

ABSTRACT

BIDGOOD, TARA LYNN. Factors that influence the distribution of antimicrobials into the interstitial fluid as determined by *in vivo* ultrafiltration and pharmacokinetic modeling in dogs. (Under the direction of Mark G. Papich.)

Successful treatment of bacterial infections requires achieving antimicrobial concentrations above the minimal inhibitory concentration (MIC) at the site of infection for a sufficient duration of time. Knowledge of pharmacokinetic parameters and the extent of protein binding of antimicrobials are important for designing appropriate dosages. Because only the protein-unbound fraction is microbiologically active, knowledge of the effect of protein binding on drug diffusion into tissues is important for evaluating MIC results in target species. Traditional antimicrobial pharmacokinetic studies have focused on determining plasma concentrations and plasma pharmacokinetic parameters of total (protein bound and unbound) drug concentrations. Since the majority of bacterial infections are in the interstitial fluid (ISF), determining unbound drug ISF concentrations is more relevant for predicting therapeutic efficacy.

We conducted three studies to test the hypothesis that the distribution of antimicrobials into the ISF can be accurately predicted from plasma concentrations by determining the extent of plasma protein binding. We compared the distribution of antimicrobials with a range of pharmacokinetic parameters and physicochemical properties that may affect drug distribution, such as protein binding, lipophilicity, systemic clearance, and volume of distribution. We also evaluated an *in vivo* ultrafiltration device as an alternative method to tissue cages and tissue biopsies for ISF collection.

The first study evaluated the plasma and ISF concentrations of meropenem after intravenous (IV) and subcutaneous (SC) administration in dogs. After *in vivo* ultrafiltration probes were placed SC, ISF and plasma samples were collected simultaneously. The results showed that ISF and plasma (total and unbound) concentrations were similar because meropenem had low protein binding. Drug concentrations at the infection site (i.e., ISF) could be reliably predicted from plasma concentrations.

The second study compared plasma (total and unbound) concentrations with ISF concentrations for meropenem and doxycycline during a constant rate infusion (CRI) in dogs. Administering a CRI enabled a comparison of plasma and ISF concentrations at steady state without the influence of drug elimination. *In vitro* protein binding and lipophilicity was higher for doxycycline compared to meropenem. At steady state there was a significant difference between total plasma and ISF doxycycline concentrations but when protein binding was taken into account, the unbound plasma and ISF concentrations were similar. At steady state there was little difference between meropenem plasma (total and unbound) and ISF concentrations. This study illustrated the strong influence of high plasma protein binding on tissue distribution of unbound drug for a drug such as doxycycline.

The third study evaluated the plasma and ISF concentrations of enrofloxacin, its metabolite ciprofloxacin, and marbofloxacin after a CRI and oral administration in dogs. High volume of distribution and lipophilicity has been two characteristics reported as important for distribution to tissues. However, despite a higher volume of distribution and greater lipophilicity, this did not result in increased penetration into the ISF for enrofloxacin compared to marbofloxacin. During steady state there was no statistical difference between unbound plasma and ISF concentrations.

The results from the three studies indicated protein binding was the primary determinant of drug distribution into the ISF from plasma. It is the unbound fraction that is microbiologically active and since most bacterial infections are confined to the extracellular space, therapeutic efficacy would be better achieved by designing dosing regimens based on pharmacokinetics of the unbound concentrations in the plasma.

The *in vivo* ultrafiltration device was shown in our studies to be a reliable, minimally invasive, and humane technique for ISF collection in the dog.

**FACTORS THAT INFLUENCE THE DISTRIBUTION OF ANTIMICROBIALS
INTO THE INTERSTITIAL FLUID AS DETERMINED BY *IN VIVO*
ULTRAFILTRATION AND PHARMACOKINETIC MODELING IN DOGS**

by

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A dissertation submitted to the Graduate Faculty of
North Carolina State University
In partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Department of Molecular Biomedical Sciences

Raleigh

2004

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DEDICATION

I am dedicating this thesis to my parents, Marlene and Bryant Bidgood, who have provided me with unconditional love, support, and encouragement throughout this challenge.

BIOGRAPHY

Tara Bidgood was born October 1971 in Edmonton, Alberta, Canada. She completed four years of undergraduate studies at the University of Alberta. She graduated from the Western College of Veterinary Medicine with a Doctor of Veterinary Medicine degree in 1997. After graduation, she worked for three years as a small animal private practitioner in Edmonton. In August of 2000, she began a graduate and residency program in pharmacology at the North Carolina State University-College of Veterinary Medicine. In 2003 she became a Diplomat from the American College of Veterinary Clinical Pharmacology (ACVCP).

ACKNOWLEDGEMENTS

I would like to thank my committee members, fellow graduate students, and technicians who helped me complete this thesis. First, I would like to acknowledge my advisor and mentor Dr. Mark Papich. Dr. Papich has had a large influence on my growth and development as a researcher and clinician. I hold in high regard his attention to detail, wide scope of knowledge, and leadership skills. I would like to thank him for believing in my research abilities and supporting me through the difficult times. It was a pleasure and honor to be under his supervision.

In addition I would like express gratitude to Dr. Jim Riviere, Dr. Ron Baynes, and Dr. Lizette Hardie for serving on my committee. Dr. Riviere has provided valuable insight and direction to this project. I would like to thank him for his contagious passion for science, for his inspirational words of encouragement, and for teaching me to always strive for higher standards. I will carry his words and wisdom with me as I go forward in my professional and personal life. I would like to thank Dr. Baynes for always having an open door and making time for constructive discussions. His insight and willingness to help were always very comforting to me. I would also like to thank Dr. Hardie for her active participation and always stressing the clinical relevance of the research ideas.

I have a tremendous amount of respect and admiration for these researchers and I hope to continue friendship and research collaborations with them in the future.

Completion of this project could not have been possible without the assistance of Butch Kukanich, Delta Plummer, Jen Davis, and Debbie Gaffney. This amazing group of people not only helped me with my research project but also provided a supportive working environment enriched with humor.

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LIST OF SYMBOLS

Abbreviations	Units	Definition
C_{p_0}	$\mu\text{g/mL}$	Concentration at time zero
C_{p_T}	$\mu\text{g/mL}$	Concentration at time T
C_{ss}	$\mu\text{g/mL}$	Concentration at steady state
$C_{p_{\min}}$	$\mu\text{g/mL}$	Minimum concentration
$C_{p_{\max}}$	$\mu\text{g/mL}$	Peak concentration
α	/h	Rate constant associated with distribution phase
$T_{1/2\alpha}$	h	Distribution half-life
β	/h	Rate constant associated with the elimination phase
$T_{1/2\beta}$	h	Elimination half-life
A	$\mu\text{g/mL}$	Intercept for distribution phase
B	$\mu\text{g/mL}$	Intercept for elimination phase
AUC	$\text{h} \cdot \mu\text{g/mL}$	Area under the curve
AUC_{niv}	$\text{h} \cdot \mu\text{g/mL}$	Area under the curve after administration by a route other than IV
AUC_{par}	$\text{h} \cdot \mu\text{g/mL}$	Partial area under the curve calculated during steady state
AUMC	$\text{h}^2 \cdot \mu\text{g/mL}$	Area under the moment curve
Cl	mL/min/kg	Total body clearance
V_{ss}	L/kg	Apparent volume of distribution at steady state
$V_{d_{\text{area}}}$	L/kg	Apparent volume of distribution of the area during the elimination phase
V_c		Apparent volume of distribution of the central compartment
K	/h	Fractional rate constant (one compartment model)
K_{01}	/h	Absorption rate
K_{10}	/h	Microconstant, elimination rate from central compartment 1
$K_{01} T_{1/2}$	h	Half-life of the absorption phase
K_{12}	/h	Microconstant, rate of movement from compartment 1 to compartment 2
K_{21}	/h	Microconstant, rate of movement from compartment 2 to compartment 1
λ_z	/h	First order rate constant of the terminal portion of the curve
$T_{1/2\lambda_z}$	h	Half-life of the terminal portion of the curve
MRT	h	Mean residence time
$T_{\max}$	h	Time to reach the peak concentration
Ro	mg/kg/h	Drug infusion rate
F	%	Fraction of drug absorbed ($\times 100$)
MIC	$\mu\text{g/mL}$	Minimum inhibitory concentration
D	mg/kg	Dose
D_L	mg/kg	Loading dose
D_m	mg/kg	Maintenance dose

LIST OF FORMULAS

One compartment Model

$$C_{p_t} = C_{p_o} e^{-K_{el} t}$$

$$T_{1/2} = \frac{0.693}{K_{el}}$$

$$K_{el} = \frac{0.693}{T_{1/2}}$$

$$\text{Total body } T_{1/2} = \frac{0.693 * V_d}{Cl}$$

$$V_d = \frac{\text{Dose}}{C_{p_o}}$$

$$Cl = V_d * K_{el}$$

$$Cl = \frac{dx/dt}{C_p} = \frac{\text{Dose}}{AUC}$$

$$AUC = \frac{C_{p_o}}{K_{el}} = \frac{\text{Dose}}{Cl_B}$$

$$R_o = C_{ss} * Cl$$

$$Cl = \frac{R_o}{C_{ss}}$$

$$V_d = \frac{R_o}{C_{ss} * K_{el}}$$

Two Compartment Model

$$C_{p_t} = A e^{-\alpha t} + B e^{-\beta t}$$

$$T_{1/2\beta} = \frac{0.693}{\beta}$$

$$T_{1/2\alpha} = \frac{0.693}{\alpha}$$

$$T_{1/2} = \frac{0.693 * V_d_{ss}}{Cl}$$

$$V_d_{ss} = V_c + \frac{(K_{12} + K_{21})}{K_{21}}$$

$$V_d_{area} = \frac{\text{Dose}}{AUC * \beta} = \frac{K_{10} * V_c}{\beta}$$

$$Cl = K_{10} * V_c = \beta * V_d_{area} = \frac{\text{Dose}}{AUC}$$

$$AUC = A/\alpha + B/\beta$$

$$K_{10} = \frac{\alpha * \beta}{K_{21}}$$

$$K_{21} = \frac{A\alpha + B\beta}{A + B}$$

$$K_{12} = \alpha + \beta - K_{21} - K_{10}$$

Non Compartment Model

$$MRT = \frac{AUMC}{AUC}$$

$$Cl = \frac{Dose}{AUC}$$

$$T_{1/2\lambda_z} = \frac{0.693}{\lambda_z}$$

$$Vd_{ss} = Cl * MRT$$

$$T_{1/2\lambda_z} = \frac{0.693 * Vd_{ss}}{Cl}$$

$$Vd_{ss} = \frac{Dose_{iv} * AUMC}{AUC^2}$$

$$F = \frac{AUC_{niv} * D_{iv}}{AUC_{iv} * D_{niv}}$$

$$Vd_{area} = \frac{Dose}{\lambda_z * AUC}$$

1. INTRODUCTION

Most bacterial infections are extravascular, therefore it is important to design dosage regimens that achieve antimicrobial concentrations above the minimal inhibitory concentration (MIC) at the infection site (i.e. the interstitial fluid (ISF)). Three studies were performed to investigate the influence physicochemical properties, specifically protein binding and lipophilicity, and pharmacokinetic parameters have on the distribution of antimicrobials to the infection site. In this manner, accounting for those factors that are important for drug distribution, plasma concentrations can be used to predict ISF concentrations, interpret MIC values, and design dosage regimens.

Most antimicrobial drugs do not distribute to all tissues in the body equally. In addition, drug distribution within tissues is usually not homogeneous. In chapter 2 of this thesis we identify the different fluid compartments of the body, their characteristics and composition, and how molecules move to and from the compartments.

The current methods used in pharmacokinetic studies to determine antimicrobial concentrations at the infection site are described in chapter 3 for the purpose of providing a review of their applications and limitations. The studies in this thesis were also performed to evaluate the use of *in vivo* ultrafiltration as an alternative to tissue biopsies and tissue fluid cages used by researchers, pharmaceutical companies, and government agencies to investigate antimicrobial distribution to tissues. The antimicrobials, meropenem, doxycycline, marbofloxacin and enrofloxacin were chosen to compare their concentrations in the plasma and ISF because they differ in physicochemical properties and pharmacokinetic parameters. Each of the drugs investigated in the three studies are described in detail in chapter 3.

2. PHYSIOLOGY REVIEW

2.1. Total body water composition: blood, intracellular fluid, extracellular fluid

The following review of fluid physiology is well documented in physiology reference materials (Bern & Levy, 1988; Guyton & Hall, 1996). In humans the total body water is approximately 60% of body weight where 33% is intracellular fluid (ICF) and 27% is extracellular fluid (ECF). The ECF is divided into 5% plasma (non cellular portion of blood), 12% interstitial fluid (ISF) and 10% transcellular fluid (TCF). The ECF is responsible for transporting nutrients to and wastes away from cells in the tissues. The blood volume constitutes 8% of the human body weight and contains 40% intracellular fluid (fluid within red blood cells) and 60% extracellular fluid (plasma). Transcellular fluid (synovial, peritoneal, pericardial and intraocular fluid) is considered a specialized ECF due to varying composition. The ECF contains sodium, chloride, calcium, bicarbonate, oxygen, carbon dioxide, glucose, fatty acids, carbohydrates, amino acids and waste products from cells. Sodium and chloride account for 90% of the extracellular solute concentration and therefore sodium measurement is used clinically to assess plasma osmolarity and ECF volume. The plasma and the ISF have similar composition except plasma has three times the protein concentration of ISF (7.3 gm/dl vs 2-3 gm/dl). The lower protein concentration in the ISF is caused by dilution by the large volume of fluid that comprises the ISF.

2.2. Structure of interstitium, cell membranes, and capillaries

The structure of the interstitium consists of proteoglycan filaments and collagen fiber bundles that provide structural support. The proteoglycan filaments are thin and coiled, and form a meshwork of hyaluronic acid and protein in between the collagen fibers. The fluid is organized between the proteoglycan filaments creating a gel like constituency. Because of

the short distance between capillaries and tissues, the exchange of fluid and small molecules is rapid and efficient.

There are differences between the membrane of capillaries and cell membranes. This difference can ultimately affect the mechanism and extent of diffusion across the membranes. The fluid mosaic model represents cell membranes as complex lipid bilayers. The lipid bilayer is organized into phospholipid heads on the aqueous outer surface and hydrophilic tails in the hydrophobic interior. Hydrophilic molecules usually enter the cell either by an integral membrane protein channel or a specific transport protein. Lipophilic molecules passively diffuse through the lipid portion of the membrane.

Capillaries are composed of a single layer of endothelial cells with a basement membrane on the outer surface. The large number of capillaries constitutes a large surface area for efficient exchange of nutrient and waste to and from cells. Intercellular clefts are thin passageways (6-7 nm) between endothelial cells allowing for diffusion of water and water-soluble solutes. The type of capillary bed influences the concentration of antimicrobials in the ECF (Barza, 1993). The majority of capillary beds in the body are porous allowing for free diffusion of molecules less than or equal to 1000 Daltons into the interstitial space containing ISF (Barza, 1993). Molecular size rather than ionization or lipophilicity is important for diffusion through capillary pores. The CNS, retina, and prostate gland are examples of regions with non-porous capillary beds. The rate-limiting step for penetration of drugs into these non-porous regions is lipophilicity since diffusion is through the capillary endothelial cells (Barza & Cuchural, 1985).

2.3. Fick's law of diffusion

The most important mechanism responsible for the exchange of ECF across capillary membranes is passive diffusion. Lipid soluble substances diffuse through the endothelial cells and therefore are not dependent on membrane pores for transport. Whether diffusion of compounds occurs through clefts (pericellular) or endothelial cells (transcellular), the rate of diffusion for both mechanisms is dependent on the compound's concentration gradient; the larger the gradient the faster the diffusion.

Diffusion across a membrane follows Fick's law:

$$\text{Rate (mg/sec)} = - \frac{(D * P)}{h} (X_1 - X_2)$$

Where D (cm/sec) is the diffusion coefficient and is dependent on membrane solubility, molecular size, and shape. P is the partition coefficient, h (cm) is the thickness of the membrane, and $X_1 - X_2$ (mg) is the concentration gradient across the membrane. The negative sign indicates the molecules move down their concentration gradient.

2.4. Starling forces

Starling forces determine the movement of fluid and molecules between capillaries and the ISF. The four Starling forces are: 1) capillary pressure (hydrostatic pressure), 2) interstitial fluid colloid pressure (usually negligible), 3) interstitial fluid pressure (hydrostatic pressure, negative), and 4) plasma colloid osmotic pressure. The first three forces drive fluid outward into the interstitium and the fourth force drives fluid into capillaries. Starling forces are responsible for establishing equilibrium between filtered and reabsorbed fluid at the capillary membrane. There is a slight excess outward force and therefore a small excess of filtration (net filtration) from the plasma to the interstitium but this is counterbalanced by the

return of fluid to the circulation by the lymphatic system. The role of the lymphatic system is to remove excess fluid, protein, and debris from the interstitial spaces and recycle it back to the circulation.

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3. LITERATURE REVIEW

3.1. Introduction

Successful antimicrobial therapy has been based on achieving adequate plasma concentrations for a sufficient duration of time, susceptibility of the bacteria, and pharmacokinetic properties of the antimicrobial. Most infections are localized in tissues that are surrounded by interstitial fluid (ISF) (Joukhadar *et al.*, 2001). There have been many pharmacokinetic studies that have shown that there may be differences between plasma and ISF drug concentrations (Bergongne-Berezin *et al.*, 1978; Brunner *et al.*, 2000; Brunner *et al.*, 2002; Joukhadar *et al.*, 2001). Since most bacterial infections are in the interstitial space, targeting therapeutic antimicrobial concentrations at the infection site (i.e., ISF) would be better associated with therapeutic success than plasma concentrations (Liu *et al.*, 2002). Direct ISF sampling is difficult due to the mesh structure of the interstitium. Therefore, pharmacokinetic-pharmacodynamic predictions of efficacy (PK-PD indices) are based on plasma/serum concentrations, because of the ease of collection of plasma/serum.

Because most capillaries are highly permeable to protein-unbound compounds, the unbound drug concentrations in the plasma and ISF are in equilibrium and it is the unbound concentration that is microbiologically active (Andes & Craig, 2002; Wise, 1986; Wise, 1986a). Albumin and less commonly α_1 -acid glycoprotein are two proteins involved with reversible binding of antimicrobials (Barza, 1994). The extent of protein binding depends on the drug, animal species, number of protein binding sites, disease status, interaction with other agents, and physicochemical properties of the drug (Lin, 1995; Ögren & Cars, 1985; Riond & Riviere, 1989). The binding of drugs to protein is nonspecific, involving hydrogen bonding, ionic, hydrophobic, and van der Waals interactions (Lázníček & Lázníčková, 1995;

Valko *et al.*, 2003). Increasing the lipophilicity of a drug generally increases the hydrophobic interaction between the drug and plasma proteins (Deschamps-Labat *et al.*, 1997; Valko *et al.*, 2003). Protein binding can vary among species, therefore extrapolation between species should be performed with caution (Lin, 1995; Riond & Riviere, 1989).

A study by Tompsett *et al.* (1947) found a correlation between the degree of protein binding and the antagonism of *in vitro* antimicrobial activity for several penicillins. Penicillins with high protein binding had lower *in vitro* antimicrobial activity than penicillins with low protein binding (Tompsett *et al.*, 1947). Since most pharmacokinetic studies provide information on total plasma drug concentrations without accounting for protein binding, the dosage regimens based on these results may result in subtherapeutic tissue levels for drugs that are highly protein bound (Liu *et al.*, 2002). It would be preferable to design dosage regimens for protein bound drugs on the basis of unbound drug concentrations at the infection site rather than total plasma concentrations (Liu *et al.*, 2002).

Since the ISF is the site of action of most antimicrobials, various models have been developed to collect and measure drug concentrations in the ISF. Pharmacological approaches to determine the concentration of antimicrobials at target tissues have included analysis of tissue biopsies and collection of ISF (Cars & Ögren, 1985; Nix *et al.*, 1991a).

3.1.1. Tissue biopsy

Determination of tissue concentrations through analysis of tissue biopsy samples has limitations. Tissues are homogenized prior to drug analysis and this process may degrade the drug molecule or destroy cellular membranes (Nix *et al.*, 1991a). Homogenization results in an average of the drug concentration contributed by the various fluid compartments of the tissue: ICF, ISF, lymph, and blood (Cars, 1991; Cars & Ögren, 1985; Nix *et al.*, 1991a).

Since there are variations in drug distribution among these compartments, the combined measurement falsely assumes the drug is evenly distributed throughout the tissue (Cars, 1991). The ECF of tissues constitutes 20-30% of the total tissue volume (Cars, 1991). Poorly lipophilic drugs with a low volume of distribution are restricted to the ECF and whole tissue concentrations of these drugs will underestimate their extracellular concentration (Cars, 1991). The intracellular compartment comprises the largest fluid volume in tissues. Therefore, whole tissue concentrations of highly lipophilic or highly tissue bound drugs will be overestimated (Cars, 1991). The total tissue concentrations of norfloxacin in rabbit muscle were shown to overestimate the ECF concentrations, suggesting that a high fraction of the drug was intracellular (Cars & Ögren, 1985). Collecting serial tissue samples from animals in order to follow the temporal relationship of drug concentrations is difficult technically and the number of samples may be limited because of humane considerations.

3.2. Interstitial fluid collection devices

There are many devices available for ISF collection: tissue fluid cages, skin blisters, cotton threads, paper disks, skin windows, *in vivo* microdialysis, and *in vivo* ultrafiltration. Tissue fluid cages have been the most common devices used to collect ISF samples for tissue distribution studies in veterinary medicine. After an extensive internet medical literature search, I found skin blisters and microdialysis to be the most common methods used for ISF collection in humans.

3.2.1. Tissue fluid cages

Tissue fluid cages have been widely used in veterinary medicine to study the distribution of drugs in a variety of species (Aliabadi & Lees, 2003; Bengtsson *et al.* 1986; Bengtsson *et al.*, 1989; Greko *et al.*, 2002; Lees *et al.*, 1986; Piercy, 1978). The cages are

implanted under the skin and after a 4-6 week recovery period, ISF sampling can begin. The tissue cage compartment contains fluid from the interstitial space and can be accessed through perforations in the tissue cage. Tissue cages have been used to assess drug distribution to the ISF. Injections of irritants or bacteria into the tissue cages have been used as a model for inflammation/infection (Higgins *et al.*, 1987; Lees *et al.*, 1987). The fluid within the tissue cage includes protein and therefore samples collected represent both bound and unbound drug concentrations (Bengtsson *et al.* 1986). A study by Bengtsson *et al.* (1986) reported lower protein levels in tissue cage fluid compared to serum, which decreased over time. An advantage of these devices is that their cage size allows collection of a large volume of fluid (Clarke, 1989a). However, the large fluid volume in the cage results in longer diffusion distances for molecules and delayed drug penetration (Clarke, 1989a). An additional disadvantage is that repetitive sampling of large volumes from the device can disrupt the rate of equilibrium between plasma and ISF within the tissue cage (Clarke, 1989a).

Clarke *et al.* (1989b) compared serum and tissue cage fluid after intravenous administration of antipyrine and phenylbutazone in calves. The tissue cage fluid concentration vs time profile of both drugs was characterized by an increase time to peak concentration and slower elimination when compared to serum (Clarke *et al.*, 1989b). The slower elimination from the tissue cage was explained by a smaller SA/V (surface area to volume) ratio of the cage and the fibrous encapsulation surrounding the cage (Clarke *et al.*, 1989b). Penetration of phenylbutazone into the tissue cages was less than antipyrine because phenylbutazone had higher serum protein binding (99.2 %) (Clarke *et al.*, 1989b). The protein concentration was lower in the tissue cage fluid than in the serum and this difference

accounted for the lower total phenylbutazone concentrations in the tissue cage fluid compared to serum. Tissue cages may be a good model for studying infectious/inflammatory processes because bacterial infections can cause encapsulation and fluid accumulation at the site of infection, and result in slow elimination and delayed peak concentrations of antimicrobials (Clarke *et al.*, 1989a).

The type of material used to construct the tissue fluid cage can influence the amount of tissue reaction around the cage (Clarke, 1989a). Vascularization and tissue in-growth within the cage, which can vary among species, can affect the rate of penetration of molecules into the cages and the fluid volume available for collection (Clarke, 1989a). Therefore, comparison of results from studies in which tissue cages were made from different material is difficult. In a comparative study among dogs, sheep, and calves, recovered implanted tissue cages from calves had a thicker connective tissue capsule and greater granulation tissue in the tissue cage holes than the other species (Piercy, 1978). In rabbits, an extensive inflammatory reaction and a large amount of tissue in-growth was seen with subcutaneous implanted steel mesh cages (Rylander *et al.*, 1978). Therefore, comparison of tissue cage data among species is difficult to interpret.

3.2.2. Blister fluid

Skin blister formation is a common method used for ISF collection in human drug distribution studies (Brunner *et al.*, 2002; Brunner *et al.*, 1998; Mouton *et al.*, 1990; Müller *et al.*, 1999b; Philip-Joet *et al.*, 1992; Ryan *et al.*, 1982). There are two methods commonly employed to induce skin blisters. The first involves placement of perspex cups on the skin surface and applying negative pressure to the skin to form a blister (Schreiner *et al.*, 1981). The fluid in the blister is an extension of the ISF and up to 1 mL of ISF can be withdrawn

from the blister at each sampling time. In the second method, cantharides patches are placed on the skin and a blister forms within 12 hours (Simon *et al.*, 1978). The cantharides induced blister fluid represents an inflammatory exudate and has higher protein content than ISF (Barza, 1981; Clarke, 1989a). Muller *et al.* (1998) reported a significant overestimation of the unbound plasma theophylline concentration in cantharides-induced blisters and doubted the reliability of this method.

3.2.3. Cotton threads, paper disks, and skin windows

Cotton threads, paper disks, and skin windows are methods not routinely employed for ISF collection because they are older and considered outdated (Cars & Ryan, 1988; Ryan *et al.*, 1982; Ryan & Cars, 1983). Cotton threads and paper disks have been surgically implanted in the subcutaneous tissue or below the muscle fascia. Both techniques absorb interstitial fluid, which can then be analyzed for drug concentrations. Erroneous results can arise because of drug adherence to the threads or evaporation of fluid from the disks (Nix *et al.*, 1991b; Ryan *et al.*, 1982). The skin window is a glass chamber that is applied over exposed dermis. Saline is instilled into the skin window chamber and unbound drug from the capillaries in the dermis equilibrate with the fluid in the chamber (Tan *et al.*, 1972). A disadvantage of this technique is that the chamber fluid has to be replaced with saline after sampling and equilibrium has to be reestablished. In addition, careful removal of the epidermis is required to prevent excessive bleeding, but contamination of the fluid with blood is still common. (Schreiner *et al.*, 1981).

3.3. Surface area to volume (SA/V) ratio

All of the above ISF collection devices have been used to relate the antimicrobial concentration at the site of infection to plasma concentrations, but there are inconsistencies in

peak concentrations, time to peak concentrations, and elimination half-lives of drugs measured in the ISF collected from different devices (Muller *et al.*, 1998). Various explanations have been provided for the different results among the above models but the most accepted theory is the one by Van Etta *et al.* (1982). Van Etta *et al.* (1982) used the geometry of a compartment, surface area to volume ratio (SA/V), as a means to evaluate the concentration of drugs in the ECF. The SA/V ratio of a compartment determines the rate of equilibrium and the extent of concentration fluctuations across a membrane. The surface area is the area available for diffusion and the volume represents the diffusion distances. Similar unbound plasma and ISF peak drug concentrations and time to peak concentrations are found in compartments with large SA/V ratios (Van Etta *et al.*, 1982). A compartment with a low ratio has a large sampling volume and a small diffusion surface area and therefore the time for molecules to reach equilibrium is longer. These findings suggest drug concentrations in the ISF should be predictable from unbound plasma concentrations for compartments with a high SA/V ratio.

3.4. Comparison of interstitial fluid collection devices

Ryan (1985) compared pharmacokinetic results from several experiments (Barza & Weinstein, 1974; Frongillo *et al.*, 1981; Walstad *et al.*, 1983), which used different devices for ISF collection. The devices were divided into compartments with large SA/V ratios (natural compartments) and small SA/V ratios (artificial compartments). They then used the Van Etta tissue geometry technique to explain the different results found among the studies (Ryan, 1985). In this comparison, devices that sampled fluid directly from the interstitium (natural compartments, $SA/V > 100$) were considered to be paper disks, cotton threads, skin windows, and lymph (Ryan, 1985). Artificial compartments ($SA/V = 0.8-11$) were the skin

blisters, fibrin clots, and skin chambers (Ryan, 1985). Artificial compartments had a small SA/V ratio because there was a large volume for collection within the sampling device. Their results showed a correlation between the device used and the pharmacokinetic parameters for plasma and ISF (Ryan, 1985). For large SA/V natural compartment models, the ISF antimicrobial pharmacokinetic parameters were similar to plasma (Ryan, 1985). In contrast, for small SA/V artificial compartment models, a lower peak concentration, increased time to peak concentrations, and a longer half-life for drugs in the ISF compared to serum were reported (Ryan, 1985). Ryan (1985) concluded that antimicrobial concentrations in ISF collected from high SA/V ratio compartments were similar to plasma concentrations, but concentrations determined from compartments with a low SA/V ratio were not reliably predicted from plasma concentrations. The effect of protein binding on distribution was not addressed in this review paper. However, because all of the drugs compared had low protein binding, similar concentrations between the ISF and plasma (total and unbound) are expected.

3.5. Microdialysis and Ultrafiltration

Two current techniques used to collect ISF are microdialysis (MD) and ultrafiltration (UF). Microdialysis and UF sample ISF directly from the interstitium and enable collection of the unbound microbiologically active drug at the infection site (i.e., ISF). An advantage of the *in vivo* UF and MD devices over skin biopsies is that they allow direct continuous collection of protein unbound molecules and fluid from the interstitial space.

Microdialysis was first used in humans to measure glucose concentrations in the ECF (Lönnroth *et al.*, 1987). In MD, a semi-permeable probe is inserted into a tissue and continuous perfusion with physiological saline allows diffusion of ISF into the MD probe

(Müller *et al.*, 1998). This method of collection is advantageous in situations where continuous fluid removal might disrupt the pharmacokinetics of the drug (Scott *et al.*, 1991). Microdialysis was a reliable method for ISF collection in a human pharmacokinetic study where theophylline concentrations were compared between serum and ISF (Müller *et al.*, 1998). Microdialysis ISF theophylline concentrations were similar to unbound plasma concentrations. On the other hand, blister fluid theophylline concentrations overestimated unbound plasma concentrations (Müller *et al.*, 1998). These investigators also measured unbound moxifloxacin concentrations in the interstitial space in humans using MD (Müller *et al.*, 1999b). The drug concentrations in the ISF were 50% lower than the plasma concentrations, which were consistent with the plasma protein binding measurement of 52% (Müller *et al.*, 1999b).

Although MD has been an important tool in drug ISF distribution studies, there are disadvantages associated with its use. A sensitive analytical detection device is required because small sample volumes and low concentrations are collected (Müller *et al.*, 1996). Human patients are required to remain in a supine position during the collection period and therefore the duration of study is limited (Müller *et al.*, 1996). Another disadvantage is that the probe is perfused continuously with fluid (perfusate), which does not allow equilibrium between the ISF and the perfusion fluid. Therefore, calculations must be performed to determine the absolute ISF concentration (Müller *et al.*, 1999b).

There have been studies measuring concentrations of molecules in the ISF using an *in vivo* ultrafiltration device in rats (Janle & Kissinger, 1998; Linhares & Kissinger, 1993), cats (Janle *et al.*, 1992), horses (Spehar *et al.*, 1998), and dogs (Bidgood & Papich, 2002; Bidgood & Papich, 2003). Similar to MD, UF probes are inserted into a tissue and ISF is collected

from a semi-permeable membrane probe. In contrast to MD, the fluid is collected under a vacuum and since no additional fluids are added or removed during the UF process, recovery calculations are not required. The ISF can then be sampled serially and analyzed directly without extraction. In addition, probes can remain in a variety of tissue for several days and ISF can be collected without restraint or discomfort to the animal (Linhares & Kissinger, 1993).

The rate of solutes crossing UF membranes depends on molecular size, protein binding, pressure gradient, medium fluid viscosity, temperature, and membrane characteristics (Janle & Kissinger, 1998; Linhares & Kissinger, 1992). The UF probe (RUF-3-12, Bioanalytical Systems Inc, West Lafayette, Ind) has 3 loops, each having 12cm of semi-permeable membrane (Figure 3.1). The three loops connect to a single non-permeable outflow tube that extends to the exterior of the animal and attaches to a 3 mL vacutainer (Figure 3.2). The vacutainer provides the negative pressure for ISF collection through the small pores in the UF probe membrane. These pores allow diffusion of water, electrolytes, and molecules of less than 30 000 Daltons. The ISF sampling rate is 1 to 5 $\mu\text{L}/\text{min}$ (Linhares & Kissinger, 1992), which is sufficient for most drug pharmacokinetic studies.

The UF probes are constructed of polyacrylonitrile fibers. These fibers were originally used to construct dialysis membranes used in renal dialysis machines. Synthetic membranes like polyacrylonitrile induced less complement activation and leukopenia than cellulosic membranes (Burhop *et al.*, 1993). In a study comparing different hemodialyzer membranes in a sheep model, polyacrylonitrile resulted in little or no neutropenia, and only temporary increases in TxB_2 occurred after a 4-hour exposure time (Burhop *et al.*, 1993). However, the probes have induced local inflammatory reactions. Polyacrylonitrile probes

inserted into the muscle of sheep caused an inflammatory reaction with granulation tissue formation after 8 days and the reaction increased with longer implantation times (Imsilp *et al.*, 2000).

Vasodilation and increased permeability accompanying inflammation causes an increase in drug delivery to the target site, but viscosity is also increased, which will slow the rate of diffusion (Barza & Cuchural, 1985). However, in studies in which UF probes were used to collect fluid from inflamed sites, there was little influence from inflammation. In a study determining the penetration of ciprofloxacin into the ISF of inflamed foot lesions of humans, there was no difference in the extent of penetration between inflamed lesions and noninflamed tissue (Müller *et al.*, 1999a). No difference was also reported in the penetration of phenoxymethylpenicillin into inflamed and noninflamed dermis in humans (Müller *et al.*, 1996).

Chronic tissue infection may change the normal extracellular environment because of fibrin barriers, abscessation, edema, or changes in blood flow. In addition, increased protein at the site of inflammation may bind some drugs and extend their half-life in the tissue (Barza & Cuchural, 1985). In chronic infections involving a walled off abscess or extensive edema, increased volume in the tissue space may change the geometry of the tissue and produce a lower SA/V ratio, which corresponds to a lower tissue fluid drug concentration (Van Etta & Peterson, 1982).

3.6. References

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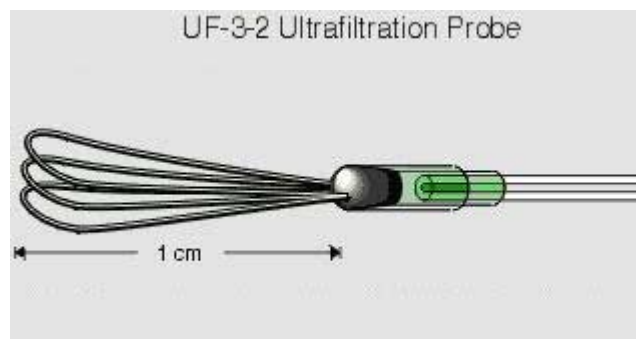


Figure 3.1. Close up of ultrafiltration probe (Bioanalytical Systems Inc, West Lafayette, Ind) showing three semipermeable loops.

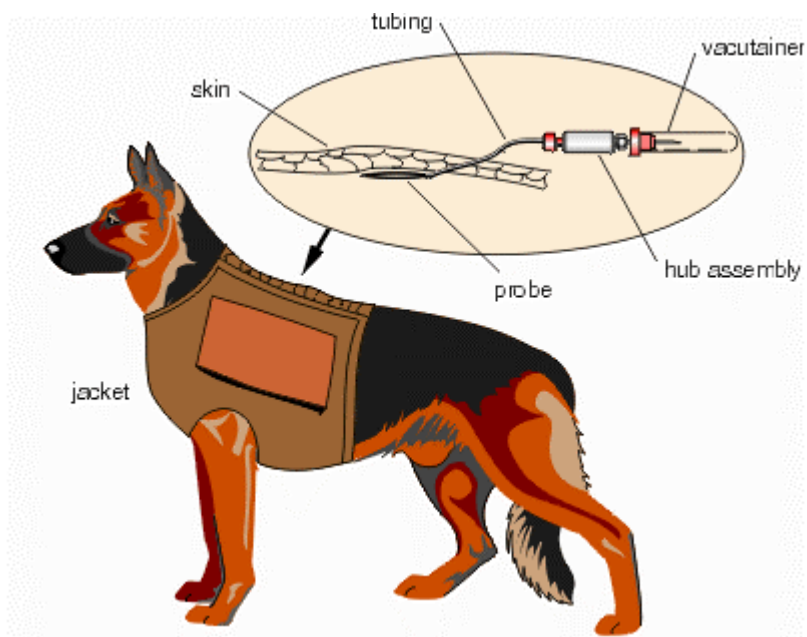


Figure 3.2. Ultrafiltration probe (Bioanalytical Systems Inc, West Lafayette, Ind) in situ.

3.7. Fluoroquinolones

3.7.1. Introduction

Quinolones were developed from the synthesis of nalidixic acid in 1962 (Escribano *et al.*, 1997; Leshner *et al.*, 1962). The usefulness of earlier drugs in this class was limited because of their narrow spectrum and rapid development of resistance by bacteria. Structural modifications over the past 3 decades have resulted in the synthesis of three new generations of quinolones. The newer quinolones have a broad spectrum of activity, high oral bioavailability, high tissue concentrations, and a long half-life (Escribano *et al.*, 1997). Enrofloxacin (Baytril[®], Bayer Corporation, KS) was the first quinolone introduced into veterinary medicine and was followed by difloxacin (Dicural[®], Fort Dodge, KS), orbifloxacin (Orbax[®], Schering-Plough, NJ), marbofloxacin (Zeniquin[®], Pfizer, NY), and danofloxacin (A180[®], Pfizer, NY). These are all members of the third generation quinolones called fluoroquinolones. The addition of one or more fluorine atom(s) distinguishes fluoroquinolones from quinolones. Recently, the fourth generation quinolones have been introduced into the US market. This new generation has high activity against gram-positive and anaerobic bacteria (Andersson & MacGowan, 2003; Piddock, 1994).

3.7.2. Mechanism of Action and Resistance

The quinolones inhibit the enzymes DNA gyrase (topoisomerase II) and topoisomerase IV in bacteria, which are important in DNA replication and transcription (Papich & Riviere, 2001; Ruiz, 2003). DNA gyrase is an ATP dependent enzyme responsible for the addition of negative supercoils by cutting and resealing the DNA (Smith, 1986). DNA gyrase consists of two A subunits (responsible for the nick and seal events) encoded by the *gyr A* gene and two B subunits (responsible for supercoiling) encoded by the

gyr B gene (Smith, 1986; Wolfson & Hooper, 1985). Topoisomerase IV is encoded by *parC* and *parE* genes (Ruiz, 2003). The A subunit of DNA gyrase is the target of fluoroquinolones in gram-negative organisms whereas topoisomerase IV is the target in some gram-positive organisms (Ruiz, 2003). Bacteria become resistant to the quinolones through chromosomal resistance rather than plasmid mediated resistance (Burman, 1977; Ruiz, 2003). Resistance mechanisms to quinolones have involved mutations in the *gyrA* gene and decreased uptake by either reduced membrane permeability or efflux pumps (Hiria, 1986; Hooper *et al.*, 1986; Ruiz, 2003; Sanders *et al.*, 1984). In veterinary clinical strains of *P. aeruginosa* isolated from canine chronic otitis externa, mutations in *gyr A* gene and an increase in efflux pump activity were found to be the primary mechanisms of resistance (Tejedor *et al.*, 2003).

3.7.3. Structure activity relationship

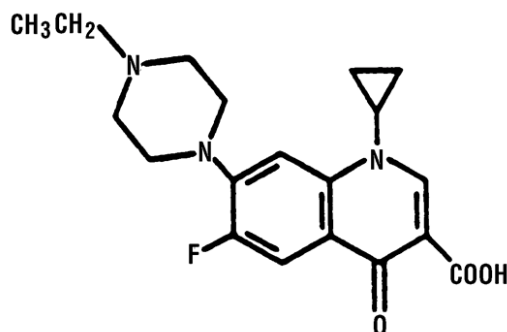


Figure 3.4. Structure of enrofloxacin

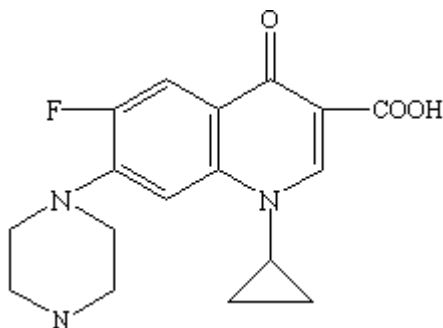


Figure 3.5. Structure of ciprofloxacin

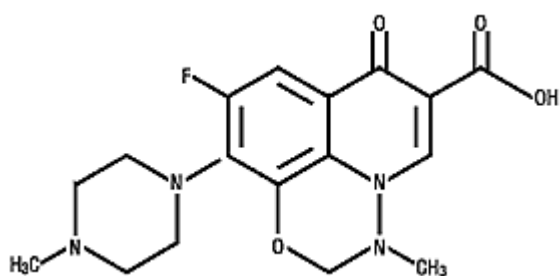


Figure 3.6. Structure of marbofloxacin

All quinolones share the 1,4 dihydro-4-oxo-pyridine-3-carboxylic acid component of their structure (Chu & Fernandes, 1989) (Figure 3.4, 3.5, 3.6). Variations among quinolones are due to different substituents at N-1, C-6, C-7, and C-8 positions (Chu & Fernandes, 1989). The C-6 fluorine of fluoroquinolones enhances bacterial penetration and inhibition of DNA gyrase (Chu & Fernandes, 1989; Domagala *et al.*, 1986; Klopman *et al.*, 1987). A C-7 substitution such as piperazine-1-yl (ciprofloxacin), 4-ethyl-piperazin-1-yl (enrofloxacin), and 4-methyl-piperazin-1-yl (marbofloxacin) increase bacterial cell penetration (Klopman *et al.*, 1987).

3.7.4. Physicochemical properties.

Fluoroquinolones are amphipathic molecules containing an acidic group (carboxylic acid, pK_{a1} = 6-7) and a basic group (tertiary amine, pK_{a2} = 7-8) (Table 3.1.) (Escribano *et al.*, 1997). At physiological pH both ionizable groups are charged and the molecule is in the zwitterionic form (Takács-Novák *et al.*, 1992). The molecular weights and pK_a for enrofloxacin, ciprofloxacin, and marbofloxacin are similar but the protein binding and partition coefficient vary among the drugs in dogs (Table 3.1.). Information on protein binding of fluoroquinolones in veterinary species is scarce. From the few studies that have measured protein binding, it is low for most drugs (Bidgood & Papich, unpublished; Papich & Riviere, 2001; Villa *et al.*, 1997). As shown in Table 3.1, depending on the technique used to determine protein binding, the results can vary within a single species.

The octanol water partition coefficient (PC) is a ratio used to measure the lipophilicity of a molecule (Takács-Novák *et al.*, 1992). Lipophilicity of the quinolone molecule is increased with the addition of methyl groups and fluorine atoms (Takács-Novák *et al.*, 1992). Lipophilicity is the highest for difloxacin, followed by enrofloxacin, danofloxacin, marbofloxacin, and ciprofloxacin (Bidgood & Papich, unpublished; Tejedor *et al.*, 2003). In a study by Tejedor *et al.* (2003), a correlation was found between lipophilicity of fluoroquinolones and efflux pump activity of *P. aeruginosa*. The less lipophilic ciprofloxacin was least affected by overexpression of efflux pump activity and had the lowest MIC against *P. aeruginosa* isolates from canine otitis externa. (Tejedor *et al.*, 2003).

Table 3.1. Physicochemical properties of fluoroquinolones.

	Marbofloxacin	Enrofloxacin	Ciprofloxacin
Molecular Weight	362.36	360.15	331.34
Octanol water partition coefficient (PC)	0.07, 0.08	3.54, 5.01	0.08, 0.07, 0.99
Protein binding (%) (dog)	9.1(method unknown), 21.8 (<i>in vitro</i> ultrafiltration) 17-33 (centrifugal ultrafiltration)	34.7(<i>in vitro</i> ultrafiltration), 27 (centrifugal ultrafiltration) 29-44 (centrifugal ultrafiltration)	18.5 (<i>in vitro</i> ultrafiltration), 44(centrifugal ultrafiltration)
Acid dissociation constants pKa 1 pKa 2	5.77 8.22	6.26 7.81	6.20 8.59

References: Bregante *et al.*, 2000; Escribano *et al.*, 1997; Papich & Riviere, 2001; Pfizer communication, 2003; Takács-Novák *et al.*, 1992; Tejedor *et al.*, 2003 (converted log PC value to PC value); Villa *et al.*, 1997. The method of determining the protein binding is shown in brackets.

3.7.5. Pharmacokinetics

The pharmacokinetics of fluoroquinolones has been extensively reviewed by Papich & Riviere, 2001. Various studies have confirmed good bioavailability in dogs after oral administration of enrofloxacin (72-84%) (Cester & Toutain, 1997; Cester *et al.*, 1996) and marbofloxacin (99-107%) (Cester *et al.*, 1996; Schneider *et al.*, 1996). Oral absorption of fluoroquinolones can be reduced by chelation from drugs such as antacids and sucralfate that contain divalent and trivalent cations (Li *et al.*, 1994; Lode, 1988). Li *et al.* (1994) reported higher MICs of ciprofloxacin against *E. coli* and *P. aeruginosa in vitro* when the concentration of cations was increased.

The volume of distribution in dogs is higher for enrofloxacin (3-7 L/kg) compared to marbofloxacin (1.5-2 L/kg) (Bidgood & Papich, unpublished; Küng *et al.*, 1993; Schneider *et al.*, 1996). However, despite these differences adequate concentrations of both

fluoroquinolones are achieved in most tissues (Boothe *et al.*, 2001; DeManuelle *et al.*, 1998; Dorfman *et al.*, 1995; Duval & Budsberg, 1995; Hawkins *et al.*, 1998; Schneider *et al.*, 1996). Tissue distribution of fluoroquinolones in dogs has been investigated using tissue biopsies (Boothe *et al.*, 2001; DeManuelle *et al.*, 1998; Frazier *et al.*, 2000; Schneider *et al.*, 1996) and tissue fluid cages (Aliabaldi & Lees, 2002; Walker *et al.*, 1990; Walker *et al.*, 1992). A study by Boothe *et al.* (2001) determined multiple tissue concentrations of enrofloxacin and its metabolite ciprofloxacin in dogs after an IV bolus injection (20 mg/kg) of enrofloxacin. Concentrations exceeding a MIC of 0.5 µg/ml were found in most tissues (Boothe *et al.*, 2001). Interpretation of the results from this study is difficult because the tissues were centrifuged through a membrane to remove the protein. Since fluoroquinolones are protein bound the tissue concentrations reported do not represent the true concentration. In addition, the tissues were homogenized which combines all tissue fluid compartments. Therefore, determining accurate concentrations in the extracellular and intracellular compartment was not possible.

There are several studies that have examined the concentrations of fluoroquinolones in canine skin biopsies (DeManuelle *et al.*, 1998; Frazier *et al.*, 2000; Kay-Mugford *et al.*, 2002; Schneider *et al.*, 1996). A study by DeManuelle *et al.* (1998) evaluated skin enrofloxacin concentrations in healthy dogs and dogs with pyoderma 3 hours and 3 days after daily enrofloxacin administration. The dogs with pyoderma had significantly higher skin enrofloxacin concentrations than healthy dogs and the total skin concentrations (µg/gram) were 12.4 times the MIC (0.5 ug/mL) of isolated *Staphylococcus* spp (DeManuelle *et al.*, 1998). Frazier *et al.* (2000) compared skin concentrations of marbofloxacin (2.75mg/kg), enrofloxacin (5mg/kg) and difloxacin (5mg/kg) in dogs after a 5-day dosing schedule.

Difloxacin had the lowest concentrations in the skin. There was no difference in skin concentrations between marbofloxacin and enrofloxacin plus its metabolite ciprofloxacin and concentrations of both drugs were above 3 µg/g by the fifth day (Frazier *et al.*, 2000). Drug concentrations determined from homogenized tissue represents an average concentration because all fluid compartments are combined. Because fluoroquinolones are able to distribute intracellularly, whole skin concentrations are overestimated.

Tissue cages in dogs and skin blisters in humans have also been used to describe tissue distribution of fluoroquinolones. Enrofloxacin and ciprofloxacin achieved high concentrations in tissue fluid cages in dogs but the peak concentration was lower and the time to peak concentration was longer than serum (Walker *et al.*, 1990; Walker *et al.*, 1992). Similar results were found in suction induced blister fluid after ciprofloxacin was administration to human volunteers (LeBel *et al.*, 1986).

The liver metabolizes all fluoroquinolones, except enrofloxacin, to inactive metabolites. In dogs, 20-43% of enrofloxacin is metabolized (de-ethylated) to ciprofloxacin by the liver (Cester & Toutain, 1997; Monlouis *et al.*, 1997). Ciprofloxacin contributes to the antimicrobial action of enrofloxacin but is subsequently metabolized to clinically insignificant metabolites. Marbofloxacin is metabolized to two inactive metabolites, marbofloxacin-N-oxide and demethyl-marbofloxacin (Lefebvre *et al.*, 1998). Excretion of most fluoroquinolones from the body is via the urine, primarily by glomerular filtration and to a small extent by renal tubular and biliary secretion (Kietzmann, 1999).

3.7.6. Pharmacodynamics

Fluoroquinolones are broad-spectrum bactericidal antimicrobials. They have activity against gram-negative, gram-positive, and some intracellular bacteria. The post antibiotic

effect (PAE), characteristic of most fluoroquinolones, is the duration of bacterial growth inhibition after the drug concentrations have fallen below the MIC (Craig & Gudmundsson, 1996). The PAE is maximized with high peak antimicrobial concentrations and long bacterial exposure times. The PAE for marbofloxacin and enrofloxacin were between 1.6 and 2.4 hours for *in vitro* canine isolates of *E. coli* and *S. intermedius* when the concentrations were four times the MIC (Spreng *et al.*, 1995).

Fluoroquinolones have concentration dependent activity, which allows for once a day dosing. Rapid bactericidal activity is found with $C_{\max}/\text{MIC}$ and $\text{AUC}_{0-24\text{h}}/\text{MIC}$ ratios of 8-10 and 125-250, respectively (Blaser, 1987; Papich & Riviere, 2001; Wright, 2000). In a study by Blaser *et al.* (1987) a two-compartment model was created to compare the *in vitro* activity of enoxacin and netilmicin against *P. aeruginosa*, *K. pneumoniae*, *E. coli*, and *S. aureus* strains after once a day and twice a day dosing. A $C_{\max}/\text{MIC}$ ratio of 6:1 had a response rate of less than 70% where a $C_{\max}/\text{MIC}$ ratio of 8:1 or higher had a response rate of 90% (Blaser *et al.*, 1987). This study also found once a day dosing achieved greater killing of *P. aeruginosa* than twice daily dosing (Blaser *et al.*, 1987). Similarly, eradication of susceptible gram-negative bacteria by ciprofloxacin was maximized when $\text{AUC}_{0-24\text{h}}/\text{MIC}$ ratios were greater than 125 (Forrest *et al.*, 1993). To achieve a clinical and microbiological cure of infections caused by some gram-positive organisms, an $\text{AUC}_{0-24\text{h}}/\text{MIC}$ ratio of 40 has been recommended (Wright *et al.*, 2000).

3.7.7. Adverse effects

Adverse effects after fluoroquinolone administration have been reported but few have been serious and most have been dose related. The most common adverse effects at high doses are nausea, vomiting, and diarrhea. Physical and histological changes in joints of

juvenile animals have been reported after administration of fluoroquinolones (Burkhardt *et al.*, 1990; Stahlmann *et al.*, 1988). After administration of 300mg/kg of difloxacin to immature dogs, eight out of ten dogs were physically lame after 3 days and nine out of ten dogs had grossly visible changes in the articular cartilage (Burkhardt *et al.*, 1990). There is variation in the effects of different fluoroquinolones on joint cartilage as well as species differences (Schlüter, 1987). Compared to rats, mice, and rabbits, dogs are the most sensitive species to cartilage damage by nalidixic acid (Schlüter, 1987). Due to the risk of cartilage lesions in juvenile animals, fluoroquinolones administration is not recommended in pregnant animals, small and medium dog breeds between the ages of 2 and 8 months of age, or large breeds less than 18 months of age.

In a study by Tsuji *et al.* (1988) quinolones inhibited binding of GABA (gamma-aminobutyric acid) to its receptors on the postsynaptic membrane in a concentration dependent manner. Because of this interaction, administration of some quinolones at high doses has caused convulsions in humans and animals (Christ, 1990; Simpson, 1985). Administration of fluoroquinolones to epileptic patients or together with the non-steroidal antiinflammatory drug, fenbufen, should be avoided due to the risk of inducing convulsions (Naora *et al.*, 1991).

Enrofloxacin has been linked to retinal degeneration in cats (Gelatt *et al.*, 2001; Wiebe & Hamilton, 2002). In a retrospective study from January 1994 to September 2000, 17 cats were diagnosed with retinal degeneration after parenteral administration (4.4-15 mg/kg twice daily) of enrofloxacin (Gelatt *et al.*, 2001). This adverse reaction to enrofloxacin is an irreversible reaction and the manufacturer now recommends using only the label dose of 5 mg/kg/day (package insert, Baytril, Bayer Corporation). Results from the use

of ciprofloxacin in dogs, cats and humans demonstrated no adverse ocular changes (Arcieri *et al.*, 1987; Schlüter, 1987).

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3.8. Carbapenems

3.8.1. Introduction

The β -lactam antimicrobials were first discovered and used in the 1940's. Carbapenems are the most recent class of parenteral β -lactams approved for use in humans. The first compound discovered in the carbapenem class was the natural product thienamycin from *Streptomyces cattleya* (Kahan, 1979). However, due to chemical instability of thienamycin, structural modifications produced other compounds. In 1985, imipenem (Primaxin[®], Merck & Co., Inc) was the first carbapenem available in the United States and

was followed by meropenem (Merrem[®], Astra-Zenica) in 1996, and ertapenem (Invanz[®], Merck & Co., Inc) in 2001. Imipenem is administered as a 30-minute intravenous infusion (package insert, Primaxin[®], Merck & Co., Inc). Because of the potential for nephrotoxicity caused by a metabolite of imipenem, it is coadministered with cilastatin, a dehydropeptidase-1 (DHP-1) inhibitor (Norrby & Gildon, 1999). Cilastatin inhibits the conversion of imipenem to a potentially toxic metabolite in the renal tubules and increases the amount of active drug in the urine. Ertapenem is administered either as a 30-minute intravenous infusion or intramuscular injection once daily (package insert, Invanz[®], Merck & Co., Inc). Meropenem can be administered as either an intravenous bolus or infusion (package insert, Merrem[®], Astra-Zenica).

3.8.2. Mechanism of action and resistance

The penicillin binding proteins (PBP) are enzymes responsible for synthesis of the bacterial cell wall. All β -lactam antimicrobials inhibit the final crosslinking step in bacterial cell wall synthesis by binding to penicillin binding proteins (PBP) inside the cell wall (Mandell & Petri, 1996). The β -lactams differ in affinity for the various PBP found in gram-negative and positive-bacteria (Georgopapadakou & Liu, 1980; Neu, 1985). Distinct bacterial morphological changes are associated with inhibition of the different PBP. Inhibition of PBP 1 produces rapid cell lysis, inhibition of PBP 2 results in spheroblast formation, and PBP 3 inhibition hinders septum formation causing elongation and endotoxin release from gram-negative bacteria (Periti & Mazzei, 1999). The carbapenems have high affinity for PBP 1 and 2 in *E.coli*, which causes rapid cell lysis (Kahan *et al.*, 1983; Neu, 1985). In addition to inhibiting the final crosslinking step, cell lysis depends on the autolytic enzymes of the bacterial cell (Mandell & Petri, 1996).

Resistance to β -lactams can occur through β -lactamase synthesis, decreased permeability of the outer bacterial membrane, and decreased affinity for PBP (Angus *et al.*, 1982; Edwards, 1995; Fernández-Cuenca *et al.*, 2003; Godfrey *et al.*, 1984). Carbapenems owe their excellent activity to remarkable stability against β -lactamases, and the ability to penetrate the outer membrane of gram-negative bacteria. When meropenem and imipenem were tested against class A and D penicillinases, class A extended spectrum β -lactamases, and class C cephalosporinases, both drugs were resistant to all enzymes (Labia *et al.*, 1989). Imipenem and meropenem diffuse through the outer membrane porin D2 where other β -lactams diffuse through porin C, E and F (Satake *et al.*, 1990). The different porin used by carbapenems for entry into bacteria and their rapid entry may be a reason for their high activity against some bacteria (Satake *et al.*, 1990). Alteration in either the 45 or 46 kD protein in the outer membrane has resulted in a decreased permeability of *P. aeruginosa* to imipenem (Büscher *et al.*, 1987; Lynch *et al.*, 1987; Quinn *et al.*, 1986).

3.8.3. Structure activity relationship

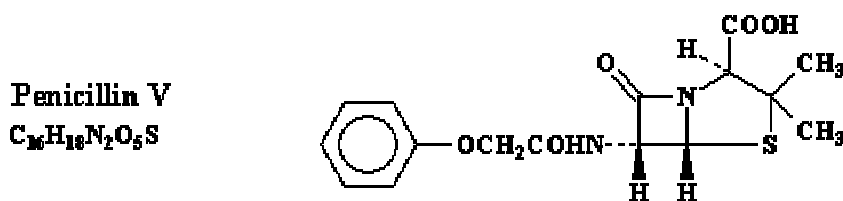


Figure 3.7. Structure of penicillin

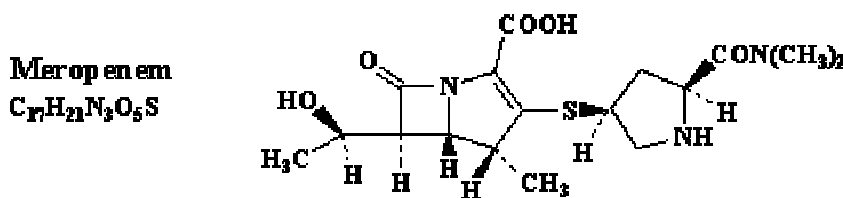


Figure 3.8. Structure of meropenem

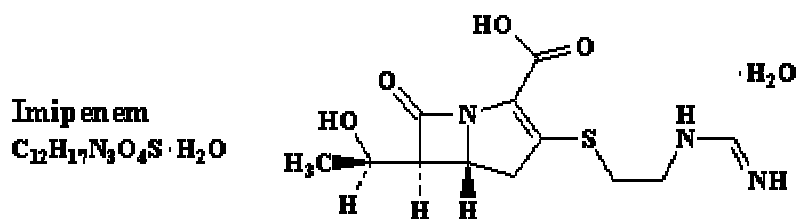


Figure 3.9. Structure of imipenem

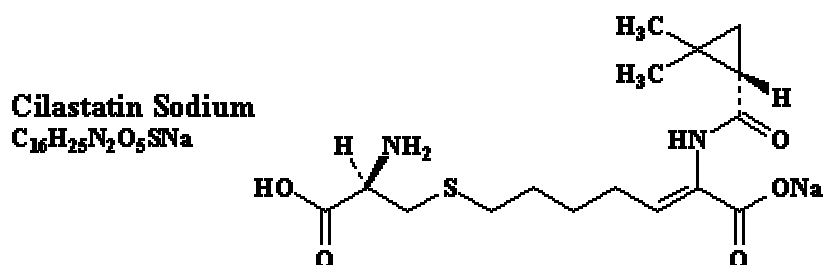


Figure 3.10. Structure of Cilastatin

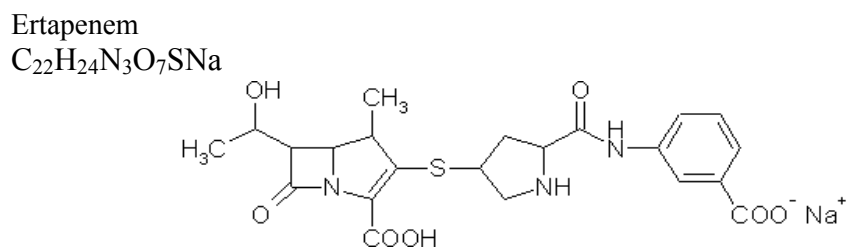


Figure 3.11. Structure of Ertapenem

The chemical structure of penicillin and the carbapenems: meropenem, imipenem-cilastatin, and ertapenem are shown in Figure 3.7, 3.8, 3.9, 3.10, and 3.11. The chemical structures of carbapenems differ from other β -lactams by substituting a carbon for a sulfur atom at position one of the thiazolidine ring as well as having a double bond between C-2

and C-3 (Hellinger & Brewer, 1999; Moellering *et al.*, 1989). The hydroxyethyl substituent at C-5 and C-6 in the trans position is responsible for the resistance of carbapenems to the β -lactamases (Kahan *et al.*, 1983). Meropenem differs from imipenem by addition of a methyl group at C-1 and a dimethylcarbamoypyrrolidinethio side chain at C-2 (Moellering *et al.*, 1989). The addition of a 1- β -methyl substituent increases meropenem activity against *P. aeruginosa* and increases its stability to DHP-1 (Edwards & Turner, 1995; Fukasawa *et al.*, 1992). The alteration at the C-2 position (dimethylcarbamoypyrrolidinethio chain) is responsible for the greater activity of meropenem against gram-negative bacteria and decreased CNS toxicity compared to imipenem (Edwards & Turner, 1995; DeSarro *et al.*, 1995).

3.8.4. Physicochemical properties

Carbapenems are zwitterions with low molecular weights (Edwards & Turner, 1995). These characteristics allow the molecule to pass easily through small porins in the outer cell membrane of gram-negative bacteria to the periplasmic space (Hellinger & Brewer, 1999; Yoshimura & Nikaido, 1985). The molecular weight is 437.51 for the trihydrate form (commercial form) and 383.46 for the anhydrous form (Mouton & van den Anker, 1995). The pKa of meropenem is 7.4 and the pH of the reconstituted commercially available product is between 7.3 and 8.3 (Nouda *et al.*, 1996). *In vitro* protein binding of meropenem is 2 % in humans (package insert, Merrem[®], Astra-Zenica) and 11.87 % in dogs (Bidgood & Papich, 2002). The *in vitro* protein binding in human plasma is low for imipenem (25%) and high for ertapenem ($\geq 95\%$) (Majumdar *et al.*, 2002; Norrby *et al.*, 1983; Sundelof *et al.*, 1997).

3.8.5. Pharmacokinetics

Unlike imipenem, meropenem and ertapenem are not metabolized extensively by DHP-1 and therefore these two carbapenems do not require administration with an inhibitor (Burman *et al.*, 1991; Fukasawa *et al.*, 1992; Hikida *et al.*, 1992; Sundelof *et al.*, 1997).

In humans the majority of meropenem is excreted as the parent molecule and only a small amount is excreted as a microbiologically inactive metabolite, ICI 213, 689 (Harrison *et al.*, 1989). In healthy humans, 79% of meropenem was excreted unchanged in the urine by both glomerular filtration and tubular secretion after 12 hours (Bax *et al.*, 1989). After a 1g dose of ertapenem, 45% was excreted unchanged in the urine (Majumdar *et al.*, 2002). Without cilastatin, only 14.7 % of imipenem is excreted in the urine (Lockley & Wise, 1985). Even with the addition of cilastatin the urinary recovery of imipenem was still lower than meropenem (55.6% vs 69.3%) (Leroy *et al.*, 1992; Lockley & Wise, 1985).

Meropenem, unlike imipenem, can be given as an IV bolus injection. When meropenem was administered after 2min, 3min, and 5 min, there were no adverse effects and peak concentrations in the blood were measured after 1- 5 minutes (Jones *et al.*, 1997). Meropenem and imipenem are characterized by a short half-life, high clearance, and low volume of distribution in dogs (Barker *et al.*, 2003; Bidgood & Papich, 2002). Compared to meropenem and imipenem, ertapenem has a longer half-life, lower clearance, lower volume of distribution, and higher protein binding in humans (Bax *et al.*, 1989; Majumdar *et al.*, 2002).

The majority of studies evaluating tissue and ISF concentrations of carbapenems are in human patients. In a study evaluating penetration of meropenem in lung tissue, bronchial mucosa, and pleural tissues, concentrations were higher than the MICs for common lower respiratory pathogens (Byl *et al.*, 1999). Tegeder *et al.* (2002) compared imipenem

concentrations in plasma and ISF collected by microdialysis from healthy and critically ill patients. Plasma concentrations were equal between the two groups but critically ill patients had significantly lower ISF concentrations and they were below the targeted MIC during most of the dosing interval (Tegeder *et al.*, 2002).

3.8.6. Pharmacodynamics

Carbapenems are broad-spectrum bactericidal antimicrobials with activity against gram-positive, gram-negative, and anaerobic bacteria (Pfaller & Jones, 1997; Watt & Naden, 1989). Their stability to β -lactamases, high affinity for penicillin binding proteins (PBP), and low development of resistance makes these antimicrobials highly active against bacteria resistant to other antimicrobials (Edwards & Turner, 1995). In humans, parenteral carbapenem monotherapy has been commonly used in the treatment of febrile neutropenia, intra-abdominal infections, septicemia, meningitis, respiratory infections, and gynecological infections (Norrby & Gildon, 1999). Carbapenem monotherapy has been used as an alternative to other regimens because of the toxicities associated with aminoglycosides, increasing resistance to 3rd generation cephalosporins, and poor activity of cephalosporins against anaerobes (Schuler *et al.*, 1995).

The β -lactam antimicrobials kill bacteria in a time-dependent manner. Increasing the duration of exposure of the bacteria to the β -lactam rather than increasing concentrations are better correlated to bactericidal activity (Craig, 1998; Gerber *et al.*, 1984). Therefore, optimal efficacy for most β -lactams is achieved when the plasma and ISF concentrations are above the MIC for the susceptible bacteria during most of the dosing interval. However, inhibitory effects of carbapenems are seen with a $T > MIC$ of 33-40% of the dosing interval compared with 50-60% for other β -lactams (Drusano & Hutchison, 1995; Turnidge, 1998;

Vogelman *et al.*, 1988). Most β -lactam antimicrobials have a PAE for gram-positive organisms but only the carbapenems have a PAE for several hours against gram-negative organisms (Bustamante *et al.*, 1984; Craig & Gundmundsson, 1996). In addition to a PAE, the MICs for carbapenems are lower than other β -lactams (Hanberger & Nilsson, 1994). These properties support intermittent administration of carbapenems at a frequency that is less than other β -lactams (Hanberger & Nilsson, 1994).

3.8.7. Adverse effects

Carbapenems are well-tolerated drugs with minimal side effects. In humans, the incidence of adverse effects from meropenem is < 3% and includes diarrhea, rash, nausea, vomiting, and injection site inflammation (Norrby & Gildon, 1999). Unlike imipenem-cilastatin, meropenem has a low incidence of nausea and vomiting from a bolus injection in humans (Jones *et al.*, 1997; Schuler *et al.*, 1995). Nausea after rapid IV bolus injections and pain after IM injections of imipenem in dogs have been reported (Barker *et al.*, 2003). In contrast, no adverse effects were reported after dogs were administered a rapid intravenous bolus and subcutaneous injection of meropenem (Bidgood & Papich, 2002). Neurotoxicity at higher doses has been reported with imipenem-cilastatin in patients with CNS and renal disorders (Calandra *et al.*, 1988). In a retrospective study of 5026 patients treated with meropenem, there was a low incidence of seizures (0.08%) (Norrby & Gildon, 1999).

3.8.8. References

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3.9. Doxycycline

3.9.1. Introduction

In 1948 Duggar discovered the product of the bacterium *Streptomyces aureofaciens*, aureomycin (chlortetracycline HCl), had broad-spectrum antimicrobial activity. Since the discovery of chlortetracycline, structural modifications to the basic molecule have produced a variety of tetracycline derivatives. Doxycycline is a second-generation derivative with improved pharmacokinetics, greater antimicrobial spectrum and activity, and reduced toxicity compared to the first generation tetracyclines.

3.9.2. Mechanism of action and resistance

The mechanism of action of tetracyclines is to attach to the 30S ribosome and inhibit the binding of aminoacyl tRNA to the mRNA-ribosome complex (Day, 1966; Franklin, 1963). The net result is inhibition of protein synthesis in bacteria by preventing additional amino acids from being joined to the peptide chain (Franklin, 1963). Human and animal cells are protected from the effects of tetracyclines at low concentrations, but at higher concentrations, protein synthesis in these cells can be inhibited causing an antianabolic effect (Beard *et al.*, 1969; Korkeila, 1971). Tetracyclines enter into bacterial cells by an energy dependent active transport process (De Zeeuw, 1968; Franklin & Higginson, 1970). Bacterial resistance to tetracyclines occurs through plasmid-mediated decreased uptake (Benveniste & Davies, 1973; Franklin & Godfrey, 1965). Therefore, in resistant bacteria, higher drug concentrations are required to transport the drug into the bacterial cell (De Zeeuw, 1968).

3.9.3. Structure activity relationship and physicochemical properties

Absorption of tetracycline in dogs is most rapid and extensive from the duodenum compared to the stomach and other parts of the intestine (Pindell *et al.*, 1958). The extent of absorption of doxycycline is higher (95%) than the first generation tetracyclines (50%) in humans and it is not influenced by food (Saivin & Houin, 1988). Di- and trivalent cations such as calcium and iron impair the absorption of tetracyclines by forming insoluble chelates (Neuvonen *et al.*, 1970; Rosenblatt *et al.*, 1966). Absorption of doxycycline is less impaired by cations than other tetracyclines (Rosenblatt *et al.*, 1966).

There are a small number of studies that have examined the distribution of doxycycline into the ISF in humans and animals. Interstitial fluid levels of doxycycline have been investigated in humans (Schreiner & Digranes, 1985), rabbits (Cars & Ryan, 1988), goats (Jha *et al.*, 1989) and dogs (Bidgood & Papich, 2003). Schreiner & Digranes (1985) compared suction induced blister fluid concentrations to serum concentrations after oral administration of doxycycline in humans. Compared to serum concentrations, there was a longer time to peak concentrations in the blister fluid (1.8 vs 2.9 hours) and concentrations were lower in the blister fluid during the entire dosing interval (Schreiner & Digranes, 1985). There were similar delayed peak ISF total drug (bound and unbound) concentrations in tissue cages after administration of intravenous doxycycline to goats. Dogs administered a constant rate infusion of doxycycline had significantly lower ISF unbound drug concentrations compared to total plasma concentrations at steady state (Bidgood & Papich, 2003). However, after the protein binding was taken into account, the ISF and unbound plasma concentrations were similar.

Schach von Wittenau & Delahunt (1966) investigated the distribution of five tetracyclines in various tissues of dogs after oral administration. The researchers reported an

increase in the ratio of total drug in homogenized tissues to unbound drug in serum with increasing lipophilicity (Schach von Wittenau & Delahunt, 1966). This ratio was high for doxycycline and therefore it was concluded that doxycycline had excellent distribution to the heart, lung, muscle, liver and kidney (Schach von Wittenau & Delahunt, 1966). These results do not accurately represent true drug concentrations in tissues because the homogenization process overestimates tissue concentrations of lipophilic drugs like doxycycline because tissue fluid compartments are combined.

Unlike tetracycline, doxycycline does not undergo extensive metabolism or renal elimination (Pindell *et al.*, 1958). In contrast, elimination of doxycycline occurs by renal, biliary, and gastrointestinal routes (Schach von Wittenau & Twomey, 1971). Greater than 90% of doxycycline was eliminated in the urine and feces of dogs and man (Schach von Wittenau & Twomey, 1971). There is less dependence on renal elimination because of doxycycline intestinal elimination and therefore it is an alternative to other tetracyclines for treating susceptible bacterial infections in patients with renal failure (Schach von Wittenau *et al.*, 1972).

3.9.5. Pharmacodynamics

Doxycycline is a broad-spectrum, bacteriostatic drug with activity against gram-negative, gram-positive, and anaerobic bacteria, and some intracellular bacteria and protozoa (Klein & Cunha, 1995; Williamson, 1968; Wong & Cox, 1948). Doxycycline is more active against staphylococci, streptococci, and anaerobic bacteria than the 1st generation tetracyclines (Williamson, 1968).

3.9.6. Adverse effects

There are several unwanted side effects that can occur after administration of tetracyclines to humans and animals. Nausea and vomiting caused by upper gastrointestinal irritation is the most common adverse affect associated with oral administration of tetracyclines (Riviere & Spoo, 2001). Esophageal ulceration has been reported after oral administration of doxycycline and other tetracyclines in humans and cats (Amendola & Spera, 1985; Carlborg et al., 1983; Crowson *et al.*, 1976). In humans, swallowing oral medication with fluids is recommended to prevent ulcer formation (Channer & Virjee, 1982; Hey *et al.*, 1982).

Both horses and cattle have collapsed following rapid IV administration of oxytetracycline and tetracycline (Gyrd-Hansen *et al.*, 1981; Potter, 1973; Riond *et al.*, 1992). Fatal cardiovascular effects have occurred in horses after intravenous administration of doxycycline (Riond et al., 1989; Riond et al., 1992). These reactions are thought to occur because calcium ions are chelated in the blood (Gyrd-Hansen *et al.*, 1981; Kohn *et al.*, 1961).

In humans tetracyclines have been associated with discoloration of teeth in young children, phototoxicity, and impaired fetal skeletal development (Blank *et al.*, 1968; Frost *et al.*, 1971; Hamp, 1967). Teeth discoloration is less likely after doxycycline administration than other tetracyclines (Lochary *et al.*, 1998). Other less common side effect reported in the literature for tetracyclines include polyneuropathy and hepatotoxicity in humans (Olsson, 2002; Whalley *et al.*, 1964) and acute renal failure in dogs (Stevenson, 1980).

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**Plasma pharmacokinetics and tissue fluid concentrations of meropenem
after intravenous and subcutaneous administration in dogs**

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2002

Published in *American Journal of Veterinary Research*, 63 (12), 1622-1628

ABSTRACT

Objective—To estimate pharmacokinetic variables and measure tissue fluid concentrations of meropenem after IV and SC administration in dogs.

Animals—6 healthy adult dogs.

Procedure—Dogs were administered a single dose of meropenem (20 mg/kg) IV and SC in a crossover design. To characterize the distribution of meropenem in dogs and to evaluate a unique tissue fluid collection method, an in vivo ultrafiltration device was used to collect interstitial fluid. Plasma, tissue fluid, and urine samples were analyzed by use of high-performance liquid chromatography. Protein binding was determined by use of an ultrafiltration device.

Results—Plasma data were analyzed by compartmental and noncompartmental pharmacokinetic methods. Mean $\pm$ SD values for half-life, volume of distribution, and clearance after IV administration for plasma samples were 0.67 ± 0.07 hours, 0.372 ± 0.053 L/kg, and 6.53 ± 1.51 mL/min/kg, respectively, and half-life for tissue fluid samples was 1.15 ± 0.57 hours. Half-life after SC administration was 0.98 ± 0.21 and 1.31 ± 0.54 hours for plasma and tissue fluid, respectively. Protein binding was 11.87%, and bioavailability after SC administration was 84%.

Conclusions and Clinical Relevance—Analysis of our data revealed that tissue fluid and plasma (unbound fraction) concentrations were similar. Because of the kinetic similarity of meropenem in the extravascular and vascular spaces, tissue fluid concentrations can be predicted from plasma concentrations. We concluded that a dosage of 8 mg/kg, SC, q 12 h would achieve adequate tissue fluid and urine concentrations for susceptible bacteria with a minimum inhibitory concentration of 0.12 μ g/mL.

INTRODUCTION

Treatment choices for patients with bacterial infections depend on the cultured or suspected bacteria, location of infection, safety and spectrum of the antimicrobial agent, and bacterial susceptibility.¹ Successful treatment of animals with bacterial infections and avoidance of antimicrobial resistance depends on appropriate selection, dosage, frequency, and duration of administration of the antimicrobial agent. Because of the increasing emergence of antimicrobial-resistant bacteria, therapeutic choices for managing animals with serious bacterial infections are being driven toward more active antimicrobials to achieve therapeutic success. The choice of antimicrobial for treating an animal with a serious bacterial infection can be limited to expensive and injectable drugs and occasionally only a few options are available because of bacterial antimicrobial resistance.

Carbapenems, a new class of β -lactam antimicrobials, offer a promising option for treating many of these infected animals when justified on the basis of microbial culture and results of antimicrobial susceptibility tests. These drugs are advantageous because of their bactericidal and postantibiotic effects that are lacking in other β -lactams. They also are more resistant to most of the β -lactamases, including some of the extended-spectrum enzymes.²⁻⁵ Their broad spectrum of activity is a result of efficient penetration into bacteria, stability to β -lactamases, and affinity toward specific penicillin-binding proteins that cause rapid lysis of bacteria.⁶ Carbapenems have activity against gram-negative and gram-positive aerobic bacteria as well as anaerobic bacteria.⁷⁻⁹

Meropenem, the second carbapenem marketed in the United States, offers distinct advantages compared with other drugs. An altered C2 chain is responsible for the increased activity against gram-negative organisms and a low incidence of seizures.¹⁰ Compared to

imipenem, meropenem has 2- to 4-fold greater activity against *Pseudomonas aeruginosa* and 2- to 32-fold increase in activity against Enterobacteriaceae in vitro.¹¹⁻¹³ Resistance to carbapenems has been minimal. Resistance of *P aeruginosa* to imipenem has been reported¹⁴⁻¹⁵ and is primarily attributed to the loss of D2 porin, an important protein for antibiotic penetration; however, meropenem is still 4-fold more active against these isolates.^{12,16-18}

In a study¹⁹ that examined the susceptibility of *P aeruginosa* in vitro, meropenem had the highest percentage of isolates with a minimum inhibitory concentration (MIC) below the breakpoint, compared with imipenem, ceftazidime, cefotaxime, piperacillin, gentamicin, amikacin, and ciprofloxacin. Compared to imipenem, meropenem causes minimal nausea and vomiting and is not nephrotoxic or neurotoxic.^{10,20-25} In a study by Dagan et al,²⁵ meropenem reached sufficient concentrations in the CSF without causing seizures, and meropenem has been used in human hospitals to treat children with meningitis. Therefore, we anticipate that safe treatment of animals with bacterial infections and concurrent CNS disease by administration of meropenem would be possible.

Meropenem has stability against dehydropeptidase-1 (DHP-1), a metabolizing enzyme in the renal proximal tubule cells. Therefore, contrary to the situation for imipenem, coadministration of the DHP-1 inhibitor, cilastatin, with meropenem is not required.

Intravenous administration of imipenem to dogs requires that the drug be dissolved in large fluid volumes so that the concentration infused is ≤ 5 mg/mL. The increased solubility and stability of meropenem allows it to be administered in a more concentrated solution (50 mg/mL). Injection of a small-volume bolus would be more convenient and advantageous in animals with fluid overload conditions or in the treatment of small or exotic animals. Drugs used for dogs with resistant bacterial infections are usually administered IV. Therefore,

treatment of dogs that have refractory infections with small-volume SC injections would also be a convenient option.

To our knowledge, we are not aware of any pharmacokinetic studies performed with meropenem in dogs to establish guidelines for administration. We also are not aware of any information on tissue penetration of meropenem or the use of an in vivo ultrafiltration device to determine the relationship between drug concentrations in plasma and tissue fluid. The objective of the study reported here was to obtain plasma pharmacokinetic data that could be used to calculate a dosage for administration of meropenem in dogs and, by comparing these values with tissue fluid concentrations of meropenem, to characterize the distribution of meropenem in the body. To obtain an understanding of the distribution of this drug in dogs, an in vivo ultrafiltration device was used to collect interstitial fluid (ISF), and the protein-free samples of ISF were then analyzed. Because of the potential for meropenem to be used to treat animals with urinary tract infections caused by resistant bacteria, another objective was to measure the concentration of meropenem in urine after SC administration.

MATERIALS AND METHODS

Animals—Five adult female Beagles (body weight, 8 to 11 kg) and 1 adult female Collie crossbred dog (body weight, 20 kg) were used in the study. The dogs were healthy as determined on the basis of results of physical examination and serum biochemical analysis. The study was reviewed and approved by the Institutional Animal Care and Use Committee at North Carolina State University.

Procedure—Dogs were randomly assigned to 2 groups (3 dogs/group). A randomized crossover design was used; thus, each dog received meropenem by each route of administration. Meropenem^a (50 mg/mL) was prepared in accordance with the

manufacturer's instructions. Dogs were given meropenem (20 mg/kg) as a single IV injection through a catheter inserted in a cephalic vein or as a single SC injection in the dorsal lumbar region. There was a 7-day washout period between treatments. Hair was clipped over the site of the SC injection to allow us to monitor signs of a localized injection reaction.

Collection of blood samples—A catheter was placed in a jugular vein of each dog 18 hours before the start of the experiment, and a catheter was placed in the cephalic vein of those dogs that were receiving meropenem via IV administration immediately before the start of the experiment. Blood samples were collected from the jugular vein into evacuated glass tubes that contained sodium heparin as the anticoagulant. Samples were collected immediately before administration (time 0) and 10, 20, and 40 minutes and 1, 2, 4, 6, 8, 12, 16, and 24 hours after administration. Blood samples were centrifuged at 1,000 X *g* for 10 minutes. Plasma was then harvested and stored at -70°C until analysis.

Collection of urine samples—Urine was collected from each dog 3 times after SC administration of meropenem (between 6 and 8 hours after administration, between 8 and 12 hours after administration, and between 12 and 24 hours after administration). Urine samples were obtained by midstream catch or cystocentesis. Aliquots (3 mL) were stored at -70°C until analysis.

Collection of tissue fluid—Tissue fluid was collected from SC tissues by use of an in vivo ultrafiltration sampling kit.^b The ultrafiltration probe contained 3 loops, each consisting of a semipermeable membrane that was 12 cm long. The 3 loops connected to a nonpermeable tube. The membrane in the loop consisted of pores that allowed water, electrolytes, and low-

molecular-weight molecules (< 30 kd) to pass. Therefore, the fluid collected was free of protein and other large-molecular-weight compounds.

A bleb of lidocaine was injected SC, and the ultrafiltration probe was inserted into the SC tissues of each dog by use of a guide needle. Once the probe was in place, the guide needle was removed. The semipermeable portion of the probe remained under the skin in the interstitial space, and the nonpermeable tube exited the skin of the dog and was attached to a 3-ml evacuated glass tube for collection of samples. The evacuated tube provided negative pressure for fluid collection. A new probe was inserted at another site, because it was necessary to combine fluid from the 2 probes to provide a sufficient volume of tissue fluid for analysis. Probes were removed immediately after collection of the final sample for each route of administration.

The ultrafiltration probes were inserted into the SC tissues 18 hours prior to the start of the experiment to allow fluid in the tissue space and the tube to reach equilibrium. Interstitial fluid was collected at time 0 and 1, 2, 4, 6, 8, 12, 16, and 24 hours after IV and SC administration. The fluid was immediately frozen at -70°C until analysis.

Analysis of meropenem concentrations—Plasma, tissue fluid, and urine samples were analyzed by reverse-phase high-performance liquid chromatography (HPLC) to determine concentrations of meropenem. Separation was achieved at 40°C by use of a C18, 4.6 X 150-mm reverse-phase column.^c This system included 2 pumps,^d a pump controller,^e an automated sampler,^f and a UV light detector. Solid-phase extraction^g was performed initially for plasma and urine samples (diluted 1:10 [vol:vol] with distilled water) followed by reverse-phase chromatography with UV detection at 296 nm. Samples of tissue fluid were analyzed directly by HPLC without extraction. The mobile phase consisted of 85% 0.01M

acetate buffer and 15% methanol (pH, 4.3) at a flow rate of 1 mL/min. Retention time for meropenem was between 4.4 and 4.6 minutes for plasma, between 4.4 and 4.9 minutes for tissue fluid, and between 4.7 and 4.8 minutes for urine. Calibration curves in the range of 0.1 to 100 µg/mL were prepared by use of pooled canine plasma, pooled canine urine, and PBS solution (pH, 7.38) prior to analyzing each set of plasma, urine, or tissue fluid samples, respectively. Minimum acceptable value for r^2 was 0.99. The assay was validated by use of a pure reference standard.^h Calibration curves and validation tests were all within 15% of the true concentration of meropenem.

Protein binding—Protein binding studies of plasma samples were performed by use of a micropartition device.ⁱ Four replicates of identical concentration were prepared by spiking pooled canine plasma with a stock solution of meropenem prepared from the pure reference standard. The replicates were incubated in a water bath (37°C for 30 minutes). One milliliter from each replicate was placed into the reservoir of each of 4 micropartition devices. The devices were centrifuged (1,500 X *g* for 30 minutes). Approximately 300 µL of ultrafiltrate was collected in the filtrate cup. Solid-phase extraction of the ultrafiltrate as described previously was performed prior to analysis by use of HPLC. The resulting concentrations represented the unbound fraction. A second set of 4 replicates of the same concentrations was analyzed (300 µL/replicate), except the micropartition step was omitted. These resulting concentrations represented the total (bound and unbound) fraction. The standard curve used to determine protein concentrations was prepared by use of 300 µL of spiked pooled canine plasma. Percentage of the bound fraction was calculated by use of the following equation:

$$\% \text{ protein binding} = \frac{\text{total concentration} - \text{unbound concentration}}{\text{total concentration}} \times 100$$

Pharmacokinetic analysis—Pharmacokinetic variables after IV administration were estimated by use of a computer software program.^j Data for IV administration was plotted with a weighting factor of $1/Y^2$, where Y is the predicted plasma concentration of meropenem. The model that provided the best fit for the data was determined by applying the Aikake information criterion.²⁶ Concentrations in tissue fluid were analyzed by use of noncompartmental modeling. Values for total body clearance, area under the curve from time 0 to infinity ($AUC_{0-\infty}$), half-life ($t_{1/2}$), mean residence time (MRT), and volume of distribution of the area during the elimination phase (Vd_{area}) were estimated by use of noncompartmental methods. The $AUC_{0-\infty}$ was estimated by use of the log-linear trapezoidal rule and extrapolated to infinity by use of the following equation:

$$AUC_{0-\infty} = C/\lambda_z,$$

where C is the concentration for the last time point and λ_z is the first-order rate constant associated with the terminal portion of the curve. Systemic availability (bioavailability [F]) was calculated by use of the following equation:

$$F = AUC_{SC}/AUC_{IV}$$

where AUC_{SC} is the area under the curve (AUC) calculated after SC administration and AUC_{IV} is the AUC after IV administration. The MRT was calculated by use of the statistical moment theory as follows:

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty}$$

where $AUMC_{0-\infty}$ is the area under the moment curve. Half-life was calculated using the formula:

$$t_{1/2} = 0.693/\lambda_z.$$

Distribution $t_{1/2}$, elimination $t_{1/2}$, microrate constants, intercepts, and rate constants were calculated from equations published elsewhere.²⁷ Microrate constants and intercepts were calculated for a 2-compartment model and averaged for 5 dogs.

Stability during storage—Stability of meropenem during refrigeration storage was evaluated. A solution (concentration of 10 $\mu\text{L/mL}$) was prepared and stored at 7°C for 53 days. An aliquot of the solution was obtained at 2-day intervals and analyzed for meropenem concentration.

Determination of meropenem dosing regimens—A computer simulation^j was used to determine meropenem dosing regimens for organisms commonly found in dogs and often associated with antimicrobial resistance.²⁸⁻³² Pharmacokinetic variables estimated in the study reported here for the 2-compartment (IV) and 1-compartment (SC) analysis were used for the simulation. Three dosing regimens were analyzed for Enterobacteriaceae (24 mg/kg, IV, q 12 h; 3 mg/kg, IV, q 8 h; 8 mg/kg, SC, q 12 h), and 2 dosing regimens were analyzed for other organisms (24 mg/kg, IV q 8 h and 12 mg/kg, SC, q 8 h). For these simulations, the MIC of meropenem for Enterobacteriaceae (0.12 $\mu\text{g/mL}$) and other organisms (1 $\mu\text{g/mL}$) were based on reports of numerous isolates from humans^{11,19,33,34} because of the lack of MIC information for meropenem in bacteria isolated from dogs.

Statistical analysis—Mixed-effects models with differing correlation structures were used to analyze the data.^k The most appropriate model was selected based on Aikake information criterion and Schwarz's Bayesian Criterion. A mixed model with 1-dependent correlation structure was selected. Also, estimated pharmacokinetic parameters (λ_z $t_{1/2}$ and $\text{AUC}_{0-\infty}$) were compared between routes of administration (IV and SC) for plasma and tissue fluid on

the basis of an ANOVA conducted on logarithmically transformed data. Values of $P \leq 0.05$ were considered significant.

RESULTS

We did not detect adverse effects in any of the dogs after IV or SC administration of meropenem. The SC injections did not appear to elicit a painful reaction, and the site of injections were not swollen or hot, and we did not elicit signs of pain when they were examined at hourly intervals coinciding with times of blood collection.

Plasma concentrations of meropenem after IV administration best fit a 2-compartmental model for 5 of 6 dogs and a 1-compartmental model for the remaining dog. A 1-compartment model with first-order input fit plasma concentrations of meropenem after SC administration in all dogs. Correlations were found among concentrations determined over time within dogs. Statistical results based on this model revealed that there were significant ($P < 0.001$) time-dependent effects for plasma and tissue fluid concentrations of meropenem after IV and SC administration. On the basis of results of the correlation test, there was a significant ($P < 0.007$) strong positive correlation between concentrations in tissue fluid and plasma for IV and SC administration (Table 4.1).

The limit of quantification (LOQ) and limit of detection (LOD) after IV administration were 0.016 and < 0.01 $\mu\text{g/mL}$, respectively, for plasma and 0.366 and 0.106 $\mu\text{g/mL}$, respectively, for tissue fluid. The LOQ and LOD after SC administration were 0.019 and < 0.01 $\mu\text{g/mL}$, respectively, for plasma and 0.43 and 0.126 $\mu\text{g/mL}$, respectively, for tissue fluid. These values are lower for plasma after both routes of administration, because plasma was concentrated 5-fold during the process of extraction and reconstitution as compared with tissue fluid, which was analyzed directly without extraction.

Stability of meropenem during refrigeration storage was evaluated. Concentrations remained within 90% of the original concentration (between 9 and 10 $\mu\text{L/mL}$) for 23 days. On day 41, the concentration measured was 7.50 $\mu\text{L/mL}$; however, by day 53, the concentration had decreased to 0.56 $\mu\text{L/mL}$. The acceptable concentration range for the meropenem injectable product is reportedly between 90 and 120% of the true concentration.³⁵

Plasma (total and free) and tissue fluid concentrations of meropenem after IV and SC administration were plotted against time on a semilogarithmic graph (Fig 4.1 and 4.2). Values above the LOD were detected, but only values above the LOQ were used in the pharmacokinetic analysis. At 8 hours after IV administration, 2 values for plasma were above the LOQ, but when averaged for all 6 dogs, the mean value was below the LOQ. Tissue fluid was not collected in 2 dogs until 2 hours after IV administration, compared to 1 hour for the remaining 4 dogs. Therefore, the maximum concentration (C_{max}) for those 2 dogs was lower because of dilution and contributed to a lower mean C_{max} when values from all 6 dogs were averaged.

Each ultrafiltration device contained tubing that was 46 cm in length and held 160 μL of fluid. After tissue fluid was collected from each dog, the amount of fluid in the 3-ml evacuated glass tube was measured and recorded. Mean rate of collection for all dogs was $2.3 \pm 0.5 \mu\text{L/min}$. Therefore, we calculated a lag time of 70 minutes for the tissue fluid to reach the evacuated tube. When we adjusted for this lag time, tissue fluid concentrations were almost identical to plasma concentrations (unbound fraction) after SC administration.

Meropenem was consistently detectable in the urine of all dogs at 8 hours but not 12 hours after administration. Mean concentration at 8 hours was 1,296 $\mu\text{g/mL}$ (range, 565 to

2,150 $\mu\text{g/mL}$). This was substantially higher than the mean plasma concentration at 8 hours (0.37 $\mu\text{g/mL}$; range, 0.1 to 1.39 $\mu\text{g/mL}$).

Results of the computer simulation for meropenem dosing regimens for organisms commonly found in dogs and often associated with antimicrobial resistance were analyzed. All dosing regimens used in the simulation resulted in meropenem concentrations above the respective MIC for longer than half of the dosing interval.

DISCUSSION

Meropenem is a carbapenem β -lactam antimicrobial with activity against infection-causing organisms that are resistant to other more commonly administered antimicrobial agents. Results from the IV and SC experiments reported here provide pharmacokinetic information to guide dosing regimens for either route of administration and to help clinicians and researchers understand the disposition of meropenem in dogs.

In healthy humans given 1 g of meropenem, IV, during a period of 2 or 3 minutes,³⁶ values for $t_{1/2}$ (mean $\pm$ SD; 0.93 ± 0.12 and 0.93 ± 0.10 minutes, respectively), volume of distribution at steady state ($V_{d_{ss}}$; mean; 0.21 and 0.21 L/kg, respectively), and clearance (mean; 3.16 and 3.10 mL/min/kg, respectively) were reported. The plasma results obtained in the study reported here for dogs administered meropenem, IV, during a period of 1 minute also showed a short $t_{1/2}$ (0.67 ± 0.07 minutes), a low $V_{d_{ss}}$ (0.337 ± 0.052 L/kg), and high clearance (6.53 ± 1.51 mL/min/kg). The mean value obtained for our dogs for $V_{d_{ss}}$ (0.337 L/kg) indicated that distribution was primarily limited to the extracellular space. Similar to the results in humans,³⁶ we did not observe adverse drug effects in the dogs of our report after an IV bolus injection. We did not detect a significant difference in AUC for plasma after IV (53.29 ± 12.04 [$\mu\text{g} \cdot \text{h}$]/mL) and SC (63.42 ± 14.24 [$\mu\text{g} \cdot \text{h}$]/mL) administration in our dogs,

and this could be explained on the basis of the high bioavailability of the drug (84%). Values for $t_{1/2}$ in tissue fluid for IV and SC routes (1.15 ± 0.57 and 1.31 ± 0.54 hours) did not differ significantly ($P = 0.076$) from values for plasma (0.67 ± 0.07 and 0.98 ± 0.21 hours, respectively). There was a significant ($P = 0.002$) difference in $t_{1/2}$ between IV and SC routes for plasma, but there was not a significant ($P = 0.852$) difference for $t_{1/2}$ between IV and SC routes for tissue fluid. Mean drug concentrations after IV administration remained > 1 $\mu\text{g/mL}$ for more than 6 hours in tissue fluid and slightly less than 4 hours in plasma.

Meropenem is eliminated by metabolism and excretion, with up to 79% of the administered dose eliminated unchanged in the urine in humans and the rest excreted as an inactive metabolite.^{25,37} Mean clearance (6.53 ± 1.51 mL/min/kg) reported here was higher than the glomerular filtration rate reported for dogs in another study (4 mL/min/kg).³⁸ This can be explained by tubular secretion in addition to glomerular filtration being responsible for elimination of the drug, which is typical for β -lactams. These findings are consistent with reports^{16,37,39-40} in humans and are supported by the reduction of renal clearance equivalent to glomerular filtration after coadministration of probenecid.^{16,37,41} Urine samples were collected once during each of 3 intervals after SC administration. The samples obtained from all dogs during the interval between 6 and 8 hours had concentrations >1 $\mu\text{g/mL}$. These results also indicated that a DHP-1 inhibitor, such as cilastatin, is not required to prevent hydrolysis in dogs, which is contrary to the situation for imipenem.

Concentrations of meropenem in tissue fluid and plasma were strongly correlated ($P < 0.001$). This can be explained by the rapid distribution of meropenem to the interstitial space. Geometry of a compartment is described by the ratio of surface area to volume, and this determines the rate and extent of equilibrium established.⁴² With a larger ratio for surface

area to volume, a greater correlation between plasma and tissue fluid should be expected.⁴² The interstitial space is considered a compartment with a large surface area-to-volume ratio. After correcting for the lag time for collection of tissue fluid, analysis revealed that tissue fluid had similar peak concentrations, time to peak concentration, and $t_{1/2}$, compared with values for plasma (Fig 4.1 and 4.2). Although protein binding (11.87%) in the dogs of our report was higher than that reported for humans (2%)¹ the value is considered low when compared with values for highly bound drugs. Pharmacokinetic characteristics of meropenem include a short $t_{1/2}$, high clearance, and low volume of distribution. Based on these characteristics, this drug would be expected to reach equilibrium quickly between tissue and plasma, and concentrations in tissue fluid (ie, ISF) should resemble concentrations in unbound plasma. The results from the study reported here confirm these assumptions, and this information can be used to predict ISF concentrations of other drugs with similar pharmacokinetic and physicochemical properties.

Concentrations and pharmacokinetic variables reported here were determined in healthy dogs. In a study⁴³ in which investigators evaluated concentrations in plasma and tissue fluid (via microdialysis) in healthy and seriously ill human patients, there was little difference in plasma concentrations or plasma AUC values between the 2 groups. However, when drug concentrations in tissue fluid and diffusion rate constants were compared, seriously ill patients had a significantly lower drug concentration in tissue fluid and slower rate of tissue distribution than did healthy patients. Because concentrations in tissues of seriously ill human patients were less than the targeted MIC for most of the dosing interval, the author of that study⁴³ suggested that making dosage adjustments on the basis of drug concentrations in plasma may be misleading. Additional studies are necessary to evaluate the

effect of disease on tissue distribution and pharmacokinetic variables in dogs. Our study reported here provides a method whereby this could be examined.

The ultrafiltration probes were easily implanted, and HPLC analysis of tissue fluid did not require an extraction step. Analyses of results reported in this study indicate that ultrafiltration is a reliable, easy, and useful method for the evaluation of drug disposition in dogs. It could potentially replace surgical collection of tissue samples, which often requires euthanizing animals to enable investigators to obtain tissue samples, or the use of tissue cages for estimating concentrations in tissues. Moreover, we believe that this method of sample collection of ISF is more representative of drug concentrations needed to counteract bacterial infections, compared with results obtained by use of whole-tissue homogenates or artificial tissue cages.

To make use of pharmacokinetic variables, correlation with in vitro MIC pharmacodynamic variables can be used to derive optimum dosages. The β -lactam antimicrobials kill bacteria in a time-dependent fashion; therefore, the amount of time for which concentrations are greater than the MIC is the most important factor when considering a dosing regimen.⁴⁴ Some recommendations have suggested administering β -lactam antimicrobials as a continuous infusion to achieve this goal.⁴⁵ Carbapenem antimicrobials differ from other β -lactams, because they have a postantibiotic effect for gram-negative and gram-positive bacteria (ie, there is continued inhibition of bacterial growth after the drug concentration decreases to less than the MIC).⁴⁵⁻⁴⁷ Duration of the postantibiotic effect depends on the dose, duration of antibiotic exposure, and organism.⁴⁸⁻⁴⁹ The required amount of time concentrations need to be above the MIC for efficacy is lower for carbapenems (33 to 40%), compared with other β -lactam antimicrobials (50 to 60%).^{17,50} In addition, bacterial

MICs for meropenem are lower than for other β -lactams, which allows use of lower doses and intermittent dosing of these antimicrobials.⁴⁶

In a report⁵¹ by the National Committee for Clinical Laboratory Standards, break points for imipenem and meropenem in humans were ≤ 4 mg/L (susceptible), 8 mg/L (intermediate), and ≥ 16 mg/L (resistant). However, published MIC values are often much lower.^{11,19,33,34} In a study²¹ in which investigators projected the plasma concentration-versus-time curve onto the MICs for *Streptococcus pyogenes*, *Neisseria meningitides*, *S pneumoniae*, *N gonorrhoeae*, *Shigella* spp, *Salmonella* spp, *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, *Listeria monocytogenes*, *Enterobacter cloacae*, and *Bacteroides fragilis*, it was revealed that meropenem administered to children at a dosage of 20 mg/kg every 8 or 12 hours was sufficient for all organisms. In that same study,²¹ patients with infections attributable to *P aeruginosa* was effectively treated by use of dosing at 8-hour intervals.

Escherichia coli, *Klebsiella* spp, and *Staphylococcus* spp are the bacteria most commonly cultured from canine urine.⁵² Antimicrobial resistance to these and other organisms can pose a therapeutic challenge. Two important therapeutic uses of meropenem would be for treating infections caused by bacteria resistant to other antimicrobials and to replace the use of multiple antimicrobials for animals with mixed infections. This would potentially decrease duration of hospitalization, reduce the use of other drugs to which resistance may develop (eg, fluoroquinolones), and decrease the use of potentially toxic drugs (eg, aminoglycosides).

We documented that meropenem has excellent penetration of tissue fluid, good concentrations in urine, and low protein binding, and it is easy to administer without adverse

effects. Because meropenem is eliminated through the renal system and has a postantibiotic effect, and the amount of time that concentrations need to be greater than the MIC is only 33 to 40% of the dosing interval, infections of soft tissues or the urinary tract that are attributable to bacteria that have a MIC of 0.12 µg/mL could be treated with dosages as low as 8 mg/kg, SC, q 12 h. More resistant infections caused by organisms such as *Pseudomonas* spp with a MIC of 1.0 µg/mL may require higher dosages of 12 mg/kg, SC, q 8 h.

FOOTNOTES

^aMerrem, Astra Zeneca, Wilmington, Del.

^bCanine Ultrafiltration Probe, Bioanalytical Systems Inc, West Lafayette, Ind.

^cZorbax SB-C18, Agilent Technologies, Wilmington, Del.

^dWaters models 590 and 591, Waters, Franklin, Mass.

^eWaters automated gradient controller, Waters, Franklin, Mass.

^fAgilent Series 1050, Agilent Technologies, Wilmington, Del.

^gSpec Plus C18AR column filters, Ansys Diagnostics, Lake Forest, Calif.

^hProvided by Astra-Zenica, Wilmington, Del.

ⁱCentrifree, Millipore, Bedford, Mass.

^jWinNonlin, version 3.1, Pharsight, Cary, NC.

^kSAS, version 8.2, SAS Institute Inc, Cary, NC.

^lMeropenem package insert, Zeneca Pharmaceuticals USA, Wilmington, Del

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Table 4.1. Mean $\pm$ SD values for pharmacokinetic variables of meropenem after IV and SC administration (20 mg/kg) to 6 dogs

Variable	IV		SC	
	Plasma	Tissue fluid	Plasma	Tissue fluid
C _{max} ($\mu\text{g/mL}$)	NA	24.32 $\pm$ 8.93	24.56 $\pm$ 6.13	10.95 $\pm$ 0.99
AUC _{0-infinity} ($\text{h} \cdot \mu\text{g}$)/mL	53.29 $\pm$ 12.04	73.71 $\pm$ 14.56	63.42 $\pm$ 14.24	43.22 $\pm$ 14.39
AUMC _{0-infinity} ($\text{h} \cdot \text{h} \cdot \mu\text{g}$)/mL	47.76 $\pm$ 16.31	183.64 $\pm$ 67.98	138.25 $\pm$ 52.83	199.26 $\pm$ 95.78
Cl _T (mL/min/kg)	6.53 $\pm$ 1.51	NA	NA	NA
Vd _{ss} (L/kg)	0.337 $\pm$ 0.052	NA	NA	NA
Vd _{area} (L/kg)	0.372 $\pm$ 0.053	NA	NA	NA
T _{max} (h)	NA	2	0.67 $\pm$ 0.21	3.67 $\pm$ 0.82
λ_z (/h)	1.05 $\pm$ 0.11	0.70 $\pm$ 0.26	0.74 $\pm$ 0.17	0.587 $\pm$ 0.17
λ_z t _{1/2} (h)	0.67 $\pm$ 0.07	1.15 $\pm$ 0.572	0.98 $\pm$ 0.21	1.31 $\pm$ 0.54
t _{1/2α} (h)*	0.08 $\pm$ 0.05	NA	NA	NA
t _{1/2β} (h)*	0.69 $\pm$ 0.08	NA	NA	NA
K ₀₁ t _{1/2} (h)	NA	NA	0.68 $\pm$ 0.18	NA
K ₁₀ t _{1/2} (h)	0.27 $\pm$ 0.18	NA	0.78 $\pm$ 0.23	NA
MRT (h)	0.88 $\pm$ 0.13	2.44 $\pm$ 0.42	2.14 $\pm$ 0.38	4.48 $\pm$ 0.79
F (%)	NA	NA	84.037	NA
K ₁₀ (/h)	4.54 $\pm$ 3.94	NA	0.88 $\pm$ 0.23	NA
K ₁₂ (/h)*	5.31 $\pm$ 4.51	NA	NA	NA
K ₂₁ (/h)*	3.12 $\pm$ 0.57	NA	NA	NA
K ₀₁ (/h)	NA	NA	1.22 $\pm$ 0.52	NA
A ($\mu\text{g/mL}$)*	218.64 $\pm$ 223.45	NA	NA	NA
B ($\mu\text{g/mL}$)*	46.50 $\pm$ 8.14	NA	NA	NA
α (/h)*	11.94 $\pm$ 8.02	NA	NA	NA
β (/h)*	1.03 $\pm$ 0.13	NA	NA	NA
Protein binding (%)	11.87	NA	NA	NA

*Calculated by use of a 2-compartment model and averaged from 5 dogs.

C_{max} = Maximum concentration. AUC_{0-infinity} = Area under the curve from time 0 to infinity, AUMC_{0-infinity} = Area under the first moment curve from time 0 to infinity. Cl_T = Total body clearance. Vd_{ss} = Volume of distribution at steady state. Vd_{area} = Volume of distribution of the area during the elimination phase. T_{max} = Time until maximum concentration. λ_z = First-order rate constant of terminal portion of the curve. λ_z t_{1/2} = Half-life of the terminal portion of the curve. t_{1/2 α} = Distribution half-life. t_{1/2 β} = Elimination half life. K₀₁ t_{1/2} = Half-life of absorption phase. K₁₀ t_{1/2} = Half-life of elimination phase. MRT = Mean residence time. F = Bioavailability. K₁₀ = elimination rate from compartment 1. K₁₂ = rate of movement from compartment 1 to compartment 2. K₂₁ = rate of movement from compartment 2 to compartment 1. K₀₁ = absorption rate. A = Intercept for elimination phase. B = Intercept for distribution phase. α = Rate constant associated with distribution phase. β = Rate constant associated with elimination phase. NA = Not applicable for the route of administration.

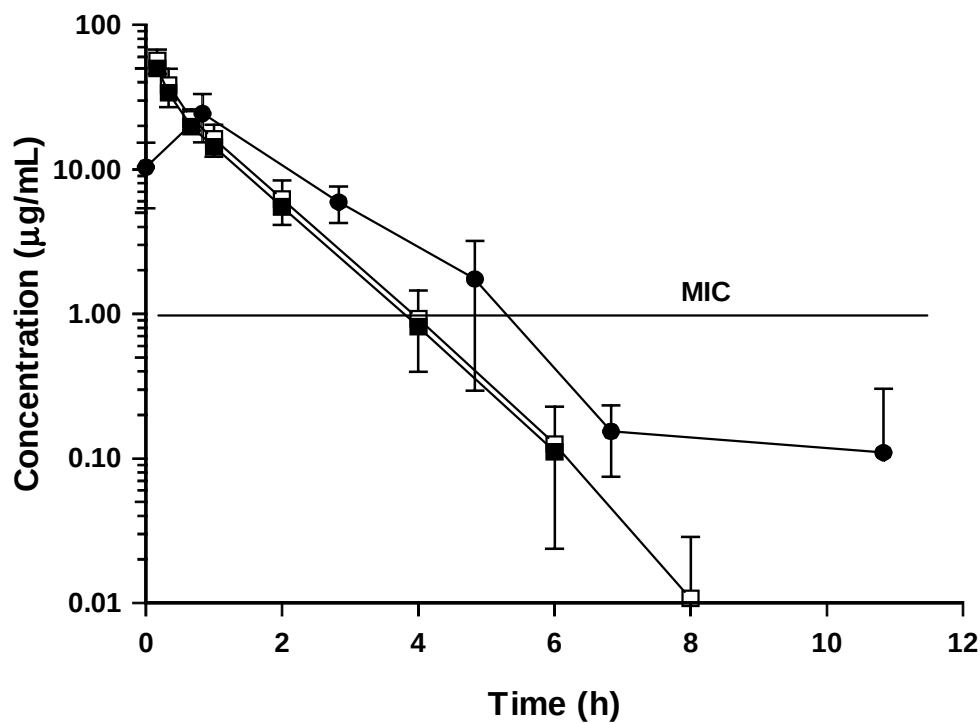


Figure 4.1. Mean $\pm$ SD concentrations of meropenem in plasma (total [open square] and free [solid square]) and tissue fluid (solid circle) concentrations after IV administration of a single bolus (20 mg/kg). Values for tissue fluid have been corrected for the lag time (70 minutes) necessary for collection into an ultrafiltration device. MIC = Minimum inhibitory concentration.

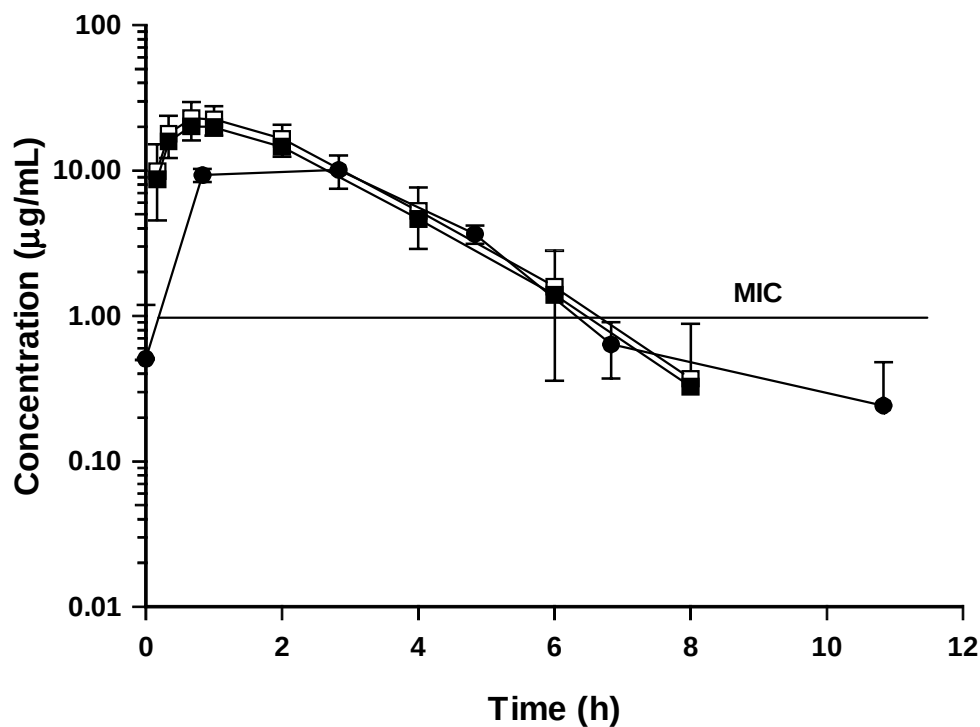


Figure 4.2. Mean $\pm$ SD concentrations of meropenem in plasma (total [open square] and free [solid square]) and tissue fluid (solid circle) concentrations after SC administration (20 mg/kg). Values for tissue fluid have been corrected for the lag time (70 minutes) necessary for collection into an ultrafiltration device.

Comparison of plasma and interstitial fluid concentrations of doxycycline and meropenem following constant rate intravenous infusion in dogs

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2003

Published in American Journal of Veterinary Research, 64 (8), 1040-104

ABSTRACT

Objective—To compare plasma (total and unbound) and interstitial fluid (ISF) concentrations of doxycycline and meropenem in dogs following constant rate IV infusion of each drug.

Animals—6 adult Beagles

Procedure—Dogs were given a loading dose of doxycycline and meropenem followed by a constant rate IV infusion of each drug to maintain an 8-hour steady state concentration. Interstitial fluid was collected with an ultrafiltration device. Plasma and ISF were analyzed by high performance liquid chromatography. Protein binding and lipophilicity were determined. Plasma data were analyzed by use of compartmental methods.

Results—Compared with meropenem, doxycycline had higher protein binding (11.87% [previously published value] vs 91.75 ± 0.63 %) and lipophilicity (partition coefficients, 0.02 ± 0.01 vs 0.68 ± 0.05). A significant difference was found between ISF and plasma total doxycycline concentrations. No significant difference was found between ISF and plasma unbound doxycycline concentrations. Concentrations of meropenem in ISF and plasma (total and unbound) were similar. Plasma half-life, volume of distribution, and clearance were 4.56 ± 0.57 hours, 0.65 ± 0.82 L/kg, 1.66 ± 2.21 mL/min/kg, respectively, for doxycycline and 0.73 ± 0.07 hours, 0.34 ± 0.06 L/kg, 5.65 ± 2.76 mL/min/kg, respectively, for meropenem. The ISF half-life of doxycycline and meropenem was 4.94 ± 0.67 and 2.31 ± 0.36 hours, respectively.

Conclusions and Clinical Relevance—The extent of protein binding determines distribution of doxycycline and meropenem into ISF. As a result of high protein binding, ISF doxycycline concentrations are lower than plasma total doxycycline concentrations.

Concentrations of meropenem in ISF can be predicted from plasma total meropenem concentrations.

INTRODUCTION

Successful antimicrobial therapy is dependent on achieving adequate drug concentrations at the site of infection for a sufficient duration, susceptibility of the organism to the antimicrobial, pharmacokinetic parameters of the drug, and the local environment. The ultimate goal of antimicrobial therapy is to obtain a clinical and bacteriologic cure without causing toxic effects or the development of resistant bacteria.¹ Most bacterial infections are in the extracellular space of tissues; therefore, to achieve therapeutic success, antimicrobial agents must be able to distribute adequately to this region.² The distribution of most drugs in the body obey Fick's law of diffusion where the rate of movement (flux) is dependent on the concentration gradient, diffusion coefficient, partition coefficient, and thickness of the membrane. Because this is a linear, or first order process, the rate of drug movement is dependent on the dose. Only small, unbound, nonionized, and lipophilic molecules can diffuse cross most cell membranes. For drugs that are protein bound, the total concentration (bound and unbound) is higher in plasma than in tissue fluid (ie, interstitial fluid [ISF]), but the unbound drug equilibrium across the capillary membrane produces equal amounts of free (unbound) drug concentrations in both compartments.³ Because only the unbound drug is microbiologically active, determining the concentrations of unbound drug at the active site is important for the evaluation of clinical efficacy.⁴

Therapeutic drug monitoring and plasma minimum inhibitory concentrations (MICs) do not account for the drug fraction bound to proteins; in addition, the total drug concentrations are usually measured in pharmacokinetics studies to predict therapeutic activity. Use of plasma total antimicrobial concentrations for predicting pharmacokinetic-pharmacodynamic relationships for some drugs may be misleading and could result in

overestimation of therapeutic efficacy.⁵ Low unbound drug concentrations at the site of action could result in a subtherapeutic effect and increase the risk for the development of antimicrobial resistance.⁵

For drugs with low plasma protein binding, the plasma total drug concentration is an accurate representation of the drug concentration at the site of action in the ISF; we have demonstrated this in a single dose study with a low protein bound β -lactam.⁶ For highly protein bound drugs, the plasma total drug concentration, without accounting for the degree of protein binding, may result in an inaccurate assessment of the drug concentration at the site of action (ie, in the ISF).⁵ Measuring the unbound concentration of these drugs at the target site (ie, in the ISF) may be a better predictor of therapeutic efficacy than plasma concentrations.⁵ To achieve a better understanding of the dynamics of drug distribution we chose to evaluate doxycycline and meropenem because they differ in their physicochemical properties, pharmacokinetic parameters, and protein binding.

Doxycycline is a second-generation tetracycline derivative and its advantages stem from having a broad spectrum of activity, formulations for oral and IV administration, limited adverse effects, good oral absorption, and being relatively inexpensive. It is a broad spectrum antimicrobial with activity against gram negative, gram positive, and anaerobic bacteria, and many intracellular organisms.^{7,8} Doxycycline's high lipophilicity is responsible for better fluid and tissue penetration, longer half-life ($t_{1/2}$), increased oral absorption, and enhanced in vitro antimicrobial activity, compared with the first generation tetracyclines.^{9,10} Doxycycline is extensively protein bound in most species.^{9,11-14} Rapid administration of doxycycline has been associated with adverse effects in some species and is thought to be the result of the chelation of calcium.¹⁵

Meropenem is a member of the carbapenem family of β -lactam antimicrobials and the pharmacokinetics and ISF concentrations from a single IV and SC administration have recently been published.⁶ Its plasma protein binding was 11.87% in that study.⁶

With a single bolus administration of drugs by any route, a lag time for drug distribution to the target site exists, making the comparison between plasma and ISF concentrations difficult. Therefore, to accurately evaluate the dynamics of drug distribution to tissues, steady state plasma concentrations are necessary by administering a constant rate IV infusion (CRI). Equilibrium between plasma and ISF can then be maintained during the IV infusion period, and samples from each compartment can be collected, analyzed, and compared pharmacokinetically. By administering a CRI, the variability and influence of the $t_{1/2}$ can be eliminated, thereby allowing for a comparison between the 2 drugs.

In a previous study,⁶ we showed that an in vivo ultrafiltration device could be used to collect ISF allowing an evaluation of the relationship between drug concentrations in plasma and ISF. The in vivo ultrafiltration device provided advantages over tissue biopsy specimens and tissue cages in the measurement of ISF concentrations in animals. Advantages of the ultrafiltration device are that subcutaneous implantation of the semipermeable probes is minimally invasive and it allows continuous collection of protein-free ISF from conscious animals. The purpose of the study reported here was to evaluate plasma (total and unbound) and ISF concentrations of 2 antimicrobials that differ in the extent of protein binding and lipophilicity and determine the effect that each of these characteristics has on the distribution between plasma and the target site (ie, the ISF) at steady state.

MATERIALS AND METHODS

Animals—Six (3 male and 3 female) healthy adult Beagles (body weight, 6 to 9 kg) were used in this study. The study was reviewed and approved by the Institutional Animal Care Use Committee at North Carolina State University. Each of the dogs was determined to be clinically normal prior to the start of the study on the basis of physical examination findings and results of CBC determination and serum biochemical analysis.

Procedure—A random crossover design approach was used where the dogs were randomly assigned to 2 groups of 3. All dogs received both treatments. A 7-day wash out period was provided between treatments. Doxycycline^a and meropenem^b were prepared as solutions (10 mg/mL) with sterile water the morning of the study. A loading dose of doxycycline (1.01 mg/kg) or meropenem (0.37 mg/kg) was administered immediately before initiating the 8-hour CRI (0.10 mg/kg/h and 0.38 mg/kg/h for doxycycline and meropenem, respectively). The loading dose for doxycycline was administered over 5 minutes, and the loading dose for meropenem was given as a bolus injection. The CRI was administered by use of a programmed electronic infusion pump^c to maintain a target concentration of 1 µg/mL during the 8-hour period. The CRI and loading dose were calculated from previously published pharmacokinetic values for dogs^{6,12} by use of established equations.¹⁶

Collection of blood samples—Two jugular catheters were placed in each dog, 1 for blood collection and 1 for administration of the loading dose and CRI. Catheters were placed 18 hours prior to drug administration and flushed with sterile saline (0.9% NaCl) solution between collection times. Blood was collected from the jugular vein in evacuated glass tubes, with sodium heparin as the anticoagulant. Sample collection times were at 0 (pretreatment time), 10, 20, 40 minutes and 1, 2, 4, 6, 8, 12, 16, 20, 24, and 28 hours during

and after the CRI for both drugs. Blood was centrifuged after collection at $1,000 \times g$ for 10 minutes and the plasma separated and stored at -70°C until analysis.

ISF collection—Interstitial fluid collection was performed with an in vivo ultrafiltration device.^d The 3 semipermeable loops of the ultrafiltration probe were inserted subcutaneously into the interstitial space with a guide needle, and the external tubing connected to a 3-mL evacuated glass tube for collection of ISF. Lidocaine (0.3 mL) was injected at the insertion point of the guide needle. A new probe at a separate site was used for each drug and administration route. Ultrafiltration probes were placed 18 hours prior to the start of the study to allow fluid in the interstitial space and the ultrafiltration probes to reach equilibrium. The membrane in the loops consists of pores allowing water, electrolytes, and low molecular weight molecules ($< 30\,000\text{ d}$) to pass. The evacuated glass tube provided the negative pressure for collection of ISF. The ISF was collected at 0 (pretreatment time), 2, 4, 6, 8, 12, 16, 20, 24, and 28 hours during and after the CRI. Interstitial fluid was collected and combined from 2 ultrafiltration probes in order to have sufficient volume for analysis. The fluid was immediately frozen at -70°C until analysis. Ultrafiltration probes were removed after the last collection time.

Protein binding—Plasma protein binding of meropenem was determined in a previous study⁶ by the same method described here. Determination of plasma protein binding of doxycycline was performed with a micropartition device.^e Three replicate concentrations (0.5, 1, and $2\text{ }\mu\text{g/mL}$) were prepared by spiking pooled canine plasma with an analytical reference standard^f and incubating them in a 37°C water bath for 30 minutes. One milliliter from each replicate was placed into a micropartition device reservoir. Devices were centrifuged for 30 minutes at $1500 \times g$. Approximately $300\text{ }\mu\text{L}$ of ultrafiltrate was recovered

in the filtrate cup. The doxycycline ultrafiltrate was processed and extracted as described for plasma prior to analysis by high performance liquid chromatography (HPLC). Resulting concentrations represent the unbound fraction. A second set of 3 spiked replicates of the same concentrations was analyzed (300 μ L), omitting the micropartition step. Resulting concentrations represent the total (bound and unbound) fraction. Concentrations were determined from a standard curve prepared with 300 μ L of spiked plasma concentrations. Percentage of the bound fraction was calculated by use of the following equation:

$$\% \text{ protein binding} = \frac{\text{total concentration} - \text{unbound concentration}}{\text{total concentration}} \times 100$$

Drug lipophilicity—To determine the lipid solubility of the 2 drugs, the octanol:water coefficient was determined by use of established methods.^{17,18} A 0.1M sodium diphosphate buffer solution (aqueous phase) was prepared by use of sodium diphosphate^g and deionized water. The pH was adjusted to 7.4 by adding a small amount of phosphoric acid.^h Five milliliters of this aqueous buffer solution was spiked with meropenem or doxycycline (10 μ g/mL) and added to an equal volume of octanolⁱ in a screw top tube. The tube was gently rocked for 1 hour at room temperature (approx 25°C) to equally disperse the drug into each phase of the tube. The tube was then centrifuged for 10 minutes at 2,000 $\times g$ for phase separation. The aqueous layer was analyzed by HPLC without extraction before and after incubation and shaking. A calibration curve prepared from spiked aqueous phase was used to determine the concentrations. The apparent partition coefficient (PC) was calculated by use of the following equation:

$$PC = \frac{\text{buffer concentration before incubation} - \text{buffer concentration after incubation}}{\text{buffer concentration after incubation}} \times \frac{\text{volume of buffer}}{\text{volume of octanol}}$$

Analysis—Plasma and ISF were analyzed by reverse phase HPLC with ultraviolet (UV) detection to determine the concentrations of meropenem and doxycycline. This system included 2 pumps,^j a pump controller,^k automated sampler,^l and an UV light detector. For meropenem, a 4.6 mm × 150 mm reverse phase column^m was used for the separation. Solid phase extractionⁿ was used to prepare plasma samples followed by reverse phase chromatography with UV detection at 296 nm. The mobile phase consisted of 85% 0.01M acetate buffer and 15% methanol at pH 4.3 and a flow rate of 1 mL/min. For doxycycline, plasma samples were prepared by first adding a releasing agent (78% water, 20% acetonitrile, 2% phosphoric acid) and vortexing the mixture. The mixture was then transferred into centrifugal filter units^o for extraction. Filter units were centrifuged at 10,000 × g for 30 minutes. The supernatant was then analyzed by HPLC with UV detection at 350 nm by use of a 4.6 mm × 150 mm reverse phase column.^p The ISF samples and fluid from the lipophilicity study for both drugs were analyzed by HPLC without extraction. Pure reference standards of each drug^{f,q} were used to prepare calibration and quality control samples. Pooled blank canine plasma and PBS solution were used for the plasma and ISF calibration curves, as well as for validation of the assay.

Pharmacokinetic analysis—A computer software program^r was used to estimate the plasma and ISF pharmacokinetic parameters. Plasma data were analyzed by compartmental methods, and the model that best fit the data was determined by use of the minimal Aikake information criterion estimation method.¹⁹ A 2 compartmental model with input for an IV bolus was used to analyze the plasma data for IV administration of doxycycline. A

weighting factor of the reciprocal of the predicted concentrations squared was used. A continuous rate infusion noncompartmental model was used to analyze the ISF data for both drugs and the plasma data for meropenem. For doxycycline, clearance from the central compartment (Cl), elimination $t_{1/2}$ ($t_{1/2\beta}$), distribution $t_{1/2}$ ($t_{1/2\alpha}$), apparent volume of distribution at steady state (Vd_{ss}), microconstants (ie, elimination rate from compartment 1 [K10]; rate of movement from compartment 1 to compartment 2 [K12]; rate of movement from compartment 2 to compartment 1 [K21]), intercept for the distribution phase (A), intercept for the elimination phase (B), rate constant associated with the distribution phase (α), rate constant associated with elimination phase (β), and area under the curve (AUC) were calculated for the compartmental model by use of published equations.¹⁶ The ISF $t_{1/2}$ was calculated with the following formula:

$$t_{1/2} = 0.693/\lambda_z$$

where λ_z is the rate constant associated with the terminal portion of the curve.

The mean residence time (MRT), which is the mean time that all molecules in a given dose spend in the body, was calculated with the following formula:

$$MRT = AUMC_{0 \rightarrow \infty} / AUC_{0 \rightarrow \infty}$$

where $AUC_{0 \rightarrow \infty}$ is the AUC from time 0 to infinity and $AUMC_{0 \rightarrow \infty}$ is the area under the moment curve from time 0 to infinity. The AUC for ISF was estimated with the log-linear trapezoidal rule and extrapolated to infinity by use of CT/λ_z , where CT is the last measured time concentration value.

Because of the limited data points during elimination, the AUC for CRI plasma meropenem concentration was calculated by determining the partial AUC (AUC_{par}) between 1 and 8 hours during the steady state period by use of the trapezoidal method. The Cl and Vd_{ss} of meropenem after the CRI was then calculated with the following formulas

$$Cl = \text{dose} / AUC_{par}$$

$$Vd_{ss} = 1.44 R_o t_{1/2} / C_{ss}$$

where R_o is the infusion rate and C_{ss} is the average concentration at steady state. The maximum concentration (C_{max}) and the time to maximum concentration (T_{max}) were determined directly from the concentration vs time curves for ISF.

Statistical analysis—Plasma unbound and total concentrations of doxycycline and meropenem were compared with ISF concentrations of doxycycline and meropenem by use of mixed effect models with different correlation structures.⁵ By comparing the Aikake information criterion and the Schwarz bayesian criterion values, the mixed model with 1-dependent correlation structure was selected.

RESULTS

Pharmacokinetic parameters for doxycycline and meropenem were estimated (Table 5.1 and 5.2). Because of the short $t_{1/2}$ and limit of detection (LOD), the last detectable plasma concentration of meropenem was at 12 hours. Therefore, we were only able to estimate the plasma $t_{1/2}$ for meropenem from 8-hour and 12-hour plasma concentrations.

The limit of quantification (LOQ) and LOD in plasma after CRI were 0.025 $\mu\text{g/mL}$ and $< 0.010 \mu\text{g/mL}$, respectively, for doxycycline and 0.016 $\mu\text{g/mL}$ and $< 0.010 \mu\text{g/mL}$,

respectively, for meropenem. The LOQ and LOD in ISF after CRI were 0.014 $\mu\text{g/mL}$ and $< 0.010 \mu\text{g/mL}$, respectively, for both drugs. Only values greater than the LOQ were used in the pharmacokinetic analysis.

Plasma (total and unbound) and ISF concentrations of doxycycline and meropenem after CRI were plotted against time (Fig 5.1 and 5.2). Because plasma unbound concentrations of doxycycline and meropenem were determined mathematically, by taking into account the extent of protein binding ($91.75 \pm 0.63\%$ for doxycycline and $11.87\%^6$ for meropenem) of the total concentrations and not through direct measurement, no SD for these mean values were included in the graphs. After administration of the loading dose or CRI of either drug, no adverse reactions were observed.

The length of the tubing connecting the ultrafiltration probe to the evacuated glass tube was custom made to a length of 11 cm by the manufacturer and held 38 μL of fluid. After ISF was collected from each dog, the amount of fluid and the duration of collection period were recorded. The mean rate of collection was $1.63 \pm 0.34 \mu\text{L/min}$. A lag time of 23 minutes was therefore determined for the collection of ISF and used to synchronize plasma and ISF samples. The ISF concentrations were adjusted for the 23-minute lag time (Fig 4.1 and 4.2) and the T_{max} values are reported as adjusted and not adjusted (Tables 1 and 2). A significant ($P < 0.001$) difference was found between ISF and plasma total doxycycline concentrations. No significant ($P = 0.395$) difference was found between ISF and plasma unbound doxycycline concentrations. Concentrations of meropenem in ISF and in plasma (total and unbound) were similar. Determination of protein binding and lipophilicity revealed that, compared with meropenem, doxycycline has higher plasma protein binding

(11.87%⁶ vs 91.75 ± 0.63 %) and lipophilicity (partition coefficients, 0.02 ± 0.01 vs 0.68 ± 0.05).

DISCUSSION

The goal of our study was to evaluate the effect of high protein binding and lipophilicity (doxycycline) versus low protein binding and lipophilicity (meropenem) on the drug distribution between plasma and ISF at steady state. Meropenem pharmacokinetic parameters and protein binding have been estimated in a previously published study⁶ by the authors.

The type of capillary bed influences the distribution of antimicrobials to the extravascular site.²⁰ Most capillary beds in the body are porous, allowing for free diffusion of molecules ≤ 1000 d across the capillaries into the ISF of the extracellular space.^{20,21} The CNS, retina, and prostate gland are examples of regions with nonporous capillary beds. The rate-limiting step for penetration to these nonporous tissues is lipophilicity because diffusion is transcellular.^{20,21} But for other tissues, protein binding limits passage across the membrane. Because the unbound fraction is responsible for microbiological activity and clinical efficacy, therapeutic failure of infections in the ISF treated with highly bound drugs ($> 90\%$ binding) can occur as a result of the unbound concentrations being less than the MIC for the organism.²² Merrikin et al⁴ evaluated the efficacy of several β -lactam antimicrobials against *Staphylococcus aureus* that were similar in MIC values and pharmacokinetic parameters but differed in the degree of protein binding. A nonlinear relationship existed between the extent of protein binding and the antimicrobial activity. When the concentration of unbound drug decreased to < 10 to 20% , the efficacy decreased substantially.⁴

In our study, doxycycline and meropenem were chosen to compare the plasma pharmacokinetics and tissue distribution at steady state, because they differ in physicochemical properties that have been cited as important for drug distribution (Table 5.3).^{23,24} Plasma protein binding was 91.75 ± 0.63 % for doxycycline and 11.87% for meropenem.⁶ Lipophilicity for doxycycline was higher (partition coefficient, 0.68 ± 0.05) than for meropenem (partition coefficient, 0.02 ± 0.01). Therefore, an aim of our study was to determine which of these factors, lipophilicity or protein binding, has a greater effect on tissue distribution.

Drug distribution is a dynamic process between plasma and the ISF. In contrast to single bolus administration of a drug,⁶ CRI allows an evaluation and comparison of the drug distribution to plasma and ISF under conditions whereby the concentration gradient is kept constant. When plasma total and ISF doxycycline concentrations were compared during the steady state period, a significant ($P < 0.001$) difference was found between the concentrations. In contrast, when the plasma unbound doxycycline concentration was compared with the ISF doxycycline concentration no significant ($P = 0.395$) difference was found. Clinically it is the unbound fraction of the drug that is microbiologically active and able to diffuse through capillary pores to the target site, the ISF. Therefore, our results indicate that determining plasma doxycycline concentrations in dogs may not reflect the microbiologically active concentration of doxycycline in tissues at steady state.

Meropenem has low protein binding (11.87%).⁶ Little difference is found between plasma total and unbound meropenem concentrations and ISF meropenem concentrations. Therefore, the use of meropenem plasma pharmacokinetics in dogs may provide a good

predictor of the microbiologically active concentration of meropenem in tissues at steady state.

The difference between plasma total concentrations and ISF concentrations for both drugs at steady state could be explained by differences in protein binding. Differences in lipophilicity appeared to have little importance for distribution to the ISF, because doxycycline had the higher lipophilicity, but lower distribution to the ISF. Differences in elimination half-life were ruled out, because distribution was measured when each drug was at steady state.

Plasma Cl and $V_{d_{ss}}$ estimated for meropenem in our study (5.65 ± 2.76 mL/min/kg and 0.30 ± 0.15 L/kg, respectively) were similar to previous reported values (6.53 ± 1.51 mL/min/kg and 0.34 ± 0.05 L/kg, respectively).¹⁴ Plasma $t_{1/2}$ of meropenem (0.73 ± 0.07 h) was also similar to a previous study⁶ (0.69 ± 0.08 hours), but the ISF $t_{1/2}$ was longer in our study (2.31 ± 0.36 h vs 1.15 ± 0.57 hours). In our study and the previous study,⁶ the $t_{1/2}$ of meropenem is longer from ISF than from plasma, owing to the rapid plasma $t_{1/2}$. For a drug in the interstitial space to be eliminated it must diffuse back into the plasma. If the time to equilibrium between tissue and plasma is longer than the time for the drug to be eliminated from plasma, a longer ISF $t_{1/2}$ of the drug will result.

The doxycycline plasma elimination $t_{1/2}$ (4.56 ± 0.57 hours) was similar to the ISF elimination $t_{1/2}$ (4.94 ± 0.67 hours), suggesting that time for equilibration between tissue and plasma is shorter or equal to plasma elimination. The plasma $t_{1/2\beta}$, Cl, and $V_{d_{ss}}$ for doxycycline was 4.56 ± 0.57 hours, 1.66 ± 2.21 mL/min/kg, 0.65 ± 0.08 L/kg, respectively, and was less than values reported in a study by Riond et al¹⁰ (6.99 ± 1.09 hours, 1.72 ± 0.17 mL/min/kg, and 0.93 ± 0.14 L/kg, respectively). The Cl is higher for meropenem ($5.65 \pm$

2.76 mL/min/kg) than doxycycline (1.66 ± 2.21 mL/min/kg) as a result of glomerular filtration and tubular secretion contributing to the elimination of meropenem.^{25,26} In humans, a strong relationship is found between protein binding, renal clearance, and $t_{1/2}$ among tetracyclines.²⁷ The highly protein bound doxycycline has a longer $t_{1/2}$ and lower renal clearance than tetracyclines with less extensive protein binding.²⁷ For β -lactams, no clear relationship is found between protein binding and $t_{1/2}$, because elimination involves tubular secretion, which is independent of protein binding.²²

Lipophilicity is an important physicochemical property for determining intracellular concentrations of tetracyclines.²⁸ Because of the high lipophilicity of doxycycline, the unbound drug in the ISF and plasma is available to cross cell membranes and attain high intracellular concentrations. High intracellular concentrations account for the higher V_{dss} (0.65 ± 0.08 L/kg), compared with that of meropenem (0.30 ± 0.15 L/kg). As the lipid solubility of tetracyclines increases, the ratio of total drug in tissues to unbound drug in serum increases.²⁸

Doxycycline binds reversibly to plasma proteins in humans, and these proteins are of low capacity; therefore, distribution depends upon the affinity of doxycycline for tissue components.¹³ Protein binding remains a controversial topic in regards to therapeutic influence. However, as reviewed by Wise,²² protein binding has an important effect on distribution, activity, and efficacy when it is high. Protein binding of different drugs can vary among species,²⁹ therefore extrapolation of plasma and tissue protein binding as well as volume of distribution and clearance, may be difficult among species.³⁰ The extent of protein binding depends on the drug, number of protein binding sites, compartment type, disease status, interaction with other agents, ionization, and lipophilicity of the drug.³ Many of these

factors are difficult to quantitate clinically, but they should be kept in mind when evaluating therapeutic efficacy.

The fluid collected from clinically normal dogs by the ultrafiltration device in our study may differ from the fluid surrounding an infected or inflamed tissue. Inflammation and infection may influence drug diffusion from capillaries by increasing the accumulation of protein, extracellular fluid, and cells. Bacteria present in the interstitial space can change the pH, protein content, and integrity of cellular barriers and, therefore, affect interstitial distribution and intracellular concentrations.³¹ Vasodilation and increased permeability accompanying inflammation causes an increase in drug delivery to the target site, but viscosity is also increased, slowing the rate of diffusion.³² Drug elimination from infected sites may be prolonged as a result of protein binding at the site of inflammation from the presence of exudates.³²

Tissue cages have been used to create a site with inflammation and infection and to study the dynamics of drug distribution.³²⁻³⁵ But compared to the sample collection method used in our study, the tissue cage model represents a compartment with a small surface area-to-volume ratio as a result of the large sample collection space.^{32,35} The in vivo ultrafiltration device samples from the interstitial space, which has a large surface area-to-volume ratio and rapid equilibrium. Because of the small surface area-to-volume ratio in tissue cages, an artificially increased time to peak concentration and longer $t_{1/2}$ are seen with these devices.^{35,36} In addition, drug concentrations measured in tissue cages are the total (bound and unbound) and may overestimate the microbiologically important fraction. However, we acknowledge that chronic tissue infection may change the usual extracellular environment as a result of fibrin barriers, abscess, edema, or changes in blood flow.³⁷ These factors are

difficult to measure and quantify; therefore, it is not known how well the ultrafiltration ISF sample collection technique actually represents the distribution of antimicrobials into infected tissues.

Doxycycline and meropenem are 2 drugs with different physicochemical, pharmacokinetic, and protein binding characteristics. In our study, the difference between plasma total concentrations and ISF concentrations of both drugs could be explained by the extent of protein binding, rather than other factors such as lipophilicity, plasma $t_{1/2}$, and acid dissociation constant. When evaluating drug plasma pharmacokinetics for antimicrobials, protein binding should be taken into account when predicting efficacy. Disease processes that change drug plasma protein binding should be considered for antimicrobials when adjusting dosage regimens for drugs with high protein binding.

FOOTNOTES

^aDoxycycline, American Pharmaceutical Partners, Los Angeles, Calif.

^bMerrem, Astra Zeneca, Wilmington, Del.

^cInfusion pump (Baxter 6201), Universal Hospital Services, Moorisville, NC.

^dCanine Ultrafiltration Probe (RUF-3-12), Bioanalytical Systems Inc, West Lafayette, Ind.

^eCentrifree, Millipore, Bedford, Mass.

^fDoxycycline Hydrochloride, Sigma Chemical Co, St Louis, Mo.

^gSodium phosphate dibasic, Sigma Chemical Co, St Louis, Mo.

^hPhosphoric acid, Sigma Chemical Co, St Louis, Mo.

ⁱl-octanol, Sigma Chemical Co, St Louis, Mo.

^jWaters models 590 and 591, Waters, Franklin, Mass.

^kWaters automated gradient controller, Waters, Franklin, Mass.

^lAgilent Series 1050, Agilent Technologies, Wilmington, Del.

^mZorbax SB-C18, Agilent Technologies, Wilmington, Del.

ⁿSpec Plus C18AR column filters, Ansys Diagnostics, Lake Forest, Calif.

^oUltrafree-MC, Millipore, Bedford, Mass.

^pZorbax SB-C8, Agilent Technologies, Wilmington, Del.

^qMeropenem, Zeneca Pharmaceuticals, Wilmington, Del.

^rWinNonlin, version 3.1, Pharsight, Cary, NC.

^sSAS, version 8.2, SAS Institute Inc, Cary, NC.

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Table 5.1. Mean ($\pm$ SD) values for pharmacokinetic parameters of doxycycline after constant rate IV infusion (0.10 mg/kg/h) in 6 dogs

Parameters	Plasma	Interstitial fluid
$AUC_{0 \rightarrow \infty}$ (h• μ g/mL)	12.09 $\pm$ 3.22	2.09 $\pm$ 0.21
$AUMC_{0 \rightarrow \infty}$ (h•h• μ g/mL)	70.25 $\pm$ 25.72	29.38 $\pm$ 3.42
Cl (mL/min/kg)	1.66 $\pm$ 2.21	NA
Vd_{ss} (L/kg)	0.65 $\pm$ 0.08	NA
λ_z (/h)	NA	0.14 $\pm$ 0.02
$\lambda_z t_{1/2}$ (h)	NA	4.94 $\pm$ 0.67
$t_{1/2\alpha}$ (h)	0.08 $\pm$ 0.05	NA
$t_{1/2\beta}$ (h)	4.56 $\pm$ 0.57	NA
K10 $t_{1/2}$ (h)	1.35 $\pm$ 0.97	NA
MRT (h)	NA	10.12 $\pm$ 1.54
C_{max} (μ g/mL)	NA	0.14 $\pm$ 0.02
T_{max} (h)	NA	11.33 $\pm$ 1.63
T_{max} adjusted (h)	NA	10.95
K10 (/h)	2.78 $\pm$ 4.05	NA
K12 (/h)	8.57 $\pm$ 7.54	NA
K21 (/h)	2.15 $\pm$ 1.37	NA
A (μ g/mL)	36.98 $\pm$ 54.51	NA
B (μ g/mL)	1.60 $\pm$ 0.32	NA
α (/h)	13.35 $\pm$ 10.10	NA
β (/h)	0.15 $\pm$ 0.02	NA
Protein binding (%)	91.75 $\pm$ 0.63	NA
Partition coefficient	0.68 $\pm$ 0.05	NA

$AUC_{0 \rightarrow \infty}$ = Area under the curve from time 0 to infinity. $AUMC_{0 \rightarrow \infty}$ = Area under the first moment curve from time 0 to infinity. Cl = Clearance from the central compartment. Vd_{ss} = Apparent volume of distribution at steady state. λ_z = First-order rate constant of terminal portion of the curve. $\lambda_z t_{1/2}$ = Half-life of the terminal portion of the curve. $t_{1/2\alpha}$ = Distribution half-life. $t_{1/2\beta}$ = Elimination half-life. K10 $t_{1/2}$ = Half-life of elimination phase. MRT = Mean residence time. C_{max} = Maximum concentration. T_{max} = Time until maximum concentration. T_{max} adjusted = Time until maximal concentration adjusted for a 23 minute lag time. K10 = elimination rate from compartment 1. K12 = rate of movement from compartment 1 to compartment 2. K21 = rate of movement from compartment 2 to compartment 1. A = Intercept for distribution phase. B = Intercept for elimination phase. α = Rate constant associated with distribution phase. β = Rate constant associated with elimination phase. NA = Not applicable for the route of administration or model used for estimations.

Table 5.2. Mean ($\pm$ SD) values for pharmacokinetic parameters of meropenem after constant rate IV infusion (0.38 mg/kg/h) in 6 dogs

Parameters	Plasma	Interstitial fluid
$AUC_{0 \rightarrow \infty}$ (h• μ g/mL)	NA	7.26 ± 1.5
$AUMC_{0 \rightarrow \infty}$ (h•h• μ g/mL)	NA	47.83 ± 8.94
AUC_{par} (h• μ g/mL)	9.92 ± 5.12	NA
Cl (mL/min/kg)	5.65 ± 2.76	NA
Vd_{ss} (L/kg)	0.30 ± 0.15	NA
λ_{λ} (/h)	0.96 ± 0.09	0.31 ± 0.04
$\lambda_{t_{1/2\lambda}}$ (h)	0.73 ± 0.07	2.31 ± 0.36
MRT (h)	NA	2.62 ± 0.45
C_{max} (μ g/mL)	NA	0.86 ± 0.18
T_{max} (h)	NA	3.00 ± 1.67
T_{max} (h) (adjusted)	NA	2.62
C_{ss} (μ g/mL)	1.39 ± 0.64	NA
Protein binding (%)	11.87	NA
Partition coefficient	0.02 ± 0.01	NA

AUC_{par} = Partial area under the curve calculated during the steady state period. C_{ss} = Mean concentration at steady state. See Table 1 for remainder of key.

Table 5.3. Physiochemical properties of doxycycline and meropenem.
pKa = acid dissociation constant.

Properties	Doxycycline	Meropenem
Molecular weight	462.46	437.51
Protein binding (%)	91.75 ± 0.63	11.87
Partition coefficient	0.68 ± 0.05	0.02 ± 0.01
pKa ^{23,24}	3.09	7.40

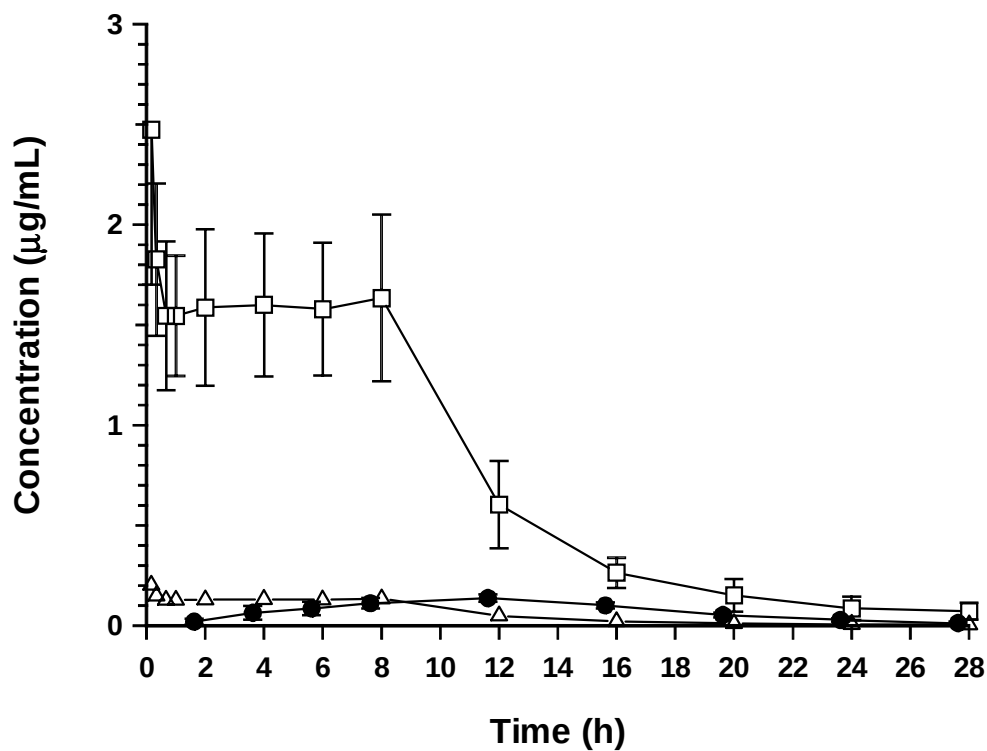


Figure 5.1. Plasma (total [open squares] and unbound [open triangles]) and interstitial fluid ([ISF; solid circles]) doxycycline concentrations versus time after constant rate IV infusion (CRI) of doxycycline in dogs. Plasma total and ISF doxycycline concentrations are expressed as mean ($\pm$ SD) values. The ISF doxycycline concentrations have been corrected for the lag time (23 minutes) necessary for collection into an ultrafiltration device.

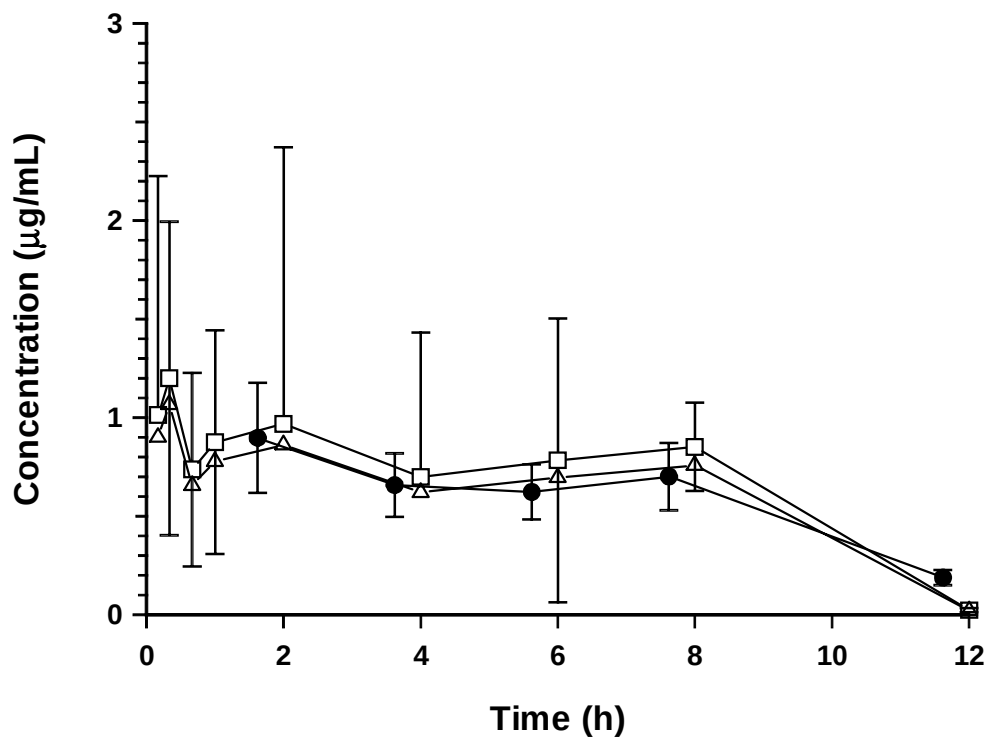


Figure 5.2. Plasma (total [open squares] and unbound [open triangles]) and ISF (solid circles) meropenem concentrations versus time after CRI of meropenem in dogs. Plasma total and ISF meropenem concentrations are expressed as mean ($\pm$ SD) values. The ISF meropenem concentrations have been corrected for the lag time (23 minutes) necessary for collection into an ultrafiltration device.

Plasma and interstitial fluid pharmacokinetics of enrofloxacin, its metabolite ciprofloxacin, and marbofloxacin after oral administration and a constant rate intravenous infusion in dogs

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Submitted for publication in the Journal of Veterinary Pharmacology and Therapeutics

ABSTRACT

Enrofloxacin and marbofloxacin were administered to six healthy dogs in separate crossover experiments as a single oral dose (5 mg/kg) and as a constant rate IV infusion (1.24 mg/kg/h and 0.12 mg/kg/h) following a loading dose (4.47 mg/kg and 2 mg/kg) to achieve a steady state concentration of approximately 1 µg/mL for 8-hours. Interstitial fluid (ISF) was collected with an *in vivo* ultrafiltration device at the same time period as plasma to assess the dynamics of drug distribution. Plasma and ISF were analyzed for enrofloxacin, its active metabolite ciprofloxacin, and marbofloxacin by high performance liquid chromatography (HPLC). Lipophilicity and protein binding of enrofloxacin were higher than marbofloxacin and ciprofloxacin. Compared to enrofloxacin, marbofloxacin had a longer half-life, higher C_{max} , and larger $AUC_{0-\infty}$ in plasma and ISF after oral administration. Establishing steady state allowed an assessment of the dynamics of drug concentrations between plasma and ISF. The ISF and plasma-unbound concentrations were similar during the steady state period despite differences in lipophilicity and pharmacokinetic parameters of the drugs.

INTRODUCTION

The fluoroquinolones are broad-spectrum antimicrobials with bactericidal activity against most gram-negative organisms. They also have good activity against gram-positive and some intracellular bacteria (Al-Nawas & Shah, 1998; Breitschwerdt *et al.*, 1999; Easmon *et al.*, 1986). These antimicrobials are used clinically by oral and parenteral administration to treat infections caused by susceptible bacteria in many species. Pharmacokinetics of fluoroquinolones have been investigated in humans (Crump *et al.*, 1983), dogs (Abadia *et al.*, 1994; Frazier *et al.*, 2000; K  ng *et al.*, 1993; Schneider *et al.*, 1996; Walker *et al.*, 1992), food animals (Aliabadi *et al.*, 2002; Bregante *et al.*, 1999; McKellar *et al.*, 1999), and horses (Bermingham *et al.*, 2000; Giguere *et al.*, 1997; Kaartinen *et al.*, 1997; Papich *et al.*, 2002). Enrofloxacin and marbofloxacin are two fluoroquinolones developed for veterinary use, registered in the U.S. for use in dogs and cats, and are frequently used in veterinary hospitals.

The spectrum of activity against organisms is similar for marbofloxacin and enrofloxacin but in dogs, some of the pharmacokinetics parameters reported in the literature show some potentially important differences (Table 6.1). The largest difference between the two drugs is the apparent volume of distribution and half-life.

The therapeutic strategy for antimicrobial drugs is to achieve plasma concentrations above the minimal inhibitory concentration (MIC) during the dosing interval. However, since most bacterial infections are extracellular, a therapeutic dosage regimen that optimizes antimicrobial concentrations at the infection site (i.e. the interstitial fluid (ISF)) would be better for predicting clinical success. Label claims on products promoting high lipophilicity and high intracellular concentrations for some drugs are of little therapeutic value when the

infection is extracellular. Therefore, measuring antimicrobial concentrations in the ISF is important for predicting therapeutic efficacy and evaluating dosages.

Enrofloxacin and marbofloxacin differ in lipophilicity and protein binding and it is one of the goals of this study to determine what effects these two characteristics have on distribution into the tissue ISF. Because the extent of plasma protein binding of fluoroquinolones has varied in the literature depending on the methodology (Bergogne-Berezin, 2002) one of the objectives of this study was to measure the protein binding of these three drugs using a consistent method – the ultrafiltration device *in vitro* – rather than rely on published values. Because drugs can bind to various proteins in plasma-albumin, α_1 -acid glycoprotein, and lipoproteins, another aim of this study was to measure specific canine albumin binding.

Pharmacological approaches to determine ISF concentrations have included analysis of tissue biopsies and tissue fluid collection. Some of these methods have included, skin blisters (Frongillo *et al.*, 1981; LeBel *et al.*, 1986; Ryan *et al.*, 1982), tissue cages (Cars *et al.*, 1981; Clarke *et al.*, 1989; Clarke *et al.*, 1989a), skin windows (Frongillo *et al.*, 1981), paper disks inserted in the tissue space (Cars & Ryan, 1988), and cotton threads implanted into tissues (Ryan & Cars, 1983; Ryan *et al.*, 1982). The approach chosen to collect the ISF can influence the concentration of drug measured and the estimates of pharmacokinetic parameters (Muller *et al.*, 1998). Anesthesia and surgery or even sacrifice of healthy animals has been performed to collect tissue samples to determine tissue distribution of drugs. Homogenization of tissue biopsy samples can overestimate lipophilic drug concentrations and underestimate hydrophilic drug concentrations due the combination of interstitial fluid,

intracellular fluid, and blood through the homogenization process (Cars, 1991; Cars & Ögren, 1985; Nix *et al.*, 1991).

Van Etta *et al.* (1982) used the surface area to volume ratio (SA/V) of a tissue, as a means to predict drug distribution to the extracellular fluid. The surface area is the area of extravascular space exposed to capillaries and the volume represents the diffusion distance (Van Etta *et al.*, 1982; Van Etta *et al.*, 1983). The larger the ratio, the greater the kinetic similarity between the vascular and extravascular space. Artificial compartments (small SA/V ratio), such as tissue cages, have a high volume due to the sampling space available within the device. Therefore, prediction of the true concentrations is unreliable from these compartments. Ultrafiltration (UF) devices sample the ISF from the natural interstitium, which has a large SA/V ratio. In the natural space, ISF sampled from UF, establishes equilibrium between plasma and ISF rapidly and the concentrations closely parallel the plasma unbound concentrations. For antimicrobials, only the protein unbound drug fraction is microbiologically active. The UF device allows the collection of protein unbound fluid from the interstitial space and direct analysis by high performance liquid chromatography (HPLC) without extraction. This study will enable a comparison of drug concentrations between the plasma and the ISF site of drug action. The UF device also provides a convenient way for continuous sampling and monitoring drug disposition in the conscious unrestrained animal (Linhares & Kissinger, 1993). To our knowledge, there have been no previous reports examining fluoroquinolone tissue distribution by means of ISF collected by an UF device *in vivo*. This device may offer an alternative to the commonly used tissue cage and tissue biopsy when evaluating tissue distribution of drugs.

To assess the dynamics of drug concentrations between the plasma and ISF, evaluations at steady state are preferred. We sought to minimize the influence of elimination rate on ISF concentration measurements by administering both drugs as constant rate infusions. At steady state, differences in half-life will not influence the comparison of the concentrations between plasma and ISF. Since the half-life is longer for marbofloxacin than enrofloxacin, this was a key variable to eliminate in this study. The goal of this study was to examine the disposition of enrofloxacin and marbofloxacin in dogs at steady state by simultaneously sampling plasma and ISF. This comparison allowed us to assess the influence of protein binding and lipophilicity on drug disposition in dogs.

Since oral administration is a common route of administration for fluoroquinolones, we chose to evaluate ISF and plasma concentrations after this route as well as after a constant rate IV infusion (CRI).

MATERIALS AND METHODS

Animals

Five female beagle dogs with body weight range of 8-11 kg and one female Collie mixed breed with a body weight of 20 kg were used in this study. The dogs were determined healthy after a physical exam and serum chemistry profile evaluation. The study was reviewed and approved by the Institutional Animal Care Use Committee at North Carolina State University.

Study Design

The study followed a two-period, four treatment crossover design with a 7 day washout period between each treatment. In the first study period enrofloxacin and marbofloxacin were given as a constant rate IV infusion (CRI). During the second study

period the dogs were given enrofloxacin and marbofloxacin as a single oral administration. The dogs were randomly assigned to 2 groups of 3 for each study period and by the end of the two studies periods each dog had received each drug by both administration routes. For the CRI the drugs were administered to each dog by first injecting a loading dose of 4.47 mg/kg for enrofloxacin and 2.00 mg/kg for marbofloxacin. Immediately following the loading dose, a CRI was started using a programmed electronic infusion pump (Baxter 6201 pump). The infusion rate was 1.24 mg/kg/h for enrofloxacin and 0.12 mg/kg/h for marbofloxacin and was administered over an 8-hour period. The loading dose and CRI were calculated to achieve a target steady state plasma concentration of 1 µg/ml from published equations (Gibaldi & Perrier, 1982) and previously published pharmacokinetic values for dogs (Küng *et al.*, 1993; Schneider *et al.*, 1996). Marbofloxacin injectable (Marbocyl SA, Vétoquinol, UK) was prepared according to the manufacturers package instructions (10 mg/mL) and the enrofloxacin solution (Baytril injection, 2.27%, Bayer Corporation) was used for intravenous administration. The loading dose was administered slowly over 5 minutes to decrease risk of potential central nervous system effects. For the oral study, enrofloxacin (Baytril chewable tablets, Bayer Corporation) and marbofloxacin (Zeniquin, Pfizer, NY, NY) were administered once at 5 mg/kg. The commercially available tablet sizes for each drug were cut in half or quarter to obtain a dose (mg) closest to the actual required 5 mg/kg dose. After the oral dose, 6 mL of water was administered by syringe to ensure the tablet was deposited in the stomach.

Blood Collection

Blood samples were collected from a jugular catheter. For the CRI a second jugular catheter was inserted for drug administration. The catheters were placed 18 hours prior to

drug administration and flushed with 0.9% sterile saline solution between collection times to maintain patency. Blood was collected from the jugular vein in evacuated glass tubes with sodium heparin as the anticoagulant. Sampling times were at 0 (pre-treatment), 10, 20, 40 minutes and 1, 2, 4, 6, 8, 12, 16, and 24 hours during and after the administration of a CRI of enrofloxacin and marbofloxacin and after oral administration of enrofloxacin. Samples were collected at 0 (pre-treatment), 10, 20, 40 minutes and 1, 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 54, and 60 hours after oral administration of marbofloxacin. The blood was centrifuged after collection at 1,000 x g for 10 minutes and the plasma separated and stored until analysis at -70°C.

Interstitial Fluid Collection

Subcutaneous ISF collection was performed with an *in vivo* ultrafiltration (UF) sampling kit (Canine Ultrafiltration Probe, Bioanalytical Systems Inc, West Lafayette, Ind). The UF probe (RUF-3-12) contained 3 loops, each having 12 cm of semi-permeable membrane. The membrane in the loop consists of pores allowing water, electrolytes, and low molecular weight molecules (less than 30 000 Daltons) to diffuse across the membrane and excluded the passage of protein and other large molecular weight compounds. The UF probe was inserted under the dog's skin into the interstitial space with a guide needle 18 hours prior to the start of the study to allow equilibrium between the ISF and the UF probe. Lidocaine was injected at the guide needle entry point and the UF probes were inserted aseptically. Once the UF probe was in place the guide needle was removed. The three loops remained under the skin in the interstitial space while the non-permeable tube extending to the exterior of the animal and attached to a vacutainer for sample collection. The vacutainer provided the negative pressure for ISF collection through the small pores in the loop membrane. A new

UF probe at a separate site was used for each drug and administration route. The ISF was collected at 0 (pre treatment), 1, 2, 4, 6, 8, 12, 16, and 24 hours during and after the administration of a CRI of enrofloxacin and marbofloxacin and after oral administration of enrofloxacin. The collection times after oral marbofloxacin administration were 0 (pre-treatment), 1, 2, 4, 6, 8, 12, 16, 24, 30, 36, 48, 54, and 60 hours. The ISF was immediately frozen at -70°C until analysis.

HPLC Analysis

Both plasma and ISF samples were analyzed by high performance liquid chromatography (HPLC) to determine the concentrations of all drugs. Separation was achieved at 40°C with a Zorbax SB-C8 4.6mm x 15mm (Agilent Technologies, Part No 883967-901, Wilmington, DE) reverse phase column and a Zorbax RX-C8 analytical guard column (Agilent Technologies, Part No 820950-915, Wilmington, DE). This system included two pumps (Waters model 590 and 591, Millipore Corp, Milford, Mass), pump controller (Waters automated gradient controller, Millipore Corp, Milford, Mass), automated sampler (Hewlett-Packard series 1100, Wilmington, DE), and an ultraviolet absorbance at 279 nm (Agilent 1100 series UV detector, Agilent Technologies, Wilmington, DE) and fluorescence detection (Agilent 1100 series fluorescence detector, Agilent Technologies, Wilmington, DE) with excitation at 280 nm and emission at 500 nm. The mobile phase consisted of 20% acetonitrile, 0.02% trifluoroacetic acid (TFA), and 80% water at a 1.0 mL/min flow rate and the injection volume was 50 µL.

Samples were prepared using solid phase extraction with an Oasis HLB 30 µm column filter (Waters, Milford, Mass). Interstitial fluid samples and fluid from the lipophilicity study were analyzed directly by HPLC without extraction. Marbofloxacin,

enrofloxacin and ciprofloxacin ISF samples were analyzed by HPLC under the conditions described. Calibration curves for all drugs were prepared in the concentration range of 0.10 µg/mL to 10 µg/mL. Pooled canine plasma was fortified with a pure reference standard of enrofloxacin (Bayer Corporation), ciprofloxacin, (United States Pharmacopoeia, Rockville, MD) and marbofloxacin, (Pfizer, UK). A new calibration curve prepared for each day's analysis was used. The calibration curve was analyzed using linear regression with a minimum accepted coefficient of correlation (r^2) of 0.99. Interstitial fluid calibration curves in the range of 0.10 µg/mL to 10 µg/mL for marbofloxacin and 0.01 µg/mL to 5 µg/mL for enrofloxacin and ciprofloxacin were prepared with phosphate buffered saline (PBS, pH 7.38) fortified with the analytical reference standards listed above.

Plasma Protein Binding

Protein binding was determined using the ultrafiltration device *in vitro*. Stock solutions of enrofloxacin, marbofloxacin, and ciprofloxacin were prepared by dissolving 2-3 mg of drug into 0.1% TFA. Three mL aliquots of pooled canine plasma were then fortified with the stock solutions to make 1 and 2 µg/mL concentrations. Replicates of the three concentrations were prepared and incubated in a water bath (37°C) for 30 minutes. The ultrafiltration device was placed into a glass tube containing 2 mL of the spiked plasma. A 3 mL vacutainer was connected to the ultrafiltration device and fluid was collected for approximately 10 minutes and then discarded. A new vacutainer was attached and fluid was collected for 30 minutes. The protein free ultrafiltrate collected was analyzed by HPLC without extraction and represented the unbound plasma drug fraction. Solid phase extraction as described previously was performed with 1 mL of the remaining spiked plasma prior to analysis by HPLC to determine the total plasma drug concentration. Total drug plasma

concentrations were determined from a calibration curve prepared from spiked plasma. Unbound plasma drug concentrations were determined from a calibration curve of spiked PBS solution.

Albumin Binding

The extent of albumin binding was determined for all drugs with the ultrafiltration device *in vitro*. A 3 g/dL solution of albumin was prepared by weighing out 0.51g of canine albumin (Sigma Chemical Co, St. Louis, MO) and adding 17mL of 0.1M sodium diphosphate buffer solution. The 0.1M sodium diphosphate buffer solution was prepared from deionized water and sodium diphosphate powder (Sigma Chemical Co, St. Louis, MO) and the pH was adjusted to 7.2 with phosphoric acid (Sigma Chemical Co, St. Louis, MO). Three milliliter aliquots of albumin solution were then spiked with the stock solutions to make 1 and 2 µg/mL concentrations. Albumin binding was determined by the same method described above for plasma protein binding. Plasma protein binding and albumin binding of each drug was determined according to the following formula:

$$\% \text{ protein binding} = \frac{\text{total concentration} - \text{unbound concentration}}{\text{total concentration}} \times 100$$

Drug Lipophilicity

The octanol water partition coefficient, a measure of the drug's lipophilicity, was measured by established methods (Ashby *et al.*, 1985; Asuquo & Piddock, 1993; Takacs-Novak *et al.*, 1992) and the technique has been described in a previously published paper by the authors (Bidgood & Papich, 2003). The aqueous phase was represented by a 0.1M sodium diphosphate buffer solution. Three replicates (5 mL) of two concentrations (2.50

µg/mL and 1.25 µg/mL) of each drug (enrofloxacin, ciprofloxacin, marbofloxacin) were prepared and added to an equal volume (5 mL) of octanol (1-octanol, Sigma Chemical, St. Louis, MO). The mixture was then gently rocked for 1 hour at room temperature to allow the drug to reach equilibrium between the two phases. Separation of the aqueous and lipid phases was achieved by centrifugation of the tubes for 10 minutes at 2,000 x g. The aqueous layer was analyzed for the drug before and after incubation and shaking. The octanol water partition coefficient (PC) was determined by the following formula:

$$PC = \frac{\text{buffer concentration before incubation} - \text{buffer concentration after incubation}}{\text{buffer concentration after incubation}} \times \frac{\text{volume of buffer}}{\text{volume of octanol}}$$

Pharmacokinetic analysis

Plasma and ISF concentrations after CRI and oral doses were plotted on a semilogarithmic graph for analysis (Figures 6.1-6.6). The model that best fit the data was selected based on the Akaike's information criterion method estimation (MAICE) (Yamaoka *et al.*, 1978). The CRI and oral pharmacokinetic parameters were estimated using a computer software program (WinNonlin Version 4.0, Pharsight Corporation, Cary, NC) (Table 2-4).

Compartmental analysis

A compartmental model was used to analyze the CRI enrofloxacin and marbofloxacin plasma data. The micro-rate constants (K_{10} , K_{01} , K_{12} , K_{21}), intercepts (A, B), rate constants (α , β), distribution half-life ($T_{1/2\alpha}$), elimination half-life ($T_{1/2\beta}$), and clearance from the central compartment (Cl) for enrofloxacin and marbofloxacin after CRI were calculated for the compartmental model using published equations (Gibaldi & Perrier, 1982). The apparent volume of distribution using the area method ($V_{d\text{area}}$) was calculated by $V_{d\text{area}} = Ro/C_{ss} \cdot \beta$, where Ro is the infusion rate, C_{ss} is the average concentration at steady state, and β is the

elimination rate constant ($\beta = 0.693/T_{1/2\beta}$). The apparent volume of distribution at steady state ($V_{d_{ss}}$) was calculated by $V_{d_{ss}} = 1.44 \cdot R_o \cdot T_{1/2\beta}/C_{ss}$.

Noncompartmental analysis

All other CRI data as well as all oral ISF and plasma data was analyzed by noncompartmental methods. The MRT (mean residence time) was calculated by the statistical moment theory: $MRT = AUMC_{0-\infty}/AUC_{0-\infty}$, where AUC is the area under curve from time 0 to infinity and AUMC is the area under the moment curve from time 0 to infinity. The AUC for the oral and CRI ISF data was estimated with the log-linear trapezoidal rule and extrapolated to infinity by using the equation CT/λ_z , where CT is the last measured time concentration and λ_z is the first order rate constant associated with the terminal portion of the curve. Half-life was calculated using the formula $T_{1/2\lambda_z} = 0.693/\lambda_z$. The maximum concentration (C_{max}) and time to maximal concentration (T_{max}) for plasma and ISF after oral administration were reported directly from the concentration vs time curves.

The oral systemic availability (F) was calculated by using the formula: $F (\%) = AUC_{po}/AUC_{iv} \cdot Dose_{iv}/Dose_{po} \times 100$, where AUC_{po} is the $AUC_{0-\infty}$ calculated after oral administration and AUC_{iv} is the partial area calculated between 1 and 8 hours during the steady state period. The $Dose_{iv}$ was the maintenance CRI dose administered between 1 and 8 hour for enrofloxacin (8.75 mg/kg) and marbofloxacin (0.81 mg/kg). The $Dose_{po}$ was 5 mg/kg for both enrofloxacin and marbofloxacin.

Statistical analysis

Mixed effects models with different correlation structures were used to analyze the data (SAS, version 8.2, SAS Institute Inc, Cary, NC). The most appropriate model was selected based on Aikake information criterion (AIC) and Schwarz's Bayesian Criterion

(BIC) values. The mixed model with one-dependent correlation structure was selected. The pharmacokinetic parameters ($C_{\max}$, $T_{\max}$, and $T_{1/2\lambda z}$) were compared between plasma and ISF for enrofloxacin and marbofloxacin. A comparison of these pharmacokinetic parameters as well as apparent volume of distribution ($V_{d\text{area}}$), total systemic clearance (Cl), and elimination rate constant (K_{10}), elimination half-life, $T_{1/2\beta}$ between enrofloxacin and marbofloxacin was also performed where applicable. The estimated parameter $T_{1/2\lambda z}$ was compared between CRI and oral administration for the drugs. These comparisons were based on an ANOVA test. Values of $P \leq 0.05$ were considered significant.

RESULTS

There were no adverse effects after administration of the loading dose, CRI maintenance dose, or after oral administration of enrofloxacin and marbofloxacin to any of the dogs.

A two compartmental model with IV bolus and IV CRI input for all dogs was fitted to the plasma CRI concentration data for enrofloxacin and marbofloxacin. A non-compartmental CRI model was used to fit the CRI ciprofloxacin plasma data and all ISF concentrations after CRI. A non-compartmental model with first order input fit the oral plasma and ISF data. Results are expressed as the arithmetic mean $\pm$ standard deviation (Table 6.2-6.4). The CRI and oral plasma (total and unbound) concentrations with corresponding ISF concentrations were plotted vs time on a semi-logarithmic graph for marbofloxacin, enrofloxacin, and ciprofloxacin (Figure 6.1-6.6). The ISF concentrations and pharmacokinetic values for enrofloxacin (PO), ciprofloxacin (PO), and marbofloxacin (CRI) were each averaged from 5 dogs. There was insufficient ISF collected from one dog in these studies for HPLC analysis therefore ISF data from the 6th dog was not included. Plasma

unbound concentrations were calculated by first determining the extent of protein binding and then subtracting this amount from the total plasma concentration.

The limit of detection (LOD) and limit of quantification (LOQ) for HPLC analysis of plasma concentrations were 0.02 $\mu\text{g/mL}$ and 0.04 $\mu\text{g/mL}$ (enrofloxacin), 0.03 $\mu\text{g/mL}$ and 0.06 $\mu\text{g/mL}$ (ciprofloxacin), and 0.05 $\mu\text{g/mL}$ and 0.12 $\mu\text{g/mL}$ (marbofloxacin). The LOD and LOQ for HPLC analysis of ISF concentrations was 0.02 $\mu\text{g/mL}$ and 0.05 $\mu\text{g/mL}$ (enrofloxacin), <0.01 $\mu\text{g/mL}$ and 0.02 $\mu\text{g/mL}$ (ciprofloxacin), and 0.06 $\mu\text{g/mL}$ and 0.19 $\mu\text{g/mL}$ (marbofloxacin). All values above the LOD were used to construct the graphs but only values above the LOQ were used for the pharmacokinetic analysis. The recovery was 94.9% for marbofloxacin and 70% for enrofloxacin. The accuracy and precision were both within a $\pm 15\%$ acceptable value.

The external tubing of each ultrafiltration device was 46 cm in length and held 160 μL of fluid. The amount of fluid collected in the vacutainer over each time period was recorded and the average collection rate was determined to be $2.11 \pm 0.85 \mu\text{L/min}$. The lag time for the fluid to collect in the vacutainer was calculated to be 75 minutes. The ISF concentrations in the Graph 6.1-6.6 were adjusted for this lag time. The T_{max} in Table 6.2-6.4 are reported as adjusted and non-adjusted times. Enrofloxacin had higher protein binding and partition coefficient compared to marbofloxacin and ciprofloxacin (Table 6.5).

CRI of enrofloxacin and marbofloxacin

At steady state there was no statistical difference between unbound plasma and ISF concentrations for marbofloxacin ($P = 0.553$), enrofloxacin ($P = 0.209$), or ciprofloxacin ($P = 0.125$). There was a significant difference between marbofloxacin and enrofloxacin plasma $T_{1/2\beta}$ ($8.30 \pm 2.61 \text{ h}$ vs $3.02 \pm 0.80 \text{ h}$, $P = 0.001$) and ISF $T_{1/2\lambda_z}$ ($8.13 \pm 1.06 \text{ h}$ vs $3.95 \pm$

0.88 h, $P = 0.002$). Both enrofloxacin and marbofloxacin had high apparent volume of distributions (3.60 ± 1.20 L/kg and 1.50 ± 0.39 L/kg) and they were statistically different ($P < 0.001$).

Oral administration of enrofloxacin and marbofloxacin

The mean plasma and ISF concentrations of marbofloxacin and enrofloxacin after oral administration are represented in Figure 6.2 and 6.4. There was a statistical difference between marbofloxacin and enrofloxacin oral plasma values for $AUC_{0-\infty}$ (42.08 ± 11.45 h* μ g/mL vs 4.46 ± 1.70 h* μ g/mL, P value = 0.001), C_{max} (3.63 ± 0.85 μ g/mL vs 1.24 ± 0.39 μ g/mL, $P < 0.001$), T_{max} (1.95 ± 1.16 h vs 0.94 ± 0.53 h, $P = 0.004$), and $T_{1/2\lambda_z}$ (7.63 ± 3.70 h vs 2.23 ± 0.89 h, $P = 0.002$). The ISF $T_{1/2\lambda_z}$ was statistically different between enrofloxacin and marbofloxacin (8.55 ± 3.22 h vs 3.12 ± 0.96 h, $P = 0.002$). Marbofloxacin and enrofloxacin peak ISF concentration (C_{max}) was 2.65 ± 0.42 μ g/mL and 0.70 ± 0.18 μ g/mL and were reached (T_{max}) at 4.80 ± 1.1 h and 4.40 ± 0.89 h. Both ISF C_{max} and T_{max} values were statistically different between the two drugs ($P = 0.001$). There was a statistical difference between CRI and oral $T_{1/2\lambda_z}$ for enrofloxacin plasma ($P = 0.019$) and ISF ($P = 0.007$), but not for marbofloxacin plasma ($P = 0.831$) or ISF ($P = 0.787$). The absolute bioavailability was $104.61 \pm 0.26\%$ for marbofloxacin and $63.22 \pm 0.24\%$ for enrofloxacin after oral administration.

Ciprofloxacin

Ciprofloxacin was detected in all samples of all the dogs administered enrofloxacin. The volume of distribution and clearance was not calculated for ciprofloxacin because it was produced as a metabolite. The plasma and ISF $T_{1/2\lambda_z}$ after oral administration was 3.41 ± 0.70 h and 5.26 ± 1.03 h and 5.17 ± 1.23 h and 6.25 ± 1.59 h after CRI, respectively. The

peak concentrations ($C_{\max}$) after oral administration were $0.36 \pm 0.10 \mu\text{g/mL}$ for plasma and $0.35 \pm 0.07 \mu\text{g/mL}$ for ISF, respectively.

DISCUSSION

Results from the CRI and oral study provide information for understanding drug distribution of three fluoroquinolones into the ISF. In order to eliminate the variables caused by declining drug concentrations over time from a bolus dose and allow an assessment of the relationship between plasma and ISF at steady state, we used an 8-hour CRI and then collected plasma and ISF samples during this time period. Ciprofloxacin concentrations did not represent a true steady state situation because it was produced as a metabolite of enrofloxacin over time, and drug doses were not calculated to produce a steady state for ciprofloxacin.

For most capillary membranes only low molecular weight protein unbound compounds are able to diffuse across the membrane (Barza & Cuchural, 1985). Equilibrium is established across the capillary for the unbound concentration via passive diffusion, therefore the concentration of free (unbound) drug concentrations should be theoretically equal in plasma and ISF (Ögren & Cars, 1985). At the site of infection (i.e., ISF) it is the unbound antimicrobial drug fraction that is pharmacologically active (Liu, 2002; Wise, 1986; Wise, 1986a). Since most bacterial infections are in the ISF of tissues rather than intracellular or in plasma, clinical efficacy of extracellular bacterial infections would be better predicted from the determination of unbound concentrations in the interstitial space (Liu, 2002). For highly protein bound drugs, if dosages are based on achieving total plasma concentrations relative to the MIC values, the target site unbound drug concentrations (in the ISF) may not achieve these MIC values and therapeutic failure may result.

Plasma (total and unbound) concentrations and ISF concentrations are graphically represented for marbofloxacin, enrofloxacin, and ciprofloxacin, after CRI in Figure 6.1, 6.3 and 6.5. During the steady state period there was no statistical difference between unbound plasma and ISF concentrations for marbofloxacin ($P = 0.553$), enrofloxacin ($P = 0.209$), or ciprofloxacin ($P = 0.125$) despite differences in pharmacokinetics parameters and physicochemical characteristics among the drugs. Compared to enrofloxacin, marbofloxacin had a statistically lower clearance (2.92 ± 0.98 mL/min/kg vs 13.33 ± 1.14 mL/min/kg, $P < 0.001$) and longer elimination $T_{1/2\beta}$ after discontinuation of the CRI (8.30 ± 2.61 h vs 3.02 ± 0.80 h, $P < 0.001$). The K_{10} for marbofloxacin (0.20 ± 0.06 /h) was statistically less than that of enrofloxacin (0.42 ± 0.08 /h) ($P < 0.001$) suggesting an increased rate of elimination from the central compartment for enrofloxacin. This is in agreement with the higher clearance values calculated for enrofloxacin when compared to marbofloxacin.

The extent of protein binding of antimicrobials is important to consider when determining and interpreting pharmacokinetics parameters (volume of distribution, clearance, half-life) and pharmacodynamic measurements (MIC) in the evaluation of therapeutic efficacy. At steady state the concentration of enrofloxacin and marbofloxacin unbound plasma and ISF concentrations were close to the target concentration of 1 μ g/mL because the protein binding for both of these drugs were low. Protein binding follows the law of mass action and degree of binding depends on the concentration of the drug and protein, binding affinity, and number of binding sites (Zlotos *et al.*, 1998). There are differences in the extent of drug protein binding among species (Aramayona *et al.*, 1994; Kaartinen *et al.*, 1995; Lin, 1995; Riond & Riviere, 1989; Villa *et al.*, 1997). Therefore, pharmacokinetic studies of

antimicrobials for animals should include a measurement of plasma protein binding because it may be clinically significant for predicting efficacy.

To determine the extent of plasma protein binding of the three fluoroquinolones we evaluated binding at drug concentrations measured during the dosing interval. In the present study, the protein binding was 34.74 $\pm$ 2.33% for enrofloxacin, 18.48 $\pm$ 2.98% for ciprofloxacin and 21.81 $\pm$ 6.26% for marbofloxacin. The results for enrofloxacin and ciprofloxacin are similar to another study, which measured albumin protein binding in human serum (34.6 $\pm$ 13.8% and 15.7 $\pm$ 4.2%) (Zlotos *et al.*, 1998). Marbofloxacin protein binding has not previously been published. Human plasma contains several different transport proteins. Drugs can be bound to various constituents in the blood, including albumin, α_1 -acid glycoprotein, and lipoproteins. Concentrations of albumin (35-50 g/dL) are higher than α_1 -acid glycoprotein (0.40 - 1 g/dL) (Roland & Tozer, 1995) but α_1 -acid glycoprotein can increase during acute inflammation. The albumin protein binding in this study showed similar results as the total plasma protein binding. These results suggest that for these fluoroquinolones, albumin is the most important protein to which they are bound. We did not measure, nor can we speculate, on the effect of diseases (for example acute inflammation that may increase α_1 -acid glycoprotein) on this binding.

The partition coefficient (PC) is a ratio of octanol to aqueous solubility and is used to measure the lipophilicity of a molecule (Buchwald & Bodor, 1998; Takács-Novák *et al.*, 1992). Enrofloxacin had a higher PC (3.54 $\pm$ 0.025) when compared to ciprofloxacin (0.081 $\pm$ 0.005) and marbofloxacin (0.074 $\pm$ 0.011). A study by Tegedor *et al.* (2003) measured the lipophilicity of several fluoroquinolones and found similar results to our study where enrofloxacin had higher lipophilicity than marbofloxacin and ciprofloxacin. High

lipophilicity for fluoroquinolones does not increase the antibacterial activity because these drugs gain access to bacterial targets through hydrophilic pores. In addition to a higher PC, enrofloxacin had a higher apparent volume of distribution than marbofloxacin ($P < 0.001$), but these higher values did not translate to increased penetration into the ISF.

The fluoroquinolones are amphoteric molecules and exist in a zwitterionic form at physiological pH (Takács-Novák *et al.*, 1992). The pKa values are similar among the three fluoroquinolones (Table 6.5). Because fluoroquinolones enter bacteria through pores, ionization does not influence passage across the membrane. Because we corrected for differences in systemic clearance with the CRI, and lipophilicity was not higher for marbofloxacin, this suggests diffusion of fluoroquinolone antibiotics from plasma to the interstitial space is influenced primarily by the degree of protein binding.

Our study also demonstrated that a true evaluation of drug diffusion into tissues should be done when the drug is at steady-state plasma concentrations rather than after a single dose. Oral plasma and ISF $C_{\max}$ and $T_{\max}$ were statistically different ($P < 0.010$) for enrofloxacin and marbofloxacin. There was also a statistical difference between the oral plasma and ISF half-life for enrofloxacin ($P = 0.029$) and marbofloxacin ($P = 0.003$). The different elimination half-lives resulted in a change in concentrations between plasma and ISF over time due to the dynamics between the two compartments. In contrast to the ISF, drug concentrations in plasma are constantly changing during intermittent dosing and therefore a comparison of ISF and plasma concentrations after a single dose administration may give false results (Gerding *et al.*, 1978). By administering these drugs by a CRI we eliminated this problem by comparing the concentrations between plasma and ISF at steady state.

After oral administration, marbofloxacin had a higher plasma $C_{\max}$ ($P < 0.001$), longer elimination $T_{1/2\lambda_z}$ ($P = 0.002$), and larger $AUC_{0-\infty}$ ($P = 0.001$) than enrofloxacin. The elimination half-life of enrofloxacin (2.23 ± 0.89 h) after oral administration was similar to other published reports (2.23 ± 0.42 h, Cester *et al.*, 1996; 2.40 ± 0.50 h, Küng *et al.*, 1993; 2.70 ± 0.10 h, Monlouis *et al.*, 1997). The elimination half-life for marbofloxacin (7.63 ± 3.70 h) was shorter than previously reported (9.77 ± 0.81 h, Cester *et al.*, 1996; 10.89 ± 0.84 h, Frazier *et al.*, 2000).

Fluoroquinolones are bactericidal concentration-dependent drugs. The surrogate markers to predict clinical effects are $C_{\max}/MIC$ or AUC_{0-24h}/MIC ratios of 8-10 and 125-250, respectively (Bergogne-Berezin, 2002; Blazer, 1987; Dudley, 1991; Papich & Riviere, 2001; Wright, 2000). The minimal inhibitory concentrations against *E. coli* have been reported as $0.02 \mu\text{g/mL}$ (Riddle *et al.*, 2000), $0.015\text{-}2 \mu\text{g/mL}$ (Pirro *et al.*, 1999) for enrofloxacin and $0.017 \mu\text{g/mL}$ (Riddle *et al.*, 2000), $0.016\text{-}0.25 \mu\text{g/mL}$ (Spreg, 1995) and $0.03\text{-}2 \mu\text{g/mL}$ (Pirro *et al.*, 1999) for marbofloxacin. Using the MIC value of $0.04 \mu\text{g/mL}$ for enrofloxacin and $0.09 \mu\text{g/mL}$ for marbofloxacin against *E. coli*, and the pharmacokinetic parameters determined in this study after oral administration of enrofloxacin and marbofloxacin (5 mg/kg), the AUC_{0-24h}/MIC ratio and the $C_{\max}/MIC$ ratio were 176.50 and 40.00 for enrofloxacin and 398.78 and 40.33 for marbofloxacin. These surrogate markers are based on total plasma concentrations not protein unbound ISF concentrations. However, a comparison of ISF shows that the AUC_{0-24h}/MIC and $C_{\max}/MIC$ were 226 and 26.25 for enrofloxacin and 413.11 and 29.44 for marbofloxacin. The explanation for the higher AUC in the ISF is because the elimination half-life for both drugs is longer in ISF and therefore the ISF AUC_{0-24h} is larger than plasma AUC_{0-24h} . The AUC_{0-24h}/MIC ratio has been associated with better

predictions of clinical effect (Wright, 2000). Marbofloxacin plasma and ISF pharmacokinetics indices are above the proposed $C_{\max}/\text{MIC}$ ranges of 8-10 and AUC/MIC of 125-250 suggested in the literature (Blazer *et al*, 1987; Forrest *et al.*, 1993). Since ciprofloxacin contributes to the antimicrobial activity of enrofloxacin, the indices were calculated combining $C_{\max}$ and $\text{AUC}_{0-24\text{h}}$ from enrofloxacin and ciprofloxacin and the resulting values for plasma and ISF were also within the suggested ranges. These results suggest adequate concentrations would be achieved in the ISF for both drugs after oral dosing of 5 mg/kg.

In conclusion, our study has provided an understanding of the distribution of fluoroquinolones into the ISF in dogs. Marbofloxacin had a higher $C_{\max}$, longer elimination half-life, and larger $\text{AUC}_{0-\text{infinity}}$ compared to enrofloxacin when given at the same oral dosage. Protein binding is the primary determinant of the extent of drug distribution from the plasma into the ISF. Our research has shown ISF collection in dogs and perhaps other species can be performed with the *in vivo* ultrafiltration device. This technique offers advantages over currently used tissue cages and tissue biopsies to determine antimicrobial concentrations at the infection site.

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Table 5.1. Pharmacokinetic parameters for enrofloxacin and marbofloxacin after oral and intravenous administration to dogs

Dose mg/kg	N	Dosing Frequency	Route	T 1/2 (h)	C _{max} (µg/mL)	T _{max} (h)	AUC (µg ^h /mL)	V _{dss} (L/kg)	Cl (L/h/kg)	Reference
Enrofloxacin										
5	4	once	IV	2.40+/-0.87	NA	NA	3.90+/-2.36(0-infinity)	7.00+/-6.40	1.63	Kung <i>et al.</i> , 1993
6	4	once	PO	2.40+/-0.50	1.16+/-0.60	0.90+/-0.80	3.90+/-2.36(0-infinity)	NA	NA	Kung <i>et al.</i> , 1993
5	8	once	IV	2.52+/-0.52	NA	NA	7.27+/-1.36 (0-infinity)	2.35+/-0.40	0.72+/-0.17	Cester <i>et al.</i> ., 1996
5	8	once	PO	2.23+/-0.42	1.12+/-0.49	1.86+/-0.93	5.34+/-1.57(0-infinity)	NA	NA	Cester <i>et al.</i> ., 1997
5	12	x 5 days	PO	4.61+/-0.84	1.75+/-0.16	1.21+/-0.08	11.65+/-3.00 (0-48h)	NA	NA	Frazier <i>et al.</i> , 2002
5.5	6	once	PO	5.79+/-2.97	1.36+/-0.79	1.71+/-0.75	7.87+/-3.51(0-12h)	NA	NA	Walker <i>et al.</i> ., 1992
			tissue cage	7.48+/-4.90	0.61+/-0.27	5.91+/-2.04	5.29+/-2.12(0-12h)	NA	NA	Walker <i>et al.</i> ., 1993
Marbofloxacin										
5	34	once	PO	4.07+/-0.18	1.41+/-0.07	1.83+/-0.16	8.74+/-0.37(0-24h)	NA	NA	Heinen, 2002
5	8	once	IV	9.83+/-1.5	NA	NA	NA	1.24+/-0.16	0.09+/-0.02	Cester <i>et al.</i> ., 1996
5	8	once	PO	9.77+/-0.81	1.35+/-0.09	1.76+/-1.44	21.95+/-1.86(0-infinity)	NA	NA	Cester <i>et al.</i> ., 1997
2.75	12	x 5 days	PO	10.89+/-0.84	2.53+/-0.05	2.13+/-0.34	35.44+/-2.27(0-48h)	NA	NA	Frazier <i>et al.</i> , 2002
2	6	once	IV	12.4+/-2.6	NA	NA	20.95+/-4.79(0-infinity)	1.37+/-0.19	0.10+/-0.02	Schneider <i>et al.</i> ., 1996
	6		PO	14.00+/-4.90	1.38+/-0.40	2.50+/-1.20	22.01+/-7.12(0-infinity)	NA	NA	Schneider <i>et al.</i> ., 1996
2	34	once	PO	9.07+/-1.9	1.47+/-0.09	1.83+/-0.17	13.07+/-1.07(0-24h)	NA	NA	Heinen, 2002

N number of animals, T ½ elimination half-life; C_{max} maximal concentration; T_{max} time to maximal concentration; AUC area under the concentration vs time curve; V_{dss} volume of distribution at steady state; Cl total systemic clearance.

Table 6.2. Pharmacokinetic parameters (mean +/- SD) for marbofloxacin after constant rate IV infusion (0.12 mg/kg/hr) and oral (5mg/kg) administration in dogs

Parameter	units	CRI Plasma	CRI ISF	PO Plasma	PO ISF
C_{max}	µg/mL	NA	1.32+/- 0.20	3.63+/-0.85	2.65+/-0.42
AUC_{0-infinity}	h*µg/mL	12.60+/-4.31	26.79 +/- 6.32	42.08+/-11.45	46.78+/-17.74
AUMC_{0-infinity}	h*h*µg/mL	NA	440.58+/-133.90	551.62+/-388.13	819.83+/-522.94
AUC_{par}	h*µg/mL	6.53+/-1.11	7.01+/-1.38	NA	NA
AUC_{0-24h}	h*µg/mL	NA	NA	35.89+/-6.37	37.18+/-11.28
Cl	mL/min/kg	2.92+/-0.98	NA	NA	NA
Vd_{ss}	mL/kg	1.53+/-0.38	NA	NA	NA
Vd_{area}	mL/kg	1.54+/-0.39	NA	NA	NA
T_{max}	h	NA	6.00+/-3.46	1.95+/-1.16	4.80+/-1.10
T_{max, adj}	h	NA	4.83+/-3.46	NA	3.55+/-1.10
λ_z	/h	NA	0.09+/-0.01	0.11+/-0.04	0.09+/-0.02
T_{1/2 λ_z}	h	NA	8.13+/-1.06	7.63+/-3.70	8.55+/-3.22
T_{1/2 α}	h	0.12+/-0.04	NA	NA	NA
T_{1/2 β}	h	8.30+/-2.61	NA	NA	NA
K₁₀ T_{1/2}	h	3.83+/-1.17	NA	NA	NA
MRT	h	NA	12.24+/-1.40	12.12+/-5.07	16.74+/-3.99
C_{ss}	µg/mL	0.93+/-0.19	NA	NA	NA
K₁₀	/h	0.20+/-0.06	NA	NA	NA
K₁₂	/h	3.51+/-2.51	NA	NA	NA
K₂₁	/h	3.05+/-1.03	NA	NA	NA
A	µg/mL	1.43+/-1.31	NA	NA	NA
B	µg/mL	1.04+/-0.17	NA	NA	NA
α	/h	6.67+/-2.58	NA	NA	NA
β	/h	0.09+/-0.03	NA	NA	NA
F	%	NA	NA	104.61+/-0.26	NA

C_{max} maximum (peak) concentration; AUC_{0-infinity} area under the curve from time 0 to infinity; AUMC_{0-infinity} area under the moment curve from time 0 to infinity; AUC_{par} partial area calculated between 1 and 8 hours during the CRI; AUC_{0-24h} area under the curve from time 0 until 24 hours; Cl total systemic clearance; Vd_{ss} apparent volume of distribution at steady state; Vd_{area} apparent volume of distribution calculated by the area method; T_{max} time to maximal concentration, T_{max,adj} time to maximal concentration adjusted for the lag time; λ_z first order rate constant of terminal portion of the curve; T_{1/2λ_z} half-life of the terminal portion of the curve; T_{1/2α} distribution half-life, T_{1/2β}, elimination half-life; K₁₀ T_{1/2} half-life of the elimination phase; MRT mean residence time; C_{ss}

concentration at steady state; K_{10} , K_{12} , K_{21} micro-distribution rate constants; A intercept for distribution phase; B intercept for elimination phase; α rate constant associated with distribution phase; β rate constant associated with elimination phase; F relative bioavailability; NA not applicable for route of administration.

Table 6.3. Pharmacokinetic parameters (mean +/- SD) for enrofloxacin after constant rate IV infusion (1.24 mg/kg/h) and oral (5 mg/kg) administration in dogs.

Parameter	units	CRI Plasma	CRI ISF	PO Plasma	PO ISF
C_{max}	µg/mL	NA	1.43+/-0.25	1.24+/-0.39	0.70+/-0.18
$AUC_{0-\infty}$	h*µg/mL	7.06+/-1.59	21.78+/-5.47	4.46+/-1.7	5.29+/-2.88
$AUMC_{0-\infty}$	h*h*µg/mL	NA	267.32+/-87.48	18.36+/-13.74	42.78+/-36.68
AUC_{par}	h*µg/mL	12.55 +/- 2.20	6.41 +/- 1.61	NA	NA
AUC_{0-24h}	h*µg/mL	NA	NA	4.45+/-1.67	5.16+/-2.62
Cl	mL/min/kg	13.33+/-1.14	NA	NA	NA
Vd_{ss}	L/kg	3.63 +/-1.17	NA	NA	NA
Vd_{area}	L/kg	3.64 +/-1.18	NA	NA	NA
T_{max}	h	NA	10.67+/-2.07	0.94+/-0.53	4.40+/-0.89
$T_{max, adj}$	h	NA	9.5+/-2.06	NA	3.15+/-0.89
λ_z	/h	NA	0.18+/-0.032	0.35+/-0.13	0.23+/-0.05
$T_{1/2\lambda_z}$	h	NA	3.95+/-0.88	2.23+/-0.89	3.12+/-0.96
$T_{1/2\alpha}$	h	0.33+/-0.27	NA	NA	NA
$T_{1/2\beta}$	h	3.02+/-0.80	NA	NA	NA
$K_{10} T_{1/2}$	h	1.70+/-0.35	NA	NA	NA
MRT	h	NA	8.13+/-0.95	3.75+/-1.46	7.28+/-1.88
C_{ss}	µg/mL	1.62+/-0.25	NA	NA	NA
K_{10}	/h	0.42+/-0.08	NA	NA	NA
K_{12}	/h	1.33+/-0.89	NA	NA	NA
K_{21}	/h	2.09+/-1.47	NA	NA	NA
		NA	NA	NA	NA
A	µg/mL	1.43+/-0.48	NA	NA	NA
B	µg/mL	1.51+/-0.36	NA	NA	NA
α	/h	3.61+/-2.30	NA	NA	NA
β	/h	0.24+/-0.06	NA	NA	NA
F	%	NA	NA	63.22 +/- 0.24	NA

Table 6.4. Pharmacokinetic parameters (mean +/- SD) for ciprofloxacin after constant rate IV infusion (1.24mg/kg/hr) and oral (5mg/kg) administration of enrofloxacin in dogs.

Parameter	units	CRI Plasma	CRI ISF	PO Plasma	PO ISF
C_{max}	µg/mL	0.65+/-0.12	0.65+/-0.12	0.36+/-0.10	0.35+/-0.07
$AUC_{0-infinity}$	h*µg/mL	9.18+/-1.91	11.15+/-2.67	2.66+/-1.03	4.30+/-1.9
$AUMC_{0-infinity}$	h*h*µg/mL	103.54+/-27.43	183.41+/-62.21	16.54+/-7.27	52.82+/-34.12
AUC_{0-24h}	h*µg/mL	NA	NA	2.61+/-0.97	3.89+/-1.51
T_{max}	h	7.00 +/-1.67	12.00	3.00+/-1.41	6.80+/-3.03
$T_{max, adj}$	h	NA	10.83	NA	5.55+/-3.03
λ_z	/h	0.14+/-0.03	0.12+/-0.02	0.21+/-0.06	0.14+/-0.03
$T_{1/2z}$	h	5.17+/-1.23	6.25+/-1.59	3.41+/-0.70	5.26+/-1.03
MRT	h	7.21+/-1.42	12.16+/-2.14	6.09+/-0.86	11.58+/-2.29

Table 6.5. Physicochemical properties of marbofloxacin, enrofloxacin, and ciprofloxacin

	Molecular Weight g/mol	Protein Binding (%)	Protein Binding (albumin) %	Octanol water Partition Coefficient (PC)	pKa1	pKa2
Marbofloxacin	362.36	21.81 +/- 6.26	26.74 +/- 5.69	0.07 +/- 0.01	5.77 +/- 0.02	8.22 +/- 0.02
Enrofloxacin	360.15	34.74 +/- 2.33	30.04 +/- 0.81	3.54 +/- 0.02	6.26 +/- 0.03	7.81 +/- -0.03
Ciprofloxacin	331.34	18.48 +/- 2.98	14.20 +/- 1.89	0.08 +/- 0.01	6.20 +/- 0.1	8.59 +/- 0.1

Protein binding and partition coefficient were determined in the present study.

Enrofloxacin and ciprofloxacin molecular weights and pKa values were reported from Escibano *et al.*, 1997

Marbofloxacin molecular weight and pKa value were obtained through a Pfizer communication, 2003

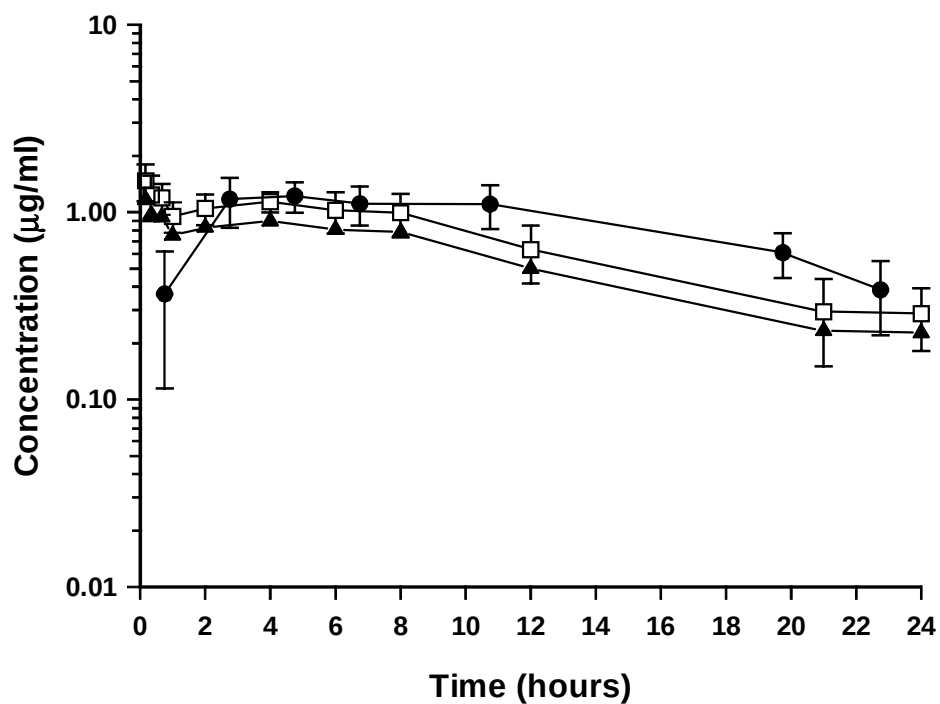


Figure 6.1. Concentrations of marbofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after constant rate IV infusion. The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration device.

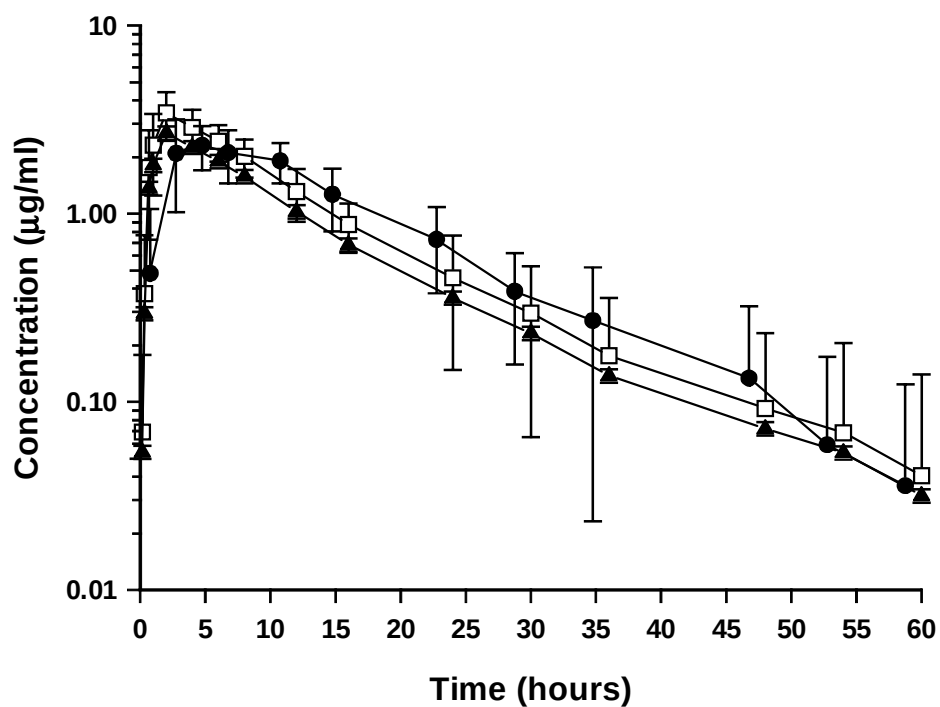


Figure 6.2. Concentrations of marbofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after oral administration (5 mg/kg). The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration device.

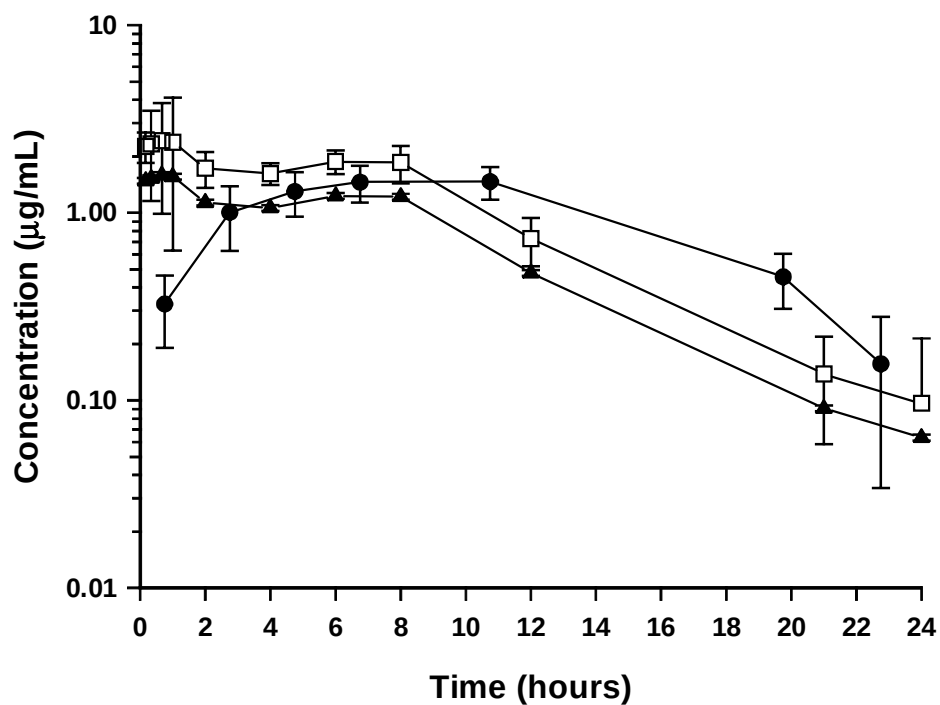


Figure 6.3. Concentrations of enrofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after constant rate IV infusion. The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration device.

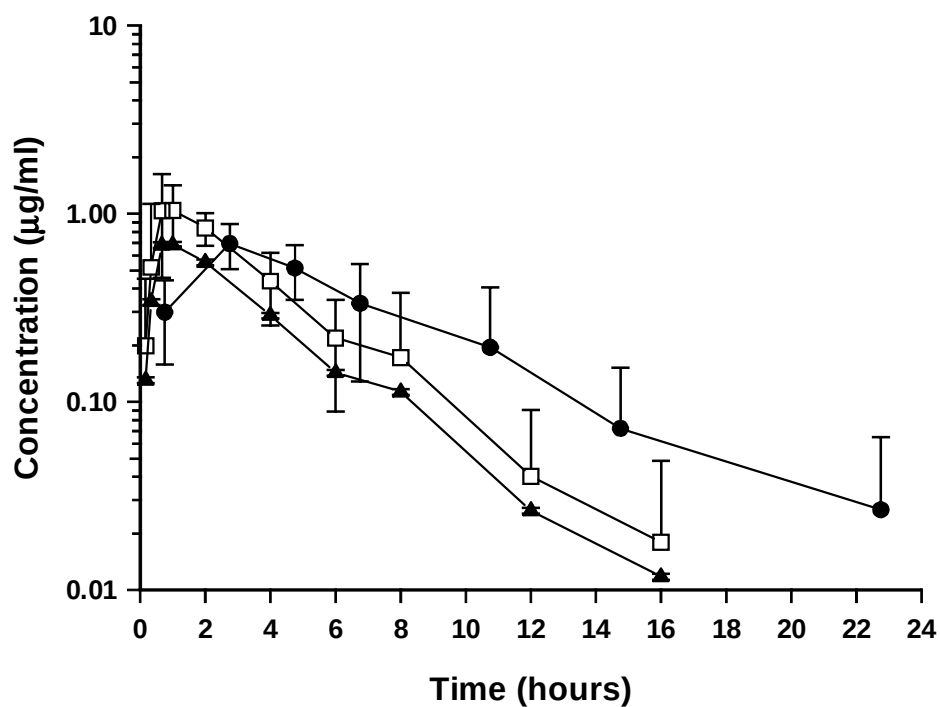


Figure 6.4. Concentrations of enrofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after oral administration (5 mg/kg). The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration device.

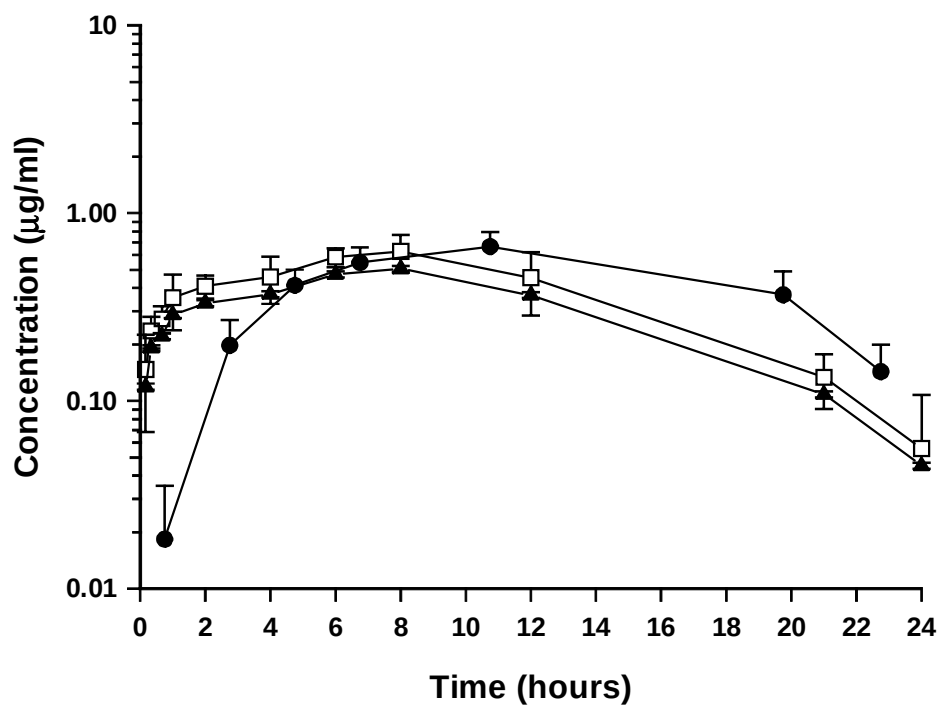


Figure 6.5. Concentrations of ciprofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after constant rate IV infusion of enrofloxacin. The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration.

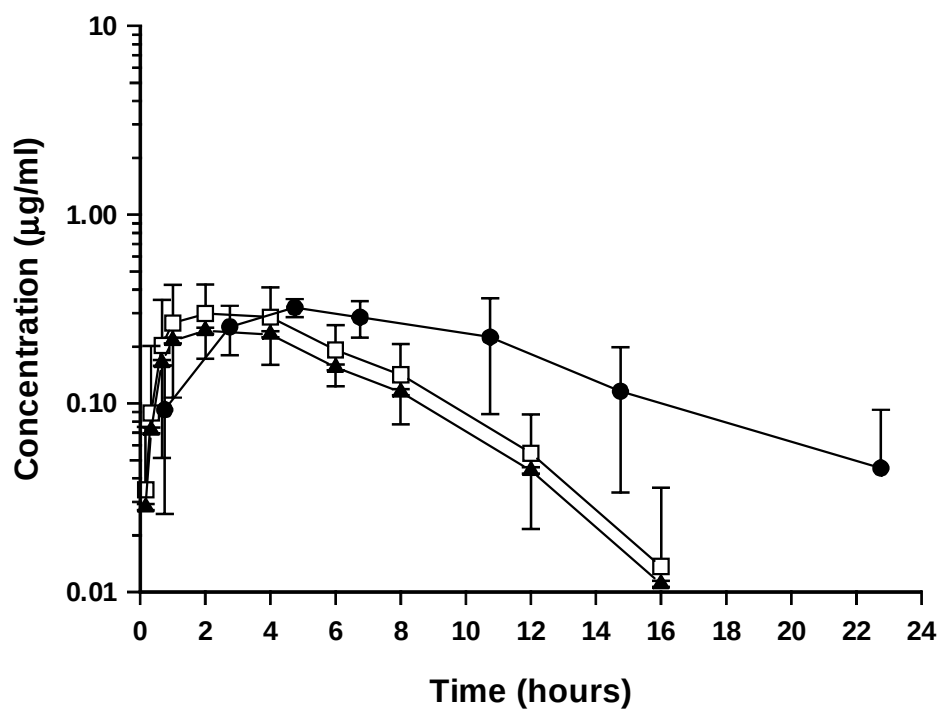


Figure 6.6. Concentrations of ciprofloxacin in plasma (total [open square] and unbound [solid triangle]) and ISF (solid circle) concentrations after oral administration (5 mg/kg) of enrofloxacin. The concentrations for total plasma and ISF concentrations are expressed as mean $\pm$ SD. Values for ISF have been corrected for the lag time (75 minutes) necessary for collection into an ultrafiltration device.

7. CONCLUSIONS

In summary, the three studies reported here have contributed to a better understanding of ISF distribution of antimicrobials in dogs. Since the majority of bacterial infections are extracellular, achieving adequate active antimicrobial concentrations above the MIC in the ISF are required for eradication of bacteria. Accurate predictions of the active antimicrobial concentration at the target site will enable the design of more precise dosage regimens for bacterial infections in dogs that perhaps can be extended to other species.

Since the protein unbound fraction of antimicrobials is the pharmacologically active fraction, determining unbound drug concentrations in the ISF is important for predicting clinical efficacy. Until recently, drug distribution to tissues in animals has been determined in studies using tissue fluid cages and collection of tissue biopsies. Both of these techniques are invasive and arguments have been presented in this thesis to show they cannot accurately determine protein unbound drug concentrations in the ISF. Ultrafiltration is a technique that samples the microbiologically active unbound ISF drug concentrations from the target site.

The single parenteral meropenem study provided clinically useful information for designing dosage regimens in dogs. Because meropenem had low protein binding, the ISF and plasma (total and unbound) concentrations were similar. Carbapenems would be a rational choice for treating bacterial infections resistant to other antimicrobials because of their high affinity for PBP, stability against β -lactamases, low development of resistance, and low toxicity. The post antibiotic effect (PAE) and low MICs of meropenem against susceptible bacteria enable convenient dosing schedules with subcutaneous and intravenous administration. We already have had several anecdotal accounts of successful treatment since publication of our results.

The study evaluating doxycycline concentrations in the ISF and plasma at steady state demonstrated that high protein binding could significantly restrict the extent of distribution of drugs into the ISF. Interstitial fluid doxycycline concentrations were significantly lower than total (bound and unbound) plasma concentrations due to the high protein binding of doxycycline. Adjusting for the degree of protein binding for highly protein bound drugs (doxycycline) is important for determining microbiologically active concentrations at the site of action (i.e. in the ISF). Designing dosage regimens for these drugs should be based on pharmacokinetics of the unbound plasma concentrations.

The study evaluating the concentrations of fluoroquinolones in plasma and ISF showed that pharmacokinetic parameters like volume of distribution and physicochemical properties like lipophilicity did not predict distribution to the ISF. All three fluoroquinolones; enrofloxacin, marbofloxacin, and ciprofloxacin had low plasma protein binding and the ISF concentrations could be predicted from total plasma concentrations at steady state.

Our studies reveal that an accurate evaluation of drug diffusion into the ISF should be performed after the plasma drug concentrations have reached steady state. Administering drugs by a constant rate infusion eliminates factors that change drug concentrations, such as half-life. This is especially important when comparing tissue distribution of drugs like enrofloxacin and marbofloxacin, which differ in elimination half-lives.

Ultrafiltration is a unique device that allows an evaluation of the distribution of various molecules in different tissues, disease states, and species. The *in vivo* ultrafiltration device used in these three studies proved to be a valuable tool for studying ISF distribution of antimicrobials that has not previously been published for studies in dogs. *In vivo*

ultrafiltration is a reliable and humane technique for collecting ISF compared to older techniques such as tissue cages and biopsies from sacrificed animals. In the studies, we did not measure the extent of binding of the drugs to tissue components and protein in the ISF and therefore, we were unable to determine the total drug concentration in the ISF, only the unbound fraction. However, measuring the unbound antimicrobial concentration rather than the total concentration is more clinically relevant since the unbound fraction is the microbiologically active fraction.

The octanol water partition coefficient (PC) values determined for the drugs in our studies did not differ to a great extent (less than one logarithmic scale). To accurately evaluate the influence lipophilicity has on the distribution of drugs from plasma into the ISF, a comparison of drugs that differ over a larger range of PC values would be required.

The pharmacokinetic studies performed with the ultrafiltration device has determined that protein binding, rather than the other physicochemical or pharmacokinetic parameters determined in the studies, was responsible for the distribution of the antimicrobials into the ISF. Therefore, designing optimal dosage regimens and predicting clinical efficacy for highly protein bound drugs, like doxycycline, should be based on plasma unbound concentrations. For low protein bound drugs; meropenem, enrofloxacin, and marbofloxacin, ISF concentrations can be predicted from total plasma concentrations at steady state.

8. FUTURE DIRECTIONS: APPLICATIONS OF MICRODIALYSIS AND ULTRAFILTRATION

These studies have focused on ISF collection of antimicrobials in the dog using *in vivo* ultrafiltration (UF). This UF technique can also be used in other species to investigate the ISF distribution of other classes of drugs. Microdialysis (MD) has been used to collect the unbound fraction of drugs in the ISF from the lung (Tomaselli *et al.*, 2003), brain (Scheyer *et al.*, 1994), neoplastic tissue (Ekstrøm *et al.*, 1997; Müller *et al.*, 1997a), bone (Thorsen *et al.*, 1996) following systemic administration, and from the subcutaneous tissues following transdermal drug application (Müller *et al.*, 1997b; Müller *et al.*, 1995; Benfeldt & Groth, 1998). Ultrafiltration has been used for ISF collection from the subcutaneous space (Janle and Kissinger, 1995), muscle (Spehar *et al.*, 1998), and bone (Janle *et al.*, 2001). Currently UF and MD ISF collection devices are being used for their potential application in describing pharmacokinetic-pharmacodynamic relationships (PK-PD) and drug distribution in critically ill patients (Delacher *et al.*, 2000; Joukhadar *et al.*, 2001; Joukhadar *et al.*, 2002; Zeitlinger *et al.*, 2003).

Pharmacokinetic surrogate markers (AUC/MIC, T>MIC, C_{max}/MIC) are used to describe the PK-PD relationships between antimicrobials and microorganisms. The PK-PD indices are used to define optimal dosage regimens for antimicrobials (Liu *et al.*, 2002; MacGowan & Bowker, 2002). These markers are based on the plasma concentration vs time profile of the antimicrobial and the MIC (Müller *et al.*, 1996). The MIC is a measurement of the activity of an antimicrobial at a single concentration against a specific inoculum of bacteria. The relationship between the host, the unbound antimicrobial concentration, and the organism is a dynamic process. An alternative method for describing the dynamic PK-

PD relationship for antimicrobials is the *in vivo*-pharmacokinetic-*in vitro*-pharmacodynamic model using MD or UF (Delacher *et al.*, 2000; Zeitlinger *et al.*, 2003). This new approach relates the pharmacokinetics of the antimicrobial to bacterial kill rates over time.

Zeitlinger *et al.* (2003) measured *in vivo* ISF fosfomycin and cefpirome concentrations by MD in septic shock patients after a single intravenous administration. The investigators then performed a PK-PD simulation by exposing strains of *P. aeruginosa* and *S. aureus in vitro* to the ISF and serum antimicrobial concentrations measured *in vivo*. This study enabled an evaluation of the unbound antimicrobial activity at the target site (i.e., ISF) (Zeitlinger *et al.*, 2003). The results showed that both serum and ISF concentrations were sufficient to kill *S. aureus* and *P. aeruginosa* strains *in vitro*.

Pharmacokinetic studies are traditionally performed on healthy patients but in disease conditions, the PK-PD activity of drugs may be altered. Pharmacokinetics of drugs can be influenced by liver disease (McLean & Morgan, 1991), anesthesia (Nimmo & Peacock, 1988), kidney disease (Bodeham *et al.*, 1988; Tam *et al.*, 2003), heart disease (Shammas & Dickstein, 1988), septic shock (Joukhadar *et al.*, 2001; Zeitlinger *et al.*, 2003), and inflammatory disease (Müller *et al.*, 1999). Both PK parameters and PD activity of drugs can be altered in septic shock. Inadequate distribution of antimicrobials to the target site may be a reason for therapeutic failure despite *in vitro* susceptibility in shock patients (Joukhadar *et al.*, 2001). Critically ill patients often have multiple organ dysfunction and drugs commonly administered to these patients often have delayed drug clearance, altered volume of distribution, and long elimination half-lives (Bodenham *et al.*, 1988). Drug absorption from peripheral sites may be reduced because of decreased tissue perfusion (De Paepe *et al.*, 2002). In human patients with septic shock, piperacillin ISF concentrations were 5-10 fold

lower than unbound plasma concentrations and these ISF concentrations were also below the MIC for bacteria associated with septic shock (Joukhadar *et al.*, 2001). An increased flux of fluid, albumin, and solutes from plasma into the interstitium due to increased capillary permeability as a response to infection may explain the observed findings (Joukhadar *et al.*, 2001). The increased albumin leakage into the interstitial space can bind drug and reduce the unbound drug available for activity (Joukhadar *et al.*, 2001). Increased fluid in the interstitial space from plasma causes a dilution effect, which will reduce the drug concentration in the ISF. In addition, administration of fluids and vasopressors to shock patients change fluid dynamics and reduce tissue perfusion, which can ultimately affect drug diffusion (Joukhadar *et al.*, 2001). Use of UF or MD in PK-PD research of antimicrobial drugs in shock patients would help predict therapeutic concentrations at target sites required for optimal therapy and reduce the risk of toxicity.

Albumin is the major protein of plasma and is important in the binding of many acidic drugs (Bodenham *et al.*, 1988). Alpha₁-acid glycoprotein is an acute phase protein that is often increased in disease (Bodenham *et al.*, 1988). Alterations in plasma protein binding of drugs occur because of changes in protein concentration, competition from endogenous compounds (urea, bilirubin), or alterations in affinity (Bodenham *et al.*, 1988; Kennedy & Van Riji, 1998; Koch-Weser & Sellers, 1976; Ögren & Cars, 1985). An increase in α_1 -acid glycoproteins in disease will increase the binding of basic drugs and decrease the unbound fraction and volume of distribution (De Paepe *et al.*, 2002). Zielmann *et al.* (1994) demonstrated an increase in unbound plasma phenytoin concentrations in critically ill trauma patients with hypoalbuminaemia. Based on these results therapeutic monitoring of drugs with narrow therapeutic drug windows should be based on unbound concentrations. Future

studies investigating tissue distribution of drugs in diseases associated with plasma protein loss (nephrotic syndrome, protein losing enteropathy) would expand our understanding of the effects of disease on drug distribution.

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