastrointestinal pH

Solubility must occur over a range of pH values. This range was chosen based on the pH of the stomach and small intestines in humans, which varies greatly, depending on the specific region of the GIT. Reported pH values in the fasting human are 1.4-2.1 in the stomach, 4.9-6.4 in the duodenum, 4.4-6.6 in the jejunum, and 6.5-7.4 in the ileum (Yu et al, 2002). In horses, the gastric pH is highly variable, particularly in the fasted state, with pH values ranging from 1.0-7.5 (Baker et al, 1993). Additionally, several periods of spontaneous alkalinization are noted, with sustained pH values of 6.0-7.5. This is thought to be caused by reflux of alkaline duodenal contents into the stomach (Merritt, 1999). Other explanations include salivary intake or variable gastric acid secretion (Baker and Gerring, 1993). The median pH of the intestinal tract in horses, as recorded at necropsy, was 5.4 and 3.3 in the anterior and posterior stomach, respectively, 6.7-7.9 in the small intestine, 7.0 in the cecum, and 7.4 in the colon (Kararli, 1995).

2.4.2 Gastrointestinal Fluid Volume

The volume of fluid available for dissolution also varies between species. The human gastrointestinal tract is exposed to approximately 5-10 liters of fluids per day (approximately 70-150 mL/kg/day) (Davenport, 1982). This includes ingested fluids, as well as gastric, duodenal and pancreatic secretions. The horse, in contrast, normally ingests approximately 24 liters per day of maintenance fluids (40-50 mL/kg/day). Gastroduodenal secretions are, on average, 1.6 liters per hour, or over 38 liters per day (Merritt et al, 1996). Daily biliary secretions in ponies total 4 L/100 kg, compared to 0.5 L/100kg in humans and 1.1-4 L/100 kg in sheep (Stevens and Humes, 1995). Pancreatic secretions are also much higher in the pony, totaling 10-12 L/100 kg, compared to
humans and sheep (1.3 and 1.1 L/100 kg, respectively). This represents a huge increase in the total fluids available for dissolution in the horse compared to humans; however one must also take into account the increase in dosage on a mg/kg basis. This will affect the dose:solubility ratio, and volume of fluid needed to dissolve a medication.

The volume of fluid (usually water) administered with an oral formulation is not standardized for veterinary species. In horses, many pharmacokinetic studies are performed by administering the drug through a nasogastric tube followed by 1-2 liters of water (approx. 2-4 mL/kg). This is similar to human studies in which the standard volume is 250 mL (~1 cup) or approximately 3.5 mL/kg. However, in equine clinical practice, most drugs are administered in an oral dosing syringe with only approximately 60 mL of water or flavoring agent, such as corn syrup or molasses. This is less than used in many published experimental studies of equine bioavailability, and may overestimate solubility, and subsequently, the bioavailability of marginally soluble drugs.

2.4.3 Effects of Feeding

Feeding can increase the gastric pH of humans to a variable degree, with ranges from 3.0-7.0 (Dressman et al, 1998). As previously stated, fasted horses have a variable gastric pH with periods of spontaneous alkalinization. Feeding blunts alkalinization, and the mean gastric pH is usually only 1-2 points higher in fed versus fasted adult horses (Sanchez et al, 1998). The increase in pH is much more dramatic in foals, where the pH can vary from <1.0 in the fasted state, to >6.0 following milk ingestion (Sanchez et al, 1998). In horses, feeding creates a layered pH effect with pH values as high as 6-7 in the squamous portion of the stomach and as low as 1-2 in the glandular portion (Merritt, 2003; Baker and Gerring, 1993). In the small intestine in humans, feeding generally
causes a slight decrease in the pH (Persson et al, 2005). In contrast to horses and people, feeding induces a larger increase in gastric acid secretion in dogs and the pH decreases rather than increases after feeding in this species (Kararli, 1995).

Feeding can also alter the gastric emptying rate, increasing contact time with the gastric contents, and delaying delivery of the drug to the intestine for absorption. Increased retention time in the stomach may be beneficial for some drugs that have an improved solubility in acidic environments (ie itraconazole), but it may be detrimental to drugs that are degraded at low pH values (ie omeprazole, penicillins). The effect of feeding on gastric emptying in horses differs from other monogastric species. There was no significant difference in the emptying of solid, non-digestible radiopaque markers in ponies following feeding, whereas the emptying of similar markers is greatly delayed by the presence of food in man (Baker and Gerring, 1994). Lorenzo-Figueras et al (2005), report no difference in the effect of fat supplementation on gastric motility in horses, which is markedly different from other species.

Many drugs are recommended to be taken with food in human medicine. This may not be applicable to the horse because the composition of diets varies so much between species. Most equine diets, such as hay or grasses, have a low fat content. This type of high roughage diet may decrease drug solubility and absorption by creating a physical barrier between the gastric fluid and the drug (Toothaker and Welling, 1980). Drugs may also adsorb to feed particles, preventing solubilization. This was demonstrated for trimethoprim-sulphachlorpyridazine combinations in the horse (Van Duijkeren et al, 1996). Decreased oral absorption of drugs in fed horses has been
demonstrated for rifampin, erythromycin and doxycycline (Baggot, 1992; Lakritz et al, 2000)

2.4.5 Gastrointestinal Anatomy

Horses are considered monogastric species, similar to dogs, humans and pigs, but unlike these other species, they are herbivores. Horses are hindgut fermenters, and synthesis of proteins, vitamins and volatile fatty acids occurs mainly in the large intestine. Another difference between horses and other species is the high surface area of squamous versus glandular epithelium in the stomach. This is necessary to provide protection from the high fibrous diet. Fiber in the diet may decrease drug dissolution in the stomach, resulting in undissolved drug being delivered to the small intestine, where the higher pH may further interfere with drug dissolution (Martinez et al, 2002b). Consequently, there may be more absorption occurring in the large intestine of horses compared to other animals. This may explain the biphasic absorption of some drugs that is seen in the horse (Baggot, 1992).

2.4.6 Bile Components

Interspecies differences in bile flow and bile salt composition can affect oral drug absorption, because some hydrophobic drugs are solubilized by bile. Bile flow in the pony averages 19 µL/min/kg (Riviere, 1999). This is higher than in humans (1.5-15 µL/min/kg), cats (11 µL/min/kg) and dogs (4-10 µL/min/kg). In contrast to other species, horses secrete bile continuously into the intestinal lumen because they lack a gall bladder. Not surprisingly, fasting increases bile reflux into the stomach (Berschneider et al, 1999). Within 14 hours of withholding food, the bile salt concentrations were high enough in the stomach to alter mucosal transport of electrolytes in the horse.
Serum bile acid composition also varies with species. Cholic acid is the major bile acid present in dogs and cattle, whereas chenodeoxycholic acid is predominant in horses and humans (Washizu et al, 1991). Horses also have a lower biliary lipid excretion than rodents, which is independent of bile salt secretion (Engelking et al, 1989). This lower lipid excretion may hinder the drug solubilization capacity of equine bile.

### 2.5 Other Factors Affecting Oral Absorption

Animal factors, independent of a drug’s solubility or permeability, can also affect the bioavailability of a drug. These factors may be more variable in diseases that can affect absorption.

#### 2.5.1 Hepatic and Intestinal Metabolism

It has been speculated that herbivores have a higher hepatic metabolic capacity, which would increase the first pass metabolism of lipophilic compounds, resulting in an overall decrease in bioavailability (Baggot, 1992). *In vitro* hepatic microsomal assays have been performed that demonstrate species variability between horses and other laboratory or food producing animals (Nebbia et al, 2003). The hepatic metabolizing enzymes in the horse are lower at birth and in the first year of life, but tend to increase over a period of at least 15 years (Nebbia et al, 2004). Overall, horses had much higher activities of cytochrome P450 (CYP) 1A and 2E1 isoenzymes, but lower activity in CYP 2B and 3A isoenzymes, when compared to other species. The lower CYP 3A activity has also been demonstrated in the ability of horses to metabolize monensin, a drug that is highly cardiotoxic in this species (Nebbia et al, 2001).
Cytochrome P450 enzymes have also been found in the intestine of horses and may cause metabolism of some drugs prior to absorption (Tyden et al, 2004). Although the activity of p-glycoprotein efflux pumps has not been characterized in equine intestinal mucosa, they likely exist. In humans and other animals, these CYP enzymes are often coupled with p-glycoprotein efflux pumps to decrease oral bioavailability of some drugs (Zhang and Benet, 2001).

2.5.2 Gastrointestinal Disease

Gastrointestinal diseases are extremely common in the horse. Diseases causing colitis may result in an increased intestinal transit time that would decrease the amount of time available for drug absorption. Diseases causing intestinal obstruction, particularly in the small intestine, may increase contact time and allow for greater absorption. The barriers to drug absorption may also be decreased from an injured gastrointestinal mucosa, which may increase intestinal permeability. Intestinal disease in horses are characterized by poor perfusion and ischemia. Decreased blood perfusion may result in decreased drug absorption. Alterations in microbial flora may occur secondary to gastrointestinal disease, and these bacteria may possess the ability to metabolize antimicrobial drugs prior to absorption, thus altering the bioavailability (Kararli, 1995). Regarding diseases, there is also the effect caused by infection. This was demonstrated in pigs infected experimentally with Escherichia coli (Jensen et al, 2004). Infected pigs had decreased systemic availability of oral amoxicillin. This effect was presumably caused by a reduction in effective surface area for absorption or via a sodium chloride secretion mechanism that interferes with the active transport of amoxicillin.
2.6 **SUMMARY**

In conclusion, the solubility and permeability characteristics of each drug are key determinants of oral drug absorption, as defined by the BCS. These characteristics have been used extensively by the FDA and pharmaceutical companies to determine the absorption capacity of generic drugs, new drug formulations, and investigational drug compounds in humans. There is a great interest in developing similar systems for use in veterinary species. Such a system would provide more drug availability to equine veterinary practitioners, facilitate new drug development by drug sponsors, and improve the FDA-CVM capability to evaluate new drugs. However the physiological differences between humans and other animals prevents direct extrapolation of data from one to the other. Investigation into the effects of these physiologic differences on drug solubility and permeability will ultimately allow for the use of the BCS in veterinary species, which may increase the number of drugs available for treatment of these patients. Once this has been accomplished, further studies may need to be performed to determine the effects of other factors, such as metabolism and disease states on oral absorption. It will be a goal of this dissertation to lay a foundation, using *in vitro* and *in vivo* studies in horses so that a BCS system can be applied to oral antimicrobial drugs in horses.
2.7 References


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Table 2-1. Solubility (C<sub>s</sub>) definitions used in calculation of the dose number (Do).

<table>
<thead>
<tr>
<th>Descriptive term</th>
<th>Solubility assigned (mg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Very Soluble</td>
<td>1000</td>
</tr>
<tr>
<td>Freely soluble</td>
<td>100</td>
</tr>
<tr>
<td>Soluble</td>
<td>33</td>
</tr>
<tr>
<td>Sparingly soluble</td>
<td>10</td>
</tr>
<tr>
<td>Slightly soluble</td>
<td>1</td>
</tr>
<tr>
<td>Very slightly soluble</td>
<td>0.1</td>
</tr>
<tr>
<td>Practically insoluble or insoluble</td>
<td>(\leq 0.01)</td>
</tr>
</tbody>
</table>
Figure 2-1. Photograph of an Ussing chamber apparatus with equine intestinal mucosa.
3. LITERATURE REVIEW: TISSUE DISTRIBUTION

After an antimicrobial drug is absorbed into the systemic circulation, active concentrations must reach the site of the infection for successful therapy. For most infections, this requires therapeutic concentrations of active drug in the extracellular space (Liu et al., 2002). Penetration can occur through pores in capillaries in most tissues, but some tissues are protected by a barrier that may limit penetration. Examples of limited perfusion include the blood-ocular barriers, the blood-brain/blood-csf barriers, and the blood-prostate barriers (Barza, 1993). The physicochemical factors that effect drug disposition into these sites will be discussed below, with particular reference to the interstitial fluid and the aqueous humor.

3.1 INTERSTITIAL FLUID

The interstitial fluid (ISF) is the major component of the extracellular fluid and is comprised of electrolytes, lipids, and a small amount of very low molecular weight proteins. This fluid bathes the major organs in the body and is therefore in direct contact with common areas of infection. The concentration of antimicrobial drugs in the ISF is one of the main indicators for successful treatment outcome. Interstitial fluid concentrations have not historically been used in pharmacokinetic-pharmacodynamic models, due to the difficulty in sampling the ISF versus the plasma (Barza, 1981).

3.1.1 Methods of Interstitial Fluid Sampling

Tissue biopsies and homogenized tissues have been used to determine the concentrations of antibiotics at sites of infection. There are several drawbacks to this technique, however. Homogenization of the tissues causes intracellular as well as extracellular concentrations to be measured together, along with drug concentrations
within the vessels in the biopsy specimen. Also, this technique measures total (both protein bound and unbound) drugs, when only the unbound drug fraction is biologically active. Other techniques have since been developed to sample the ISF and determine active drug concentrations within the tissue fluid: skin blister studies, tissue cages, microdialysis and ultrafiltration.

Skin blister studies use suction or cantharidin-induced blisters to simulate the ISF (Bergan, 1981; Wise et al, 1980). The suction method produces a more typical transudate, whereas the cantharidin blisters produce more of an exudate, with higher protein concentrations (Barza, 1981). There are several drawbacks to this method, however. A high protein content, variable size of the blisters, and a small surface area:volume (SA/V) ratio make the blister fluid an imperfect measure of ISF drug pharmacokinetics (Blaser et al, 1991). The small SA/V ratio results in a lower maximum concentration, a longer time to maximum concentration, and a prolonged half-life of the drug when compared to sampling methods that have a large SA/V ratio (Clarke et al, 1989).

Tissue cages are one of the most common methods for measuring ISF drug concentrations, particularly in veterinary species. Several papers have been published using this technique in horses (Landoni and Lees, 1996; Lees and Landoni, 2002; Ensink et al, 2003; Ensink et al, 2005). Many authors prefer this method because infection can be introduced into the chambers to simulate a disease process and predict clinical efficacy. However, as with the skin blister technique, there is a small surface area for diffusion compared to the volume within the tissue cage. Additionally, these chambers can induce an inflammatory response leading to increased protein levels, encapsulation
and vascularization that may change drug penetration into the cage (Clarke et al, 1989). While this may more closely mimic the *in vivo* situation in diseases causing abscessation, it will not necessarily predict the drug concentrations in the ISF fluid in cases of naturally-occurring infections.

To overcome the problems associated with the small SA/V ratio found in skin blisters and tissue chambers, microdialysis and ultrafiltration techniques were developed. These techniques continuously sample the ISF directly from the extracellular space. Both methods use small probes that are inserted into the subcutaneous tissues or directly into specific organs. These probes have a semipermeable membrane that allows for the collection of small molecules (<30,000 Da) and therefore only the protein unbound drug fraction is collected.

For microdialysis studies, a perfusate is continuously flushed through the probe, therefore the drug becomes diluted and a very sensitive analytical technique is required to determine the concentrations in the samples. Calibration of the assay must be performed to account for the dilution caused by the perfusate (Müller, 2000). In spite of these drawbacks, microdialysis is an accurate, minimally invasive, reproducible method for studying drug concentrations in the ISF (Joukhadar and Müller, 2005).

Ultrafiltration is similar to microdialysis, except it uses vacuum pressure to collect samples rather than a fluid flow system; therefore, the samples can be analyzed directly without corrections made for perfused fluid volumes. Ultrafiltration systems collect ISF at a rate of 1-5 µL/min (Linhares and Kissinger, 1992). The probes induce minimal inflammatory responses therefore can be left in an animal for several days without adverse effects (Linhares and Kissinger, 1993). Previous reports have published the use
of these probes to measure small molecules such as glucose and electrolytes in several veterinary species, including horses (Spehar et al, 1998). More recently, these probes have been successfully used to determine antimicrobial drug concentrations in the dog (Bidgood and Papich, 2003; Bidgood and Papich, 2005) and the horse (Davis et al, 2005). Unlike the studies performed using tissue cages, a delayed peak and prolonged terminal half-life of tissue fluid concentrations were not observed from samples collected with ultrafiltration.

### 3.1.2 Effects of Protein Binding on ISF Drug Concentrations

In sites without protective barrier membranes, plasma protein binding is the major determinant of drug concentrations within the ISF (Barza, 1981). These sites contain fenestrated capillaries that allow molecules with molecular weights of up to 1000 to freely pass from the intravascular to the extravascular space (Hogben, 1971). The effects of protein binding on antimicrobial drug concentrations have been studied in humans (Liu et al, 2002), dogs (Bidgood and Papich, 2003; Bidgood and Papich, 2005) and horses (Davis et al, 2005). These studies have demonstrated that plasma protein binding provides a reliable estimate of free (active) drug concentration in the ISF, which may be more predictive of therapeutic success than total plasma concentrations.

Inflammation causes vasodilation and increased capillary permeability, which would be expected to increase drug concentrations at the site. However, since plasma proteins also enter into the inflamed sites, the protein unbound concentration of the drug will remain the same. This may be one explanation why total trimethoprim/sulfadiazine concentrations in tissue chambers are adequate for the treatment of susceptible bacteria, but they are not clinically efficacious (Ensink et al, 2005). In contrast, a drug with low
protein binding, such as procaine penicillin G, is able to completely eliminate infection in tissue chambers (Ensink et al, 2003). This is supported by work in humans that has shown subtherapeutic concentrations of unbound drug in the interstitial fluid of inflamed tissues using a microdialysis technique (Joukhadar et al, 2001). Microdialysis experiments in human subjects have also demonstrated that the protein unbound drug concentrations are similar between inflamed and non-inflamed tissues (Müller et al, 1996; Müller et al, 1999). Another consideration is that the viscosity of the fluids are also increased which can decrease the rate of drug diffusion (Barza and Cuchural, 1985).

3.2 AQUEOUS HUMOR

Following systemic administration of an antibiotic, drug concentrations in the aqueous humor are often much lower than plasma concentrations. This has been demonstrated extensively in humans and other laboratory animals, particularly the rabbit; however there is currently limited data on ocular penetration of antimicrobials in the horse. The difference between aqueous humor and plasma drug concentrations can be explained by the blood-aqueous barrier (BAB).

3.2.1 Anatomy and Physiology of the BAB

The purpose of the BAB is to prevent the movement of large molecules, particularly proteins, from entering the aqueous humor and interfering with vision (Novack and Leopold, 1988). There is newer evidence that suggests the BAB also is important in preventing movement of molecules between the anterior and posterior chambers (Freddo, 2001). The BAB consists of both an epithelial and endothelial portion. This includes tight junctions present at the apico-lateral surface of the non-pigmented epithelium in the ciliary as well as nonfenestrated capillaries within the iris.
vasculature (Freddo, 2001; Hirsch et al, 1995; Cunha-Vaz, 1979). Evidence also exists supporting the presence of p-glycoprotein efflux pumps as a component of the BAB, just as is found in the blood-brain barrier, which may contribute to decreased drug penetration into the aqueous humor (Kajikawa et al, 1999).

Several physicochemical characteristics of drugs have been correlated with the rate of drug penetration into the eye. These include lipophilicity, protein binding and molecular weight. The drugs that penetrate the BAB most readily are low molecular weight, lipophilic, protein unbound drugs (Langham, 1951; Barza, 1981; Barza, 1993).

There are several classic examples of this in studies that examined the ocular penetration of drugs within the same class of antimicrobials that have similar protein binding, but variations in lipophilicity. Doxycycline reaches higher drug concentrations in the eye compared to other tetracyclines, although maximum concentrations are restricted because of high protein binding (Salminen, 1977). Among the fluoroquinolones, a strong linear correlation was found between lipophilicity and % ocular penetration (Liu et al, 1998).

Protein binding restricts ocular penetration of drugs, if the % plasma or tissue binding is high. This was demonstrated in the study by Tang-Liu and Liu (1987). They examined the ocular penetration of flurbiprofen, a highly protein bound drug, before and after plasmapheresis. Ocular penetration of flurbiprofen was increased, as was the unbound drug in the aqueous, following plasmapheresis which decreased plasma protein levels by 60%. Similar to the effects of inflammation in the ISF, inflammation or infection in the eye may increase the total drug concentration in the aqueous humor, but
the unbound fraction of drug may not be affected due to the simultaneous increase in aqueous protein concentrations.

Similar to intestinal permeability, most uptake of drug across the BAB is a passive process that is governed by the concentration gradient across the membrane. However, active transport processes may exist that counteract the effects of the BAB, which may explain the penetration of certain non-lipophilic substances into the eye. These include glucose transporters (Tserentsoodol et al, 1998) and peptide transporters (Basu et al, 1998).

3.2.2 Pharmacokinetics and Modeling of Drug Penetration into the Aqueous Humor

Recent works have examined pharmacokinetics of drugs into and out of the eye. In most studies that have examined drug concentrations in the aqueous humor, the small number of samples collected does not always allow for an adequate assessment of drug concentration versus time curves. To overcome this, microdialysis probes have been developed that allow for repeated sampling of aqueous humor from the same eye (Macha and Mitra, 2001). This technique has provided a better estimate of drug penetration, elimination, and the total area under the curve for drugs distributed into the eye (Rittenhouse and Pollack, 2000). Microdialysis has been used in rabbits and dogs, but there is no information available on the viability of this method for use in the horse.

Drug concentrations determined by either repeated paracentesis or microdialysis may be affected by an alteration in the aqueous humor protein concentrations secondary to either of these invasive procedures. Removal of >20% of the aqueous humor at one or multiple sampling points can increase the protein concentration up to 10 fold, and the protein can remain elevated for up to 1 day after sampling (Novack and Leopold, 1988).
After insertion of the microdialysis probe, aqueous humor protein concentrations increased up to 30 mg/mL over baseline values, remained elevated for greater than 150 minutes, and were still slightly elevated at 5 days post-placement (Rittenhouse et al, 1999). High aqueous protein concentrations may decrease the protein unbound drug fraction and overestimate the active drug concentrations.

In addition to improved sampling techniques, newer pharmacokinetic models are being explored that may accurately predict ocular concentrations of drugs. Originally, a two-compartment model was used consisting of a vascular and aqueous humor compartment but it provided inaccurate predictions (Walker et al, 1982). Subsequently, a new model was developed that was a three-compartment model, consisting of a vascular, aqueous humor and iris stroma compartment. The iris stroma can serve as a time-delayed reservoir for drugs entering the aqueous humor (McLaren et al, 1993). Another model was developed by Tojo (2004) that assumes a modified cylindrical shape and uses Fick’s laws of diffusion to predict movement within the structures of the eye following topical and systemic administration. Because there is little data available on the pharmacokinetics of a drug in the anterior chamber of the horse, none of these models have been applied to this species.

3.2.3 Comparison of the Blood-Aqueous Barriers with Other Anatomical Barriers

The BAB and blood-brain barrier (BBB) are similar in several respects. Each barrier consists of tight junctions between the cells that limit the penetration of large molecules (Barza, 1993), however the junctions between the cells in the BAB are less restrictive than the BBB (Novack and Leopold, 1988). The BBB consists not only of the tight junctions in the endothelial cells of the capillaries within the brain and spinal cord, it
also has a second component consisting of tight junctions in the epithelial cells of the choroid plexus (Barza, 1993). This latter part is known as the blood-cerebrospinal fluid barrier. The BBB has functional as well as anatomic barriers. The p-glycoprotein efflux pumps as well as active transport pumps can influence drug penetration into the site.

Antimicrobial penetration of the BBB in horses has received little attention. With the exception of streptomycin (Kottman et al, 1969), peak concentrations were typically higher in the CSF when compared to the aqueous humor (Zakopal et al, 1970; Dowling et al, 1995; Latimer et al, 2001). This may be due to the decreased surface area available for drug diffusion into the eye, compared to the CSF. Nevertheless, drugs that penetrate well into the aqueous humor should also penetrate well into the CSF, making the aqueous humor a more practical site for sampling in the horse. As with drug penetration into the aqueous humor, drug penetrations into the CSF is also governed by lipophilicity, and to a lesser extent, protein binding and molecular weight (Péhourcq et al, 2004; Radouane et al, 1996; Nau et al, 1994).

Blood-prostate barriers also exist, although they may not be clinically significant in the horse due to the low incidence of prostatic disease in this species. This barrier also consists of tight junction in the capillary beds. As with penetration of drugs across the BAB and the BBB, lipid solubility and protein binding play an important role, however there is an additional element to the BPB. A pH gradient is present between the prostatic fluid and the plasma, which means that the pKa of the drug also becomes an important limiting factor (Charalabopoulos et al, 2003; Wagenlehner et al, 2005).
3.3 SUMMARY

Once a drug is absorbed, it can be distributed to tissues, bound to plasma or tissue proteins, metabolized or excreted. Plasma protein binding and drug lipophilicity are two of the major physicochemical characteristics that will determine the fate of a drug in the systemic circulation. For drug distribution into non-protected sites, such as the ISF, plasma protein binding and tissue geometry (SA/V ratio) is the major determinant of the rate and extent of tissue distribution. For drug penetration into protected sites, such as the aqueous humor and the cerebrospinal fluid, lipophilicity is the most important determinant, although protein binding may play a secondary role.
3.4 References


4. PHARMACOKINETICS AND TISSUE DISTRIBUTION OF ITRACONAZOLE AFTER ORAL AND INTRAVENOUS ADMINISTRATION TO HORSES

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Objective—To determine the pharmacokinetics of itraconazole after IV or oral administration of a solution or capsules to horses and to examine the disposition of itraconazole into the interstitial fluid (ISF), aqueous humor, and polymorphonuclear leukocytes after oral administration of the solution.

Animals—6 healthy horses.

Procedure—Horses were administered itraconazole solution at a rate of 5 mg/kg by nasogastric tube, and samples of plasma, ISF, aqueous humor, and leukocytes were obtained. Horses were then administered itraconazole capsules (5 mg/kg) and plasma was obtained. Three horses were administered itraconazole (1.5 mg/kg, IV) and plasma samples were obtained. All samples were analyzed by high-performance liquid chromatography. Plasma protein binding was determined. Data were analyzed by compartmental and noncompartmental pharmacokinetic methods.

Results—Itraconazole reached higher mean ± SD plasma concentrations after administration of the solution (0.41 ± 0.13 µg/mL) versus the capsules (0.15 ± 0.12 µg/mL). Bioavailability after administration of capsule relative to solution was 33.83 ± 33.08%. Similar to other species, itraconazole has a high volume of distribution (6.3 ± 0.94 L/kg) and long half-life (11.3 ± 2.84 hours). Itraconazole was not detected in the ISF, aqueous humor, or leukocytes. Plasma protein binding was 98.81 ± 0.17%.

Conclusions and Clinical Relevance—Itraconazole administered orally as a solution had higher, more consistent absorption than orally administered capsules and attained plasma concentrations that are inhibitory against fungi that infect horses. Administration
of itraconazole solution (5 mg/kg, PO, q 24 h) is suggested for clinical trials to test efficacy of itraconazole in horses.

INTRODUCTION

Itraconazole is a broad-spectrum, orally administered, triazole antifungal drug used for the treatment of humans and other animals with infections caused by *Aspergillus* spp, *Histoplasma* spp, and *Blastomyces* spp.\(^1\) It acts by blocking the action of the cytochrome P450 enzyme 14α-demethylase, which converts lanosterol to ergosterol in the cell membrane of fungi.\(^2\) This leads to alterations in the cell membrane of fungi, inhibition of cell growth, and, ultimately, cell death. Itraconazole has a higher affinity for the fungal cytochrome P450 enzymes than do other azole antifungal drugs; however, it may also inhibit mammalian enzymes, and drug-drug interactions are possible.\(^3\) In people and laboratory animals, itraconazole is highly protein bound, extremely lipophilic, and capable of attaining high concentrations in tissues, including the lungs, kidneys, brain, skin, and esophagus, with concentrations often higher in the tissues than in plasma.\(^4,5\) Its major metabolite, hydroxyitraconazole, has equal antifungal activity in vitro, although conversion to this metabolite is not necessary for efficacy.\(^3\)

Itraconazole reportedly is effective in the treatment of horses with mycotic rhinitis, osteomyelitis, and mycosis of the diverticulum of the auditory tube (ie, guttural pouch).\(^6-8\) It has been administered for up to 6 months without adverse effects as determined during physical examination or serum biochemical analysis.\(^6-8\) One of the most frequent clinical manifestations of fungal infections in horses is keratomycosis. *Aspergillus* spp are the most commonly isolated organisms from horses with keratomycosis, and 1 report\(^9\)
revealed 100% susceptibility of these isolates to itraconazole. Fungal invasion of the cornea can be devastating, requiring surgical correction or resulting in blindness or loss of the globe. Topically applied antifungal compounds are the mainstay of treatment for keratomycoses; however, absorption of topically applied drugs is usually < 5% because of poor penetration of the drug through the cornea and tear washout. Therefore, in animals that do not respond to topical medications or that have involvement of the posterior segment of the eye, adjunctive systemic administration of antifungal compounds may be beneficial.

Our laboratory group has conducted pharmacokinetic studies in horses to determine appropriate doses of fluconazole. Although fluconazole has excellent absorption and tissue distribution after oral administration as well as favorable pharmacokinetic properties in horses, it has weak activity against Aspergillus spp. This may be overcome in clinical situations because of high plasma and intraocular concentrations of the drug. Ketoconazole also does not have substantial activity against Aspergillus spp and is not absorbed in horses after oral administration because of insolubility of the drug in the equine stomach. Of the azole antifungals, only itraconazole and voriconazole have substantial in vitro activity against filamentous fungi, such as Aspergillus spp. Activity against Fusarium spp, another group of fungi commonly isolated from horses with keratomycosis, is limited, and many strains are resistant. Pharmacokinetics and dosing recommendations have not been determined for horses; therefore, the dosages used in horses have been extrapolated from dosages used in dogs and range from 2.6 to 5.0 mg/kg, PO, q 12 h. Studies in which investigators examined the in vitro
susceptibilities of itraconazole revealed a **minimum inhibitory concentration (MIC)** of 0.125 to 32 µg/mL against *Fusarium spp*[^14] and an **MIC at which growth of 90% of the organisms are inhibited (MIC\textsubscript{90})** of 0.03 to 0.12 µg/mL against *Aspergillus spp.*[^15]

Three formulations of itraconazole are currently marketed for use in humans: an orally administered solution,[^b] orally administered capsules,[^c] and a product formulated for IV injection.[^d] A solution is also marketed in Europe for oral administration to cats.[^e] Human pharmacokinetic studies[^3,16] have revealed that oral administration of the solution resulted in improved absorption and bioavailability with no substantial increase in the incidence of adverse effects. Intermittent pulse dosing has been used in humans and cats for the treatment of dermatophytosis, which resulted in similar or better outcomes than for once-daily dosing.[^17,18]

The purpose of the study reported here was to describe pharmacokinetics after oral administration of the solution and capsules and IV administration of itraconazole in horses and to establish the comparative bioavailability of the 2 oral formulations. Protein-bound and -unbound concentrations of itraconazole are reported to be important for efficacy as determined on the basis of an in vitro study[^19]; therefore, plasma protein binding was determined in the horses. We also intended to further classify the tissue distribution of itraconazole in horses and evaluate whether the activity of the drug at the tissue site was potentially related to unbound concentrations within aqueous fluids, concentrations delivered to the site of infection via polymorphonuclear leukocytes, or related to intracellular concentrations and total tissue concentrations. To do this, we
collected interstitial fluid, aqueous humor, and leukocytes from a representative sample of the horses after oral administration of the itraconazole solution and determined the drug concentrations within those matrices. Information from this part of the study was used to establish whether efficacy may be related to total drug concentrations in plasma or unbound drug concentrations within each of those compartments.

MATERIALS AND METHODS

Animals—Six healthy adult horses (3 males and 3 females) that weighed between 424 and 615 kg were used in the study. Horses included 2 Quarter Horses, 1 Hanoverian, 1 Thoroughbred, 1 Arabian, and 1 Standardbred horse. Three of the 6 horses were used for collection of samples of interstitial fluid, aqueous humor, and leukocytes. On the basis of preliminary results obtained from these horses, these samples were not collected from the remaining 3 horses. Horses were housed in stables beginning the day prior to drug administration and for the duration of the study. Water was available ad libitum throughout the study. This study was approved by the North Carolina State University Institutional Animal Care and Use Committee.

Drug administration—By use of a 2-way crossover design, itraconazole in the form of a commercially available solution or capsules was administered orally. Three horses received the solution first, followed by the capsule formulation. The other 3 horses received the capsules first, followed by the solution. A minimum 2-week washout period was allowed between administrations. Food was withheld for 12 hours before and 4 hours after drug administration. The solution was administered via nasogastric tube at a dosage
of 5 mg/kg. For administration of the capsules, the capsules were opened and the contents mixed with corn syrup in a dosing syringe (administration volume, 60 mL). Oral administration was achieved within 15 minutes after capsule contents were mixed with corn syrup. The amount of drug administered was rounded to the nearest 100 mg, and the actual dosage range for the capsules was 5.05 to 5.14 mg/kg.

In addition, 3 horses were randomly chosen to receive IV injections of itraconazole at a dosage of 1.5 mg/kg to allow for the calculation of absolute bioavailability and total systemic clearance. Only 3 horses were administered the drug in this manner because of the high cost of the IV preparation (approx $586/dose). For IV administration, itraconazole injection was mixed in accordance with the manufacturer’s directions to achieve a final concentration of 3.33 mg/mL. It was slowly administered IV; infusion times ranged from 14 to 30 minutes, depending on the volume injected.

**Collection of blood samples**—Blood samples were collected via a catheter inserted in the jugular vein. Samples were collected into heparinized tubes 0 (before treatment), 15, and 30 minutes and 1, 2, 4, 8, 12, 24, 48, and 72 hours after oral administration. For the IV administration, 2 catheters were inserted (1 in each jugular vein). Itraconazole was administered via 1 of these catheters, and blood samples were collected into heparinized tubes via the other catheter. Samples were collected 0, 10, 20, and 40 minutes and 1, 2, 4, 8, 12, 24, and 48 hours after the end of the IV infusion.

After collection, all tubes were immediately centrifuged at approximately 1,000 X g for 10 minutes. Plasma was harvested, and samples were then stored at –70°C until assayed.
**Collection of samples of tissue fluid** — Collection of tissue fluid from the subcutaneous tissues was performed by use of an in vivo ultrafiltration sampling kit that has been tested by our laboratory group. The probe was inserted into the subcutaneous tissues of 3 of 6 horses 12 hours before drug administration to allow a state of equilibrium to develop. Interstitial fluid was collected immediately before (time 0), 2, 4, 8, 12, and 24 hours after oral administration of the solution at a dosage of 5 mg/kg. Immediately after collection, the fluid was stored at −70°C until analyzed.

**Collection of samples of aqueous humor** — Samples of aqueous humor were collected 2.5 hours (1 sample) or 4.5 hours (2 samples) after oral administration of the itraconazole solution. These time points was chosen to coincide with peak plasma concentrations predicted on the basis of results of preliminary studies and to allow for a 30-minute equilibrium period between plasma and tissues. For sample collection, the horses were sedated by IV administration of detomidine (0.01 to 0.015 mg/kg) and manually restrained by application of a nose twitch. Auriculopalpebral, supraorbital, and retrobulbar nerve blocks were performed, and a topical anesthetic was applied to the eye. The eye was cleansed with dilute (10%) aqueous iodine solution. Aqueous humor was aspirated by inserting a sterile 27-gauge, 0.5-in needle through the conjunctiva and sclera in the dorsolateral limbus of the eye until the needle penetrated the anterior chamber. Once in the anterior chamber, gentle suction by use of a 1-mL tuberculin syringe was used to aspirate approximately 200 to 300 µL of fluid, and then the needle was withdrawn. Samples were frozen at −70°C until analyzed.
**Isolation of polymorphonuclear leukocytes**—Leukocytes were harvested from blood samples collected from 3 horses immediately before (time 0) and 1, 4, 8, and 24 hours after itraconazole administration. Leukocytes were harvested by a method published elsewhere. The remaining cell pellets were frozen at –70°C until analyzed.

**Plasma protein binding**—Protein binding was determined by use of a microcentrifugation system. Pooled plasma obtained from 6 healthy horses that did not receive any itraconazole was used in the analysis. Aliquots of equine plasma were fortified (ie, spiked) with itraconazole to make solutions containing 2, 1, and 0.5 µg/mL. Three replicates of spiked plasma samples per concentration were prepared and incubated in a water bath at 37°C for 30 minutes. Each spiked plasma sample was added to a microcentrifugation system and centrifuged at 1,000 X g for 10 minutes. A protein-free ultrafiltrate was obtained in the reservoir of the system, extracted, and analyzed as described for the drug analysis. The percentage of nonspecific binding of the drug to the microcentrifugation device and filter was determined in vitro. There was no measured adherence of the drug to the device, and adherence to the filter was < 5%. Protein binding was determined by use of the following equation:

\[
\text{protein binding percentage} = \left(\frac{\text{total concentration} - \text{unbound concentration}}{\text{total concentration}}\right) \times 100.
\]

**Drug analysis**—Concentrations of itraconazole and its active metabolite, hydroxyitraconazole, were analyzed by use of reverse-phase high-performance liquid chromatography (HPLC) with UV detection after solid-phase extraction by use of an
assay developed and validated in our laboratory. The HPLC apparatus consisted of a pump,\textsuperscript{i} autosampler,\textsuperscript{j} UV detector,\textsuperscript{k} and computer for data collection and analysis.\textsuperscript{l} Solid-phase extraction of itraconazole in plasma was performed by use of cyano-bonded extraction cartridges.\textsuperscript{m} Cartridges were initially conditioned with 1 mL of methanol and 1 mL of 0.05M dibasic potassium phosphate buffer (pH, 7.5). One milliliter of plasma was then extracted and washed with a solution of 0.05M dibasic potassium phosphate buffer:methanol (95:5). Samples were eluted with 1 mL of methanol, evaporated under a stream of nitrogen gas for 25 minutes at 40°C, and reconstituted with 200 µL of mobile phase solution prior to injection onto the HPLC. A reverse-phase column\textsuperscript{n} was used for separation. The mobile phase solution consisted of distilled water and HPLC–grade acetonitrile in a 50:50 mixture with 0.02% trifluoroacetic acid. Ultraviolet detection was performed at 263 nm, and injection volume was 50 µL/sample.

Tissue fluid samples were analyzed directly by use of HPLC without extraction because they were derived from a cell-free, protein-free matrix. A liquid-liquid extraction technique that used heptane:isoamyl alcohol (9:1) was modified from another published report\textsuperscript{23} for use on the samples of leukocytes and aqueous humor. Briefly, for leukocyte samples, the cell pellet was diluted with 200 µL of 0.05M dibasic potassium phosphate buffer (pH, 7.5). The heptane:isoamyl alcohol mixture (800 µL) was added to the cell-buffer mixture. Samples were then sonicated for 15 seconds and vortexed for 15 seconds. Then, the samples were centrifuged for 15 minutes at 13,500 X g. An aliquot (500 µL) of the top organic layer was pipetted into a glass test tube and evaporated under a stream of nitrogen gas for 20 minutes. The resulting samples were then reconstituted with 200 µL of mobile phase solution and injected directly into the HPLC. For samples of aqueous
humor, the extraction process was identical, with the exception that 800 µL of heptane:isoamyl alcohol was added directly to 200 µL of aqueous humor.

Standard curves were prepared by use of pure itraconazole and hydroxyitraconazole reference standards dissolved in 100% acetonitrile. When frozen at –70°C in a plastic container, the standards were stable for > 2 months. Standard curves were prepared fresh daily in pooled plasma obtained from untreated horses (for plasma and protein-binding analyses), mobile phase solution (for samples of tissue fluid), aqueous humor from untreated horses (for samples of aqueous humor), and 0.05M dibasic potassium phosphate buffer (for leukocyte samples). Standard curves were prepared daily. Calibration curves were linear between the concentrations of 2 and 0.0156 µg/mL, with a coefficient of determination of > 0.99 and all values within 20% of the expected range. The lower limit of quantification was the lowest concentration that was determined to be on a linear regression line on the calibration curve. This value was 0.0156 µg/mL for plasma, 0.05 µg/mL for interstitial fluid, and 0.025 µg/mL for aqueous humor and leukocytes. At concentrations of 1.0, 0.125, and 0.0625, the accuracy of the HPLC assay was within (mean ± SD) 3.66 ± 3.03% of the true value, and intra-assay precision was within 4.35 ± 1.86% of the mean.

Pharmacokinetic analysis—Drug concentrations were analyzed by use of standard pharmacokinetic methods to determine the drug disposition for each drug in each horse. A computer program was used to determine pharmacokinetic variables in accordance with methods described elsewhere. Pharmacokinetic variables were calculated
separately for each horse, and the results for all horses were reported as the mean ± SD. Both noncompartmental and compartmental methods were used.

Noncompartmental analysis was used to calculate the area-under-the-curve (AUC) for the plasma concentration-versus-time data for each drug formulation. Each AUC was calculated by use of the trapezoidal rule. Absolute bioavailability (F) for oral administration of the solution and capsules was then calculated for the 3 horses that also received IV infusions of itraconazole. Values for F were calculated by use of the following equation:

\[ F = \frac{\text{AUC}_{\text{oral}} \times \text{Dose}_{\text{IV}}}{\text{Dose}_{\text{oral}} \times \text{AUC}_{\text{IV}}} \]

where AUC_{oral} is the AUC after oral administration, Dose_{IV} is the dose administered IV, Dose_{oral} is the dose administered orally, and AUC_{IV} is the AUC after IV administration. Relative F after oral administration of the capsules was determined by dividing the AUC after oral administration of the capsules by the AUC after oral administration of the solution (ie, the ratio between the 2 drug formulations).

RESULTS

Oral administration of the solution resulted in more consistent absorption and a higher maximum concentration, compared with results after oral administration of the capsule formulation (Figure 1). Mean ± SD elimination half-life after oral administration of the solution (11.3 ± 2.84 hours) was almost twice that after oral administration of the capsules (7.97 ± 3.11 hours) or IV administration (6.52 ± 0.20 hours). Concentrations of itraconazole were initially detected 15 minutes after oral administration of the solution in
2 of 6 horses and 30 minutes after oral administration of the oral solution in the remaining 4 horses and were still detectable 48 hours after administration in 4 of 6 horses. Drug was not detected until 1 hour after oral administration of the capsules in 4 of 6 horses and 2 hours after administration in the remaining 2 horses. At time points at which the drug was not detected or was below the limit of quantification, the concentration was considered 0 for the calculations. Relevant noncompartmental pharmacokinetic variables for each formulation were summarized (Table 1).

For compartmental analysis of the data obtained after IV administration of the drug, a 2-compartment model was determined to provide the best fit as determined on the basis of evaluation of the plasma concentration-versus-time data plotted on a logarithmic scale as well as the Aikake inclusion criteria (Figure 2). An infusion time was added to the calculations by use of the following equation:

\[ C = A(e^{-\alpha t} - e^{-\alpha(t-t^*)}) + B(e^{-\beta t} - e^{-\beta(t-t^*)}), \]

where \( C \) is the plasma concentration of itraconazole, \( A \) is the intercept of the distribution phase, \( e \) is the base of natural logarithm, \( \alpha \) is the slope of the distribution phase, \( t \) is time after the end of the itraconazole infusion, \( t^* \) is \( t \) minus the duration of the infusion (\( ti \)) for \( t > ti \) and 0 for \( t < ti \), \( B \) is the intercept of the elimination phase, and \( \beta \) is the slope of the elimination phase.

Pharmacokinetic variables for a 2-compartment analysis after IV administration of itraconazole were summarized (Table 2). Following IV administration of the drug, mean ± SD apparent volume of distribution was 6.3 ± 0.94 L/kg. Mean absolute F after oral
administration of the solution and capsules was 64.96 ± 26.34% and 12.18 ± 5.6%, respectively. Relative F of the capsule formulation, compared with F of the solution, was highly variable at 33.83 ± 33.08%.

No adverse effects were observed during the study following oral or IV administration of itraconazole. The metabolite, hydroxyitraconazole was not detected in any samples.

Itraconazole concentrations were less than the detection limit of the assay in all of the samples of interstitial fluid, aqueous humor, and leukocytes analyzed; therefore, these samples were obtained from only 3 of the 6 horses in the study. Subcutaneous insertion of the ultrafiltration probe was easily accomplished in awake, sedated horses. Fluid collection rates ranged from 74.2 to 113.3 µL/h. Probes were tolerated well for up to 5 days with no adverse reactions evident at the insertion site. Results of in vitro plasma protein binding analyses revealed that itraconazole was highly protein bound in these horses (98.81 ± 0.17%).

**DISCUSSION**

Analysis of results of the study reported here indicated that itraconazole solution is absorbed following oral administration at a dosage of 5 mg/kg in horses and maintains plasma concentrations above the targeted MIC of 0.06 µg/mL against *Aspergillus* spp for >12 hours in all horses in the study and > 24 hours in 2 horses in the study. Plasma concentrations did not consistently reach values sufficiently high to treat horses with *Fusarium* infections; therefore, this drug is not recommended for the treatment of horses infected with this pathogen unless adequate susceptibility can be documented. The
capsule formulation was variably absorbed with a lower peak concentration and smaller AUC, compared with results for the solution. Overall F after oral administration of the solution was high (64.96 ± 26.34%), whereas the F after oral administration of the capsules was much lower (12.18 ± 5.6%). Relative F of the capsules, when compared with F for the solution, was 33.83 ± 33.08%. The high degree of variability was attributable to 1 horse that had almost 100% bioequivalence between capsules and solution.

These findings in horses are similar to data obtained from human pharmacokinetic trials in which oral administration of the solution resulted in improved absorption and F without a substantial increase in the incidence of adverse effects. The physicochemical properties of itraconazole can be used to explain this phenomenon. Itraconazole is a highly lipophilic (logarithm of the partition coefficient, 5.66) weak base; as such, it will become minimally soluble in aqueous solutions only at a low pH. The capsule formulation relies on the natural acid environment of the stomach to dissolve the drug into a solution prior to absorption; therefore, its absorption is subject to variability. The pKa for this weak base is < 2 for the base and 3.7 for the piperazine portion of the molecule. At these pKa values, it will only be protonated (and therefore solubilized in aqueous solutions) at an extremely low pH. The pH in the stomach of horses may not be sufficiently acidic and may be too variable to ensure consistent oral absorption. A newer oral formulation is a combination of itraconazole with cyclodextrins. Cyclodextrins are commonly used permeability enhancers made up of oligosaccharides with a hydrophilic outer surface and a lipophilic inner surface.
absorbed to any major degree after oral administration and are rapidly cleared from the system by renal excretion after IV administration, therefore they do not add substantially to the toxic effects of most drugs, although gastrointestinal tract disturbances have been reported\textsuperscript{28,29} after long-term administration. The main function of cyclodextrins is to form complexes with extremely lipophilic drugs, such as itraconazole, and allow them to remain soluble in solution. Because dissolution is the rate-limiting step in the absorption of most orally administered lipophilic drugs, this formulation would be expected to be absorbed better than would the capsule formulation when factors such as gastric pH may be a limiting factor. However, because of the low concentration of drug in the solution (10 mg/mL), a large volume must be administered orally (250 mL for a 500-kg horse treated at a dosage of 5 mg/kg), which makes administration inconvenient for horse owners. The IV formulation is not considered practical for administration to horses because of the high cost per dose, large volume administered, instability of the compound, and need for a slow infusion.

In humans, itraconazole has patterns toward nonlinear pharmacokinetics as determined on the basis of comparisons of the AUC for various doses.\textsuperscript{30} Steady-state conditions in the plasma are not reached until up to 10 days after oral administration in humans and 21 days after oral administration in cats,\textsuperscript{31,32} and the elimination half-life after multiple doses is often prolonged, indicating a saturable mechanism for excretion at clinical doses or inhibition of metabolism through inhibition of cytochrome P450 enzymes.\textsuperscript{16,33} The longer half-life observed with oral administration of the solution, compared with that for the IV formulation in the horses of the study reported here, was most likely attributable to a
prolonged absorption phase and a potential flip-flop phenomenon, as evidenced by discrepancies in the terminal elimination phase between IV administration and oral administration of the solution. Elimination in humans is mainly through metabolism by the liver and biliary excretion of itraconazole and its metabolites. More than 30 metabolites of itraconazole have been identified in humans, and 1 of these (hydroxyitraconazole) has comparable activity in vitro to that of the parent compound. In humans, this metabolite often reaches concentrations that are 2 to 3 times higher than itraconazole in the plasma. Hydroxyitraconazole was not detected in any of the samples analyzed in our study, indicating that metabolizing pathways must differ between horses and humans.

Another favorable feature of itraconazole in other species is that it reaches concentrations in the tissues that are often several fold higher than plasma concentrations and may last up to 4 weeks after cessation of treatment in some organs, particularly the skin and esophagus. In those studies, investigators determined concentrations of the drug in whole-tissue biopsy specimens that measured bound and unbound concentrations of the drug. Itraconazole is highly bound to plasma protein (> 99%) and tissues in humans, and analysis of results of the study reported here indicated that plasma protein binding was similar in horses (98.81%). Because protein binding may influence the distribution of drugs to tissue fluid, we therefore used an in vivo ultrafiltration technique to determine the free, unbound concentrations of itraconazole in the interstitial fluid of horses. These probes have been used by our laboratory group to measure drug concentrations in tissue fluid in dogs and have revealed that those concentrations correlate well with unbound
drug concentrations in plasma. The correlation of unbound drug concentrations in plasma and unbound drug concentrations in interstitial fluid was also confirmed in the study reported here because itraconazole was not detected in any of the interstitial fluid samples, indicating that free drug concentrations in the interstitial tissue fluid were negligible and free drug in plasma represented < 2% of total drug. However, the lack of unbound drug in the interstitial fluid does not imply that drug concentrations of itraconazole in horses are not therapeutic. The large volume of distribution observed in our study is consistent with, but not absolute proof of, high intracellular concentrations. It may also have been attributable to high tissue binding.

Although protein-unbound drug is considered to be the only active component for many antibacterial drugs, this may not be true for some antifungal drugs, including itraconazole and other triazole antifungals (eg, fluconazole and voriconazole). By use of a skin-blister technique, investigators documented in 1 study that at doses commonly considered to be effective in clinical cases, the free drug concentration in the skin is far below reported MIC values for Candida spp and dermatophytes. In vitro techniques for examining antifungal activity revealed that itraconazole had comparable efficacy against Candida albicans when incubated with or without 4% human serum albumin. Similar results were detected when a microdilution technique was used. On the basis of results for those techniques, the antifungal activity of itraconazole is not related entirely to free drug concentrations; therefore the lack of unbound drug detected in our interstitial fluid samples would not necessarily predict therapeutic failure. In fact, a significant effect was documented between total plasma concentrations (bound plus unbound) and antifungal
efficacy as a function of *Aspergillus fumigatus* burden in the lungs of immunosuppressed rabbits.\textsuperscript{37} Significant correlations have also been found between efficacy and maximum and minimum total plasma concentrations as well as the AUC in neutropenic rabbits with invasive pulmonary aspergillosis, supporting time- and concentration-dependent pharmacodynamic relationships.\textsuperscript{30} In the study reported here, orally administered itraconazole solution yielded a plasma concentration of total drug that was typically between 3.4 and 13.6 times the reported MIC\textsubscript{90} for *Aspergillus* spp.

Although protein-bound itraconazole may be active in some tissues, the high amount of protein binding will influence distribution to fluid compartments that have a relative lack of protein, such as the aqueous humor. Itraconazole was not detected in the aqueous humor samples taken from noninflamed eyes in the horses of the study reported here. This is consistent with reports of topical application of 1\% itraconazole ointment with or without dimethyl sulfoxide in horses.\textsuperscript{38} The topical treatment resulted in high corneal concentrations and was effective for clearing infection from 8 of 10 eyes with fungal keratitis.\textsuperscript{38,39} However, topical application (with or without dimethyl sulfoxide) did not yield measurable itraconazole concentrations in the aqueous humor. This is most likely a consequence of the high tissue-binding affinity and low aqueous solubility of the drug. Investigators also did not detect itraconazole in the aqueous or vitreous humor of noninflamed eyes of rabbits after systemic administration; however, the drug was detectable at low concentrations in the cornea following administration of a single dose.\textsuperscript{40} When endophthalmitis was induced with *C albicans*, drug was detectable in all compartments of the eye examined, although the concentrations were significantly lower.
than for ketoconazole or fluconazole.\textsuperscript{40} Therefore, we can conclude that, despite the high lipophilicity of itraconazole, it does not penetrate an intact blood-aqueous barrier. This is in contrast to fluconazole, which readily penetrates into the aqueous humor of the noninflamed eyes of horses.\textsuperscript{11,a}

Itraconazole was also not detectable in the samples of leukocytes analyzed in our study. In an in vitro study\textsuperscript{41} of alveolar macrophages isolated by use of bronchoalveolar lavage from New Zealand White rabbits, investigators revealed a rapid, passive uptake of tritium-labeled itraconazole when incubated in serum-free media. This uptake was drastically reduced when incubated with 5\% to 100\% serum, indicating an inhibitory effect of serum protein binding on cellular uptake of the drug.\textsuperscript{41} The lack of detectable drug in our study may have been attributable to the relatively low plasma concentrations, high plasma protein binding in vivo, or instability of the drug in the cells as a result of metabolism by intracellular enzymes (eg, myeloperoxidase). Because most fungal infections are not intracellular, the lack of penetration of itraconazole into WBCs would not be expected to have a negative effect on clinical efficacy; however, it does potentially eliminate this as a route of drug delivery to the site of infection.

We conclude that concentrations of itraconazole in aqueous fluids (eg, aqueous humor and interstitial tissue fluid) and leukocytes are negligible. We also conclude that itraconazole solution administered orally at a dosage of 5 mg/kg every 24 hours will yield total (bound and unbound) plasma concentrations that are inhibitory against fungi known to infect horses. This regimen is suggested for use in clinical trials conducted with
itraconazole for the treatment of horses with susceptible fungal infections. However, oral administration of the solution may be impractical because of the cost and large volume of drug required. Itraconazole capsules are more variable in the extent of absorption and cannot be recommended for use in horses at a dosage of 5 mg/kg. Higher or more frequent dosing with the capsules would be necessary to attain pharmacokinetic patterns similar to those for oral administration of the solution. Use of the IV formulation at a dosage of 1.5 mg/kg once daily would achieve concentrations considered to be therapeutic for the treatment of most infections with *Aspergillus* spp, but the expense may prohibit its use in most horses. Multiple-dosing studies are needed to determine the time needed to reach steady-state conditions and whether the half-life of itraconazole would increase or itraconazole would accumulate in tissues, which would allow for intermittent dosing in horses, similar to its use in other species. The safety of long-term oral administration of a cyclodextrin solution also needs to be assessed.

**FOOTNOTES**


b. Sporanox (itraconazole) oral solution (10 mg/mL), Janssen Pharmaceuticals, Titusville, NJ.

c. Sporanox (itraconazole) capsules (100 mg), Janssen Pharmaceuticals, Titusville, NJ.
d. Sporanox (itraconazole) injection (10 mg/mL), Janssen Pharmaceuticals, Titusville, NJ.

e. Itrafungol (itraconazole) oral solution (10 mg/mL), Janssen-Cilag Ltd, High Wycombe, UK.

f. Canine ultrafiltration probe (RUF-3-12), BAS Bioanalytical Systems, West Lafayette, Ind.


h. Centrifree micropartition system, Amicon, Beverly, Mass.

i. Waters pump, Millipore Corp, Milford, Mass.

j. Agilent series 1100, Agilent Technologies, Wilmington, Del.

k. Agilent series 1050 variable wavelength detector, Agilent Technologies, Wilmington, Del.

l. Agilent series 1100 Chemstation software, Agilent Technologies, Wilmington, Del.

m. Bond-Elut CN-E extraction cartridges (1 mL), Varian Inc, Harbor City, Calif.

n. Zorbax RX-C8 4.6 X 150-mm reverse-phase column, Agilent Technologies, Wilmington, Del.

o. Research Diagnostics, Inc. Flanders, NJ

p. WinNonlin, version 4.0, Pharsight Corp, Mountain View, Calif.
REFERENCES


Table 4.1—Mean ± SD values for noncompartmental pharmacokinetic variables after IV administration of a single dose of itraconazole (1.5 mg/kg) or after oral administration of a single dose of itraconazole (5 mg/kg) in the form of a solution or in capsules to horses.*

<table>
<thead>
<tr>
<th>Pharmacokinetic variable</th>
<th>IV</th>
<th>Oral Solution</th>
<th>Capsules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cmax (µg/mL)</td>
<td>NA</td>
<td>0.41 ± 0.13</td>
<td>0.15 ± 0.12</td>
</tr>
<tr>
<td>Tmax (h)</td>
<td>NA</td>
<td>3.33 ± 1.03</td>
<td>3.33 ± 1.15</td>
</tr>
<tr>
<td>Vdarea (L/kg)</td>
<td>6.30 ± 0.94</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>MRT (h)</td>
<td>6.94 ± 0.05</td>
<td>13.83 ± 2.73</td>
<td>12.59 ± 4.21</td>
</tr>
<tr>
<td>AUC ([h X µg]/mL)</td>
<td>2.27 ± 0.28</td>
<td>4.73 ± 1.85</td>
<td>1.60 ± 1.66</td>
</tr>
<tr>
<td>AUMC([h X [h X µg]/mL])</td>
<td>16.13 ± 2.08</td>
<td>67.44 ± 36.32</td>
<td>23.79 ± 28.95</td>
</tr>
<tr>
<td>λ (/h)</td>
<td>0.11 ± 0.00</td>
<td>0.06 ± 0.02</td>
<td>0.10 ± 0.05</td>
</tr>
<tr>
<td>t1/2α (h)</td>
<td>6.52 ± 0.20</td>
<td>11.30 ± 2.84</td>
<td>7.97 ± 3.11</td>
</tr>
<tr>
<td>F (%)</td>
<td>NA</td>
<td>64.96 ± 26.34</td>
<td>12.18 ± 5.60</td>
</tr>
</tbody>
</table>

*Itraconazole was administered IV to 3 horses and orally (solution and capsules) to 6 horses. Cmax = Maximum concentration. Tmax = Time to Cmax. Vdarea = Apparent volume of distribution (area method). MRT = Mean residence time. AUC = Area under the concentration-vs-time curve. AUMC = Area under the first moment-vs-time curve. λ = Rate constant of the terminal phase. t1/2α = Half-life of the terminal phase. F = Systemic availability. NA = Not applicable.
Table 4.2—Mean ± SD values* for pharmacokinetic variables after IV administration of itraconazole (1.5 mg/kg) to 3 horses.

<table>
<thead>
<tr>
<th>Pharmacokinetic variable</th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µg/mL)</td>
<td>0.69 ± 0.17</td>
</tr>
<tr>
<td>α (/h)</td>
<td>1.02 ± 0.36</td>
</tr>
<tr>
<td>B (µg/mL)</td>
<td>0.17 ± 0.01</td>
</tr>
<tr>
<td>β (/h)</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>t_{1/2α} (h)</td>
<td>0.75 ± 0.32</td>
</tr>
<tr>
<td>t_{1/2β} (h)</td>
<td>6.54 ± 0.51</td>
</tr>
<tr>
<td>k_{10} (/h)</td>
<td>0.38 ± 0.11</td>
</tr>
<tr>
<td>k_{12} (/h)</td>
<td>0.47 ± 0.22</td>
</tr>
<tr>
<td>k_{21} (/h)</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>V_{dss} (L/kg)</td>
<td>4.47 ± 0.23</td>
</tr>
<tr>
<td>Cl (mL/min/kg)</td>
<td>11.14 ± 1.33</td>
</tr>
</tbody>
</table>

*Values were determined by use of a 2-compartment model.

A = Coefficient of the distribution phase. α = Rate constant of the distribution phase. B = Coefficient of the elimination phase. β = Rate constant of the elimination phase. t_{1/2α} = Half-life of distribution. t_{1/2β} = Half-life of elimination. k_{10} = Elimination rate from compartment 1. k_{12} = Rate of movement from compartment 1 to compartment 2. k_{21} = Rate of movement from compartment 2 to compartment 1. V_{dss} = Apparent volume of distribution at steady state. Cl = Systemic clearance.
Figure 4.1—Mean ± SD values for the concentration-vs time data after oral administration of itraconazole (5 mg/kg) in the form of a solution (solid triangles) or in capsules (solid circle) to 6 horses.
Figure 4.2—Mean ± SD values for plasma concentration-vs-time data after IV administration of itraconazole at a dosage of 1.5 mg/kg to 3 horses. Infusion of itraconazole required 14 to 30 minutes. Time 0 = Completion of IV administration.
5. PHARMACOKINETICS AND TISSUE DISTRIBUTION OF CEPHALEXIN AFTER ORAL ADMINISTRATION OF SINGLE AND MULTIPLE DOSES IN HORSES

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ABSTRACT

The purpose of this study was to determine the pharmacokinetics and tissue fluid distribution of cephalexin in the adult horse following oral and intravenous administration. Cephalexin hydrate (10 mg/kg) was administered to horses IV and plasma samples were collected. Following a washout period, cephalexin (30 mg/kg) was administered intragastrically. Plasma, interstitial fluid, aqueous humor and urine samples were collected. All samples were analyzed by high pressure liquid chromatography (HPLC). Following IV administration, cephalexin had a plasma half-life ($t_{1/2}$) of 2.02 hr and volume of distribution ($V_{dss}$) of 0.25 L/kg. Following oral administration, the average maximum plasma concentration ($C_{max}$) was 3.47 µg/mL and the apparent half-life ($t_{1/2}$) of 1.64 hr. Bioavailability was approximately 5.0%. The $AUC_{ISF}:AUC_{plasma}$ ratio was 80.55% which corresponded to the % protein unbound drug in the plasma (77.07%). The $t_{1/2}$ in the ISF was 2.49 hr. Cephalexin was not detected in the aqueous humor. The octanol:water partition coefficient was 0.076 ± 0.025. Cephalexin was concentrated in the urine with an average concentration of 47.59 µg/mL. No adverse events were noted during this study. This study showed that cephalexin at a dose of 30 mg/kg administered orally at 8 hour dosage intervals in horses can produce plasma and interstitial fluid drug concentrations that are in a range recommended to treat susceptible gram-positive bacteria ($MIC \leq 0.5 \mu g/mL$). Due to the low oral bioavailability of cephalexin in the horse, the effect of chronic dosing on the normal intestinal bacterial flora requires further investigation.
INTRODUCTION

Cephalosporins are β-lactam antibiotics that have been in use since the 1960's. Injectable cephalosporins such as cefazolin and ceftiofur have been used in the horse, but the large volumes of injection, cost of the drugs, and pain from injections make them undesirable for long-term use. Oral antibiotic regimens are desirable to avoid the pain and irritation caused from repeated intravenous or intramuscular injections. The administration of oral cephalosporin antibiotics in the adult horse has received little attention. Dosing regimens for oral cefadroxil in foals have been published, however absorption in the adult horse was both poor and inconsistent (Duffee et al., 1989; Wilson et al., 1985). Similarly, cephadrine has been studied in neonatal foals for oral dosing, but no data exists on the drug in adult horses and cephradine is currently unavailable (Henry et al., 1992). A recent study on cefpodoxime showed adequate oral absorption of the drug, however colic was noted in 2 of 6 adult horses following a single dose (Carillo et al., 2005).

Cephalexin is a first generation cephalosporin antibiotic that is frequently used in human and small animals for the treatment of upper respiratory diseases, urinary tract infections and pyoderma (Papich, 1984, 1987 & 1988). It is rapidly bactericidal and has a broad spectrum of activity that includes gram-positive organisms, such as *Streptococcus* and *Staphylococcus* spp., anaerobic bacteria and some gram-negative organisms, notably *E.coli*, *Proteus* and *Klebsiella* spp. (Griffith & Black, 1970), although gram-negative enteric bacilli are inherently more resistant.

Previous studies have examined the pharmacokinetics of cephalexin following intravenous and intramuscular administration in horses (Lees et al., 1990; Villa et al.,
However, no data currently exists on oral absorption or tissue distribution of cephalixin in horses. The purpose of this study is to characterize the pharmacokinetics of cephalixin after oral administration, determine the oral bioavailability of cephalixin in the horse to assess the practicality of oral dosing regimens. An additional objective was to examine the tissue distribution of cephalixin and correlate these properties with plasma protein binding and lipophilicity of the drug.

MATERIALS AND METHODS

Animals

Six healthy adult horses were used in this study. One horse was initially used in a pilot study to determine if an oral cephalixin dose of 30 mg/kg was appropriate to proceed with a larger study. This horse was not available for the remainder of the study, though the data obtained from the plasma samples collected during the pilot study were added to the data obtained from the remaining 5 horses. Breeds used included 2 Thoroughbreds, 1 Quarterhorse, 1 Standardbred, 1 Arabian and 1 Dutch Warmblood. Three males and 3 females were used with weights ranging from 407-639 kg. All horses had a normal complete blood count and physical exam prior to inclusion in the study. The horses were housed in individual box stalls with access to free choice water for a minimum of 12 hours prior to drug administration. Horses were fasted for 12 hours prior to and 2 hours following drug administration. This study was approved by the Institutional Animal Care and Use Committee (IACUC) of North Carolina State University.
Drug administration

A two-way crossover design was used for this study. For the oral dosing study, generic cephalixin capsules (500 mg) were broken open and the contents dissolved in 500 mL of water and administered via a nasogastric tube as a single dose of 30 mg/kg. The nasogastric tube was then rinsed with 1 L of water to ensure adequate delivery of the drug. Following a two-week washout period, the drug was administered via an IV catheter at a dose of 10 mg/kg of cephalixin base. The intravenous dose was prepared by dissolving cephalixin hydrate powder (95.07% pure) obtained from Sigma-Aldrich (St. Louis, MO) and Spectrum Chemicals and Laboratory Supplies (New Brunswick, NJ) in sterile water to a concentration of 10 mg/mL. All solutions were kept refrigerated at 8°C and administered to the horses within 24 hours. The solutions were tested for potency and stability by high pressure liquid chromatography (HPLC) prior to administration and again at 48 hours following reconstitution. Treatments were not randomized due to the initial lack of availability of the cephalixin hydrate powder.

Blood collection

Blood samples were collected via a jugular catheter at 0, 10, 20 and 40 minutes, and 1, 1.5, 2, 3, 4, 6, 8, 10 and 12 hours after dosing. For the IV study, two catheters were placed and the plasma samples were collected through the catheter opposite to that from which the drug was administered. The samples were immediately centrifuged, and the plasma harvested and stored at -70°C until analysis. Samples were analyzed within one month of collection.
Urine Collection

Urine was collected from 3 horses at 8 hours following drug administration by free catch or urinary catheterization. In addition, one horse was catheterized prior to drug administration to collect blank urine for baseline analysis.

Interstitial fluid sampling

Interstitial fluid sampling was done via a subcutaneously placed tissue probe (UF-3-12 Ultrafiltration Probes, Bioanalytical Systems, West Lafayette, IN) at 0, 2, 4, 6, 8 and 12 hours after the oral dose. Samples were collected from 5 of the 6 horses that received oral cephalexin. Tissue probes were placed in the subcutaneous space immediately cranial to the scapula approximately 18 hours prior to the experiment to allow for equilibrium and removed at the end of the experiment.

Aqueous Humor Aspiration

Aqueous humor samples were collected from 4 of the horses at either 2 (n=3) or 3 (n=1) hours following oral administration of cephalexin. The procedure was performed in the standing horse following sedation, topical anesthesia and local nerve blocks. The eye was cleansed with a dilute aqueous iodine solution (10%) and rinsed with sterile saline. A sterile 27g, ½” needle was inserted into the anterior chamber at the dorsolateral limbus and 0.2-0.5 mL of aqueous humor was aspirated. Plasma samples were collected at times simultaneous with ocular fluid collection and all samples were frozen at -70°C and stored for analysis.

Protein binding assay

Protein binding was determined using a microcentrifugation system (Centrifree™ Micropartition system, Amicon, Beverly, MA) as previously described (Bidgood &
Papich, 2003). Pooled plasma collected from six healthy horses was fortified with cephalixin hydrate to make either a 5 or a 2.5 µg/mL solution and placed in a water bath at room temperature for 30 minutes. Three replicates of each concentration were pipetted into a microcentrifugation system and centrifuged at 1,000 x g for 10 minutes. The ultrafiltrate was then analyzed by HPLC to determine the unbound plasma concentration. Samples that did not undergo microcentrifugation were assayed to determine total (bound and unbound) drug concentration. Protein binding of each drug was determined according to the following formula:

\[
\text{% Protein Binding} = \frac{(\text{total}) - (\text{unbound})}{(\text{total})} \times 100
\]

**Drug Lipophilicity:**

The octanol:water partition coefficient, a measure of drug lipophilicity, was measured by established methods (Purcell *et al.*, 1973; Ashby *et al.*, 1985; Asuquo & Piddock, 1993) and a procedure previously published from our laboratory (Bidgood & Papich, 2002 & 2003). Phosphate buffer (0.1M) was prepared from deionized water and dibasic sodium phosphate. The pH was adjusted to 7.4 using 85% phosphoric acid. This aqueous buffer solution was fortified with cephalixin hydrate at 50, 20 and 10 µg/mL and added to an equal volume of octanol (l-octanol, Sigma Chemical, St. Louis, MO) in a screw top tube. The tube was gently rocked for 1 hour at room temperature to equally disperse the drug into each phase of the tube and then centrifuged for 10 minutes at 2,000 x g. The drug concentration in the aqueous layer was analyzed by HPLC before and after incubation and shaking. The partition coefficient (PC) was determined by the formula below.
PC = (Concentration pre-incubation) – (Concentration post-incubation) 
(Concentration post-incubation)

Drug analysis

Cephalexin concentrations in all samples were determined by HPLC with ultraviolet detection at 265nm using a method developed in our laboratory. Calibration curves were prepared by fortifying blank plasma, buffer solution, urine, interstitial fluid, or aqueous humor with cephalexin. The matrix used was specific for the samples analyzed. Our laboratory uses previously published guidelines for method validation (USP-NF 2004). A Zorbax RX-C8 4.6mm x 150mm reverse phase column (Agilent Technologies, Wilmington, DE) was used for separation. The mobile phase consisted of 0.01M sodium acetate buffer, acetonitrile and methanol in a ratio of 85:10:5 fortified with 0.001% triethylamine. The pH was adjusted to 3.0 using 85% phosphoric acid. Solid phase extraction using Sep-Pak Vac C18 extraction cartridges (Waters Corporation, Milford MA) was performed on all plasma samples. The cartridges were initially conditioned with 1 mL of methanol and 1 mL of 0.01 M sodium acetate buffer. The plasma was then extracted and the cartridges washed with 1 mL of 0.01 M sodium acetate buffer, followed by elution with 1 mL of methanol into clean glass tubes. Each sample was evaporated and reconstituted with 200 µL of mobile phase and injected into the HPLC. The injection volume was 25 µL. Calibration curves were linear between the concentrations of 50 µg/mL and 0.1 µg/mL, with a coefficient of determination (r²) of > 0.99 and calibration samples back-calculated to be within +/- 15% of the true concentration. The lower limit of quantification (LOQ) was the lowest concentration on a linear regression line from the calibration curve (0.1 µg/mL). Accuracy of the HPLC assay was within 3.16 ± 2.72% of the true value, and precision was within 3.87 ± 1.28%
of the mean. Drug concentrations in aqueous humor, buffer solution used for the lipophilicity studies and interstitial fluid samples were analyzed without extraction. The limit of detection for these samples was 0.1µg/mL, with an injection volume of 50 µL.

The urine samples were analyzed using a liquid:liquid extraction technique by adding 500 µL of sample to 500 µL of ethanol:acetonitrile:water (40:10:50). The resulting mixture was added to a microcentrifugation system (Centricon 30, Amicon Corp., Danvers, MA) and centrifuged at 3500 rpm (850 x g) for 30 minutes in a fixed rotor centrifuge. The ultrafiltrate was analyzed by HPLC without extraction as described above. The limit of detection for these samples was 20 µg/mL, with an injection volume of 25 µL.

**Pharmacokinetic analysis**

Drug concentrations were analyzed with the help of a computer program (WinNonlin, Version 4.0, Pharsight) to determine pharmacokinetic parameters for each horse. Compartmental analyses were performed on the data obtained from the plasma samples and noncompartmental analysis was performed on the data obtained from the ISF samples. Pharmacokinetic parameters measured included absorption rate, elimination rate, maximum concentration ($C_{max}$), time to maximum concentration ($T_{max}$), half-life ($t_{1/2}$) and area under the curve (AUC). Data obtained following analysis of plasma concentrations were plotted on a log-linear graph to determine the best-fit model for compartmental analysis (Figure 1). Based on this and on the Aikaike’s inclusion criteria (AIC), a two-compartmental model was chosen for analysis of the intravenous data (Yamaoaka et al., 1978). The model used was characterized by the equation:
$C = Ae^{-\alpha t} + Be^{-\beta t}$

where $C$ is the plasma concentration of cephalexin; $t$ is time after cephalexin administration; $A$ and $\alpha$ are the intercept and slope, respectively, of the distribution phase; $B$ and $\beta$ are the intercept and slope, respectively, of the elimination phase; and $e$ is the base of natural logarithm. A weighting factor of $1/y^2$ was used for curve fitting. The IV drug was administered as a slow bolus within 10 minutes and it was not necessary to include an infusion time in the calculation.

Data obtained after oral administration best-fitted a one-compartment model and first-order elimination with an added lag time characterized by the equation:

$$C = \frac{k_{01}FD}{V(k_{01} - k_{10})} \left( e^{-k_{10}(t-t_{lag})} - e^{-k_{01}(t-t_{lag})} \right)$$

where $C$ is the plasma concentration, $t$ is time, $t_{lag}$ is the estimated lag time, $k_{01}$ is the oral absorption rate, $k_{10}$ is the elimination rate constant, $F$ is the fraction of drug absorbed, $D$ is the non-intravenous dose, and $V$ is the volume of distribution. A weighting factor of $1/y^2$ was used. Extent of absorption (bioavailability) was calculated using the formula:

$$F = \frac{(AUC_{oral})(DOSE_{IV})}{(AUC_{IV})(DOSE_{oral})}.$$

**RESULTS**

No adverse effects were observed in any of the horses after administration of either oral or intravenous cephalexin. The cephalexin solution for injection was stable for a minimum of 48 hours and drug potency was assessed to be within ± 5% of the intended
10 mg/mL. Cephalexin was rapidly excreted following intravenous administration of cephalexin hydrate with a clearance (Cl) of $3.4 \pm 0.73$ mL/kg/min and an elimination half-life ($t_{1/2}^\beta$) of $2.02 \pm 0.46$ hr. The volume of distribution at steady-state was low ($V_{dss}$ $0.25 \pm 0.04$ L/kg). Other relevant pharmacokinetic parameters are summarized in Table 1.

Following oral administration, cephalexin was detected in the plasma of 5 out of 6 horses at 10 minutes and in all 6 horses at 20 minutes (Figure 2). Maximum plasma concentrations were $3.47 \pm 1.21$ µg/mL and were reached at $0.97 \pm 0.31$ hr after dosing. The variability in the plasma concentrations was primarily caused by one horse that had low oral absorption. Pharmacokinetic parameters for oral cephalexin are summarized in Table 2. The bioavailability of cephalexin was $5.0 \pm 2.8\%$.

Cephalexin was detected in all but one of the ISF samples analyzed. The ultrafiltration devices used were 46 cm in length and held 160 µL of fluid. The average collection rate for all horses was $1.37 \pm 0.63$ µL/min; therefore a 117 min (1.95 hr) lag time was calculated and used to adjust the ISF concentration versus time profile. Maximum concentrations measured in the ISF were $1.65 \pm 0.7$ µg/mL. The $t_{1/2}$ in the ISF was slightly longer than the $t_{1/2}$ in plasma ($2.26 \pm 0.59$ hr versus $1.64 \pm 0.53$ hr). A summary of the relevant pharmacokinetic parameters is provided in table 3. Plasma protein binding was $22.93 \pm 7.94\%$. The $AUC_{isf}:AUC_{plasma}$ ratio was $80.55 \pm 11.42\%$ which corresponded to the predicted concentration based on the percentage of protein unbound drug in the plasma ($77.07\%$). The $C_{max \ isf}:C_{max \ plasma}$ ratio was $45.93 \pm 4.88\%$.

Cephalexin was below the limit of detection in all aqueous humor samples analyzed. Cephalexin urine concentrations averaged $47.59 \pm 11.39$ µg/mL (range 35.74-
58.46 µg/mL) at 8 hours following drug administration. There were no obvious differences noted between sample collection methods for urine collection. The octanol:water partition coefficient of cephalexin was 0.076 ± 0.025.

**DISCUSSION**

The results of this study indicate that although cephalexin has a low systemic bioavailability in the horse following oral administration, adequate concentrations are reached to treat susceptible gram-positive infections, particularly *Streptococcus* and *Staphylococcus* species. The antibacterial activity of cephalexin depends on the amount of time drug concentrations are maintained above the MIC (T > MIC) for susceptible bacteria. Current recommendations for cephalosporins suggest that concentrations be maintained above the MIC for at least 50% of the dosing interval to achieve maximum activity (Turnidge, 1998). The reported MIC for most gram-positive organisms and *Pasteurella multocida* is ≤ 1.0 µg/mL, while for susceptible gram-negative organisms, the MIC may be as high as 8 µg/mL (Brown *et al.*, 2004; Griffith and Black, 1970). The CLSI (formerly NCCLS) breakpoint for susceptible bacteria is ≤ 8 µg/mL (NCCLS, 2002). Based on our preliminary data, a dose of 30 mg/kg administered orally every 8 hours will attain this goal for many gram-positive infections. Because of the higher MIC for gram-negative infections (*E. coli,* and *Klebsiella pneumoniae* for example), it is not possible to devise practical dosing regimens for these infections, except those involving the urinary tract.

Another oral cephalosporin antibiotic has recently been studied in the horse (Carrillo *et al.*, 2005). Cefpodoxime proxetil (Simplicef®, Pharmacia Upjohn,
Kalamazoo, Michigan) is a third-generation oral cephalosporin labeled for the treatment of skin infections in the dog. Similar to cephalexin, cefpodoxime was quickly absorbed in the adult horse, however the elimination half-life of cefpodoxime was longer (3.8 h). Maximum concentrations reached following a single oral dose of cefpodoxime were 1.27 μg/mL. This was significantly lower than the C_{max} reported in this study, although adequate comparisons cannot be made between these two studies since a lower dose of cefpodoxime (10 mg/kg) was used and the bioavailability of the drug was not reported. Also, 2 of the 6 horses in the cefpodoxime study developed mild colic that may or may not have been attributable to the drug. The recommended dose regimen for cefpodoxime in horses was 10 mg/kg orally every 12 hours for the treatment of bacterial infections. Based on this dosing regimen and the dosing regimen for cephalexin recommended from this study, as well as the price of the drugs in the pharmacy at the NCSU Veterinary Teaching Hospital, a daily dose of cefpodoxime proxetil would be approximately 13 times more expensive than that of cephalexin. Cefpodoxime proxetil does not appear to have advantages over cephalexin for the treatment of gram-positive infections in horses and cephalexin is more cost effective. Additional studies would be necessary to determine the safety of cephalexin in horses. Although we did not observe any adverse effects from either oral or IV administration in these horses, only single doses were administered.

Cephalexin has been shown to be actively absorbed from the small intestine via di- and tripeptide transporters in humans and rats (Berlioz et al., 2000). These same transporters may not be present in the equine intestine, which would account for the low systemic bioavailability in horses. Gastrointestinal side-effects have been infrequent
after cephalaxin administration in animals and humans, which is attributed to rapid absorption in the proximal small intestine, leaving only a small residual effect on colonic flora (Berlioz et al., 2000). Because of the low oral bioavailability in the horse, it is uncertain whether or not there would be adverse affects on the bacteria flora in the gastrointestinal tract.

Cephalaxin is excreted almost exclusively by the kidneys through both glomerular filtration and tubular excretion (Gower & Dash, 1969). It is minimally metabolized and 80-100% of the active drug is reported to be recovered in the urine at 6-8 hours following oral dosing in humans, with concentrations reaching 500-1000 µg/mL in the urine (Griffith, 1983). This property suggests that it would be a rational choice for the treatment of urinary tract infections in the horse. The urine concentrations reported here were approximately 13.23 ± 4.73 times greater at 8 hours than the maximum plasma concentrations. Since only a single time point was used, these samples do not represent the total amount of drug excreted into the urine. Furthermore, all of the horses had urinated at least once during the time between drug administration and sample collection. Therefore, it is possible that urine concentrations were actually higher than the values reported here. The majority of cases of cystitis and pyelonephritis in the horse are caused by bacteria normally susceptible to cephalaxin at these concentrations, including *E. coli*, *Klebsiella*, *Proteus*, *Streptococcus* and *Staphylococcus* species (Schott, 2004).

Unless an antibiotic can reach the site of infection, it will be ineffective. Although cephalosporins do not concentrate intracellularly (Brown & Percival, 1978), many bacterial infections in horses are in the extracellular milieu. Studies of antibiotics in the dog performed in our laboratory have shown that plasma protein binding is the main
determinant of extracellular drug concentrations (Bidgood & Papich, 2002 & 2003). Cephalexin has low protein binding which allows for rapid equilibration between plasma and tissue fluid compartments. Concentrations attained in the pleural fluid, interstitial fluid, and synovial fluid are comparable to concentrations found in plasma (Nightingale, 1986; Jalava et al., 1977; Cadorniga et al., 1979). Protein binding is also low in the horse (22.93 ± 7.94%) and, based on the \( \text{AUC}_{\text{isf}}:\text{AUC}_{\text{plasma}} \) ratio (80.55 ± 11.42%), appears to be the major determinant of drug distribution into the ISF. The \( \text{C}_{\text{max isf}}:\text{C}_{\text{max plasma}} \) ratio was 45.93 ± 4.88%. As observed in Figure 2, it is likely that if ISF samples were collected earlier a higher ISF peak concentration would have been detected. Nonetheless, a \( \text{C}_{\text{max isf}} \) lower than unbound plasma concentrations is entirely expected given the rapid clearance of cephalexin from plasma. A similar pattern was observed with cephaiprin administered to horses and the low peak concentration in tissue fluids was attributed to the short serum half-life (Short et al., 1987).

This study demonstrated the advantages of the ISF sampling method used here compared to the traditionally-used tissue cages for studies in animals. In dogs, sampling from a tissue cage showed accumulation of cephalexin in the ISF and a prolonged half-life compared to the plasma (Cadorniga et al., 1979). This is also consistent with the study in horses (Short et al., 1987) in which cephaiprin was administered intravenously and the samples collected from tissue cages showed lower concentrations and a longer elimination half-life of drug compared to serum. This is primarily caused by the geometry of the implanted cage compared to natural body tissues, which lowers the surface area – volume ratio into which the drug diffuses (Clarke et al., 1989; Ryan, 1985; Ryan & Cars, 1983). The tissue cage is an unnatural body compartment and produces
artificially low tissue fluid concentration data compared to a more natural tissue environment. Our tissue sampling method was not influenced by tissue geometry or ‘dead space’. Because unbound antibiotics can freely diffuse through microchannels (fenestrations) in capillaries, the limiting factor for diffusion into ISF is primarily the extent of protein binding, as demonstrated here. Although there was a slightly longer half-life observed for cephalexin elimination from ISF in this study, this is not unexpected given the time for flux between vascular and extravascular fluid compartments. Another difference between the ultrafiltration technique presented here and tissue cage measurements is that the tissue cages measure total (protein bound and unbound) drug concentrations whereas the probes used in this study measure only the unbound fraction of the drug. Only the unbound fraction of an antibiotic is active (Wise, 1986) and studies that report total (protein-bound and –unbound) drug concentrations in tissues produce an inaccurate impression of the drug activity at the site. In addition, our results showed that measurements of plasma concentration and the corresponding plasma protein binding can be used to predict tissue fluid concentrations for cephalexin in horses, and perhaps other antibiotics. Our calculation of the unbound plasma drug concentration closely paralleled the drug concentration in ISF as measured by AUC, and by comparing individual time points (Figure 2).

Although plasma protein binding can be used to predict drug diffusion through porous capillaries, drug lipophilicity is more accurate to predict drug diffusion across membranes and non-fenestrated capillaries, similar to those found in the blood-aqueous barrier. As predicted from the low octanol:water partition coefficient (PC 0.076 ± 0.025; log P -1.12), cephalexin was not detected in any of the aqueous humor samples analyzed.
This is characteristic of a poorly lipophilic drug. Cephalexin has previously been shown to penetrate into the aqueous humor of non-inflamed rabbit eyes at concentrations as high as 15-20% of plasma concentrations (Gager et al., 1969). However, the difference between the study in rabbits and in these horses is that the rabbits were administered a higher dose (50 mg/kg) and the aqueous levels peaked at 1 hour after dosing, which was earlier than the sampling times of the horses in this study.

In conclusion, cephalexin may be a useful antibiotic for the treatment of gram-positive and urinary tract infections in the horse. Despite the low oral bioavailability of cephalexin, adequate concentrations were achieved in the plasma and interstitial fluid for the treatment of susceptible gram-positive bacteria (MIC ≤ 0.5 µg/mL) at a dose of 30 mg/kg. To maintain therapeutic concentrations, we estimate that oral administration every 8 hours would be necessary. Multiple dose studies are necessary to establish the safety of cephalexin for long-term use in horses. We further showed that plasma protein binding can be used to predict drug penetration into the interstitial fluid – the site of antibacterial drug action.
REFERENCES


Table 5.1. Two-compartmental pharmacokinetic parameters for cephalexin hydrate (10 mg/kg) given intravenously.

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>IV (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µg/mL)</td>
<td>58.29 ± 9.92</td>
</tr>
<tr>
<td>α (hr⁻¹)</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>B (µg/mL)</td>
<td>4.89 ± 1.34</td>
</tr>
<tr>
<td>β (hr⁻¹)</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>t₁/₂α (hr)</td>
<td>0.44 ± 0.06</td>
</tr>
<tr>
<td>t₁/₂β (hr)</td>
<td>2.02 ± 0.46</td>
</tr>
<tr>
<td>k₁₀ (hr⁻¹)</td>
<td>1.26 ± 0.15</td>
</tr>
<tr>
<td>k₁₂ (hr⁻¹)</td>
<td>0.24 ± 0.04</td>
</tr>
<tr>
<td>k₂₁ (hr⁻¹)</td>
<td>0.46 ± 0.12</td>
</tr>
<tr>
<td>Vdss (L/kg)</td>
<td>0.25 ± 0.04</td>
</tr>
<tr>
<td>Vdarea (L/kg)</td>
<td>0.59 ± 0.16</td>
</tr>
<tr>
<td>Cl (mL/kg/min)</td>
<td>3.4 ± 0.73</td>
</tr>
<tr>
<td>AUC₀-∞ (hr*µg/mL)</td>
<td>50.7 ± 9.87</td>
</tr>
<tr>
<td>AUMC(hr<em>hr</em>µg/mL)</td>
<td>64.01 ± 19.25</td>
</tr>
</tbody>
</table>

A = coefficient of the distribution phase; α = slope of the distribution phase; B = coefficient of the elimination phase; β = slope of the elimination phase; t₁/₂α = half-life of distribution; t₁/₂β = half-life of elimination; k₁₀ = first-order elimination rate constant; k₁₂ = first-order transfer rate constant describing distribution between the central and peripheral compartments; k₂₁ = first-order elimination rate constant; k₁₂ = first-order transfer rate constant describing distribution between the peripheral and central compartments; Vdss = volume of distribution at steady-state; Vdarea = apparent volume of distribution; Cl = systemic clearance; AUC = area under the concentration-time curve; AUMC = area under the first moment-time curve.
Table 5.2. One-compartmental pharmacokinetic parameters for cephalexin (30 mg/kg) given via nasogastric tube.

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>(mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>0.97 ± 0.31*</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>3.47 ± 1.21*</td>
</tr>
<tr>
<td>$k_{01}$ (hr$^{-1}$)</td>
<td>4.55 ± 2.17</td>
</tr>
<tr>
<td>$k_{10}$ (hr$^{-1}$)</td>
<td>0.48 ± 0.23</td>
</tr>
<tr>
<td>$k_{01} t_{1/2}$ (hr)</td>
<td>0.18 ± 0.09</td>
</tr>
<tr>
<td>$k_{10} t_{1/2}$ (hr)</td>
<td>1.64 ± 0.53</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (hr*µg/mL)</td>
<td>8.59 ± 4.03</td>
</tr>
<tr>
<td>F (%)</td>
<td>5.0 ± 2.8</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$ = time to maximum concentration; $C_{\text{max}}$ = maximum concentration; $k_{01}$ = first-order absorption rate constant; $k_{10}$ = first-order elimination rate constant; $k_{01} t_{1/2}$ = half-life of absorption; $k_{10} t_{1/2}$ = half-life of elimination; $AUC_{0-\infty}$ = area under the concentration-time curve extrapolated to infinity; F = systemic bioavailability.

* Derived from the plasma concentration curves
Table 5.3. Noncompartmental pharmacokinetic parameters of cephalexin hydrate (30 mg/kg) in the ISF of 5 horses following oral dosing.

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>ISF (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>2.05</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>1.65 ± 0.7</td>
</tr>
<tr>
<td>$AUC_{0-\infty}$ (hr*µg/mL)</td>
<td>6.85 ± 3.91</td>
</tr>
<tr>
<td>$AUMC$ (hr<em>hr</em>µg/mL)</td>
<td>29.6 ± 21.37</td>
</tr>
<tr>
<td>$\lambda$ (hr$^{-1}$)</td>
<td>0.33 ± 0.11</td>
</tr>
<tr>
<td>$t_{1/2\lambda}$ (hr)</td>
<td>2.26 ± 0.59</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$ = time to maximum concentration; $C_{\text{max}}$ = maximum concentration; $AUC$ = area under the concentration-time curve; $AUMC$ = area under the first moment-time curve; $\lambda$ = slope of the terminal phase; $t_{1/2\lambda}$ = half-life of terminal phase
Figure 5.1. Plasma concentration-versus-time curve for cephalexin hydrate (10 mg/kg) given intravenously. Data represent mean concentration ± standard deviation for 5 horses.
Figure 5.2. Concentration-versus-time curves for plasma, ISF and the plasma unbound fraction in horses given 30 mg/kg cephalexin by nasogastric tube. ISF concentrations are denoted by bars at single time points. The time points reported for the interstitial fluid concentrations have been corrected for by the calculated lag time (1.95 hr).
6. PHARMACOKINETICS AND TISSUE DISTRIBUTION OF DOXYCYCLINE AFTER ORAL ADMINISTRATION OF SINGLE AND MULTIPLE DOSES IN HORSES

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ABSTRACT

Objective: To determine pharmacokinetics, safety, and penetration into the interstitial fluid (ISF), polymorphonuclear leukocytes (PMNL), and aqueous humor of doxycycline after oral administration of single and multiple doses in horses.

Animals: 6 healthy adult horses.

Procedure: The effect of feeding on drug absorption was determined. Plasma samples were obtained after administration of single or multiple doses of doxycycline (20 mg/kg) via nasogastric tube. Additionally, ISF, PMNL, and aqueous humor samples were obtained after the final administration. Horses were monitored for adverse reactions.

Results: Feeding decreased drug absorption. After multiple doses, time to maximum concentration was 1.63 ± 1.36 hours, maximum concentration was 1.74 ± 0.3 µg/mL, and elimination half-life was 12.07 ± 3.17 hours. Plasma protein binding was 81.76 ± 2.43%. The ISF concentrations correlated with the calculated percentage of protein-unbound drug. Maximum concentration was 17.27 ± 8.98 times as great in PMNLs, compared with plasma. Drug was detected in the aqueous humor at 7.5 to 10% of plasma concentrations. One horse developed signs of acute colitis and required euthanasia.

Conclusions and Clinical Relevance: Results suggest that doxycycline administered at a dose of 20 mg/kg, PO, every 24 hours will result in drug concentrations adequate for treating intracellular bacteria and bacteria with a minimum inhibitory concentration ≤ 0.25 µg/mL. For bacteria with a minimum inhibitory concentration of 0.5 to 1.0 µg/mL, more frequent doses of 20 mg/kg, PO, every 12 hours may be required; extreme caution should be exercised with these higher doses until more safety data are available.
INTRODUCTION

Tetracycline antimicrobials have a long history of use in veterinary medicine as broad-spectrum agents active against many organisms of importance to equine medicine.\(^1\) Doxycycline is a structural isomer of tetracycline that is available in formulations for oral and parenteral administration. The oral formulation, although not approved for veterinary species, is generic and inexpensive. Intravenous administration of doxycycline to horses has caused collapse and sudden death, apparently secondary to cardiovascular effects.\(^2\) Oral administration of other tetracyclines has been associated with gastrointestinal problems such as changes in the bacterial flora and diarrhea.\(^3,4,5,6\)

However, these problems, to date, have not been reported with oral administration of doxycycline in horses. In other species, doxycycline is bound to ingesta in the gastrointestinal tract, which may decrease its effect on the resident microflora.\(^1\) The oral formulation has been well tolerated and has been recommended for use at a dose of 10 mg/kg administered twice daily.\(^7\)

One of the most common uses of doxycycline in horses is treatment for ehrlichial infections, such as equine granulocytic ehrlichiosis caused by Anaplasma phagocytophilum (formerly Ehrlichia equi) and Potomac horse fever caused by Neorickettsia risticii (formerly Ehrlichia risticii).\(^8,9\) Because these organisms have a predilection for intracellular infection of WBCs (PMNLs for \(A\) phagocytophilum and monocytic cells for \(N\) risticii), the intracellular concentrations of the drug may be more predictive of efficacy than plasma concentrations.\(^10\) Another important use of doxycycline in horses is for treatment for leptospirosis, which has been implicated in abortion outbreaks and recurrent uveitis.\(^11,12\) Results of previous studies indicate that
doxycycline attains adequate concentrations in endometrial tissue. However another study reported that while the drug was detectable in the vitreous humor, it did not reach measurable concentrations in the aqueous humor of the eye. Studies in humans reveal that ocular penetration is possible with higher doses and more frequent administration.

Orally administered doxycycline in horses does not result in plasma concentrations necessary for inhibition of some important pathogens. Research reveals that doxycycline is highly protein bound in plasma, which may limit the distribution. Low doses may limit the effective concentrations at the target site. Specific studies are necessary to determine the extent of protein binding in horses, the extracellular distribution, and intraocular and intracellular penetration. We hypothesized that intraocular penetration and intracellular penetration into leukocytes is possible in horses, but it may require adjustment of the dosage regimens that are presently recommended.

The purpose of the study reported here was to determine the pharmacokinetics of doxycycline after single and multiple administrations at a dose of 20 mg/kg PO every 12 hours in horses. Plasma protein binding and interstitial fluid, intraocular, and intracellular doxycycline concentrations were also determined. Previous reports of sudden death from IV administration of doxycycline in horses precluded an accompanying IV study to determine variables such as systemic clearance, volumes of distribution, and oral systemic availability; therefore, allometric principles were applied to the data to estimate these variables.
MATERIALS AND METHODS

Pilot Study

Initially, a pilot study was performed to determine the safety of orally administered doxycycline at 20 mg/kg and to measure the effects of feeding on doxycycline absorption in the horse. One horse was administered doxycycline hyclate at a dose of 20 mg/kg via nasogastric tube while being maintained on a normal ration of grain and being allowed access to free choice timothy hay. Plasma samples were drawn at 0 (pre-treatment), 15, and 30 minutes and 1, 2, 4, 8, 12 and 24 hours after administration. After a suitable washout period (> 2 weeks), the same horse was given doxycycline hyclate (20 mg/kg) via nasogastric tube after feed was withheld for 12 hours. Plasma samples were drawn at 0, 15, 30, and 45 minutes and 1, 2, 3, 4, 6, 8, 12, 24, and 48 hours. Drug concentrations were analyzed by use of high pressure liquid chromatography (HPLC). The peak plasma concentration (C<sub>max</sub>), time to peak plasma concentration (T<sub>max</sub>), and the area under the plasma concentration-time curve (AUC) for fed versus nonfed conditions were compared. Because withholding feed yielded better results in the pilot study, food was withheld prior to drug administration in the remainder of the studies.

Horses

For the single and multiple dose studies, 6 adult horses donated for research purposes (3 males and 3 females) that weighed from 424 to 615 kg were used. The horses were determined to be healthy on the basis of results of a CBC, physical examination, and ophthalmic examination performed prior to inclusion in the study. The horses were housed in individual box stalls at least 12 hours prior to drug administration. Feed
consisted of a commercially available 12% pelleted feed and grass hay. This study was approved by the Institutional Animal Care and Use Committee of North Carolina State University.

Drug administration

For all parts of this study, generic doxycycline hyclate tablets containing 500 mg of doxycycline base per tablet were used. The dose was 20 mg of doxycycline base/kg of body weight. The tablets were crushed, suspended in 500 mL of water, and administered via nasogastric tube; the tube was then flushed with 1.5 L of water to clear any drug residue from the tube. For the single-dose study, feed was withheld for 12 hours prior to drug administration and 2 hours after drug administration. For the multiple-dose study, doxycycline hyclate was administered as described at 12-hour intervals, 5 times. Feed was withheld for 12 hours prior to the initial administration. Horses were fed their normal ration of grain and 1 flake of timothy hay 2 hours after each administration. Any feed that was not consumed was removed 2 hours later so feed was withheld for at least 8 hours prior to the next administration.

Blood collection

For pharmacokinetic analysis, blood samples were collected with a 14 gauge, 6 inch jugular catheter at 0, 15, 30, and 45 minutes, and 1, 2, 3, 4, 6, 8, 12, 24 and 48 hours after administration of the single dose and after the final administration of the multiple-dose study. The samples were immediately centrifuged at 600 x g and the plasma was harvested and stored at -70°C until analysis.
Polymorphonuclear leukocyte isolation

Polymorphonuclear leukocytes (PMNLs) were harvested from blood samples collected at 0 (prior to any drug administration), 1, 4, 8 and 24 hours after administration of the final dose in the multiple-dose study via density gradient centrifugation as described. Final cell counts were determined with a hemacytometer. The cell suspensions were centrifuged at 170 x g at 25°C for 7 minutes, decanted, and resuspended to a concentration of 20 x 10^6 cells/mL. Cell suspensions were centrifuged at 240 x g at 25°C for 10 minutes and the supernatant was discarded. The resulting cell pellet was stored at -70°C until analysis. To calculate the concentration of doxycycline in cells, a volume of 450 fL was assumed to be the mean volume of a PMNL. Ratios between the C_{max} and AUC of the doxycycline concentration in the PMNL versus plasma were used to measure the extent of drug accumulation in the PMNLs.

Interstitial fluid sampling

Interstitial fluid was sampled via a subcutaneously placed tissue probe at 1, 2, 4, 8, 12, and 24 hours after administration of the final dose in the multiple-dose study. Horses were sedated with xylazine hydrochloride (0.03 to 0.04 mg/kg, IV) to permit insertion of the probe. A 12 cm x 12 cm area of the neck was clipped and aseptically prepared and skin and subcutaneous tissues were anesthetized with 2% lidocaine hydrochloride. The probe was inserted through an introducer needle and sutured in place. Collection of fluid was achieved via a sterile vacutainer tube attached to the exposed end of the probe. Tissue probes were placed a minimum of 12 hours prior to the final administration and removed at the end of the experiment. Interstitial fluid was collected and stored prior to analysis at -70°C.
Aqueous humor

Aqueous humor samples were collected at either 2 or 4 hours after the final administration of doxycycline in the multiple-dose study. Samples were obtained from 3 horses at each time point. For humane collection of aqueous humor samples, the horses were sedated with detomidine (0.01 to 0.015 mg/kg, IV) and the eye was anesthetized by topical administration of 0.2 mL of anesthetic followed by auriculopalpebral, supraorbital, and retrobulbar nerve blocks performed with 2% lidocaine. The eye was cleansed with a dilute (10%) aqueous iodine solution and rinsed with sterile saline (0.9% NaCl) solution. A sterile 27 gauge, 1/2 inch needle was inserted into the anterior chamber at the dorsolateral or dorsoventral limbus and 0.2 to 0.5 mL of aqueous humor was aspirated. Plasma samples were collected at times that corresponded with ocular fluid collection and all samples were stored at -70°C prior to analysis.

Protein binding assay

Protein binding was determined via ultrafiltration with a microcentrifugation system as described. Protein binding of doxycycline was determined according to the following formula:

\[
\text{Protein binding (\%)} = \frac{[\text{total}] - [\text{unbound}]}{[\text{total}]} \times 100
\]

Where [total] is the drug concentration of bound and unbound doxycycline, prior to ultrafiltration, and [unbound] is the drug concentration collected in the filtrate. The percentage free drug was determined by subtracting the percentage protein bound from 100%.
Drug analysis

Doxycycline concentrations in all samples were determined via HPLC with ultraviolet detection. The HPLC apparatus consisted of a pump, autosampler, UV detector, and computer for data collection and analysis. A C8 reverse phase column was used for separation. Doxycycline plasma samples and PMNL samples were prepared via a protein precipitation method validated and used in our laboratory. Tissue fluid and aqueous humor samples were analyzed directly via HPLC without extraction. Calibration curves were prepared daily in plasma, phosphate buffered saline solution, blank ISF, or aqueous humor for plasma, PMNLs, tissue fluid, and aqueous humor samples, respectively. The limit of quantification (LOQ) for this assay was 0.05 µg/mL for the plasma and PMNL samples and 0.01 µg/mL for the tissue fluid and aqueous humor samples.

A computer program was used to determine pharmacokinetic values. Drug concentrations after administration of the single dose were analyzed by use of noncompartmental pharmacokinetic methods to determine drug disposition for each horse. Noncompartmental and compartmental analyses were performed on the plasma data obtained after multiple-dose administration. For compartmental analyses, doxycycline best fit a 1-compartment model described by the equation:

\[
C = \frac{k_{01}FD}{V(k_{01} - k_{10})} (e^{k_{10}t} - e^{k_{01}t})
\]

Where C is the plasma concentration, t is time, k_{01} is the oral absorption rate, k_{10} is the elimination rate constant, F is the fraction of drug absorbed, D is the non-IV dose, and V is the volume of distribution. This model assumes that there is first-order absorption and that k_{01} > k_{10}. A weighting factor of 1/y^2 was added for the best fit.
Noncompartmental analyses were also performed on the data obtained from the ISF and PMNL samples. Mean plasma, tissue fluid, and PMNL concentrations; C$_{\text{max}}$; T$_{\text{max}}$; T$_{1/2}$; and area under the curve (AUC) are reported. Tissue fluid:plasma, intraocular:plasma, and intracellular:plasma concentration ratios were calculated for protein-bound and –unbound concentrations. An accumulation ratio (RA) was calculated from the following equation:

$$RA = \frac{1}{1-e^{-\lambda z \tau}}$$

where $\lambda z$ is the slope of the elimination phase and $\tau$ is the administration interval.

**Allometric analysis**

The allometric equation used was $Y = a (BW)^b$, where $Y$ is the calculated systemic clearance, $a$ is an allometric constant (the y-axis intercept for an animal of 1 kg), $BW$ is body weight in kg, and $b$ is the allometric exponent. Principles of allometric scaling have already been established for doxycycline in animals for a wide range of body weights.$^{21}$ To determine systemic clearance ($Y$) for horses, a value of $b = 0.75$ was used, which corresponds to values from other species determined for doxycycline.$^{21}$, and BW used was the mean weight of the horses in this study. On the basis of these results, the AUC for a dose administered IV was determined with the equation $AUC = Dose/Y$. Oral bioavailability was then calculated as $F = AUC_{\text{oral}}/AUC_{\text{IV}}.$
RESULTS

Pilot study

Feeding resulted in lower $C_{\text{max}}$, longer $T_{\text{max}}$, shorter elimination half-life ($t_{1/2}$), steeper slope to the terminal phase ($\lambda$), and smaller AUC, compared with that in horses for which feed was withheld (Table 1).

Single Dose Study

The plasma concentration-versus-time curve for doxycycline after administration of a single dose was determined (Figure 1). After oral administration, doxycycline was detected in 5 of 6 horses at 15 minutes and in all horses at 30 minutes. Drug was still present in the plasma at 24 hours in all horses, but was not detected in any of the horses at 48 hours. Mean $T_{\text{max}}$ was $1.54 \pm 1.3$ hours, although 3 of the 6 horses had a second peak at 3 to 4 hours (Table 2). One horse had signs of mild discomfort (increased respiration and sternal recumbency) for approximately 1 hour after drug administration. This resolved without treatment.

Multiple Dose Study

The concentration-versus-time curves for doxycycline in the plasma, ISF and PMNLs after multiple oral administrations were determined (Figure 2). Mean $T_{\text{max}}$ was similar to the single dose study ($1.63 \pm 1.36$ hours [Table 2]) and 3 of 6 horses again had a secondary peak at 3 to 4 hours.

Doxycycline was detected in all ISF samples analyzed (Table 3). The ultrafiltration devices used were 46 cm in length and held 160 µL of fluid. Mean collection rate was $1.13 \pm 0.2$ µL/min; therefore, a 141-minute (2.35-hour) lag time was used to adjust the ISF concentrations to the correct time reference. Results of the in vitro
plasma protein binding assays revealed that doxycycline was less highly protein bound in horses than other species, with mean percentage protein binding of 81.76 ± 2.43% (free drug, 18.24%). The $C_{\text{max,plasma}}/C_{\text{max,plasma}}$ and $AUC_{\text{plasma}}/AUC_{\text{plasma}}$ ratios were 17.88 ± 3.49% and 24.84 ± 7.54%, respectively. Substantial drug accumulation was detected in the plasma in the multiple dose study (RA, 1.96). When an administration interval of 24 hours was used, the calculated RA was 1.32.

Mean half-life of doxycycline in the PMNLs was similar to the mean half-life in plasma (13.01 ± 9.2 hours vs 12.07 ± 3.17 hours, respectively). Maximum PMNL concentrations occurred later than maximum plasma concentration (3.33 ± 1.03 hours vs 1.63 ± 1.36 hours, respectively). The $C_{\text{max}}$ and AUC of doxycycline in equine PMNLs were 17.27 ± 8.98 and 14.58 ± 6.64 times as high, compared with plasma $C_{\text{max}}$ and AUC, respectively.

Doxycycline was detected in the aqueous humor samples from all 6 horses. Mean aqueous humor concentrations were 0.11 ± 0.01 µg/mL at 2 hours and 0.095 ± 0.016 µg/mL at 4 hours. These concentrations represented 7.5 ± 0.3% and 10.9 ± 1.8% of the corresponding plasma concentrations, respectively.

On the basis of the results of allometric analysis, the systemic clearance of doxycycline in horses was estimated to be 0.683 mL/kg/min. By use of this value, an estimated AUC for a dose of 20 mg/kg administered IV to a horse is 488 µg · h/mL. Compared with the AUC from a single orally administered dose, an estimated systemic absorption (F) for doxycycline after oral administration to a horse is only 2.7%.

Four of the 6 horses had no adverse effects from drug administration. During the multiple-dose study, 1 horse developed moderate anorexia after the first and second dose
and had signs of mild abdominal discomfort after the second dose; however this resolved without treatment. No diarrhea was seen. This was the same horse that had mild colic signs after the single-dose experiment. Fifty-two hours after the final dose was administered, a different horse developed diarrhea, fever, and leukopenia (2 x 10^3 WBCs/µL; reference range, 6.0 to 12.5 WBCs/µL). The horse was administered fluids IV and PO and received nonsteroidal anti-inflammatory drugs and α2-receptor agonists. Twenty-four hours later, clinical signs of laminitis in the forefeet were detected and the horse had signs of progressive abdominal discomfort. Euthanasia was performed and the horse was submitted for necropsy. Grossly, a peritoneal exudate was evident, with severe, diffuse gas and fluid distention of the cecum and large colon. Multifocal transmural venous infarctions were evident in the cecum. Serosal petechiae and ecchymoses were seen on the cecum, large colon, adrenal glands, subcutis, and pleural surfaces. Mild separation of the laminae at the dorsal and palmar aspects of the third phalanx was seen in both forelimbs. The final diagnosis was ulcerative typhlitis with regional ileus, disseminated intravascular coagulopathy, and acute laminitis. No etiologic agents were detected. Bacteriologic culture of the cecum, small and large colon, liver, and mesenteric lymph nodes for Salmonella spp yielded negative results as did testing for Clostridium perfringens enterotoxins.

**DISCUSSION**

Results of this study indicated that doxycycline is poorly absorbed in horses, although it reaches adequate plasma concentrations, has good tissue penetration after oral administration, and should be considered for further study as a treatment of infections
caused by bacteria susceptible to tetracyclines. It has a long half-life, making a once
daily administration regimen possible. Because plasma concentrations reported in a
study of orally administered doxycycline in horses using a dose of 10 mg/kg every 12
hours were low, a dose of 20 mg/kg every 12 hours was chosen for this experiment to
achieve plasma doxycycline concentrations of 1 to 2 µg/mL, which is within the range
that would be effective for many susceptible bacteria. Mean plasma concentrations
achieved after administration of 5 consecutive doses in our study were approximately 4
times greater than those reported in the previous study (1.74 ± 0.3 µg/mL vs 0.42 ± 0.05
µg/mL), even though only twice the dose was used. The most likely explanation for this
is that the horses in the previous study were allowed free choice access to hay and the
horses in this study had food withheld for a minimum of 8 hours prior to each
administration. In humans and other species, oral absorption of doxycycline is not
substantially affected by food intake, presumably because of the decreased affinity of
doxycycline for chelating anions, such as calcium and magnesium, when compared to
other tetracycline antibiotics. However, in our small pilot study, feeding inhibited
absorption and decreased plasma concentrations by almost one-half. We suspect that the
high roughage content of equine diets may create a physical barrier that blocks drug
absorption by the intestinal wall. Additionally, doxycycline has an isoelectric point
and maximum lipid solubility at a pH of 5.5, indicating optimal absorption occurs in the
duodenum. Feeding may delay gastric emptying and therefore delay absorption.

Because of risk of cardiovascular toxicosis, we could not conduct studies of IV
administration in these horses to establish rates for systemic clearance and volumes of
distribution and determine oral systemic availability. To provide an estimate of IV
pharmacokinetic values, one can use principles of allometric scaling to extrapolate an IV plasma curve from values determined in other animals. A previous report of allometric analysis used to determine pharmacokinetic values for doxycycline in several species, not including horses, revealed a good fit on the basis of plasma protein binding and free drug concentrations. Pharmacokinetic values of clearance and half-life have been determined for dogs, for which protein binding is similar to that seen in horses. Clearance was chosen because it had the least variability of all parameters reported in dogs. The bioavailability of orally administered doxycycline in horses determined by use of this method was low (2.7%). This agrees with estimates made on the basis of values of Vd/F and CL/F and explains why concentrations were so low in relation to doses administered orally to other animals.

Doxycycline concentrated in equine PMNLs in this study. Tetracyclines are commonly reported to concentrate intracellularly and doxycycline has a higher affinity for intracellular accumulation than other tetracyclines. In vitro analysis of the penetration of radiolabelled doxycycline into isolated human PMNLs revealed a cellular-to-extracellular concentration ratio of 13. In our study in horses, the ratio was 17 at peak concentrations and 14.6 for the total area-under-the-curve. Previous studies reveal that 50% of the intracellular drug is released from the cells after incubation in an antimicrobial-free medium, indicating that 50% is irreversibly bound to the cell.

The percentage binding to plasma proteins as measured by use of an ultrafiltration technique was 81.6% in this study. This was lower than that reported for cats (99%) but closer to values reported for dogs of 92%, 75 to 86%, and 91%. A high degree of plasma protein binding is an important factor that affects diffusion of
drug from plasma to the interstitial fluid. The concentration unbound drug in the plasma should theoretically equal the unbound drug concentration in the interstitial tissue space at steady state. This was true in our study, in which the extent of protein binding was proportional to the interstitial fluid concentration. Plasma unbound drug concentration was 18.4% and the values in interstitial fluid were 17.88 ± 3.4 and 24.84 ± 7.54% of the plasma concentrations, based on the $C_{\text{max}}$ and the AUC, respectively.

Steady state conditions were confirmed by comparing the AUC$_{0-12}$ of the last dose to the AUC$_{0-\infty}$ of the single dose study, which was approximately equal (13.35 vs 12.2 hr•µg/mL).

In this study, doxycycline penetrated the intact blood-aqueous barrier. This is in direct contrast to the findings of Gilmour et al (2005). In that study, doxycycline was not detected in the aqueous humor. There are several explanations for this discrepancy. A lower dose was administered in the other study (10 mg/kg vs 20 mg/kg), resulting in lower plasma drug concentrations and less drug available for diffusion into the eye. That study also used a bioassay for drug detection, which is less specific and less sensitive than the HPLC method used in the present study. The LOQ for the bioassay was 0.15 µg/mL, which is higher than the concentrations found in the present study. We report aqueous concentrations between 7.5 to 10.9% of plasma concentrations at the times they were measured. This was similar to findings in humans (concentration in the noninflamed eye, 11 to 13% of plasma concentrations). Concentrations are higher when the eye is inflamed and therapeutic concentrations are reached after administration of a single dose. In rabbits, doxycycline has better penetration into ocular tissues than tetracycline. The authors of that study speculated that this was attributable to the
increased lipophilicity of doxycycline, compared with tetracycline, which allowed for easier movement of the drug across the blood-ocular barriers. Doxycycline has been used in equine medicine to treat anterior uveitis in horses with suspected infections with *Leptospira* spp. Several other ocular uses are attributed to its anticollagenolytic activity, anti-inflammatory activity, and ability to enhance corneal repair.\(^{34,35}\)

The development of diarrhea in 1 of the horses in this study was an unexpected event. Orally and parenterally administered oxytetracycline in horses has been associated with fatal diarrhea and increased or prolonged shedding of *Salmonella* organisms,\(^{3,36}\) although no cases of diarrhea after treatment with doxycycline have been reported. *Clostridium perfringens, Salmonella* spp, and coliform bacteria have been implicated as contributing factors in cases of colitis induced by oxytetracycline; in other cases no etiologic agent was identified.\(^{3,36}\) Culture for *Salmonella* spp in feces and gastrointestinal tissues yielded negative results in the horse of this report, as did testing for *C. perfringens* toxin type B. Although positive results would increase the likelihood of antimicrobial-associated diarrhea in this case, negative results do not rule that diagnosis out.

Antimicrobial-associated diarrhea has not been associated with doxycycline administration in humans or other species, because it has minimal effect on gastrointestinal flora after repeated administration, especially compared with other antimicrobials such as clindamycin and erythromycin.\(^{37}\) Doxycycline also preserves the host’s resistance to bacterial colonization, compared with clindamycin and erythromycin.\(^{38}\) Doxycycline had no effect on colonic motility in an in vitro model of rabbit and guinea pig colon.\(^{39}\) Development of diarrhea in our horse may be attributed to
the low oral bioavailability of doxycycline, which leads to an increased amount of drug in
the intestine. The two previous reports on doxycycline administration in horses\textsuperscript{7,13} did
not observe gastrointestinal problems associated with drug administration. Each of these
studies used lower doses and it is possible that a higher dose used in our study increased
the risk of a gastrointestinal adverse reaction.

We have formulated administration and dose regimens on the basis of the
calculated accumulation ratios that would maintain trough plasma concentrations greater
than the reported minimum inhibitory concentration-90\% (MIC\textsubscript{90}) of common equine
pathogens throughout the administration interval.\textsuperscript{7,40} For those bacteria with an MIC \leq
0.25 \mu g/mL, a dose of 20 mg/kg, PO, every 24 hours will achieve this goal. This would
include many susceptible \textit{Streptococcus} spp, \textit{Staphylococcus} spp, \textit{Pasteurella} spp,
\textit{Rhodococcus equi}, \textit{Actinobacillus equuli}, and most ehrlichial organisms.\textsuperscript{7,40} Those
bacteria that reside within PMNLs would also be susceptible with this regimen because
of the high degree of accumulation of doxycycline within these cells. For less susceptible
bacteria with an MIC of 0.5 to 1.0 \mu g/mL, a dose of 20 mg/kg, PO, every 12 hours
would be needed to maintain the trough in the necessary range. As revealed in our study,
doxycycline is absorbed better if food is withheld for a minimum of 8 hours before and 2
hours after doxycycline administration, although this may not be possible in practice,
particularly if twice daily administration is used.

Although absorption after oral administration appears to be low, with doses used
in this study concentrations in cells and tissues are in a range that may give therapeutic
results in horses. The value of determining plasma protein binding for studies with
antimicrobials in horses was also apparent. The unbound drug in the plasma diffused
through tissue capillaries and into the interstitial space. The ISF drug concentrations closely paralleled the unbound concentrations in plasma. Oral administration of doxycycline results in concentrations in PMNLs that are higher than in plasma and therefore may be well suited to treat horses with infections caused by susceptible intracellular organisms. Doxycycline penetrates an intact blood-aqueous barrier and may be useful in the treatment of several ocular diseases. Because of development of severe, acute colitis in 1 of the 6 horses used in this study, we recommend that further clinical and safety studies that use similar doses for longer intervals be performed on horses prior to using this administration regimen.
Footnotes

a Doxycycline hyclate tablets, 500 mg, West-ward Pharmaceutical Corporation, Eatontown, NJ

b Canine ultrafiltration probe (RUF-3-12), BAS Bioanalytical Systems, West Lafayette, IN

c Sedazine®, Fort Dodge, Animal Health, Fort Dodge, IA

d Lidocaine hydrochloride injectable-2%, Phoenix Scientific, Inc, St Joseph, MO.

e Dormosedan®, Pfizer Animal Health, Exton, PA

f Proparacaine Hydrochloride Ophthalmic Solution, Falcon Pharmaceuticals, Ltd., Fort Worth, TX

g Centrifree™ Micropartition system, Amicon, Beverly, MA

h Waters Pump, Millipore Corp., Milford, MA

i Agilent Series 1100, Agilent Technologies, Wilmington, DE

j Agilent series 1050 variable wavelength detector, Agilent Technologies, Wilmington, DE

k Agilent Series 1100 Chemstation software, Agilent Technologies, Wilmington, DE

l Zorbax RX-C8 4.6mm x 150mm reverse phase column, Agilent Technologies, Wilmington, DE

m WinNonlin, Version 4.0, Pharsight Corporation, Mountain View, CA
REFERENCES


8. Maurin M, Bakken JS, Dumler JS. Antibiotic susceptibilities of *Anaplasma* (Ehrlichia) phagocytophilum strains from various geographic areas in the United


Table 6.1. Pharmacokinetic variables in a horse administered doxycycline hyclate (20 mg/kg) PO after feeding or withholding of feed.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Fed</th>
<th>Feed withheld</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>4</td>
<td>0.75</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>0.432</td>
<td>0.966</td>
</tr>
<tr>
<td>AUC (h*µg/mL)</td>
<td>4.459</td>
<td>10.671</td>
</tr>
<tr>
<td>$\lambda$ (h$^{-1}$)</td>
<td>0.123</td>
<td>0.042</td>
</tr>
<tr>
<td>$t_{1/2\lambda}$ (h)</td>
<td>5.647</td>
<td>16.542</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$ = time to maximum concentration; $C_{\text{max}}$ = maximum concentration; AUC = area under the concentration-time curve; AUMC = area under the first moment-time curve; $\lambda$ = slope of the terminal phase; $t_{1/2\lambda}$ = half-life of terminal phase.
Table 6.2. Mean ± SD values for pharmacokinetic variables in 6 horses after single or multiple (n = 5) oral administrations of doxycycline hyclate (20 mg/kg)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Single dose</th>
<th>Multiple doses</th>
</tr>
</thead>
<tbody>
<tr>
<td>$T_{\text{max}}$ (h)</td>
<td>1.54 ± 1.3</td>
<td>1.63 ± 1.36</td>
</tr>
<tr>
<td>$C_{\text{max}}$ (µg/mL)</td>
<td>0.91 ± 0.25</td>
<td>1.74 ± 0.30</td>
</tr>
<tr>
<td>$k_{01}$ (h$^{-1}$)</td>
<td>---</td>
<td>2.56 ± 2.1</td>
</tr>
<tr>
<td>$k_{10}$ (h$^{-1}$)</td>
<td>---</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>$k_{01} t_{1/2}$ (h)</td>
<td>---</td>
<td>0.18 ± 0.18</td>
</tr>
<tr>
<td>$k_{10} t_{1/2}$ (h)</td>
<td>---</td>
<td>10.39 ± 1.06</td>
</tr>
<tr>
<td>AUC$_{0-12}$ (h*µg/mL)</td>
<td>---</td>
<td>12.2 ± 1.46</td>
</tr>
<tr>
<td>AUC$_{0-\infty}$ (h*µg/mL)</td>
<td>13.35 ± 2.71</td>
<td>24.83 ± 5.05</td>
</tr>
<tr>
<td>AUMC(h*µg/mL)</td>
<td>88.78 ± 17.21</td>
<td>361.53 ± 298.74</td>
</tr>
<tr>
<td>$\lambda$ (h$^{-1}$)</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.02</td>
</tr>
<tr>
<td>$t_{1/2\lambda}$ (h)</td>
<td>11.81 ± 3.51</td>
<td>12.07 ± 3.17</td>
</tr>
</tbody>
</table>

$T_{\text{max}}$ = time to maximum concentration; $C_{\text{max}}$ = maximum concentration; $k_{01}$ = first-order absorption rate constant; $k_{10}$ = first-order elimination rate constant; $k_{01} t_{1/2\alpha}$ = half-life of absorption; $k_{10} t_{1/2\beta}$ = half-life of elimination; Vd$_{\text{area/F}}$ = apparent volume of distribution corrected for bioavailability; Cl/F = systemic clearance corrected for bioavailability; MRT = mean residence time; AUC$_{0-12}$ = area under the concentration-time curve from 0 to 12 hours; AUC$_{0-\infty}$ = area under the concentration-time curve extrapolated to infinity; AUMC = area under the first moment-time curve; $\lambda$ = slope of the terminal phase; $t_{1/2\lambda}$ = half-life of terminal phase.
Table 6.3. Mean ± SD values for pharmacokinetic variables in interstitial fluid (ISF) and polymorphonuclear leucocytes (PMNLs) in 6 horses after multiple oral administrations of doxycycline hyclate (20 mg/kg).

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>ISF (mean ± SD)</th>
<th>PMNL (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( T_{\text{max}} ) (hr)</td>
<td>---</td>
<td>3.33 ± 1.03</td>
</tr>
<tr>
<td>( C_{\text{max}} ) (µg/mL)</td>
<td>0.31 ± 0.08</td>
<td>29.02 ± 13.01</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>12.01 ± 1.1</td>
<td>18.81 ± 12.71</td>
</tr>
<tr>
<td>AUC (hr*µg/mL)</td>
<td>5.24 ± 1.3</td>
<td>299.95 ± 81.93</td>
</tr>
<tr>
<td>AUMC(hr<em>hr</em>µg/mL)</td>
<td>62.01 ± 10.94</td>
<td>2579.85 ± 995.52</td>
</tr>
<tr>
<td>( \lambda ) (hr(^{-1}))</td>
<td>---</td>
<td>0.07 ± 0.03</td>
</tr>
<tr>
<td>( t_{1/2\lambda} ) (hr)</td>
<td>---</td>
<td>13.01 ± 9.2</td>
</tr>
</tbody>
</table>

\( T_{\text{max}} \) = time to maximum concentration; \( C_{\text{max}} \) = maximum concentration; MRT = mean residence time; AUC = area under the concentration-time curve; AUMC = area under the first moment-time curve; \( \lambda \) = slope of the terminal phase; \( t_{1/2\lambda} \) = half-life of terminal phase; F = systemic bioavailability
Figure 6.1. Mean ± SD plasma concentration-versus-time curve in 6 horses after oral administration of a single dose of doxycycline hyclate (20 mg/kg).
Figure 6.2. Concentration-versus-time curves for doxycycline in polymorphonuclear leukocytes (-♦-), plasma (-▲-), unbound plasma (-●-) and interstitial fluid (-■-) after 5 oral administrations in 6 horses. Concentrations are reported as the mean ± SD with the exception of the unbound plasma concentrations, which is a calculated value.
7. THE PHARMACOKINETICS OF ORBIFLOXACIN IN THE HORSE FOLLOWING ORAL AND INTRAVENOUS ADMINISTRATION.

Jennifer L. Davis, Mark G. Papich, and Allan Weingarten

Accepted for publication in the Journal of Veterinary Pharmacology and Therapeutics
Abstract

The purpose of this study was to determine the pharmacokinetics and physicochemical characteristics of orbifloxacin in the horse. Six healthy adult horses were administered oral and intravenous orbifloxacin at a dose of 2.5 mg/kg. Plasma samples were collected and analyzed by high pressure liquid chromatography (HPLC) with ultraviolet detection. Plasma protein binding and lipophilicity were determined in vitro. Following IV administration, orbifloxacin had a terminal half-life ($t_{1/2}$) of 5.08 hr and a volume of distribution ($V_d_{ss}$) of 1.58 L/kg. Following oral administration, the average maximum plasma concentration ($C_{max}$) was 1.25 µg/mL with a $t_{1/2}$ of 3.42 hr. Systemic bioavailability was 68.35%. Plasma protein binding was 20.64%. The octanol:water partition coefficient (pH 7.4) was 0.2 ± 0.11. No adverse reactions were noted during this study. Dosing regimens were determined from the pharmacokinetic-pharmacodynamic parameters established for fluoroquinolone antibiotics. For susceptible bacteria, an oral dose of approximately 5 mg/kg once daily will produce plasma concentrations within the suggested range. This dose is suggested for further studies on the clinical efficacy of orbifloxacin for treatment of susceptible bacterial infections in the horse.
Introduction

Fluoroquinolone antibiotics are frequently used in veterinary medicine for the treatment of gram-negative infections, although activity against some gram-positive organisms and Mycoplasma sp. has been demonstrated (Papich & Riviere, 2001; Hannan et al., 1997). These drugs act by preventing DNA supercoiling through the inhibition of bacterial DNA gyrase (topoisomerase II) and topoisomerase IV. The pharmacokinetics of several of the fluoroquinolones have previously been reported in the horse, including enrofloxacin (Giguère et al., 1996; Papich et al., 2002; Epstein et al., 2004; Kaartinen et al., 1997), ciprofloxacin (Dowling et al., 1995), marbofloxacin (Bousquet-Melou et al., 2002; Carretero et al., 2002; Peyrou et al., 2004), fleroxacin (Rebuelto et al., 2000), and one of the new 8-methoxy fluoroquinolones, moxifloxacin (Gardner et al., 2004). Side effects are rarely associated with these drugs in horses, with the exception of cartilage damage in foals (Bermingham et al., 1998; Vivrette et al., 2001), seizures following intravenous bolus of high doses, and diarrhea in adult horses following oral administration of moxifloxacin (Gardner et al., 2004). However, because there are no drugs of this class approved by FDA for horses, there are no target animal species safety studies available.

Orbifloxacin is a broad-spectrum fluoroquinolone antibiotic labeled for use in the United States for the treatment of skin, soft tissue and urinary tract infections in dogs, and skin and soft tissue infections in cats. One previous study has examined the pharmacokinetics and tissue distribution of orbifloxacin following oral administration in horses (Haines et al., 2001). However, that study did not include an IV dose, therefore there are no reports on intravenous orbifloxacin in horses, or a measure of the systemic
availability after an oral dose. We hypothesized that since this drug is both lipophilic and soluble, it has characteristics defined by the Biopharmaceutics Classification System (BCS) (Amidon et al., 1995; Martinez et al., 2002) as a Class I drug (highly soluble, highly permeable). A Class I drug theoretically may have advantages for oral absorption in horses because the high solubility makes the drug amenable to compounding in aqueous formulations and achieving good dissolution in the equine gastrointestinal tract, and the high permeability will favor gastrointestinal absorption, which has notoriously been a problem for oral antibiotics in horses.

The purpose of this study was to characterize the pharmacokinetics of orbifloxacin after intravenous and oral administration, and to determine the oral bioavailability of orbifloxacin in the horse. It was also our objective to measure protein binding and lipophilicity so that the effect of these indices on pharmacokinetics could be examined. In addition, the pharmacokinetic data was used to derive practical dosing regimens that might be examined for clinical use in horses.

Materials and Methods

Animals and Dosing:

Six mixed-breed male and female horses, aged 4-10 years, weighing approximately 450kg were used. All horses received both oral and intravenous orbifloxacin at a dose of 2.5 mg/kg in a crossover fashion. The oral formulation was administered as crushed tablets (68, 22.7 and 5.7 mg) for more accurate dosing. The crushed tablets were suspended in 500 mL of water and administered via nasogastric tube. The intravenous formulation was prepared by Schering-Plough Animal Health as a
5% injectable solution. The IV dose was administered via jugular catheter that was placed 2 hours before the start of the study. The injection was administered slowly over 3-5 minutes and the catheter was flushed with 10 mL of sterile saline solution after administration.

All the horses were kept in an enclosed horse barn in individual box stalls during the study. They were fed ad libitum mixed grass hay and water throughout the study, as well as 2.3 kg non-medicated sweet feed (Purina Sweetena® grain mix) per horse per day. However, approximately 12 hours prior to dosing, all food was removed from the stalls and they did not have access to feed until 4 hours after drug administration. The dosing studies were performed at the Schering-Plough Animal Health Corporation’s Elkhorn Research Center in Elkhorn, NE. There was a 14 day washout period between treatments. One horse had to be euthanized during the study due to reasons unrelated to drug administration, therefore there was only data from the oral dose study available for this animal. Horses were monitored for adverse drug reactions during the study.

Sample Collection:

Blood samples were collected via a jugular catheter 20 minutes prior to drug administration, 10, 15, 20, 30, 45, 60, 90 minutes, and 2, 3, 4, 6, 8, 12, 18, 24, 30 hours following administration of the intravenous drug. Samples were collected from a catheter in the jugular vein opposite the vein used for drug administration. After oral administration, samples were collected at 0, 15, 30, 60, 90 minutes, and 2, 3, 4, 6, 8, 12, 18, 24, 30 hours. The samples were collected into heparinized (green-top) tubes and were
centrifuged (2800 x g for 10 to 12 minutes) and the plasma was harvested and frozen until HPLC analysis.

Protein Binding Study:

Pooled plasma from six healthy horses was harvested for use in in vitro protein binding studies. Aliquots of equine plasma were fortified with orbifloxacin at concentrations of 0.3, 1, and 3.0 µg/ml. Three replicates of fortified samples per concentration were prepared for analysis and added to a microcentrifugation system (Centrifree® Micropartition System, Amicon, Beverly, MA). Samples were incubated in a water bath at 37°C for 30 minutes and then centrifuged at 1,000 x g for 20 minutes. Approximately 500 µL of protein-free ultrafiltrate was obtained in the reservoir of the system. The ultrafiltrate, as well as unprocessed plasma was analyzed for orbifloxacin. Plasma protein binding was determined according to the following formula:

% Protein Binding = \frac{(\text{total}) - (\text{unbound})}{(\text{total})} \times 100

Drug Lipophilicity:

The octanol:water partition coefficient, was measured by established methods (Purcell et al., 1973; Ashby et al., 1985; Asuquo & Piddock, 1993) and a procedure previously published from our laboratory (Bidgood & Papich, 2003). Dibasic sodium phosphate buffer (0.1M) was prepared and the pH was adjusted to 7.4 using 85% phosphoric acid. This aqueous phase was then fortified with orbifloxacin at 5, 2.5 and 1 µg/mL. An equal volume of octanol (l-octanol, Sigma Chemical, St. Louis, MO) was added to the fortified buffer in a screw-top tube. The tube was slowly agitated for 1 hour at room temperature to allow equilibrium of the drug within each phase of the tube and
then centrifuged for 10 minutes at 2,000 x g. The aqueous layer was analyzed for orbifloxacin concentrations before and after incubation and drug concentrations were determined from a calibration curve prepared in the phosphate buffer. The partition coefficient (PC) was determined by the formula below:

\[
PC = \frac{\text{[Buffer before incubation]} - \text{[Buffer after incubation]}}{\text{[Buffer after incubation]}}
\]

**HPLC Analysis:**

The HPLC apparatus consisted of a pump (Waters Model 600 Pump, Millipore Corp., Milford, MA), autosampler (Hewlett Packard Series 1050 Autosampler, Hewlett-Packard, Palo Alto, CA), variable wavelength ultraviolet detector (Hewlett Packard Series 1050 UV detector, Hewlett-Packard, Palo Alto, CA), and computer for data collection and analysis (Hewlett Packard HPLC2D ChemStation). The column was a Zorbax RX-C8, 4.6 mm x 15cm (MAC-MOD Analytical Inc., Chadds Ford, PA), with a Zorbax RX-C18, 4mm x 1.25cm guard column (MAC-MOD Analytical Inc., Chadds Ford, PA). All solvents and reagents were HPLC grade. The reference standard for orbifloxacin was supplied by Schering-Plough Corporation.

Orbifloxacin was eluted with a mobile phase consisting of 77% distilled water, 23% acetonitrile, and 0.02% trifluoroacetic acid (TFA) added as a pH modifier. The flow rate was 1.0 ml/min. Ultraviolet detection was performed at a wavelength of 279 nm. Retention time for orbifloxacin with these conditions was approximately 6 to 7 min. Stock solutions of 1 mg/ml orbifloxacin were prepared by dissolving a pure reference standard of orbifloxacin in 0.1% TFA. The stock solution was further diluted in a mixture of 0.1% TFA and methanol (85:15 v/v) to make concentrations ranging from 500
µg/ml to 5 µg/ml. These dilutions were then added to blank (unfortified) equine plasma to produce a calibration curve from 0.05 µg/ml to 5.0 µg/ml. An untreated plasma sample was used as a blank.

The samples from the study, as well as the calibration plasma samples were prepared by solid-phase extraction (Waters Oasis® HLB Extraction Cartridge, Waters Corporation, Milford, MA) using a vacuum manifold (Visiprep®, Supelco Bellefonte, PA). The cartridge was conditioned by first adding 1.0 mL of methanol, followed by 1.0 mL of distilled water. The plasma sample (500 µL) was aspirated through the extraction cartridge and the cartridge washed with 5% methanol in water (v/v). A clean glass tube was placed under the cartridge and the sample eluted with 1.0 mL methanol. This tube was evaporated under a stream of nitrogen (30 psi for 15 min at 40°C). The dry residue in the tube was reconstituted with 200 µL of a mixture of 0.1% TFA:methanol (85:15 v/v), vortexed, and transferred to an HPLC injection vial. The injection volume was 50 µl.

Calibration and quality assurance were performed according to guidelines described by Shah et al. (1992). Seven calibration samples and a blank (unfortified) sample were analyzed daily. In order for the calibration curve to be accepted, the curve had to be linear with a coefficient of determination ($r^2$) value of at least 0.99 and calibration standards within 15% of the true value. Blank samples from each animal were analyzed to ensure that there were no interfering chromatographic peaks at the retention time window for orbifloxacin. Precision and accuracy were determined by analyzing five replicates at a low, medium, and high concentration within the concentration range for the study. Precision was measured as the % deviation about the
mean and accuracy was determined as the % deviation from the true value. Limit of
detection (LOD) and limit of quantitation (LOQ) were determined by analyzing blank
samples. The LOD and LOQ were taken from the mean background noise plus 3 times
the standard deviation of the background noise for LOD and 10 times the standard
deviation of the background noise for the LOQ.

Pharmacokinetic analysis:

Pharmacokinetic analysis was performed using standard methods as described by
Gibaldi and Perrier (1982) using a specialized computer program, (WinNonlin, Pharsight
Corporation, Mountain View, CA). Plasma concentration-versus-time curves were first
plotted on a semilogarithmic graph to determine the appropriateness of initial models
selected. Plasma samples with concentrations below the LOQ were not included in the
analysis.

Plasma concentrations of orbifloxacin were analyzed using both
noncompartmental and compartmental analyses. For the IV administration, a 2-
compartment open model, with elimination from the central compartment described by
the following formula was used:

\[ C(t) = Ae^{-\alpha t} + Be^{-\beta t} \]

Where \( C(t) \) is the plasma concentration at time = \( t \), \( e \) is the base of the natural logarithm,
\( A \) is the intercept and \( \alpha \) is the rate constant for the distribution phase of the curve; \( B \) is the
intercept and \( \beta \) is the rate constant for the terminal portion of the curve. A weighting
factor of \( 1/y^2 \) was used, where \( y \) is the predicted plasma concentration.
For the oral administration data, a 1-compartment open model with first order input was used for 5 of the 6 horses. For the sixth horse, a two-compartment model with first-order input provided a better fit. Systemic bioavailability was calculated from the ratio of total AUC from the oral dose to the total AUC from the IV injection and expressed as %F (%F = [AUC_{po}/AUC_{iv}]*100). The maximum plasma concentration (C_{MAX}) and time of maximum plasma concentration (T_{MAX}) were reported as the observed values.

Noncompartmental analysis was used to determine model-independent parameters such as area under the plasma concentration-time curve (AUC) using the trapezoidal method, area under the first moment curve (AUMC), mean residence time (MRT), systemic clearance (Cl), and volume of distribution using the area method (VdAREA) for both the oral and IV data. For calculation of the total AUC, the terminal portion of each curve was estimated using the last time point and the terminal slope.

Optimum dosing regimens for intravenous orbifloxacin were calculated using the established pharmacodynamic parameters for fluoroquinolones, pharmacokinetic parameters calculated from this study, and the equation:

\[
\text{Dose} = \frac{\text{AUC}}{\text{Clearance}}
\]

Because there is no commercial IV formulation available, for this calculation we used the oral clearance (Cl/F) from our study. For the AUC, we used a value for AUC that was 100 x the MIC for gram-negative organisms, and 50x MIC (AUC/MIC ratio of 50) for gram-positive organisms. These values were based on pharmacokinetic-pharmacodynamic (PK-PD) parameters published (Craig, 1998; Wright 2000; Ambrose
& Grasela, 2000; Drusano et al., 1993). The MIC value used was taken from published studies on susceptibility of equine pathogens to orbifloxacin (Haines et al., 2001).

**Results**

The HPLC method was sensitive, specific and repeatable. There were no interfering chromatographic peaks from blank samples in the window for orbifloxacin. The average LOD and LOQ was determined to be 0.017 µg/ml and 0.034 µg/mL, respectively. The lowest calibration standard for which precision and accuracy were within 15% was 0.05 µg/ml. Intra-assay and inter-assay accuracy and precision were determined at concentrations of 0.05, 0.5 and 5 µg/mL. The mean intra- and inter-assay accuracy was within 3.81 ± 0.31% and 4.36 ± 4.62% of the true value, respectively. The mean intra- and inter-assay precision was within 4.81 ± 1.76% and 9.3 ± 3.79% of the mean, respectively.

The relevant noncompartmental pharmacokinetic parameters are summarized in Table 1. Tables 2 and 3 show the pharmacokinetic parameters derived from compartmental analysis of the oral and intravenous data, respectively. Mean plasma concentrations are shown in Figure 1. Oral orbifloxacin was well absorbed with an average systemic bioavailability of 68.35 ± 27.32% and a C_{MAX} of 1.25 ± 0.5 µg/ml. This was skewed by one horse that had a bioavailability of 28.83% and a C_{MAX} of 0.48 µg/ml. Bioavailability from only 5 out of the 6 horses could be assessed because one of the horses died prior to the intravenous dosing. The terminal half-life of orbifloxacin after intravenous and oral administration was 5.1 ± 2.1 hr and 3.4 ± 1.2 hr, respectively. There were no adverse effects from drug administration noted during this study.
Protein binding was performed with three replicates at each concentration. The average percent plasma protein binding of orbifloxacin in equine plasma at concentrations of 0.3, 1.0 and 3.0 µg/ml was 20.64 ± 3.69% (range 17-24%). Protein binding tended to be lower at higher concentrations. The average octanol:water partition coefficient at concentrations of 1.0, 2.5 and 5.0 µg/ml was 0.2 ± 0.11.

Discussion

The results of this study indicate that orbifloxacin is well absorbed following oral administration to horses and could offer a potentially useful treatment for infections caused by susceptible bacteria. Orbifloxacin has a high bioavailability (68 ± 27%) and rapid peak (TMAX of 1.2 hr). However, extent of oral absorption could only be measured in 5 out of 6 of the horses and one animal had a lower absorption that skewed the results. One other report of orbifloxacin pharmacokinetics in the horse has been previously published. In this study, a single oral dose of 7.5 mg/kg was administered. Systemic bioavailability was not calculated however, because an intravenous study was not performed in that study. Comparison of the AUC from that study with the AUC reported here (19.8 ± 1.7 vs. 6.16 ± 2.35, respectively) shows an approximate 3-fold increase when horses were administered 3 times the dose. This suggests linear first-order kinetics of orbifloxacin in the horse.

Oral bioavailability of other fluoroquinolones in the horse has varied depending on the drug administered. Oral absorption of ciprofloxacin is erratic and poor, with a calculated bioavailability of 6.8% (Dowling et al., 1995). Bioavailability of enrofloxacin tablets at 2.5 mg/kg and 5 mg/kg was 57% and 62.5% respectively (Giguère
et al., 1996) however, this value was probably overestimated because this study used a bioassay which has been shown to overestimate the AUC of enrofloxacin and ciprofloxacin by up to 71% following oral administration (Küng et al., 1993). Marbofloxacin bioavailability at the recommended dose of 2 mg/kg was comparable to that of enrofloxacin at 62% (Bousquet-Melou et al., 2002).

Orbifloxacin apparent volume of distribution was consistent with a drug that distributes out of the extracellular fluid. The mean $V_{\text{d,AREA}}$ was $2.35 \pm 0.55$ L/kg and volume of distribution at steady-state ($V_{\text{d,SS}}$) was $1.58 \pm 0.42$ L/kg. This is larger than the volume reported for orbifloxacin in dogs and cats (Schering-Plough Technical Monograph) but similar to other studies of enrofloxacin in horses (Papich et al., 2002; Kaartinen et al., 1997; Epstein et al., 2004).

In this study, plasma protein binding was 17 to 24%. As demonstrated for marbofloxacin and enrofloxacin in dogs (Bidgood & Papich, 2005) this low degree of protein binding will not inhibit drug distribution to the interstitial fluid – the site of action for most antibacterial drugs and interstitial fluid drug concentrations are expected to equal the plasma concentrations. Orbifloxacin has been shown to penetrate well into the synovial fluid, peritoneal fluid, and endometrial tissues of horses (Haines et al., 2001). Mean cerebrospinal fluid concentrations were approximately 25% of mean serum concentrations at 3 hours following administration of a single oral dose of 7.5 mg/kg to normal adult horses (Haines et al., 2001). Peak urine concentrations were more than 200 times the peak serum concentrations in that study (Haines et al., 2001).

Comparison of the terminal half-lives between intravenous and oral orbifloxacin in Figure 1 shows that oral administration did not affect the terminal half-life. As
observed from the graph, the intravenous and oral plasma concentration curves were practically parallel after absorption was complete. The half-life of elimination calculated in this study for horses was 5.08 ± 2.06 hr (harmonic mean 4.44 hr) following intravenous administration. This was in a similar range as for dogs and cats. The elimination half-life following oral administration reported here is considerably shorter than that reported in the one previous study of oral orbifloxacin pharmacokinetics in horses (3.42 ± 1.24 vs. 9.06 ± 1.33 hr, respectively) (Haines et al., 2001). This is most likely due to differences in the method of calculation used. When the mean serum values for orbifloxacin reported in the study by Haines et al (2001) are analyzed using the same methods as those here, the elimination half-life is 5.81 hr, which is closer to the oral elimination half-life and similar to that reported in this study for intravenous orbifloxacin. The study by Haines et al (2001) also used a bioassay for determination of serum orbifloxacin concentrations. There is no information available describing any active metabolites of orbifloxacin in the horse, however, they may have been detected by the bioassay and may have contributed to differences in results.

The octanol:water partition coefficient has been correlated to drug permeability, and hence, drug absorption across the intestinal mucosa. Orbifloxacin had a relatively low octanol:water partition coefficient, however this low value did not impair oral drug absorption in these horses. The oral absorption reported here was at least as high as, or higher than, other fluoroquinolones that have been studied in horses. The water solubility of this drug also is an advantage. Drug oral absorption is facilitated when it is able to dissolve in the GI fluids. Poor dissolution can result in poor systemic absorption. The good water solubility for orbifloxacin contributes to the favorable oral absorption and
will also make this drug amenable to compounding in various aqueous formulations. Our laboratory has shown that compounded formulations of orbifloxacin tablets are stable and potent for at least seven days, as long as the vehicle does not contain metal cations (e.g., Al\(^{+3}\), Fe\(^{+3}\), Ca\(^{+2}\)), which can chelate and prevent oral absorption (KuKanich & Papich, 2003). Orbifloxacin has criteria that may define it as a Class I drug according to the Biopharmaceutic Classification System (BCS). A Class I drug (highly soluble, highly permeable) has optimum characteristics to favor oral absorption, which was demonstrated in this study.

Pharmacokinetic-pharmacodynamic (PK-PD) indices have been used to predict optimum dosing strategies. For the fluoroquinolone antimicrobials, either the \(C_{\text{max}}:\text{MIC}\) ratio, or the \(AUC:\text{MIC}\) ratio have been used to predict antibacterial success. As reviewed by Hyatt et al (1995), Lode et al, (1998), and recently by Wright et al (2000), McKellar et al (2004), and Papich & Riviere (2001) investigators have shown that either index may predict clinical cure in studies of laboratory animals, and in a limited number of human clinical studies. There are no published studies involving horses that indicate which of these parameters may be the best predictor of clinical cure, or what the respective target ratios might be.

Most experts agree that a \(C_{\text{max}}:\text{MIC}\) of 8-10, or a \(AUC:\text{MIC}\) of greater than 100-125 have been associated with a cure. There is a generally-held assumption that, when the \(C_{\text{max}}:\text{MIC}\) ratio cannot be maximized, the \(AUC:\text{MIC}\) ratio may be a better index of therapeutic success (Ambrose & Grasela, 2000; Drusano et al 1993). Some authors cite a \(AUC:\text{MIC}\) ratio of >100 (Craig, 1998). As reviewed by Wright et al (2000), there is
evidence that for some clinical situations, including treatment of gram-positive infections, AUC:MIC ratios as low as 30-55 are adequate for a clinical cure.

Susceptible *E. coli, Klebsiella pneumoniae, Salmonella* sp., *Actinobacillus equuli* and some *Enterobacter* sp. from horses have MIC values ≤ 0.12 µg/mL (Haines *et al.*, 2001). The formula: Dose = AUC/Clearance can be used to calculate a dose from the desired AUC. Clearance used here is the oral clearance (CL/F) from our study which was 0.444 L/kg/hr (± .355). To achieve an AUC of 100x the MIC (12 hr*µg/mL) the dose required would be 5.3 mg/kg once daily orally (rounded off to 5 mg/kg). If the desired AUC/MIC ratio for gram-positive bacteria is 50, the dose of 5 mg/kg would be similar for gram positive bacteria with an MIC of 0.25 µg/mL, such as some strains of *Staphylococcus aureus* (Haines *et al.*, 2001). Although our study only examined the pharmacokinetics of 2.5 mg/kg, we believe that it is justified to predict higher doses from our study because the pharmacokinetics appear to be linear with higher doses, as we presented earlier. Interestingly, these doses are comparable to those recommended in the previous study of 5 mg/kg for bacteria with an MIC of 0.12 µg/mL cited from another study (Haines *et al.*, 2001).

In conclusion, this is the first study that compared oral and IV orbifloxacin in horses. We found that it is well-absorbed compared to other fluoroquinolones. Based on the principles of dosing for fluoroquinolone antimicrobials, we recommend an oral dose of 5 mg/kg once daily for additional studies for treating gram-negative bacteria with MIC values ≤ 0.12 µg/mL and gram-positive bacteria with MIC values ≤ 0.25 µg/mL. We also found that orbifloxacin has low protein binding, which will facilitate distribution to
interstitial fluids, and good solubility, which is an advantage for compounding in aqueous vehicles and achieving good oral absorption.
References


Table 7.1. Noncompartmental pharmacokinetic parameters for horses administered a single dose of intravenous or oral orbifloxacin (2.5 mg/kg).

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>IV (mean ± SD)</th>
<th>Oral (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMAX (hr)</td>
<td>---</td>
<td>1.21 ± 0.6</td>
</tr>
<tr>
<td>CMAX (µg/mL)</td>
<td>---</td>
<td>1.25 ± 0.5</td>
</tr>
<tr>
<td>VdMAX (L/kg)</td>
<td>2.35 ± 0.55</td>
<td>---</td>
</tr>
<tr>
<td>MRT (hr)</td>
<td>5.81 ± 2.11</td>
<td>5.2 ± 0.85</td>
</tr>
<tr>
<td>AUC₀−∞ (hr*µg/mL)</td>
<td>9.04 ± 0.9</td>
<td>6.16 ± 2.35</td>
</tr>
<tr>
<td>AUMC₀−∞ (hr<em>hr</em>µg/mL)</td>
<td>53.86 ± 25.82</td>
<td>32.48 ± 13.33</td>
</tr>
</tbody>
</table>

TMAX = time to maximum concentration; CMAX = maximum concentration; VdAREA = apparent volume of distribution (area method); MRT = mean residence time; AUC = area under the concentration-time curve; AUMC = area under the first moment-time curve
Table 7.2. Pharmacokinetic parameters of oral orbifloxacin (2.5 mg/kg) using a one-compartment model (n = 6).

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>(mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_0$ (hr$^{-1}$)</td>
<td>3.66 ± 4.59</td>
</tr>
<tr>
<td>$k_1$ (hr$^{-1}$)</td>
<td>0.22 ± 0.07</td>
</tr>
<tr>
<td>$k_0$ $t_{1/2}$ (hr)</td>
<td>0.41 ± 0.30</td>
</tr>
<tr>
<td>$k_1$ $t_{1/2}$ (hr)</td>
<td>3.42 ± 1.24</td>
</tr>
<tr>
<td>F (%)</td>
<td>68.35 ± 27.32</td>
</tr>
</tbody>
</table>

$k_0$ = first-order absorption rate constant; $k_1$ = first-order elimination rate constant; $k_0$$t_{1/2}$ = half-life of absorption; $k_1$$t_{1/2}$ = half-life of elimination; F = systemic availability
Table 7.3. Pharmacokinetic parameters of intravenous orbifloxacin (2.5 mg/kg) using a two-compartment model (n = 5).

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>IV (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µg/mL)</td>
<td>3.09 ± 1.21</td>
</tr>
<tr>
<td>α (hr⁻¹)</td>
<td>2.25 ± 1.87</td>
</tr>
<tr>
<td>B (µg/mL)</td>
<td>1.07 ± 0.61</td>
</tr>
<tr>
<td>β (hr⁻¹)</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>t₁/₂α (hr)</td>
<td>0.62 ± 0.57</td>
</tr>
<tr>
<td>t₁/₂β (hr)</td>
<td>5.08 ± 2.06</td>
</tr>
<tr>
<td>k₁₀ (hr⁻¹)</td>
<td>0.48 ± 0.19</td>
</tr>
<tr>
<td>k₁₂ (hr⁻¹)</td>
<td>1.17 ± 1.09</td>
</tr>
<tr>
<td>k₂₁ (hr⁻¹)</td>
<td>0.76 ± 0.68</td>
</tr>
<tr>
<td>Vdₚₛ (L/kg)</td>
<td>1.58 ± 0.42</td>
</tr>
<tr>
<td>Cl (L/kg/hr)</td>
<td>0.28 ± 0.03</td>
</tr>
</tbody>
</table>

A = coefficient of the distribution phase; α = distribution phase rate constant; B = coefficient of the elimination phase; β = elimination phase rate constant; t₁/₂α = half-life of distribution; t₁/₂β = half-life of elimination; k₁₀ , k₁₂, k₂₁ = microdistribution rate constants; Vdₚₛ = apparent volume of distribution at steady-state; Cl = systemic clearance
Figure 7.1. Plasma concentration versus time curves for oral (solid circle, n = 6) and intravenous (solid triangle, n = 5) orbifloxacin following a single dose of 2.5 mg/kg.
8. PHARMACOKINETICS OF VORICONAZOLE IN THE HORSE AFTER ORAL AND INTRAVENOUS ADMINISTRATION.

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Abstract

Objectives: The purpose of this study was to characterize the pharmacokinetics of voriconazole in the horse following oral and intravenous administration and to determine the in vitro physicochemical characteristics of the drug that may affect oral absorption and tissue distribution.

Animals: Six adult horses.

Procedure: Horses were given 1 mg/kg of IV or 4 mg/kg of oral voriconazole and plasma concentrations were measured using high pressure liquid chromatography (HPLC). The in vitro plasma protein binding and octanol:water partition coefficient were also measured.

Results: Voriconazole is well absorbed following oral administration in the horse with a systemic bioavailability of 91.63 ± 16.55%. The elimination half-life following a single oral dose was 13.11 ± 2.85 hr and the maximum plasma concentration was 2.43 ± 0.4 µg/mL. Plasma protein binding was 31.68% and the octanol:water partition coefficient was 64.69. No adverse reactions were noted during this study.

Conclusions and Clinical Relevance: Voriconazole has excellent oral absorption and a long half-life in horses. Based on the results of this study, we can conclude that voriconazole at a dose of 4 mg/kg PO q24h will attain plasma concentrations adequate for treatment of fungi with a MIC ≤ 1 µg/mL. Calculations based on the pharmacokinetic parameters of voriconazole indicate that a dose of 1.5-2 mg/kg orally for pathogens with an MIC ≤ 0.5 µg/mL should be used for further study. Chronic dosing studies and clinical trials are needed to determine the safety and efficacy of voriconazole in the horse.
Introduction

Fungal infections in horses are rare, however when they occur, they are often difficult to treat due to a lack of available medications, expense, and a long duration of treatment. Several antifungal agents have previously been studied in the horse, however poor absorption, cost or lack of appropriate spectrum of activity preclude their use in many instances. Ketoconazole is not absorbed orally unless administered in an acidic vehicle, such as hydrochloric acid. Itraconazole oral solution has good systemic bioavailability, however it requires a large volume of administration and the high cost of the drug is a deterrent for its use. Fluconazole is well absorbed following oral administration, and is available in generic formulations; however it is ineffective against filamentous fungi such as *Aspergillus* spp. and *Fusarium* spp. Amphotericin B requires an IV infusion for administration and would be impractical for use in horses.

Voriconazole is a second-generation triazole antifungal drug registered for use in humans for the treatment of invasive aspergillosis and serious fungal infections caused by *Scedosporium apiospermum* and *Fusarium* spp. Similar to the other currently availableazole and triazole antifungals, voriconazole inhibits the fungal cytochrome P450-dependent 14α-sterol demethylase which is essential for formation of ergosterol in the fungal cell wall. Voriconazole is similar in structure to fluconazole; however the substitution of a fluoropyrimidine ring for one of the triazole moieties and the additional of a methyl group to the propanol backbone increases the spectrum of activity and potency as well as the fungicidal activity against some species of molds, including *Aspergillus* spp. In human clinical trials, voriconazole has been shown to be superior to
amphotericin B for the treatment of invasive aspergillosis and it is safer than amphotericin B in patients with renal dysfunction. The pharmacokinetics of voriconazole in the horse have not been reported. The purpose of this study was to characterize the pharmacokinetics of voriconazole after oral and intravenous administration to adult horses. Additionally, we studied the physicochemical properties of the drug that influence oral absorption and tissue distribution, including lipophilicity, and plasma protein binding. The data obtained from this study was then used to determine a dosing regimen for oral voriconazole in the horse.

Materials and Methods

Animals:

Six healthy adult horses, 4 mares and 2 geldings, were used in this study. The weights of the horses ranged from 430-631 kg (mean 526 kg). Breeds used included 2 Arabians, 1 Thoroughbred, 1 Quarterhorse, 1 Standardbred, and 1 Dutch warmblood. All horses were considered to be healthy based on a complete blood count and physical exam prior to inclusion in the study. The horses were housed in individual box stalls 18 hours prior to drug administration and for the duration of sampling. They received their normal ration of grass hay and a 10% pelleted feed with the exception that food was withheld for 12 hours prior to and 4 hours after drug administration. All horses had access to fresh water at all times during the study. This study was approved by the North Carolina State University Institutional Animal Care and Use Committee (IACUC).
Drug Administration:

Horses were administered either intravenous or oral voriconazole in a randomized crossover design. There was a minimum 3 week washout period between drug administrations. For intravenous drug administration, a commercially available injectable formulation of voriconazole was used. The drug is packaged as 200 mg vials of lyophilized powder complexed with cyclodextrins to improve solubility. Each vial was reconstituted with 19 mL of sterile water for injection and then further diluted to a final volume of 250 mL in 0.9% sodium chloride. The final drug concentrations ranged from 1.72-2.52 mg/mL (mean 2.10 mg/mL) to comply with the manufacturer’s dosing recommendations of 0.5-5.0 mg/mL. The drug was then administered at a dose of 1 mg/kg via a 16 g, 3 ¼ inch jugular catheter as a slow intravenous bolus over 5-6 minutes.

For the oral drug study, voriconazole powder (99.4% pure) was obtained from the manufacturer. The powder was weighed out at a dose of 4 mg/kg for each horse, mixed with 60 mL of corn syrup and administered as a suspension via an oral dosing syringe.

Drug Stability Testing:

Prior to drug administration, the stability and potency of the voriconazole powder in the vehicle it was to be administered in was tested. To accomplish this, voriconazole powder was mixed with corn syrup into a suspension with a final concentration of 33 mg/mL. This concentration was based on a 4 mg/kg dose for a 500 kg horse given in a total volume of 60 mL. Once mixed with the vehicle, the drug was stored at 8°C and protected from light. For drug analysis, the original suspension was diluted 1:1000 with a mixture of 50% water and 50% acetonitrile to a final concentration of 33 µg/mL. The
voriconazole concentrations were determined immediately after mixing, and again at 24 and 48 hours after mixing, as described below.

Sample Collection:

Blood samples were collected via a 14 g, 5 ¼ inch jugular catheter. For the intravenous study, the samples were collected from the jugular opposite that which the drug was administered. For both the intravenous and oral studies, samples were collected prior to drug administration (time 0), and at 10, 20, 40 minutes and 1, 1.5, 2, 3, 4, 6, 8, 12, 24, 36, and 48 hours after drug administration. The blood was transferred to lithium heparin tubes, immediately centrifuged and the plasma was harvested. The plasma samples were frozen and stored at -70°C until analysis.

Protein Binding Study:

Plasma from 6 healthy untreated adult horses was harvested and pooled for use in plasma protein binding studies. Three replicates of plasma were fortified with voriconazole at concentrations of 2.5 and 1 µg/mL. The samples were prepared for analysis and added to an in vitro microcentrifugation system. Samples were incubated for 30 minutes in a water bath at 37°C and then centrifuged at 1,000 x g for 20 minutes. A protein-free ultrafiltrate was obtained in the reservoir of the system. This ultrafiltrate, as well as unprocessed plasma was then analyzed for voriconazole concentrations. Plasma protein binding was determined according to the following formula:

\[
\% \text{ Protein Binding} = \frac{(\text{total}) - (\text{unbound})}{(\text{total})} \times 100
\]

Drug Lipophilicity:

The octanol:water partition coefficient, was measured by established methods and a procedure previously published from our laboratory. The aqueous phase
consisted of a dibasic sodium phosphate buffer (0.1M) with the pH adjusted to 7.4 using 85% phosphoric acid. The buffer was then fortified with voriconazole at 5 and 2 µg/mL and added to an equal volume of octanol in a screw top tube. The tube was rotated for 1 hour at room temperature to allow equilibrium of the drug between the lipid and aqueous phases and then centrifuged for 10 minutes at 2,000 x g. The aqueous layer was analyzed for voriconazole both before and after incubation. Drug concentrations were determined from a calibration curve prepared in the phosphate buffer. The partition coefficient (PC) was determined by the formula below:

\[
PC = \frac{[\text{Buffer before incubation}] - [\text{Buffer after incubation}]}{[\text{Buffer after incubation}]}
\]

Drug Analysis:

Voriconazole concentrations from the oral and intravenous plasma samples, as well as for the plasma protein binding and lipophilicity studies, were determined by high pressure liquid chromatography (HPLC) with ultraviolet detection at 263 nm. All plasma samples were subjected to solid phase extraction prior to injection onto the HPLC system. Cyano-bonded cartridges were used for extraction. The cartridges were attached to a vacuum manifold and initially conditioned with 1 mL of methanol and 1 mL of water. The plasma sample (1 mL) was then extracted and the cartridge was washed with 1 mL of a 95:5 (v/v) mixture of water and methanol. The samples were then eluted into clean glass tubes using 1 mL of 100% methanol. The resulting eluate was evaporated at 40°C for 25 minutes under compressed air. The evaporated samples were reconstituted with 200 µL of mobile phase which consisted of 50:50 (v/v) water and acetonitrile with 0.02%
trifluoroacetic acid added to adjust the pH to 2.38. A C8 reverse-phase column\textsuperscript{6} was used for separation. The flow rate was 1.0 mL/min and the injection volume was 25 µL.

Calibration curves were made daily from pooled plasma of untreated horses. A blank sample was processed and analyzed at the beginning of each run to check for interfering peaks. Calibration curves were linear between the concentrations of 10 µg/mL and 0.025 µg/mL, with a coefficient of determination (r\textsuperscript{2}) of > 0.99 and all values within 15% of the expected range. The lower limit of quantification (LOQ) was set as the lowest concentration that was consistently linear based on regression analysis of the calibration curve. Under these conditions, this value was 0.025 µg/mL for voriconazole in plasma. At concentrations of 5.0, 1.0, and 0.5 µg/mL, the accuracy of the HPLC assay was within 2.71 ± 1.83% of the true value, and intra-assay precision was within 2.48 ± 1.54% of the mean. The average percent recovery of the drug from the plasma was 103.11 ± 3.77%.

Pharmacokinetic Analysis:

The plasma concentrations of voriconazole were analyzed by compartmental pharmacokinetic methods, using a specialized computer program\textsuperscript{9}. Our laboratory uses standard methods for analysis as described by Gibaldi & Perrier.\textsuperscript{12} For the IV administration, a 2-compartment open model, with elimination from the central compartment described by the following formula was used:

\[
C(t) = Ae^{-\alpha t} + Be^{-\beta t}
\]

Where \(C(t)\) is the plasma concentration at time = t, \(e\) is the base of the natural logarithm, \(A\) is the intercept and \(\alpha\) is the rate constant for the initial steep phase (distribution) of the
curve; B is the intercept and β is the rate constant for the terminal (elimination) portion of the curve. A weighting factor of $1/\hat{y}^2$ was used, where $\hat{y}$ is the predicted plasma concentration.

For the oral administration data, a 1-compartment open model with first order input described by the following equation was used:

$$C = \frac{k_{01}FD}{V(k_{01} - k_{10})} (e^{k_{10}t} - e^{k_{01}t})$$

Where t is time, $k_{01}$ is the oral absorption rate, $k_{10}$ is the elimination rate constant, F is the fraction of drug absorbed, D is the non-intravenous dose, and V is the apparent volume of distribution. This model assumes that there is first-order absorption and that $k_{01} > k_{10}$. A weighting factor of $1/\hat{y}$ was used to obtain the best fit. Systemic bioavailability was calculated from the ratio of total area under the plasma concentration versus time curve (AUC) from the oral dose to the total AUC from the IV injection and corrected for dose and half-life. This value was expressed as %F:

$$\%F = \frac{(AUC_{po})(\text{Dose}_{iv})(t_{1/2iv})}{(AUC_{iv})(\text{Dose}_{po})(t_{1/2po})} * 100$$

The half-life was incorporated into the equation because of differences observed between the plasma elimination half-lives following oral and intravenous administration. The maximum plasma concentration ($C_{\text{max}}$) and time of maximum plasma concentration ($T_{\text{max}}$) were reported as the observed values from the plasma concentration-versus-time curves. Linearity of the kinetics were determined based in the AUC:dose ratio for the intravenous and oral studies.
Results

Voriconazole was well tolerated in all the horses used in this study after oral and intravenous administration and no adverse effects were noted. Following intravenous administration, the clearance of voriconazole was low (1.89 ± 0.46 mL/kg/min) and the volume of distribution at steady state (Vdss) was high (1.35 ± 0.06 L/kg). The terminal half-life (t1/2) was 8.89 ± 2.31.

The drug was stable in the corn syrup mixture for a minimum of 48 hours when stored under refrigerated conditions. Potency was within 5% of the predicted values. There was minimal drug loss during oral administration to all 6 horses. Following oral administration, the systemic bioavailability was almost complete at 91.63 ± 16.55%. The maximum plasma concentration reached (Cmax) was 2.43 ± 0.4 at 2.92 ± 1.2 hr following administration. The t1/2 was 13.11 ± 2.85 hr. Other relevant pharmacokinetics parameters for intravenous and oral voriconazole are summarized in Tables 1 and 2, respectively. Mean ± SD plasma concentration-versus-time curves for intravenous and oral voriconazole are depicted in Figure 1.

The in vitro plasma protein binding of voriconazole in the horse was 31.68 ± 1.92%. The octanol:water partition coefficient of voriconazole, at a pH of 7.4, was high (64.69 ± 0.38). This is equivalent to a logarithm of the partition coefficient (logP) of 1.81.

Discussion

The results of this study indicate that voriconazole is well absorbed following a single oral dose of 4 mg/kg, reaching a Cmax of 2.43 ± 0.4 µg/mL at approximately 3
hours following administration. Oral absorption of many drugs has been correlated to
drug solubility and permeability. Those drugs that have high solubility and
permeability are more readily absorbed and often have a high oral bioavailability. These
principles can be applied to antifungal drug absorption in the horse. Both ketoconazole
and itraconazole are classified as practically insoluble, with a solubility of less than 0.01
mg/mL and this solubility is highly pH-dependent. Both of these drugs have poor oral
bioavailability in the horse, unless they are administered in highly acidic formulations
designed to increase solubility. Fluconazole and voriconazole, in contrast, are
classified as slightly soluble, with a solubility of 1-10 mg/mL, and the solubility is less
dependent on gastrointestinal pH. The improved solubility also makes these drugs
more amenable to compounding with aqueous vehicles. This was demonstrated for
voriconazole in this study in which the oral dosing formulation was shown to be stable
when stored, and did not diminish oral absorption. Both fluconazole and voriconazole
show systemic availability in horses of 90-100%. Drug permeability has been correlated
with the logP of the drug. Of the azole antifungal drugs, itraconazole, ketoconazole,
and voriconazole are considered highly permeable with a logP of 5.16, 3.78, and 1.81,
respectively. Therefore, both the high solubility and high permeability of voriconazole
are favorable physicochemical properties that support our observation of excellent
bioavailability in the horse.

The apparent volume of distribution seen here following administration of
intravenous voriconazole was high (Vd_{area} 1.29 \pm 0.08 L/kg). This may be related to the
low plasma protein binding measured in this study (31.68 \pm 1.92\%), since only free,
unbound drug is available to diffuse into the tissues and interstitial fluid. The protein
binding measured in the horse is lower than that reported for other species. Values ranged from 45% in the guinea pig to 67-78% in the mouse. Values were 51% and 58% in dogs and humans, respectively. Compared to the other triazole antifungals in the horse, the plasma protein binding of voriconazole is higher than fluconazole (12.3%, unpublished data), and significantly lower than itraconazole (>98%). Previously published data from our laboratory has shown that protein binding is the major determinant of drug distribution into the interstitial fluid. Therefore, protein binding of the magnitude described in this report should not impede penetration of voriconazole to the interstitial space – the site of drug action when treating a fungal infection.

The half-life of voriconazole reported here was long following both oral and intravenous administration (13.11 ± 2.85 hr and 8.89 ± 2.31 hr, respectively), which is convenient for a proposed once-daily dosing regimen. The differences in the half-lives between oral and IV administration may be caused by nonlinear pharmacokinetics with the higher oral dose, or by prolonged absorption due to stomach emptying and GI transit. We did not administer a range of doses for each route to examine whether or not saturable metabolism occurs in horses.

The half-life of voriconazole is longer in the horse than in other species studied. Since the volumes of distribution are similar across species (1.35 L/kg in the horse, 1.3 L/kg in the dog and 2.1 L/kg in the rat), this can be related to a lower systemic clearance in the horse (1.89 mL/min/kg) compared to the dog (24 mL/min/kg) and rat (30 mL/min/kg). Clearance of voriconazole in other species is mainly dependent on metabolism (roffey). The major metabolizing enzyme identified in humans is the cytochrome P450 (CYP) isoenzyme 2C19. Autoinduction, as well as inhibition, of
these enzymes following multiple doses has been reported and the effects appear to be dependent on the species studied.\textsuperscript{5,17} Future studies are necessary to determine the levels and activities of the metabolizing enzymes, as well as the effects of chronic dosing, in horses.

Dosing regimens for antibacterial drugs are often based on the pharmacokinetic/pharmacodynamic (PK/PD) markers predictive of clinical outcome, including the time above the minimum inhibitory concentration (T>MIC), the ratio of the AUC for 24 hours after dosing to the MIC (AUC\textsubscript{0-24}:MIC), or the C\textsubscript{max}:MIC ratio.\textsuperscript{18} Unfortunately, these parameters are not as well defined for antifungal drugs. A limited number of \textit{in vivo} and \textit{in vitro} studies have examined the PK/PD of voriconazole against different species of fungi. Voriconazole exhibits fungistatic activity against most \textit{Candida} isolates, with maximum activity attained at 3 times the MIC \textit{in vitro}.\textsuperscript{19,20} In an \textit{in vivo} neutropenic murine model of systemic candidiasis, the PK/PD parameter most closely associated with efficacy was an AUC\textsubscript{0-24}:MIC ratio of 20-25.\textsuperscript{15} This is similar to results obtained with other triazole antifungals, including fluconazole and the newer agents, posaconazole and ravuconazole,\textsuperscript{21,22,23} suggesting that PK/PD parameters are similar among the classes of antifungals. Reported MICs for \textit{Candida} spp are usually $\leq$ 0.39 $\mu$g/mL.\textsuperscript{316,24} In this study, the AUC\textsubscript{0-24}:MIC ratio for horses at a dose of 1 mg/kg intravenously and 4 mg/kg orally was 21 and 91, respectively. Either of these doses would therefore be expected to attain plasma concentrations consistent with an effective outcome in the treatment of systemic candidiasis.

In contrast to \textit{Candida} spp., voriconazole shows fungicidal activity against \textit{Aspergillus} isolates \textit{in vitro}.\textsuperscript{25} Maximum fungicidal activity was seen at 2-4 times the
MIC in \textit{in vitro} experiments.\textsuperscript{19} The reported MICs for \textit{Aspergillus} sp. are usually \(\leq 0.5\ \mu g/mL\). Therefore, using \(0.5\ \mu g/mL\) as the target MIC, and the average \(C_{\text{max}}\) calculated from the horses in this study (2.43 \(\mu g/mL\)), we achieved a \(C_{\text{max}}:\text{MIC}\) ratio of 4.86, which should be adequate for the treatment of aspergillosis at an oral dose of 4 mg/kg.

Furthermore, plasma concentrations remained above the target MIC for greater than 24 hours in all 6 horses. Alternatively, the AUC\(_{0-24}:\text{MIC}\) ratios for \textit{Aspergillus} spp. were approximately 16 and 71 for the intravenous and oral doses, respectively, which may be low for the IV dose, but higher than necessary for the oral dose.

The values calculated from our data for the oral dose are well above the AUC\(_{0-24}:\text{MIC}\) ratio cited earlier for treating candidiasis. Therefore, lower oral doses may be possible than the 4 mg/kg used in this study. To obtain an AUC\(_{0-24}:\text{MIC}\) ratio of 25, the equation \(D = \text{AUC}_{0-24} \times \text{Cl}\) can be used, where \(D\) is the dose, \(\text{AUC}_{0-24}\) is the desired AUC (12.5) and \(\text{Cl}\) is the clearance obtained from the IV data. Assuming a MIC of \(0.5\ \mu g/mL\) for the treatment of aspergillosis, the calculated dose would be 1.4 mg/kg intravenously once daily. By correcting for bioavailability, we can then recommend an oral dose of 1.5 mg/kg, once daily.

A similar approach can be used to estimate dosages of voriconazole for other conditions that may occur in horses. For example, \textit{Histoplasma}, \textit{Blastomyces}, and \textit{Coccidioides} spp.\textsuperscript{26} have MICs \(\leq 0.03\ \mu g/mL\), \textit{Cryptococcus neoformans}\textsuperscript{27,28,29} has a MIC \(< 1\ \mu g/mL\), and \textit{Fusarium} spp. have MICs in the range of 1-4 \(\mu g/mL\).\textsuperscript{24}

In conclusion, voriconazole has high systemic availability after oral administration in the horse. Because it also has low protein binding, high lipophilicity, and high antifungal activity, voriconazole may be a useful drug for the treatment of
fungal infections in the horse. Chronic dose studies are needed to determine the effects of multiple dosing on pharmacokinetics and safety. Based on the results of this study, an oral dose of 4 mg/kg once daily would be more than adequate for the treatment of fungi with an MIC ≤ 1 µg/mL in horses. For fungi with MICs ≤ 0.5 µg/mL we recommend a dose of 1-1.5 mg/kg intravenously or 1.5-2 mg/kg orally once daily for future studies.
Footnotes

a  Vfend® IV, package insert, Pfizer

b  Vfend® I.V., Pfizer Ltd, Sandwich, Kent, United Kingdom

c  Pfizer Ltd, Global Research and Development, Sandwich Laboratories, Sandwich, Kent, United Kingdom

d  Centrifree® Micropartition System, Amicon, Beverly, MA

e  l-octanol, Sigma Chemical, St. Louis, MO

f  Bond-Elut CN-E (1 mL), Varian Incorporated, Harbor City, CA

g  Zorbax RX-C8 4.6mm x 150mm reverse phase column, Agilent Technologies, Wilmington, DE

h  WinNonlin Professional Version 4.1, Pharsight Corporation, Cary, NC
References


Table 8.1. Two-compartmental pharmacokinetic parameters for voriconazole (1 mg/kg) given intravenously to 6 horses.

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>IV (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A (µg/mL)</td>
<td>0.62 ± 0.26</td>
</tr>
<tr>
<td>α (hr⁻¹)</td>
<td>3.73 ± 2.5</td>
</tr>
<tr>
<td>B (µg/mL)</td>
<td>0.71 ± 0.04</td>
</tr>
<tr>
<td>β (hr⁻¹)</td>
<td>0.08 ± 0.02</td>
</tr>
<tr>
<td>t₁/₂α (hr)</td>
<td>0.25 ± 0.12</td>
</tr>
<tr>
<td>t₁/₂β (hr)</td>
<td>8.89 ± 2.31</td>
</tr>
<tr>
<td>k₁₀ (hr⁻¹)</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td>k₁₂ (hr⁻¹)</td>
<td>1.55 ± 1.26</td>
</tr>
<tr>
<td>k₂₁ (hr⁻¹)</td>
<td>2.11 ± 1.41</td>
</tr>
<tr>
<td>Vd₁₂ (L/kg)</td>
<td>1.35 ± 0.06</td>
</tr>
<tr>
<td>Vd₁₂ seam (L/kg)</td>
<td>1.29 ± 0.08</td>
</tr>
<tr>
<td>Cl (L/kg/hr)</td>
<td>0.11 ± 0.03</td>
</tr>
<tr>
<td>AUC₁₀⁻∞ (hr*µg/mL)</td>
<td>9.23 ± 2.01</td>
</tr>
<tr>
<td>AUC₁₀⁻24 (hr*µg/mL)</td>
<td>8.17 ± 1.04</td>
</tr>
<tr>
<td>AUC:dose</td>
<td>9.23</td>
</tr>
<tr>
<td>AUMC(hr<em>hr</em>µg/mL)</td>
<td>120.89 ± 53.2</td>
</tr>
</tbody>
</table>

A = coefficient of the distribution phase; α = rate constant for the distribution phase; B = coefficient of the elimination phase; β = rate constant for the elimination phase; t₁/₂α = half-life of distribution; t₁/₂β = half-life of elimination; k₁₀, k₁₂, and k₂₁ are the microdistribution rate constants; Vd₁₂ = apparent volume of distribution at steady-state; Vd₁₂ seam = apparent volume of distribution; Cl = systemic clearance; AUC = area under the concentration-time curve; AUMC = area under the first moment-time curve.
Table 8.2. One-compartmental pharmacokinetic parameters for voriconazole (4 mg/kg) given via nasogastric tube to 6 horses.

<table>
<thead>
<tr>
<th>Pharmacokinetic Variable</th>
<th>(mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tmax (hr)</td>
<td>2.92 ± 1.2*</td>
</tr>
<tr>
<td>Cmax (µg/mL)</td>
<td>2.43 ± 0.4*</td>
</tr>
<tr>
<td>k₀₁ (hr⁻¹)</td>
<td>1.14 ± 0.41</td>
</tr>
<tr>
<td>k₁₀ (hr⁻¹)</td>
<td>0.06 ± 0.01</td>
</tr>
<tr>
<td>k₀₁ t₁/₂ (hr)</td>
<td>0.69 ± 0.28</td>
</tr>
<tr>
<td>k₁₀ t₁/₂ (hr)</td>
<td>13.11 ± 2.85</td>
</tr>
<tr>
<td>Cl/F (L/kg/hr)</td>
<td>0.09 ± 0.03</td>
</tr>
<tr>
<td>AUC₀-∞ (hr*µg/mL)</td>
<td>50.81 ± 16.07</td>
</tr>
<tr>
<td>AUC₀-24 (hr*µg/mL)</td>
<td>35.54 ± 7.39</td>
</tr>
<tr>
<td>AUC:dose</td>
<td>12.7</td>
</tr>
<tr>
<td>F (%)</td>
<td>91.63 ± 16.55</td>
</tr>
</tbody>
</table>

T<sub>max</sub> = time to maximum concentration; C<sub>max</sub> = maximum concentration; k₀₁ = first-order absorption rate constant; k₁₀ = first-order elimination rate constant; k₀₁ t₁/₂<sub>α</sub> = half-life of absorption; k₁₀ t₁/₂<sub>β</sub> = half-life of elimination; Cl/F = clearance corrected for bioavailability; AUC<sub>0-∞</sub> = area under the concentration-time curve extrapolated to infinity; F = systemic bioavailability.

* Derived from the plasma concentration curves
Figure 8.1. Plasma concentration versus time curves for oral (circle, 4 mg/kg) and intravenous (triangle, 1 mg/kg) voriconazole following a single dose in 6 horses.
9. MUCOSAL PERMEABILITY OF WATER-SOLUBLE DRUGS IN THE EQUINE JEJUNUM: A PRELIMINARY INVESTIGATION.

Jennifer L. Davis, Dianne Little, Mark G. Papich, and Anthony T. Blikslager

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Abstract

Ussing chambers have been used to study the mucosal permeability of drugs in humans, rats and other species. This data can then be used to develop in vitro/in vivo correlations (IVIVC) for drugs based on the Biopharmaceutics Classification System (BCS). Due to the poor oral bioavailability of many drugs in the horse, this method may be useful for screening drugs prior to development to determine if they warrant further study. Cephalexin (CPX), marbofloxacin (MAR), metronidazole (MTZ) and fluconazole (FCZ) were chosen for this study based on the wide range of physicochemical properties and bioavailability in the horse. Permeability was ranked as follows: MTZ>FCZ>MAR>CPX. This correlated with the bioavailability ($R^2 = 0.633447$), the LogP ($R^2 = 0.648517$), as well as the molecular weight ($R^2 = 0.851208$) of the drugs. Metronidazole induced a decrease in the tissue transepithelial resistance, suggestive of the possibility of tissue toxicity, which may have falsely increased its permeability. The low permeability of cephalexin across the tissue may indicate a lack of active transporters that are found in other species. From this study, we can conclude that the Ussing chamber is a viable method for determining mucosal permeability in the horse.

Keywords: Equine, antimicrobials, permeability, oral absorption, Ussing chamber
**Introduction**

Oral absorption of equine drugs has been unpredictable because the factors that contribute to oral absorption have not been well characterized. The limited information available indicates that oral absorption of many antimicrobials is lower for horses compared to other animals (Papich, 2001). This poor bioavailability can lead to subtherapeutic treatments and increased amount of drug present in the cecum and colon which may have a detrimental effect on the normal bacterial flora of the equine intestine, leading to a potentially fatal antibiotic-associated colitis.

Intestinal permeability and aqueous solubility are the two main factors affecting oral drug absorption. A biopharmaceutical classification system (BCS) has been developed for humans to classify drugs into one of four categories based on these properties (Amidon et al, 1995; Kasim et al., 2004). Drugs that are categorized as Class I (high solubility, high permeability) may be eligible for bioequivalence waivers for development of new drug formulations and generic products, which minimizes the expense as well as the number of animals used in premarket testing. To investigate the utility of a BCS for horses, in vitro-in vivo correlations (IVIVC) need to be developed in order to determine which in vitro drug properties can predict in vivo performance. These in vitro measurements can then be used to screen drugs prior to their administration to horses, which will decrease the time and expense devoted to studying drugs that ultimately have poor oral bioavailability. Numerous systems have been developed to determine the in vitro or in situ permeability of drugs in a variety of species. The most commonly used systems are cultured cells (Caco-2 immortal cell lines), in situ perfusion techniques, and Ussing chambers (Barthe et al,1999). However, no cell lines analogous
to human Caco-2 cells exist for horses, and the in situ techniques may be technically difficult to perform in horses.

The transport velocity, or effective permeability ($P_{\text{eff}}$), expressed as $\text{cm} \cdot \text{sec}^{-1}$, is one of the key variables that controls the overall absorption rate (Lennernäs, 1997). If $P_{\text{eff}}$ can be estimated for medications used in horses, it is possible to classify drugs according to the BCS and thereby predict extent of oral absorption, as demonstrated for drug absorption in people (Lennernäs, 1997). The purpose of this study was to investigate the use of the Ussing chamber as a method for determining the mucosal permeability of water-soluble drugs in the equine small intestine and to calculate the $P_{\text{eff}}$ for those drugs. These studies were conducted during steady-state conditions so that tissue binding did not influence $P_{\text{eff}}$. Fluconazole, metronidazole, marbofloxacin and cephalexin were chosen because they represent a broad range of physicochemical characteristics and their bioavailability has been previously reported in the horse. It was assumed that, because these drugs are administered to horses as immediate-release medications, drug absorption occurs in the proximal small intestine. Therefore, $P_{\text{eff}}$ measured in the jejunum would provide the best estimate of small intestinal permeability. The permeability was then correlated to the physicochemical properties of the drugs, including the bioavailability, lipophilicity and molecular weight.

**Materials and Methods**

**Drugs**

Fluconazole, metronidazole, marbofloxacin and cephalexin were used in this experiment because of their water solubility and wide range of physicochemical properties and
reliable data for bioavailability in the horse (Table 1). The oral bioavailability for each
drug was obtained from the literature (Davis et al, 2005; Bousquet-Melou et al, 2002;
Latimer et al, 2001; Steinman et al, 2000; Sweeney et al, 1986). There are two studies
that report the bioavailability of metronidazole, therefore these values were averaged for
statistical analysis. All drugs were dissolved in 10 mL of Equine Ringer’s solution at
equimolar concentrations (0.00816 M). Additional higher concentrations of
metronidazole (7.5 mg/mL, 0.04 M) and cephalexin (15 mg/mL, 0.04M) were prepared
for additional experiments to determine the effect of dose on toxicity or permeability.

Ussing Chamber Experiments

Tissue was harvested from the proximal jejunum of 3 adult horses within 20 minutes of
euthanasia. Tissue was also harvested from 3 additional horses to test the effect of
increased doses of cephalaxin and metronidazole. The horses used were research horses
from terminal studies unrelated to the gastrointestinal tract. The tissue was immediately
placed in equine Ringer’s solution specifically formulated to mimic the pH (7.4) and
electrolyte composition of equine plasma. The solution was oxygenated (95% O₂/5%
CO₂) while the mucosal tissue was dissected from the muscular and serosal layers and
while the tissues were in the chambers. The mucosa was then mounted in a 3.14 cm²
aperture Ussing chamber and allowed to equilibrate for 30 minutes (Argenzio et al.,
1993; Tomlinson & Blikslager, 2005). Short circuit current (Isc) and spontaneous
potential difference (PD) was measured every 15 minutes. These values were then used
to calculate transepithelial resistance (TER, Ω.cm²) as previously described (Tomlinson
& Blikslager, 2005). The drug solutions were added to the mucosal side of the Ussing
chamber following the 30 minute equilibration period (time 0). Samples were collected from the mucosal (donor) side of the chamber at 0 and 120 minutes to ensure that there was no drug loss in the solution. Samples were collected from the serosal (receiver) side of the chamber at 0, 30, 60, 90 and 120 minutes after the beginning of the experiment for determination of drug flux across the mucosal membrane. Sample volume was 200 µL.

Sample Analysis

All samples were analyzed by high pressure liquid chromatography (HPLC). HPLC methods were modified from previous reports from our laboratory for cephalexin (Davis et al., 2005), marbofloxacin (Bidgood & Papich, 2005), and fluconazole (Latimer et al., 2001). Metronidazole was separated on a reverse phase C18 column (Zorbax RX-C8 4.6mm x 150mm, Agilent Technologies, Wilmington, DE) using a mobile phase of 0.05M sodium acetate:ACN (82:18) with 4 mL tetrabutylammonium hydroxide added (pH adjusted to 5.1 using 85% phosphoric acid) at a flow rate of 1 mL/min. Ultraviolet detection was performed at 320 nm.

Serosal samples were diluted 1:100 in equine Ringer’s solution prior to HPLC injection. Concentrations of each drug were determined from a calibration curve in Equine Ringer’s solution made prior to each run. Calibration curves were considered linear over a range of 0.1-100 µg/mL for all drugs if the coefficient of determination (R²) was greater than 0.99 and the calculated values for each concentration were within 15% of the true value.
Lipophilicity

The octanol:water partition coefficient (PC) was determined as a measure of drug lipophilicity. Values for cephalexin (Davis et al., 2005) have already been reported from our laboratory. Values for marbofloxacin, metronidazole and fluconazole were obtained for this experiment under identical conditions to those previously published. All values were determined using an aqueous phase of 0.1M sodium diphosphate buffer at a pH of 7.4. Drug was dissolved in the aqueous buffer and then incubated with an equal volume of l-octanol (Sigma Chemical, St. Louis, MO) for 1 hour. Drug concentrations were determined in the buffer before and after incubation. The PC was then determined by the following equation:

\[
PC = \frac{(\text{Concentration pre-incubation}) - (\text{Concentration post-incubation})}{(\text{Concentration post-incubation})}
\]

Values are reported as the log of the PC. Each drug was assayed in triplicate at three different concentrations.

Permeability Calculations

The effective permeability (Peff) was calculated using the following equation:

\[
Peff = (dC/dt) \cdot \left[ \frac{1}{A \cdot C_0} \right]
\]

where \(dC/dt\) is the appearance rate on the serosal side of the membrane, calculated from the slope of the concentration versus time curve for the individual drug from 30-120 minutes; \(A\) is the surface area of the mucosal segment in the Ussing chamber, and \(C_0\) is the initial concentration of drug in the mucosal chamber. The initial sampling point was omitted from the calculation of the slope because steady-state conditions had not yet been attained.
Statistics

Statistical analyses were performed using specialized computer software (SigmaStat version 3.11, Systat Software, Inc, Richmond, CA). The TER values were analyzed using a two-way repeated measures ANOVA followed by post-hoc analysis for differences between treatments using a Holm-Sidak test. Linear regression analysis was performed using Fig.P version 2.98 (Fig.P Software Corporation, Durham, NC) to determine the correlation between the permeability and bioavailability, Log P, or molecular weight.

Results

For all reported samples, the drug concentration in the mucosal chamber was stable over time, indicating there was no degradation of the drug in the equine Ringer’s solution. Permeability across the jejunal mucosa was linear for all of the drugs studied (Figure 1). The calculated P_{eff} showed that permeability across the jejunal mucosa was highest for metronidazole, followed by fluconazole, marbofloxacin, and cephalexin (Figure 2).

Transepithelial resistance was calculated as a measure of mucosal integrity. Any chamber that had an abnormally low TER value (greater than 2 standard deviations from the mean of the remaining data) was excluded from the experiment. Similarly, any chamber that had abnormally high drug concentrations measured at time 0 was excluded from analysis. The mean TER measurements for each drug are depicted in Figure 3. The TER was lower for metronidazole at both doses for all time points after addition of the
drug. A significantly decreased TER was noted for the high dose metronidazole
treatment when compared to marbofloxacin and fluconazole (P < 0.05). A trend for
significance was seen between high dose metronidazole and cephalexin (P = 0.08). The
differences between the high dose metronidazole and low dose metronidazole were not
significant (P = 0.279). A significant effect of time was seen for the high and low doses
of metronidazole, beginning at 15 and 45 minutes, respectively.

The flux of drug across the equine jejunum was correlated to the bioavailability of
the drug ($R^2 = 0.633447$) as measured in in vivo experiments (Figure 4a). There was a
similar correlation of permeability with the Log P of the drug ($R^2 = 0.648517$) (Figure
4b). If metronidazole is excluded from the regression analysis because it exhibited
unusually high permeability of the mucosa, the correlation between $P_{eff}$ and
bioavailability and $P_{eff}$ and log P becomes much stronger with values of $R^2 = 0.934913$
and 0.958787, respectively. A strong correlation was also seen between the $P_{eff}$ and the
molecular weight of the drugs ($R^2 = 0.851208$) (Figure 4c).

The permeability of cephalexin across the jejunum was low, as would be expected
from its low bioavailability in the horse. This was a concentration-dependent effect
because we demonstrated that higher concentrations of cephalexin (0.04 µmol) added to
the mucosal chamber produced an increase in $P_{eff}$ ($3.68 \times 10^{-5}$ vs $1.95 \times 10^{-5}$).

**Discussion**

This study demonstrates that the Ussing chamber can be used to measure the transport
velocity (effective permeability, $P_{eff}$) of drugs across the mucosa of the equine jejunum.
As reviewed by Lennernäs (1997), the $P_{eff}$ can be used to predict human in vivo drug
permeability and it also appears that $P_{\text{eff}}$, measured with the technique described here, can be used to predict in vivo drug permeability for horses. This technique can be used to screen drugs that are intended for oral administration to horses. The Ussing chamber method is currently the only in vitro method described for horses to measure $P_{\text{eff}}$. There is no equivalent to the cultured cell lines (eg, Caco-2 cells) used to predict $P_{\text{eff}}$ for drugs in humans and permeability does not correlate well across species. In addition there have been questions about the ability of the Caco-2 intestinal cell line for predicting absorption in the small intestine since they were initially derived from colonic adenocarcinoma (Artursson, 1991).

In situ intestinal perfusion models are impractical for horses since they require specialized equipment and prolonged anesthesia. In situ techniques have been developed for rats and humans, and they have the advantages of an intact blood supply, innervation and drug clearance capacity (Stewart et al, 1995). Interspecies differences have been noted and may be attributable to differences in the Peff, diffusion coefficients, diffusion distances and partitioning into mucosal membranes (Lennernas, 1997).

We chose the Ussing chamber method because it has previously been validated in our laboratories to determine the effects of anti-inflammatory drugs on mucosal recovery following ischemia and reperfusion in the horse (Tomlinson & Blikslager, 2005; Campbell & Blikslager, 2000). This method has been shown to be effective for studying both transcellular and paracellular permeability of different compounds in the horse. Tissue is normally viable for up to 4 hours in the chambers and can therefore be used for sampling during steady-state conditions. Because it uses equine specific tissue, this method provides the most accurate model for drug permeability in this species. Since the
intestinal mucosa is intact and viable, the Ussing chamber permeability measurements account for effects carrier mediated transport mechanisms (Lennernas et al, 1997) as well as mucosal efflux pumps, such as p-glycoprotein (Gotoh et al, 2005), and metabolizing enzymes, such as cytochrome P450. It does not, however account for post-absorption metabolism, the effect of blood flow to the intestine or the effect of the mucus layer that covers the mucosa in vivo, all of which can only be adequately assessed using in vivo studies. Also, it is difficult to study poorly water-soluble drugs because they cannot be mixed in the aqueous bathing solution and organic solvents may injure the mucosal tissue.

When comparing the overall effective permeability for the 120 minute sampling period, the compounds were ranked as follows (from highest permeability to lowest): metronidazole > fluconazole > marbofloxacin > cephalexin. This relationship was well correlated with the bioavailability ($R^2 = 0.633447$). If metronidazole is not included in the linear regression analysis, this relationship becomes much stronger ($R^2 = 0.934913$) and the permeability rank is the same order for in vivo oral absorption in horses (Table 1).

We feel it is justified to omit metronidazole from the calculation because at these concentrations there is evidence that metronidazole causes tissue injury. The high permeability of metronidazole demonstrated in this experiment may be due to a direct cytotoxic effect on the mucosal epithelial cells that increases with increasing doses, as evidenced by the decreased TER measurements for those chambers. Nitroimidazoles are reported to cause a dose dependent cytotoxicity through reduction of the nitro group which generates free radical anion intermediates that possess toxic activity (Rauth, 1984).
Oxygen is protective against the nitroreduction reaction therefore this cytotoxicity is increased in the presence of hypoxia (Mohindra & Rauth, 1976). Although the mucosa is continuously oxygenated while mounted in the Ussing chambers, the significant effect of time on the decreasing TER of both the high and low dose metronidazole most likely indicates a hypoxia in the mucosal tissue in the chambers that was dependent on the cytotoxicity of this compound. The lack of a significant time effect for the other compounds studied tends to support the fact that significant hypoxia does not occur without the presence of a noxious stimuli.

Cephalexin is one of the few drugs that are classified as low permeability based on the Log P, but high permeability based on oral bioavailability in humans (Kasim et al, 2004). The justification for this is the presence of carrier mediated transport mechanisms in the human. Studies in Caco-2 cell lines that overexpress the proton-coupled oligopeptide transporter (PEPT1), as well as rat in situ intestinal perfusion studies, have shown a direct correlation between PEPT1 protein expression and the uptake and permeability of cephalexin (Chu et al, 2001). The $P_{eff}$ reported using the rat in situ intestinal perfusion model was $3.89 \pm 1.63 \times 10^{-5}$ cm/s, which is still much higher than the $P_{eff}$ reported here of $1.95 \times 10^{-5}$ cm/min. Increasing the dose 5-fold caused an increase in the $P_{eff}$. The increase in $P_{eff}$ was not proportional to the increase in concentration, however; suggesting that saturation of permeability can occur as known for this mode of transport (Paintaud et al, 1992). This low permeability of cephalexin in the horse compared to the rat and the human indicates that the PEPT1 transporter protein is minimally expressed or not present in the equine jejunum. This can also be used to explain the low bioavailability of cephalexin, as well as other β-lactam antibiotics in the
adult horse, including cefadroxil (Wilson et al, 1985), amoxicillin (Wilson et al, 1988) and ampicillin (Ensink et al, 1996), all of which have been demonstrated to have affinity for PEPT1 (Luckner & Brandsch, 2005).

Permeability across intestinal membranes has previously been correlated to the lipophilicity of the drug, as measured by Log P, Log D, or CLogP (Kasim et al, 2004; Winiwarter et al, 1998; Winiwarter et al, 2003). A similar trend was reported here \((R^2 = 0.648517)\). As with the bioavailability measurements, this relationship becomes much stronger when metronidazole is removed from the analysis \((R^2 = 0.958787)\). All of the Log P measurements reported here were performed in our laboratory at a pH of 7.4, the same pH that was used for the solutions placed in the chambers. This pH was used to approximate that of the intestinal fluid in the horse. Although there are no specific reports of jejunal fluid pH in the horse, it is assumed to be similar to that of the duodenum, which is between 6.0 and 7.5 (Merritt, 1999) and within the range for humans, between 5.3 and 8.1 (median 7.2) (Lindahl et al, 1997).

We do not believe that pH across this range would have affected permeability of fluconazole or metronidazole. Both are weak bases with a pKa of 1.76 and 2.6, respectively (Trissel, 2002) and therefore ionization would be pH insensitive across a range of 5-8. Since both of these drugs exist predominantly in an unionized form in this pH range, optimal membrane permeability would be achieved. In contrast, both marbofloxacin and cephalixin are zwitterionic compounds with both an acidic and a basic side group. Marbofloxacin has pKa’s of 5.8 and 8.2 (Bidgood & Papich, 2005) and an isoelectric point of 7.0. Fluoroquinolone antibiotics reach maximum lipophilicity at or near their isoelectric point, when the majority of drug is in the form of a zwitterion (Sun
et al, 2002; Sun et al, 2003). Since the pH of our drug solution was near the isoelectric point of marbofloxacin, we can assume that near maximal permeability was achieved. Cephalexin has reported pKa’s of 2.81 and 6.62, with an isoelectric point of 4.7 (Hatanaka et al, 1995). In contrast to marbofloxacin, cephalexin has its lowest lipophilicity at the isoelectric point (Hatanaki et al, 1995). At a pH of 7.4, cephalexin would mainly be in an anionic state, not in a zwitterionic state, and would therefore be expected to have high lipophilicity and permeability as well.

The strongest correlation found in this study was between $P_{\text{eff}}$ and molecular weight ($R^2 = 0.851208$), although this should be interpreted with caution due to the limited range of molecular weights used. This correlation is most likely related to the route of drug permeability across the membrane. There are two routes possible, the transcellular route and the paracellular route (Barthe et al 1999). Most drugs are transported across intestinal mucosa via passive transcellular transport (Winiwarter et al, 1998) and this is dependent on the lipophilicity of the compound. The paracellular pathway requires transport across the mucosa via the tight junctions between cells, and therefore it is size and charge dependent (Pade & Stavchansky, 1997). Paracellular transport is applicable primarily to small molecular weight (<200 daltons), hydrophilic drugs (Winiwarter et al, 1998). Of the drugs studied here, only metronidazole has a low enough molecular weight to be transported paracellularly, therefore its high permeability may be due to absorption via both pathways, in addition to its relatively high lipophilicity, high unionized fraction, and cytotoxicity.

In conclusion, our study demonstrates that the Ussing chamber can be used to measure $P_{\text{eff}}$ in equine small intestine. The $P_{\text{eff}}$ measured correlated with in vivo oral
absorption and the drug’s lipophilicity. It may be a valuable in vitro method for estimating absorption applying BCS criteria to drugs for horses, and to screen potential candidates for oral administration.
References


Figure 9.1. Concentration versus time curves for equimolar concentrations of each drug measured in the serosal compartment of the Ussing chamber. The coefficient of determination ($r^2$) for each drug was >0.99 between 30 and 120 minutes.
Figure 9.2. Calculated effective permeability ($P_{eff}$) across the equine jejunum for equimolar concentrations of each drug over a 120 minute flux period.
Table 9.1. Physicochemical properties of the drugs used in this experiment that may affect drug permeability.

<table>
<thead>
<tr>
<th>Drug</th>
<th>MW</th>
<th>LogP</th>
<th>pKa</th>
<th>Isoelectric point</th>
<th>Acid/Base</th>
<th>F</th>
<th>Peff (x 10^{-5})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cephalexin</td>
<td>365.14</td>
<td>-1.12</td>
<td>2.81 &amp; 6.62</td>
<td>4.7</td>
<td>Zwitterion</td>
<td>5%</td>
<td>1.95</td>
</tr>
<tr>
<td>Marbofloxacin</td>
<td>362.36</td>
<td>-0.76</td>
<td>5.8 &amp; 8.2</td>
<td>7.0</td>
<td>Zwitterion</td>
<td>62%</td>
<td>3.86</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>171.15</td>
<td>-0.02</td>
<td>2.6</td>
<td>---</td>
<td>Weak base</td>
<td>79.5%</td>
<td>9.14</td>
</tr>
<tr>
<td>Fluconazole</td>
<td>306.27</td>
<td>0.54</td>
<td>1.76</td>
<td>---</td>
<td>Weak base</td>
<td>101%</td>
<td>6.7</td>
</tr>
</tbody>
</table>

MW = molecular weight; Log P = the log of the octanol:water partition coefficient; F = bioavailability; P_{eff} = effective permeability in cm/s
Figure 9.3. Mean transepithelial resistance data for 180 minutes that the mucosa was mounted in the Ussing chamber.
Figure 9.4. Linear regression analysis of the in vitro $P_{\text{eff}}$ versus the in vivo bioavailability (a) Log P (b) and molecular weight (c) of cephalexin (CPX), marbofloxacin (MAR), metronidazole (MTZ), and fluconazole (FCZ).
Figure 9.4b.
Figure 9.4c.
10. CONCLUSIONS

There were two main goals of the research presented here. The first one was to determine which physicochemical characteristics of a drug could be used to predict intestinal permeability using an in vitro system, as well as oral absorption in vivo. The second goal was to determine which physicochemical characteristics of the drug could be used to predict the distribution of the drug into the tissues once it was absorbed. By achieving these goals, our studies successfully described, for the first time, the key variables that are important for oral administration and distribution of antimicrobials in the horse.

10.1 Oral Absorption

The goal of the Biopharmaceutical Classification System (BCS) for oral immediate-release dosage formulations in people is to identify the key variables controlling drug absorption, and thereby make it possible to classify drugs and simplify drug development, approval, registration, and availability (Amidon et al. 1995). For people, these key variables have now been identified through extensive in vitro and in vivo studies. Classification in accordance with BCS can be based on the variables of solubility and permeability. Techniques have been devised to measure each of these variables. We have now established, for the first time, through the work contained in this dissertation that these variables can be defined for horses. The research presented here supports the theory that oral drug absorption can be predicted based on the solubility and permeability characteristics of the drug. For the pharmacokinetic studies we performed, we were able to provisionally classify 5 different antimicrobial drugs into the four different BCS classes prior to drug administration. As predicted, those drugs with a Class
I designation (voriconazole and orbifloxacin) were well absorbed, whereas those with a Class III (cephalexin) or Class IV (doxycycline) designation were poorly absorbed. Additionally, the formulation of the product can greatly affect the oral absorption, mainly by altering the solubility of the drug. This is an important variable for highly permeable drugs that have low solubility (Class II). The representative example of this is itraconazole. Itraconazole is a highly lipophilic drug that is practically insoluble in water (Class II), and what solubility it does have is highly dependent on the pH of the fluid. By changing the drug formulation from a capsule to a solution, the BCS shifts from a Class II to a Class I. We were able to demonstrate that this increased oral drug absorption in the horse from 12% to 64%.

Drug permeability as measured by lipid solubility (partition coefficient, LogP), must be validated using tissues from the species of interest. To further support our findings, we measured the transport velocity (effective permeability – $P_{eff}$) of representative drugs in the Ussing chamber system. Other investigators have found good correlations between permeability ($P_{eff}$) and lipophilicity (LogP) of the drug. We found similar results here with more lipophilic drugs having a higher permeability. There was also a good correlation between permeability and molecular weight, although the range of molecular weights used in this study was very small. Additionally, bioavailability and permeability were well correlated, which further supports our theory that a BCS for horses would be a valid and useful tool for predicting drug absorption.

The Ussing chamber appears to be a valid method for studying the permeability of hydrophilic drugs in the horse. It can be used to study the direct effects of a drug on the
intestinal epithelium, as with metronidazole. Drug transporter properties can also be investigated, as was seen with cephalexin.

10.2 Tissue Distribution

The subcutaneous ultrafiltration probe proved to be a simple, minimally invasive method for collecting interstitial fluid in the horse. The interstitial fluid samples were easily analyzed by HPLC without extraction. This method may not be appropriate for all drugs however, since certain drugs may bind to the material of the probe and not be detectable in the ISF samples, as was the case with voriconazole. *In vitro* testing is therefore recommended prior to using these probes *in vivo*. Based on the results of our studies, we can conclude that the major determinant of drug distribution into the interstitial fluid is plasma protein binding (Figure 9.1).

The aqueous humor was chosen as a prototype for all anatomically protected sites in the body because it is easily accessible in the standing, sedated horse using appropriate nerve blocks, and there is an adequate volume of fluid available for testing. Aqueocentesis was successful in all but one attempt from these horses. All horses underwent multiple procedures, with no long term adverse effects noted in any of the animals. Two horses developed fibrin clots in the eyes following aqueocentesis which resolved without treatment, and two horses developed corneal ulcers from self-induced trauma to the eye, which resolved with medical treatment. Aqueous humor is easily analyzed by HPLC without extraction. Because other sites with limited penetration (eg, CSF, prostate) have similar anatomic or functional barriers, we believe that this technique
can be used to make predictions of drug penetration into other privileged sites in the horse.

The results of the data presented here, combined with data extracted from the literature, suggest that the major determinant of drug distribution into the aqueous humor of the horse is lipophilicity (Figure 9.2), with some exceptions. The drug with the highest lipophilicity studied (itraconazole) did not reach detectable levels in the aqueous humor after oral administration. The most likely explanation for this is that drug with extremely high lipid solubility or affinity for tissue proteins may trap the drug in the ocular tissues, preventing diffusion in an aqueous media such as the aqueous humor. Also, since itraconazole is so highly protein bound in the plasma, this would limit the amount of free drug available for diffusion. In conclusion, drug distribution into the interstitial fluid is primarily governed by the extent of plasma protein binding. Drug distribution into the aqueous humor, and other sites with specific anatomic barriers, is influenced most strongly by the lipophilicity of the drug. These findings are summarized in Figure 9.3.
Figure 10.1 Linear regression analysis of the relationship between plasma protein binding and the ratio of the $\frac{AUC_{\text{isf}}}{AUC_{\text{plasma}}}$ of three drugs in the horse.
Figure 10.2  Linear regression analysis of the relationship between lipophilicity (Log P) and aqueous humor drug concentration (expressed as a percent of the plasma concentration) in the horse.
Figure 10.3 Schematic flow of medication from absorption across mucosa, across capillary membranes, via fenestrated pores or across physiologic barriers, to the site of action of antimicrobial drugs.
Figure 10.4 Factors affecting oral absorption of solid dose forms.
11. FUTURE DIRECTIONS

The work presented here establishes a foundation for future work to identify factors that can predict drug absorption and distribution in the horse. Future work should expand on the studies conducted for this dissertation to include more drugs and drug classes to develop *in vitro-in vivo* correlations (IVIVC). This will allow classification of drugs for horses based on the principles of the BCS. We can also make better therapeutic decisions regarding treatment of infections based on the characterisitics of the drug and the site of the infection.

11.1 Oral Absorption

Future work regarding drug solubility classifications should center on developing an equation for the dose number (\(D_0\)) in horses, based on a modification of the one used in humans. This may require standardization of the volume used to medicate horses. Because most horses are administered medications designed for human or small animal therapy, this requires administration of multiple tablets, making the maximum dose strength of an individual tablet unimportant for this species. Additionally, the majority of pharmacokinetic studies are performed using intragastric administration of the drug with 1-2 liters of water, which is much higher than the 250 mL used in human medicine. Conversely, in clinical practice, the drugs are often administered in a 60 cc syringe. We may realize that 60 mL is inadequate for dissolution of some medications, which compromises oral absorption. Once these factors have been accounted for, the classification of high versus low solubility will need to be redefined specifically for the horse.
Permeability studies using the Ussing chamber technique was limited for this dissertation because of availability of tissues and oral absorption data. Additional drugs should be studied. At this time, we have only examined hydrophilic drugs in the Ussing chamber. This is due to the difficulty of getting hydrophobic drugs into solution for addition to the chambers without using solvents that can damage the intestinal mucosa, reduce tissue transepithelial resistance and thereby falsely increase the permeability measurement. Other investigators have used low concentrations of solvents such as DMSO ($\leq 1\%$), absorption enhancers such as cyclodextrins, and fasted state simulated intestinal fluids (Ingels & Augustijns, 2003; Sharma et al, 2005; Ingels et al, 2004). Other authors suggest that permeability will be similar regardless of whether or not the drug is in solution or suspension (Gotoh et al, 2005). These approaches should be examined for equine intestine to develop methods to study a wider range of drugs in the horse.

Other factors such as pH of the bathing solution and effects on drug permeability in the Ussing chamber model also require additional investigation. Our preliminary study used only one pH (7.4), which is at the high end of the pH range reported for gastrointestinal fluids in the horse (Merritt, 1999). By changing the pH of the solution, we may be changing the ratio of ionized to unionized drug, which may increase or decrease the permeability, depending on the specific pKa and acid/base characteristics of that drug.

Other regions of the gastrointestinal tract should also be studied. Drug permeability has been shown to differ based on the region of the intestine studied in other species (Jezyk et al, 1992). Our preliminary investigation looked at duodenal versus
jejunal permeability and found differences between those 2 segments of the intestine. Other segments, including the ileum and small colon should be investigated, as well as the differences in drug permeability between the proximal, middle and distal jejunal segments.

Although a good correlation between lipophilicity and permeability was found in our study, other physicochemical characteristics of the drugs should be examined. These would include the number of hydrogen bond donors, polar surface area, and the octanol/water distribution coefficient at pH 5.5 and 6.5 (Log D$_{5.5}$ or Log D$_{6.5}$), all of which have been shown to correlate well with permeability and absorption data in humans (Winiwarter et al, 1998). Once the optimum correlative parameter has been determined in the horse, a large database can be created that combines the solubility and permeability data into a BCS for horses.

Finally, this work only focused on the factors of the drug that affect oral absorption, without investigating the factors of the individual horse on oral absorption. The presence or absence of specific drug transporters in the equine intestine, such as the oligopeptide transporters for beta-lactam absorption, and the relative expression of metabolizing enzymes and drug efflux pumps, should be determined. The effects of feeding, increased gastrointestinal transit time (in cases of colitis), decreased gastrointestinal transit time or blood flow (in post-operative colic), influence of infection, and the drug-drug interactions between orally administered drugs are all areas that require further investigation in the horse.
11.2 Tissue Distribution

The future goals for studying tissue distribution in the horse include increasing the number and classes of drugs studied as well as investigations of other tissues. The interstitial probes may be useful for collecting other samples, including pleural, peritoneal, and synovial fluid. This would allow us to gain valuable information on drug distribution and elimination from these sites, without the need for multiple needle aspirates. This may lead to better drug selection for infections in those areas. The effect of infection or inflammation on drug distribution into these sites also should be characterized. Tissue cage studies in a variety of animals have shown that drug concentrations are higher in exudate samples, presumably due to increased blood flow to the area, increased ‘leakiness’ from the vasculature and increased protein content of the exudate. Tissue cage studies have measured total drug in the samples without separating the unbound/active fraction, although some of these studies (Clarke et al, 1996) used a microbiological assay as well as HPLC. The microbiological assay presumably would test for (active) unbound drug. Therefore, even though total drug concentration might be higher, the activity of the drug at the site of infection might still be governed by the protein binding properties.

Further work regarding ocular drug concentrations in the horse should also focus on increasing the number and classes of drugs studied, as well as the effects of infection/inflammation. Since drugs often distribute differently into the anterior and posterior chambers, the vitreous concentrations of drugs needs to be examined as well. Our studies collected only one sample from each horse at one timepoint. While this gives us an insight into the ability of a drug to penetrate the eye, it does not allow us to
determine the rate or extent of distribution of a drug into the eye or the rate of drug elimination from the eye. In order to study this, specialized microdialysis probes have been used by some researchers, which allow for multiple samples to be taken from both the anterior and posterior chambers so that a concentration versus time profile for a drug can be created. These probes have been successfully used in rabbits (Macha & Mitra, 2001), and the feasibility of using them in horses should be examined. Other researchers have developed a mathematically based models for determining the pharmacokinetics of ocular drug delivery (Tojo, 2004). These models can be adapted for the horse based on differences in the size and shape of the anterior chamber and then be used as a predictive model for drug distribution and elimination in the eye. The correlation between CSF drug levels and ocular drug levels should also be examined to validate the assumption that aqueous humor drug concentrations can be used as a surrogate marker for drug penetration into other sites such as the CNS.
11.3 References


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