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CLASSICAL AND POPULATION PHARMACOKINETICS OF ANTIMICROBIALS, SEDATIVES AND ANAESTHETICS IN VETERINARY MEDICINE

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Abstract

Pharmacokinetics is defined as *the use of mathematical models to quantitate the time course of drug absorption and disposition in man and animals*. This discipline is the branch of pharmacology that aims to describe, through mathematics, the interactions and physiological processes (i.e. absorption, distribution, metabolism and excretion – the ADME process) that the drugs undergo after administration. When applied to a clinical situation, pharmacokinetics (PK) provides the clinician with important information on optimal drug dosages for each single patient.

In the present thesis, the focus moved to the importance of classical PK and its applications, starting from the theory and use of compartmental analysis and at the end arriving to illustrate the more recent notions of this discipline, popPK and PBPK, which are the most innovative topics in the clinical and translational pharmacology field.

The classical PK usually, translated clinically, is represented by quick studies which have a significant relevance in understanding ADME process of drugs with their desired, collateral or adverse effects. The population PK approach aims to investigate the influence of the interindividual variability in a target population of subjects, evaluating the populations characteristics that influence the fate of a drug after administration. The time required for the development of a popPK study applied to the clinical setting is much longer than a classical PK study.

Three studies of classical clinical PK and one population PK study were included in this thesis.

The first study concerned dexmedetomidine (DEX) and aimed to define the kinetic profile of this sedative following intravenous administration in a group of dairy calves, comparing its pharmacological and clinical effects with those of another α-2 agonist, xylazine, for minor surgical procedures.

The second study was related to the simultaneous administration, as preanaesthetics, of a mixture of DEX and methadone, an analgesic with a remarkable sedative efficacy belonging to the class of opioid μ-agonists. The aim was to establish the pharmacokinetic profile of this co-administration in dogs by oral transmucosal route and compare it with the intramuscular kinetic profile of the same drugs combination.

The third research addressed the species *Panthera tigris*. Since the literature concerning the non-domestic animals' PK is lacking and needy of new information, specifically, the objective was to compare the kinetic profile of a simultaneous administration of DEX and ketamine, an injectable

anaesthetic antagonist of NMDA receptors, following IM administration for chemical restraint in two groups of tigers.

The population PK study wanted to determine the popPK profile of cefazolin administered in a clinical setting for prophylactic purposes in 78 dogs, of different breed, age, weight, sex, body condition scores and health status, undergoing different surgical procedures. The ultimate goal was the definition of Clinical Breakpoints for this antimicrobial administered to the canine patient according to the guidelines of the Veterinary Committee on Antimicrobial Susceptibility Testing (VetCAST).

Finally, during these three years of PhD program, another popPK work has been started. This study is in collaboration with the veterinary anaesthesia Operative Unit of the University of Padua, which requested our laboratory for performing propofol quantification. The aim was the definition of the kinetic population profile of propofol, administered for the induction and maintenance of general anaesthesia with modern TCI technique, in order to determine a popPK model that would be applicable to dog anaesthesiology. Until now, propofol quantification has been accomplished, but the final popPK model is still under investigation (for this reason data are not reported in the thesis).

In conclusion, the use of appropriate pharmacokinetic modelling was important to perform different types of studies which helped to provide the veterinary pharmacological literature with innovative data with wide clinical implications.

Riassunto

La farmacocinetica è definita come *l'impiego di modelli matematici per quantificare il corso dell'assorbimento e della disposizione dei farmaci nell'uomo e negli animali*. Questa disciplina è la branca della farmacologia che punta a descrivere, tramite la matematica, le interazioni e i processi fisiologici (cioè assorbimento, distribuzione, metabolismo ed escrezione – il processo ADME) che i farmaci subiscono dopo la somministrazione. Quando applicata a un contesto clinico, la farmacocinetica fornisce al clinico importanti informazioni circa i dosaggi migliori di un farmaco per ogni singolo paziente.

Nella presente tesi, l'attenzione è partita dall'importanza della farmacocinetica classica e delle sue applicazioni, cominciando dalla teoria e dall'uso dell'analisi compartimentale, arrivando alla fine a illustrare le più recenti nozioni di questa disciplina, la farmacocinetica di popolazione e la farmacocinetica basata sulla fisiologia, che sono gli argomenti più innovativi nel campo della farmacologia clinica e traslazionale.

Traslata in ambito clinico, la farmacocinetica classica è solitamente rappresentata da studi rapidi che hanno rilevanza significativa nella comprensione del processo ADME dei farmaci, con i loro effetti desiderati, collaterali e avversi. L'approccio della farmacocinetica di popolazione mira a indagare l'influenza della variabilità interindividuale in una specifica popolazione target di soggetti, valutando le caratteristiche della popolazione che influenzano il destino di un farmaco dopo la somministrazione. Il tempo richiesto per lo sviluppo di uno studio di farmacocinetica di popolazione applicato in ambito clinico è più lungo rispetto a uno studio di farmacocinetica classica.

In questa tesi sono stati inclusi tre studi di farmacocinetica clinica classica e uno studio di farmacocinetica di popolazione.

Il primo studio ha riguardato la dexmedetomidina (DEX) e lo scopo è stato definire il profilo cinetico di questo sedativo dopo somministrazione endovenosa in un gruppo di vitelli, confrontandone gli effetti farmacologici e sedativi con quelli di un altro α-2 agonista, la xilazina, in corso di procedure chirurgiche minori.

Il secondo studio ha interessato la somministrazione simultanea, come preanestetici, di una combinazione di DEX e metadone, un analgesico con notevole efficacia sedativa appartenente alla classe degli oppioidi μ-agonisti. L'obiettivo è stato stabilire il profilo farmacocinetico di questa co-somministrazione per via transmucosale orale nel cane, comparandolo con il profilo cinetico intramuscolare della stessa combinazione di farmaci.

La terza ricerca ha riguardato la specie *Panthera tigris*. Dal momento che la letteratura inerente la farmacocinetica degli animali non-domestici è carente e bisognosa di nuove informazioni, lo scopo di questo lavoro è stato, specificatamente, quello di confrontare il profilo cinetico di una somministrazione simultanea di DEX e ketamina, un anestetico iniettabile antagonista dei recettori NMDA, dopo somministrazione intramuscolare di due gruppi di tigri per ottenere il contenimento farmacologico.

Lo studio di farmacocinetica di popolazione ha mirato a determinare il profilo cinetico di popolazione della cefazolina, somministrata in contesto clinico a scopo profilattico a 78 cani di differente razza, età, peso, sesso, stato di nutrizione e stato di salute, sottoposti a diverse procedure chirurgiche. L'obiettivo finale è stata la definizione del Breakpoint Clinico per questo antibiotico somministrato al paziente canino in accordo con le lineeguida del Veterinary Committee on Antimicrobial Susceptibility Testing (VetCAST).

Infine, durante questi tre anni di dottorato, è stato iniziato un altro studio di farmacocinetica di popolazione. Questo lavoro è in collaborazione con l'Unità Operativa di anestesia veterinaria dell'Università di Padova, la quale ha richiesto il nostro laboratorio per lo svolgimento delle analisi e la quantificazione del propofol. Lo scopo è stato la definizione del profilo farmacocinetico di popolazione del propofol, somministrato per l'induzione e il mantenimento dell'anestesia generale mediante moderna tecnica TCI, per determinare un modello di popolazione che fosse applicabile all'anestesia canina. Per adesso, la quantificazione del propofol è stata completata, ma la determinazione del modello è ancora in corso (per questa ragione i dati non sono stati riportati nella tesi).

In conclusione, è possibile dire che l'impiego di modellizzazioni farmacocinetiche appropriate è stato importante per svolgere tipi di studio diversi che hanno contribuito a fornire alla letteratura farmacologica veterinaria dati innovativi con ampie implicazioni cliniche.

Introduction

1.1 CLASSICAL PHARMACOKINETICSn

1.1.1 ADME PROCESSES

Absorption, distribution, metabolism, and elimination represent the physiological processes that control the drug fate time course in the body, the so-called "*ADME process*". Pharmacokinetics (PK), the study of the time course of drug concentrations in the body, provides a means of quantitating ADME parameters. When applied to a clinical situation, PK provides the clinician with important information to delineate beneficial drug dosage schedules for each single patient. In the research and premarketing phase of drug development, PK is an essential tool in determining effective safe dosage forms and regimens. To understand PK principles allows rational therapeutic decisions to be made. In food animals, PK provides the concepts for utilizing the withdrawal time to prevent violative drug residues in edible tissues of food-producing animals (Riviere, 2008).

1.1.1.1 Absorption

Absorption is the drug movement from the administration site into the bloodstream. Despite the anatomical and physiological differences among species, the biology of drug absorption and distribution and, in some cases, even elimination is very similar in that it involves drug molecules crossing the biological membranes. These membrane barriers often directly or indirectly define the nature of compartments or other mathematical modules in pharmacokinetic models. Biological spaces are defined by the restrictions on drug movement imposed by these barriers. Membranes define homogeneous tissue compartments and must be crossed in all processes of drug absorption and disposition.

The cellular membranes are primarily lipid bilayers into which are embedded proteins that may reside on either surface (intra- or extracellular) or traverse the entire structure. The lipid leaflets are arranged with hydrophilic (polar) head groups on the surface and hydrophobic (nonpolar) tails forming the interior. The proteins location in the lipid matrix is primarily a consequence of their hydrophobic regions residing in the lipid interior and their hydrophilic and ionic regions occupying the surface. Changes in the lipids fluidity alter protein conformations, which then may modulate their activity. In some cases, aqueous channels form from integral proteins that traverse the membrane. In other cases, these integral proteins may actually be enzymatic transport proteins that function as active or facilitative transport systems. The primary way for drugs to cross these lipid membranes is by passive diffusion through the lipid environment. Thus, in order for a drug to be absorbed or distributed throughout the body, it must be able to pass through a lipid membrane. In some absorption sites and in many capillaries, fenestrated pores exist, which allow some flow of small molecules. This is contrasted to some protected sites of the body (e.g., brain, cerebral spinal fluid) where additional membranes (e.g., glial cells) may have to be traversed before a drug arrives at its target site. These specialized membranes could be considered a general adaptation to further protect susceptible tissues from hostile lipophilic chemicals (Riviere, 2008).

1.1.1.2 Distribution

Distribution is the set of processes that determine the attainment of the site of action by the xenobiotic absorbed into the bloodstream at a high enough concentration for a sufficient period of time to elicit a biological response (Riviere, 2008).

Cardiac output, regional blood flow, capillary permeability, and tissue volume determine the rate of delivery and potential amount of drug distributed into tissues. Initially, liver, kidney, brain, and other well-perfused organs receive most of the drug; delivery to muscle, most viscera, skin, and fat is slower, and this second distribution phase may require minutes to several hours before the concentration of drug in tissue is in equilibrium with that in blood (Buxton and Benet, 2011).

Distribution can be considered as “absorption” into the tissues from the blood. Thus, the driving concentration is now dependent upon blood flow, the surface area for “absorption into tissues” is dependent upon capillary density and tissue mass, the relevant partition coefficient is the blood/tissue ratio, and plasma/tissue protein binding influences the process.

There are several tissues to which an active compound may be distributed, some of them capable of eliciting a pharmacologic or toxicologic (intended versus unintended) response while others serve only as a sink or depot for the chemical. Sinks may also be formed as a result of chemical binding to tissue or plasma proteins. The toxicologic significance of such sinks is that chemicals will be distributed to, and in some cases stored, in these tissues and only slowly released back into the bloodstream for ultimate elimination. Such tissue binding may actually protect against acute adverse effects by providing an “inert” site for toxicant localization. Storage may, however, prolong the overall residence time of a compound in the body and promote accumulation during chronic exposure, two processes that would potentiate chronic toxicity. Distribution of chemicals to peripheral tissues is dependent on four factors: 1) the physiochemical properties of the compound (pKa, lipid solubility, molecular weight) are most important in determining its propensity to distribute to a specific tissue; 2) the concentration gradient established between the blood and tissue; 3) the ratio of blood flow to tissue mass, and 4) the affinity of the chemical for tissue constituents (Riviere, 2008).

1.1.1.3 Metabolism

Drugs and other xenobiotics metabolism into more hydrophilic metabolites is essential for their elimination from the body, as well as for termination of their biological and pharmacological activity (Buxton and Benet, 2011). In fact, the primary mechanism by which a molecule can be excreted from the body is by becoming less lipophilic and more hydrophilic, the latter property being required for excretion in the aqueous fluids of the urinary or biliary systems. If a compound lipophilicity hinders the possibility of an easy excretion, the liver and other organs may metabolize it to more hydrophilic metabolites that have a limited distribution (and thus reduced access to sites for activity) in the body and can be more easily excreted (Riviere, 2008).

Drug metabolism or biotransformation reactions are classified as either phase I functionalization reactions or phase II conjugation reactions. Phase I reactions introduce or expose a functional group on the parent molecule. Generally, this process results in the loss of pharmacological activity, although there are examples of retention or enhancement of activity. In rare instances, metabolism is associated with an altered pharmacological activity. Prodrugs are pharmacologically inactive compounds designed to amplify the amount of the active species that reaches its site of action, which are converted rapidly to biologically active metabolites through phase I reactions (Buxton and Benet, 2011).

Phase II conjugation reactions lead to the formation of a covalent linkage between a functional group on the parent molecule or phase I metabolite and endogenously derived glucuronic acid, sulfate, glutathione, amino acids, or acetate. These highly polar conjugates are often inactive and are excreted rapidly in urines and feces (Riviere, 2008).

Furthermore, exists another mechanism, the phase III metabolism (named also enterohepatic cycle) with which some parent drugs and numerous drug metabolites derived from hepatic metabolism are excreted through the bile into the intestinal tract. These molecules, after they arrived into the intestine, can be excreted via feces although, more commonly, they are subject to reabsorption into the small intestine and, subsequently, can be reabsorbed into blood forming the so-called drug enterohepatic cycle (Riviere, 2008).

The enzyme systems involved in the biotransformation of drugs are localized primarily in the liver, although every tissue has some metabolic activity. Other organs with significant metabolic capacity include the gastrointestinal tract, kidneys, and lungs. Following oral administration of a drug, a significant portion of the dose may be metabolically inactivated in either the intestinal epithelium or the liver before the drug reaches the systemic circulation. This so-called first-pass metabolism significantly limits the oral availability of highly metabolized drugs.

Within a cell, most drug-metabolizing activity is found in the smooth endoplasmic reticulum and the cytosol, although drug biotransformation also can occur in the mitochondria, nuclear envelope, and plasma membrane. The enzyme systems involved in phase I reactions are located primarily in the endoplasmic reticulum, whereas the phase II conjugation enzyme systems are mainly cytosolic (Buxton and Benet, 2011).

Among the reactions catalyzed by drug metabolism enzymes in the hepatic endoplasmic reticulum, cytochrome (Cyt) P450-dependent mixed-function oxidation is the most studied. This reaction catalyzes the hydroxylation of hundreds of structurally different drugs and compounds, whose only common feature is a relatively high lipophilicity. The enzyme consists of a isoenzymes family embedded in the endoplasmic reticulum membrane. Its name derived from the fact that the cytochrome is a pigment that exhibits a maximal absorbance wavelength of 450 nm when reduced and complexed with carbon monoxide (Riviere, 2008).

Species differences in drug metabolism are, in most cases, the primary source of variation in drug disposition and in drug activity or toxicity. Metabolism is necessary for the animal or human body to free itself of lipophilic xenobiotics as an efficient defense mechanism against adverse effects. Therefore, any process or factor that modifies the drug/metabolite concentration at a site of action will cause an altered activity or toxicity profile (Riviere, 2008).

1.1.1.4 Excretion

Drugs are eliminated from the body either unchanged by the process of excretion or converted to metabolites. Excretory organs (lung excluded), eliminate polar compounds more efficiently than substances with high lipid solubility. Lipid-soluble drugs thus are not readily eliminated until they are metabolized to more polar compounds (Buxton and Benet, 2011).

The main route for drug elimination from the body is the kidney. Drugs can also be eliminated in bile, sweat, saliva, tears, milk, and expired air; however, for most therapeutic drugs these routes are not quantitatively important as mechanisms for reducing total body burden of drug. The degree of lipid solubility and extent of ionization in blood determines how much of drug will be excreted by the kidney. For drugs that are first biotransformed by the liver, the more water-soluble metabolites are then ultimately excreted by the kidney (Riviere, 2008).

Drugs are normally excreted into the urine through the processes of glomerular filtration, active tubular secretion and/or reabsorption, and/or passive, flow-dependent, nonionic back diffusion.

The sum of these processes determines the ultimate elimination of a specific drug by the kidney. If a drug is reabsorbed back from the tubular fluid into the blood, its global renal excretion will be reduced. In contrast, if a drug is secreted from the blood into the tubular fluid, its global excretion will be increased (Buxton and Benet, 2011).

As an exocrine function of the liver, bile excretion is present in almost all vertebrates and it represents a secondary (compared to urinary excretion) but also relevant route of excretion of molecules from the body. In addition to its physiological functions (to serve as the excretory route for products of biotransformation, to facilitate the intestinal absorption of ingested lipids such as fatty acids, cholesterol, lecithin, and/or monoglycerides and to serve as a major route for cholesterol elimination), bile is also pharmacologically and

toxicologically important since some heavy metals and enzymes are also excreted via the biliary system. Bile secretion is very important to chemical/drug transport and elimination under both physiological and pathological conditions. (Riviere, 2008).

There are different biliary transport pathways for organic anions, cations, and neutral compounds. Organic anions and cations can be actively transported into bile by carrier systems similar to those involved in the renal tubule. These are nonselective transport systems, and ions with similar electrical charge may compete for the same transport mechanisms. Additionally, another carrier system, whose activity is sex-dependent, may be involved in the active transport of steroids and related compounds into bile. In contrast to renal excretion, amphiphatic drugs (those having both polar and nonpolar properties) are preferentially excreted in the bile. The drug (or metabolite) excreted into the small intestine can enter into the enterohepatic cycle. In general, different factors are able to influence the type of excretion through which molecular weight is a key determinant of the extent to which drug/metabolite molecules are transported into bile. The molecular weight cutoff required for biliary excretion is greater than that for renal excretion, being from 300–500 Da in most species. If the molecular weight is lower, the compound may be preferentially excreted in urine. Molecules with weights from 3–500 to 850 Da may be eliminated via both the renal and biliary routes. Excretion of molecules larger than 850 Da occurs mainly via the biliary active transport system. However, molecular weight is not the only factor determining the route of excretion. Physicochemical properties of the drug (polarity/ lipophilicity, structure) are also important to the extent of biliary excretion of a drug/metabolite, with amphiphatic drugs being well secreted by the biliary route (Buxton and Benet, 2011).

1.1.2 CLINICAL PHARMACOKINETICS AND PK PARAMETERS

Pharmacokinetics is best defined as "*the use of mathematical models to quantitate the time course of drug absorption and disposition in man and animals"* (Riviere, 2008).

This discipline has allowed dosages of drugs to be tailored to individuals or groups to optimize therapeutic effectiveness, minimize toxicity, and avoid unsafe tissue residues in the case of food-producing animals (Riviere, 2008).

The fundamental principle of clinical PK is that a relationship exists between the pharmacological effects of a drug and an accessible concentration of the drug (e.g., in blood or plasma). In most cases, the concentration of drug at its sites of action will be related to the concentration of drug in the systemic circulation. The pharmacological effect that results may be the clinical effect desired, a toxic effect, or in some cases an effect unrelated to the known therapeutic efficacy or toxicity. Clinical PK attempts to provide both a quantitative relationship between dose and effect and a context within which to interpret measurements of concentrations of drugs in biological fluids and their adjustment through changes in dosing for the patient's benefit. The importance of PK in patient care is based on the improvement in therapeutic efficacy and the avoidance of adverse effects that can be attained by application of its principles when dosage regimens are chosen and modified (Buxton and Benet, 2011).

The four most important parameters governing drug disposition are *bioavailability*, the fraction of drug absorbed as such into the systemic circulation; *volume of distribution,* a measure of the apparent space in the body available to contain the drug based on how much is given versus what is found in the systemic circulation; *clearance,* a measure of the body efficiency in eliminating drug from the systemic circulation; and *elimination* half-life*,* a measure of the rate of removal of drug from the systemic circulation (Baggot, 2001).

1.1.2.1 Bioavailability

The extent of drug absorption is defined as absolute systemic availability and is denoted in pharmacokinetic equations as the fraction of an applied dose absorbed into the body (*F*) that escaped any first-pass metabolism reaching the systemic circulation (Riviere, 2008; Buxton and Benet, 2011).

If one is estimating the extent of drug absorption by measuring the resultant concentrations in either blood or excreta, one must have an estimate of how much drug normally would be found if the entire dose were absorbed. To estimate this amount, an intravenous dose is required since this is the only route of administration that guarantees that 100% of the dose is systemically available ($F = 1.0$) and the pattern of disposition and metabolism can be quantitated. Parameters used to measure systemic availability are thus calculated as a ratio relative to the intravenous dose.

For most therapeutic drug studies, systemic absorption is assessed by measuring blood concentrations. The amount of drug collected after administration by the route under study is divided by that collected after intravenous administration. When drug concentrations in blood (or serum or plasma) are assayed, total

absorption is assessed by measuring the area under the concentration-time curve (AUC) using the trapezoidal method. This is a geometrical technique that breaks the AUC into corresponding trapezoids based on the number of samples assayed. The terminal area beyond the last data point (a triangle) is estimated and added together with the previous trapezoidal areas (Baggot, 2001). Absolute systemic availability then is calculated as Equation 1:

$$F\ (\%) = \frac{AUC \quad Dose_{\ route\ iv}}{AUC_{iv}\ Dose_{route}} \qquad \text{(Eq. 1)}$$

Calculation of *F* provides only an estimate of the extent, and not rate, of drug absorption. Finally, so-called relative systemic availability may be calculated for two extravascular formulations where the data for the reference product is in the denominator and the test formulation in the numerator (Riviere, 2008).

1.1.2.2 Clearance

Clearance is a concept widely used to measure the efficiency of drug elimination from an organ or the whole body. There are two definitions of renal clearance that are used to define equations to calculate this parameter from real data. The first is "*the volume of blood cleared of a substance by the kidney per unit of time"*, that is, the volume of blood required to contain the quantity of drug removed by the kidney during a specific time interval. The second definition is "*the rate of drug excretion relative to its plasma concentration"* (Riviere, 2008).

Assuming complete bioavailability, the steady-state concentration of drug in the body will be achieved when the rate of drug elimination equals the rate of drug administration, situation explained with Equation 2:

$$Dosing\ Rate = Cl \cdot C_{SS} \qquad \text{(Eq. 2)}$$

where *Cl* is clearance of drug from the systemic circulation and C_{SS} is the steady-state concentration of drug.

If the desired steady-state concentration of drug in plasma or blood is known, the rate of clearance of drug will dictate the rate at which the drug should be administered (Buxton and Benet, 2011).

Principles of drug clearance are similar to those of renal physiology, where, e.g., creatinine clearance is defined as the rate of elimination of creatinine in the urine relative to its concentration in plasma. At the simplest level, clearance of a drug is its rate of elimination by all routes normalized to the concentration of drug *C* in some biological fluid where measurement can be made with Equation 3:

$$Cl = Rate\ of\ elimination/C \qquad \text{(Eq. 3)}$$

Thus, when clearance is constant, the rate of drug elimination is directly proportional to drug concentration. Note that clearance does not indicate how much drug is being removed, but rather the volume of biological

fluid such as blood or plasma from which drug would have to be completely removed to account for the clearance per unit of body weight (e.g., mL/min per kg) (Riviere, 2008).

Clearance of drug by several organs is additive. Elimination of drug from the systemic circulation may occur as a result of processes that occur in the kidney, liver, and other organs. Division of the rate of elimination by each organ by a concentration of drug (e.g., plasma concentration) will yield the respective clearance by that organ. Added together, these separate clearances will equal systemic clearance (Equation 4):

$$Cl_{body} = Cl_{renal} + Cl_{hepatic} + Cl_{other} \qquad \text{(Eq. 4)}$$

Systemic clearance may be determined at steady state by using Equation 2. For a single dose of a drug with complete bioavailability and first-order kinetics of elimination, systemic clearance may be determined from mass balance and the integration of Equation 3 over time with Equation 5:

$$Cl = Dose/AUC \qquad \text{(Eq. 5)}$$

where *AUC* is the total area under the curve that describes the measured concentration of drug in the systemic circulation as a function of time (from zero to infinity) (Baggot, 2001).

1.1.2.3 Volume of Distribution

The extent of distribution of a compound is termed its volume of distribution (V_d) and is considered one of the most important PK parameters given its usefulness in considering processes of drug disposition. The volume of distribution relates the amount of drug in the body to the concentration of drug (*C*) in the blood or plasma depending on the fluid measured. This volume does not necessarily refer to an identifiable physiological volume but rather to the fluid volume that would be required to contain all of the drug in the body at the same concentration measured in the blood or plasma (Equation 6):

$$V = Dose_{(mg)}/C_{(mg/L)} \qquad \text{(Eq. 6)}$$

The V_d is actually a proportionality constant relating the plasma concentration to administered dose (Buxton and Benet, 2011).

A drug volume of distribution therefore reflects the extent to which it is present in extravascular tissues and not in the plasma. It is reasonable to view V_d as an imaginary volume, since for many drugs the volume of distribution exceeds the known volume of any and all body compartments. For drugs that are bound extensively to plasma proteins but that are not bound to tissue components, the volume of distribution will approach that of the plasma volume because drug bound to plasma protein is measurable in the assay of most drugs. In contrast, certain drugs have high volumes of distribution even though most of the drug in the circulation is bound to albumin because these drugs are also sequestered elsewhere (Riviere, 2008).

The volume of distribution may vary widely depending on the relative degrees of binding to high-affinity receptor sites, plasma and tissue proteins, the partition coefficient of the drug in fat, and accumulation in poorly perfused tissues. As might be expected, the volume of distribution for a given drug can differ according to patient's age, gender, body composition, and presence of disease (Buxton and Benet, 2011).

Several volume terms are commonly used to describe drug distribution, and they have been derived in a number of ways. The volume of distribution defined in Equation 6 considers the body as a single homogeneous compartment. Clearance of drug from this compartment occurs in a first-order fashion, as defined in Equation 3; that is, the amount of drug eliminated per unit of time depends on the amount (concentration) of drug in the body compartment. Equation 7 describe the decline of plasma concentration with time for a drug introduced into this central compartment:

$$C = [Dose/V_d] \cdot [e^{-kt}] \qquad \text{(Eq. 7)}$$

where *k* is the rate constant for elimination that reflects the fraction of drug removed from the compartment per unit of time. This rate constant is inversely related to another fundamental PK parameter, the half-life of the drug with the relation $k\ t_{1/2} = 0.693 = \ln 2$ (Baggot, 2001).

1.1.2.4 Half-life

Half-life is the pharmacokinetic parameter used to measure the overall rate of drug elimination. Following the intravenous injection of a single dose, the half-life expresses the time required for the plasma concentration, as well as the amount of drug in the body, to decrease by 50% through elimination processes, that is, during the elimination phase of the disposition curve. At therapeutic dosage, the majority of drugs are eliminated by first-order kinetics, which implies that a constant fraction (50%) is eliminated each clinically relevant half-life. The clinically relevant half-life is that associated with the therapeutic range of plasma concentrations of the drug and it is used in the selection of a dosage interval. Another application of this pharmacokinetic parameter is in the comparison of the rate of drug elimination in different species (Baggot, 2001).

Useful applications of half-life include selection of the dosage interval associated with a dosage regimen, prediction of the time required to attain a steady-state (plateau) concentration during constant intravenous infusion, and species comparison of the overall rate of elimination of a drug (or marker substance for an elimination process). Because of the availability in research papers of half-life values for drugs and the hybrid nature of half-life, this parameter is generally used for interspecies allometric scaling of drug elimination. Although useful under certain circumstances, the predictive value of this application of half-life depends upon knowledge of the elimination process for the drug and the judicious selection of the species to be included in the scaling technique. It should be appreciated that half-life is a composite (hybrid) pharmacokinetic parameter which reflects the decline of systemic drug concentrations during a dosing

interval at steady-state as depicted, expressing the relationship between the volume of distribution and the systemic (body) clearance of the drug (Equation 8) (Baggot, 2001; Riviere, 2008).

$$t_{1/2} \cong 0.693 \cdot V_{SS}/Cl_{body} \qquad \text{(Eq. 8)}$$

Bioavailability, clearance, volume of distribution and half-life are the fundamental parameters of pharmacokinetics. One of the main clinical applications of pharmacokinetic principles is to construct dosage regimens. Diseases that change any of these primary pharmacokinetic parameters would be expected to change the plasma concentrations achieved after dosing, and thus drug effect. For example, renal disease that reduced GFR, might reduce body clearance (Cl_B) for drugs primarily eliminated by the kidney. Similarly, liver disease might alter disposition of hepatically cleared drugs. In contrast, diseases that resulted in severe elimination and fluid accumulation could alter V_d. Both of these scenarios would increase half-life and alter plasma concentrations (Riviere, 2008).

1.1.3 PHARMACOKINETIC MODELS

As mentioned above, "*pharmacokinetics is the use of mathematical models to quantitate the time course of drug absorption and disposition in man and animals"* (Riviere, 2008). This discipline aims to describe, through mathematics, the interactions and physiological processes that the drugs undergo after being administered. The most widely used modeling paradigm in comparative and veterinary medicine is the compartmental approach. In this analysis, the body is viewed as being composed of a number of so-called equilibrium compartments, each defined as representing nonspecific body regions where the rates of compound disappearance are of a similar order of magnitude. Specifically, the fraction or percent of drug eliminated per unit of time from such a defined compartment is constant. Such compartments are classified and grouped on the basis of similar rates of drug movement within a kinetically homogeneous but anatomically and physiologically heterogeneous group of tissues. These compartments are theoretical entities that allow formulation of mathematical models to describe a drug behavior over time with respect to movement within and between compartments. These models are nothing than a set of equations described to determine the *pharmacokinetic profile* of a drug. Depending on the physiological, paraphysiological or pathophysiological situation taken into consideration, the type of drug and the type of administration, and on the experimental or clinical scenario, the kineticist decides to consider specific variables from which different kinetic profiles will be determined through different mathematical models (Bonate, 2011).

1.1.3.1 One-compartment open models

The simplest compartment model is when one considers the body as consisting of a single homogeneous compartment. In this case, the entire dose of drug is assumed to move out of the body at a single rate (Riviere, 2008). The specific model, illustrated in Figure 1, is best theorized as instantly dissolving and homogeneously mixing the drug in a beaker from which it is eliminated by a single rate process described by the rate constant K, now termed K_{el}.

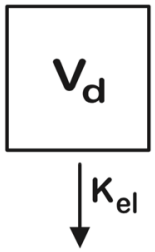

Figure 1 - One-compartment open pharmacokinetic model (from Riviere, 2008)

Since the drug leaves the system, the model is termed *open*. Equation 9 is the pharmacokinetic equation for the one-compartment open model in terms of concentrations which are experimentally accessible by sampling blood:

$$C_p = \frac{X_0}{V_d \cdot e^{-K_{el}t}} = C_{p_0} \cdot e^{-K_{el}t} \quad \text{(Eq. 9)}$$

A semilogarithmic plot seen after intravenous administration using this model is depicted in Figure 2. V_d quantitates the apparent volume into which a drug is dissolved, since the true volume is determined by the physiology of the animal, the relative transmembrane diffusion coefficients, and the chemical properties of the drug being studied (Bonate, 2011).

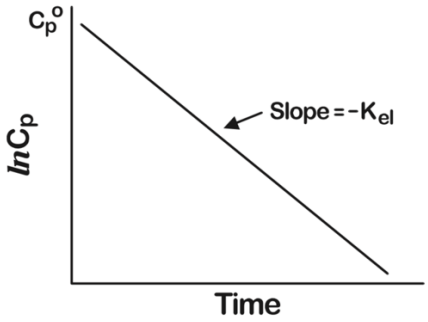

Figure 2 - Semilogarithmic concentration-time profile for a one-compartment drug with slope is $-K_{el}$ and intercept Cp_0 (from Riviere, 2008)

From this simple analysis, and using the model in Figure 1, a number of useful pharmacokinetic parameters may be defined. Assuming that an experiment such as depicted in Figure 2 has been conducted using a dose of D and values for K_{el} and V_d have been determined, $t_{1/2}$ can be calculated from Equation 9 as below:

$$K = 0.693/t_{1/2} \qquad \text{(Eq. 10)}$$

In the case in which the drug is administered by an extravascular route, the kinetist has to consider that the drug must be absorbed from the dosing site into the bloodstream, as illustrated in Figure 3 (Riviere, 2008).

Figure 3 - One-compartment open pharmacokinetic model with first-order absorption (from Riviere, 2008)

Thus, the resulting semilogarithmic concentration-time profile, showed in Figure 4, is characterized by an initial rising component that peaks and then undergoes the log-linear decline.

The rate of the drug absorption is governed by the rate constant K_a. When the absorption phase is finally complete, elimination is still described by K_{el}. The overall elimination half-life can still be calculated using K_{el} if this terminal slope is taken after the peak (C_{max}) in the linear portion of the semilogarithmic plot (providing $K_a >> K_{el}$). However, calculation of V_d and Cl becomes more complicated since K_a is present, and unlike an intravenous injection, it cannot be assumed that all of the drug has been absorbed into the body (Bonate, 2011).

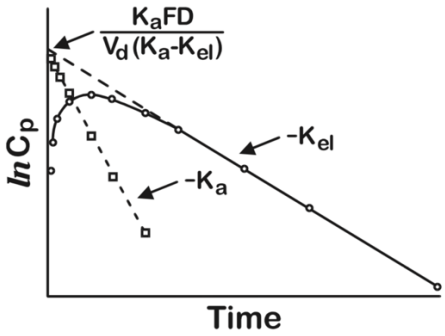

Figure 4 - Semilogarithmic plot of plasma concentration versus time using a one-compartment open pharmacokinetic model with first-order absorption. The profile is decomposed into two lines with slopes $-K_a$ and $-K_{el}$ (from Riviere, 2008)

Here below is given the Equation 11 that represents the expression of the model described in Figure 4.

$$C_p = \frac{K_a \cdot F \cdot D}{V_d \cdot (K_a - K_{el})} \cdot [e^{-K_{el}t} - e^{K_a t}] \qquad \text{(Eq. 11)}$$

Figure 4 illustrates the process in which an observed semilogarithmic profile is plotted as a composite of its absorption phase (controlled by K_a) and the elimination phase (controlled by K_{el}) and where F is the bioavailability, V_d is the volume of distribution and D is the dose of the drug administered (Riviere, 2008).
In contrast to the intravenous scenario, the time zero intercept is now a more complex function, which is dependent upon the fraction of administered dose that is systemically available and thus able to be acted on by the elimination process described by the rate constant K_{el}.

1.1.3.2 Two-compartment models

Many drugs are not described by a simple one-compartment model since the plasma concentration time profile is not a straight line. This reflects the biological reality that for many drugs, the body is not a single homogeneous compartment, but instead is composed of regions that are defined by having different rates of drug distribution. Such a situation is well described by a two-compartment model, in which the drug initially is distributed in the central compartment and by definition is eliminated from this compartment. The difference is that now the drug also distributes into other body regions at a rate that is different from that of the central compartment (Figure 5) (Bonate, 2011).
There are many factors that determine the rate and extent of drug distribution into a tissue (e.g., blood flow, tissue mass, blood/tissue partition coefficient, etc.). When the composite rates of these flow and diffusion processes are significantly different from K_{el}, then the concentration-time profile will reflect this by assuming a biexponential nature. For many drugs, the central compartment may consist of blood plasma and the extracellular fluid of highly perfused organs such as the heart, lung, kidneys, and liver. Distribution to the rest of the body occurs more slowly, which provides the physiological basis for a two-compartment model. Such a peripheral compartment is defined by a distribution rate constant (K_{12}) out of the central compartment and a redistribution rate constant (K_{21}) from the peripheral back into the central compartment. This is a pharmacokinetic concept where the distribution rate constants are significantly slower than K_{el} and thus become the rate-limiting factor defining the terminal slope of a biexponential concentration-time profile (Baggot, 2001).

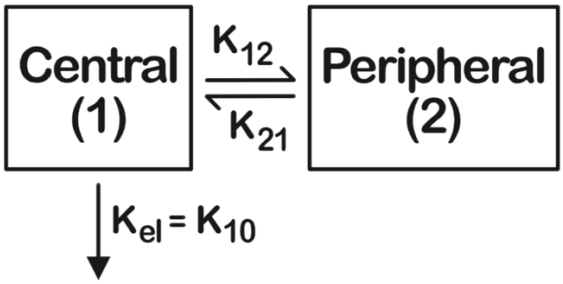

Figure 5 - Generalized open two-compartment pharmacokinetic model after intravenous administration with elimination (Kel) from the central compartment. K_{12} and K_{21} represent intercompartmental micro-rate constants (from Riviere, 2008)

One of the most common scenarios is the one described by a two-compartment model after intravenous administration (Figure 5) (Riviere, 2008). The fundamental principle involved is that the observed serum concentration time profile is actually the result of two separate pharmacokinetic processes that can be described by two separate exponential terms, written with Equation 12a:

$$Cp = A \cdot e^{-\alpha t} + B \cdot e^{-\beta t} \quad \text{(Eq. 12a)}$$

In this case we have terms with slopes (α and β) and corresponding intercepts (*A* and *B*). The concentration-time profile on semilogarithmic plot is illustrated in Figure 6. By definition, $\alpha >> \beta$ and thus β is the terminal slope (Riviere, 2008).

In the two-compartment model of Figure 5, this equation describes drug movement in terms of the mass of drug in compartment one (central) and two (peripheric).

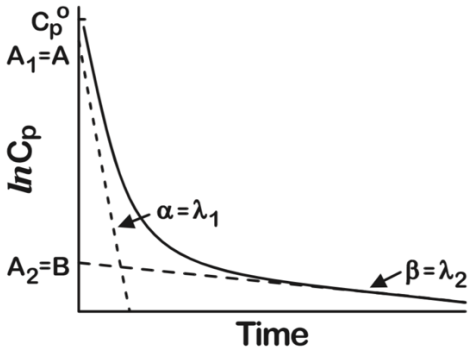

Figure 6 - Semilogarithmic plasma concentration versus time profile of a drug described by a two-compartment open model (parameters are defined in the text) (from Riviere, 2008)

Multicompartmental models have their own syntax. A preferred nomenclature uses the Greek letter λ_n, with n = 1, 2, 3, . . . progressing from the most rapid to the slowest rate process. The corresponding intercept terms are denoted as A_n. This nomenclature describes any multicompartmental model without implying a physiological basis to the underlying mechanism responsible for the different rates observed (Bonate, 2011). Given the above, the biexponential equation (Equation 12a) for a two-compartment model may now be transformed into Equation 12b:

$$Cp = A \cdot e^{-\lambda 1 t} + A \cdot e^{-\lambda 2 t} \quad \text{(Eq. 12b)}$$

The actual rate constants describing flux between compartments are now termed micro-rate constants and denoted by k_{xy}, where compound moves from x → y. When the origin or destination of a compound is outside of the body, x or y is denoted as 0, respectively. K_a thus becomes k_{01} and K_{el} becomes k_{10}. With a two-compartment model, three V_d may be calculated; the volume of the central compartment V_c or V_1, the

peripheral compartment V_p or V_2, and the total volume of distribution in the body V_t or $V_1 + V_2$. The only estimate of V_t which can be broken into its component central and peripheral volumes is the volume of distribution at steady-state, V_{dss}.

The power and essence of pharmacokinetic analysis is that the physiological processes driving drug disposition can be quantitated by using differential equations describing drug flux into and out of observable compartments, with most models structured to reflect the central compartment, which is monitored via blood sampling as the primary point of reference (Riviere, 2008).

The extravascular dose administered as input into a two-compartment model is reported in Figure 7.

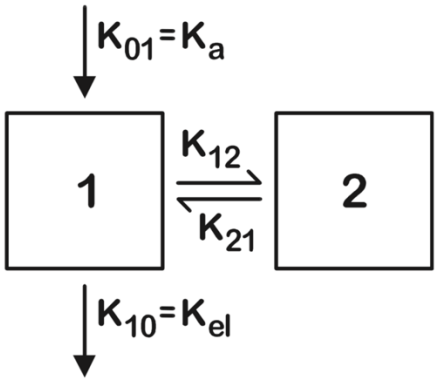

Figure 7 - Generalized open two-compartment pharmacokinetic model with first-order absorption (K_{01}) into and elimination (K_{el}) from the central compartment. K_{12} and K_{21} represent intercompartmental constants reflecting distribution (from Riviere, 2008)

There are a number of approaches to solve this model. An example of the equation (Equation 13) describing such a plasma profile would be:

$$C_p = \frac{k_{01} \cdot D}{V_1} \cdot \left[A_1' \cdot e^{-\alpha\lambda 1 t} + A_2' \cdot e^{-\lambda 2 t} - A_3' \cdot e^{-K_{01} t}\right] \qquad \text{(Eq. 13)}$$

In this case, the intercepts (A_n') are different than those obtained from an intravenous study (A_n) and significantly more complex since the "driving" concentrations in compartments one and two are now dependent upon the fraction absorbed in a fashion analogous to the terms of Equation 10 seen for absorption in a one-compartment model. However, these equations are now easily analyzed using modern computer software (Bonate, 2011).

1.1.3.3 Multicompartmental models

The last level of compartmental model complexity is the three-compartment model showed in Figure 8.

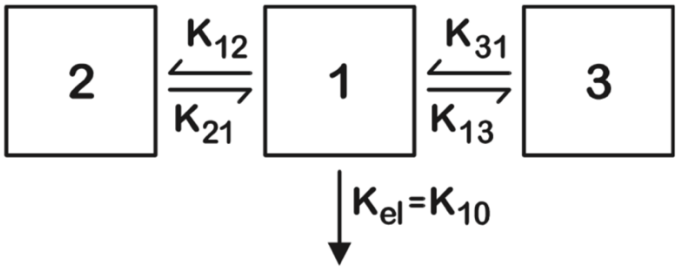

Figure 8 - Three-compartment pharmacokinetic model after intravenous administration (parameters are defined in text) (from Riviere, 2008)

In this case, the drug distributes into two different compartments from the central compartment, one with rates faster (k_{12}/k_{21}) and the other with rates slower (k_{13}/k_{31}) than k_{10}. This model is applicable to many three-compartment drugs encountered in veterinary medicine (e.g., aminoglycosides, tetracyclines, persistent chlorinated hydrocarbon pesticides).

These types of models are generally employed when experiments are conducted over long time frames and concentration-time profiles monitored to low concentrations. If the data are truncated at earlier times, a normal two-compartment model is adequate to describe the data. However, if the goal of a study is, for example, to describe the tissue residue depletion profile of a drug in a food-producing animal, the tissue concentration-time profile would be of interest since it is the tissue where legal tolerances are established. This makes such complicated models useful in food animal veterinary medicine (Baggot, 2001).

Models consisting of more than three compartments have been used when the data are of sufficient quality (sensitive analytically method, sufficient samples) to warrant such an analysis. The poly-exponential equation (Equation 14) describing an *n*-compartment models is:

$$Cp = \sum_{i=1}^{n} A_i \cdot e^{-\lambda it} \quad \text{(Eq. 14)}$$

Compartmental modeling concepts and techniques have defined the discipline of pharmacokinetics representing extremely useful tools. One- and two-compartment analyses form the basis for most models used in human as well as veterinary and comparative medicine. These two models also serve as the foundation upon which many of the other techniques are based (Riviere, 2008).

1.1.3.4 Noncompartmental models

Over the last two decades, there has been generalized adoption of noncompartmental methods in veterinary pharmacokinetics (Bonate, 2011).

This approach is for the most part actually an application of well-developed statistical moment theory. The noncompartmental approach involves primarily calculation of the Slopes, Heights, Areas and Moments (SHAM) of plasma concentration time curves. Statistical moment theory describes drug behavior based on the mean or average time an administered drug molecule spends in a kinetically homogeneous space, a concept identical to that of a compartment. The difference is that no specific inferences are being made about the structure of these spaces (Riviere, 2008).

Rather than being based on diffusion, these models are based on probability density functions that define drug disposition in terms of the probability of the drug being in a specific location. Instead of determining rates in terms of rate constants or half-lives, they describe processes in terms of statistical moments; the most useful is the mean residence time (MRT).

These are based on plasma concentration data and are determined by calculating areas under concentration versus time curves. MRT is calculated with Equation 15:

$$MRT = \frac{\int_0^\infty tC(t)dt}{\int_0^\infty C(t)dt} = \frac{AUMC}{AUC} \qquad \text{(Eq. 15)}$$

The denominator of this equation is the AUC, the numerator is known as the area under the [first] moment curve (AUMC), which is the concentrationtime-time (CT-T) profile.

The primary task to solve non-compartmental models is the direct estimation of the moments from data. This essentially is determining the relevant AUCs and moments from the C-T profile. The simplest and most commonly used method for estimating area under any curve is the trapezoidal rule (Figure 9) (Bonate, 2011).

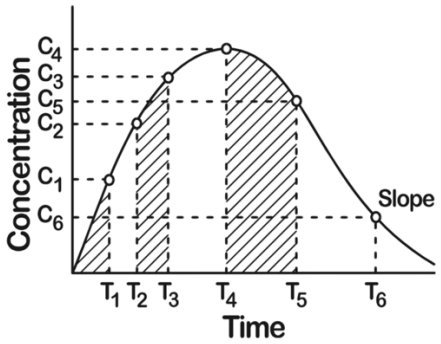

Figure 9 - Breakdown of a plasma concentration versus time curve into trapezoids used to calculate the area under the curve. The terminal area from T_6 to T_∞ is calculated from extrapolating the terminal slope (from Riviere, 2008)

The summation is over N trapezoids, formed by N+1 data points. This algorithm is quick and, if enough data points are available, relatively accurate. It is also a simple algorithm to implement on a computer. The area under each pair of connected points describes a trapezoid (except when one of the points has zero value, it is a triangle). The area under the entire curve is then the sum of the areas of the individual trapezoids, which can easily be calculated with Equation 16:

$$AUC = \sum_{n=1}^{N} \frac{C_n + C_{n+1}}{2} \cdot (t_{n+1} - t_n) \qquad \text{(Eq. 16)}$$

The area under the final triangle is estimated by the AUC and must be estimated to infinite time. Generally, this portion of the AUC should be less than 20% of the total.

The statistical moment methods provide a tool for calculating many of the common pharmacokinetic parameters that are routinely encountered in veterinary medicine (Figure 10). This includes the concept of bioequivalence, as well as generating parameters that are used to construct dosage regimens and assess the effect of disease of drug effects. Today, noncompartmental analysis is the primary method by which pharmacokinetic parameters are now determined in veterinary medicine (Riviere, 2008).

$Cl_B = Dose / AUC$
$Cl_D = V_c \lambda_1 - Cl_B$
$Vd_{ss} = (Dose \times AUMC) / AUC^2$
$V_c = Dose / Cp_0$
$MRT_{iv} = AUMC/AUC = V_d(ss) / Cl_B$
$MAT = MRT_{route} - MRT_{iv}$
$T1/2 = 0.693\ MRT = 0.693\ Cl_B / Vd_{ss}$
$T1/2\ (\lambda) = 0.693 / \lambda$
$F = (AUC_{route})\ (Dose_{iv}) / (AUC_{iv})\ (Dose_{route})$
$AUC = \Sigma\ A_i / \lambda_i$
$AUMC = \Sigma\ A_i / (\lambda_i)^2$
$Cp_0 = \Sigma\ A_i$

Figure 10 - Noncompartmental equations for calculating common pharmacokinetic parameters from an analysis of a concentration-time profile described by a polyexponential equation of the form $f(t)=A_ie^{-\lambda t}$ (from Riviere, 2008)

1.1.3.5 Nonlinear models

Most pharmacokinetic models incorporate the common assumption that drug elimination from the body is a first-order process, and the rate constant for elimination is assumed to be a true constant, independent of drug concentration. In such cases, the amount of drug cleared from the body per unit time is directly dose or concentration-dependent, the percentage of body drug load that is cleared per unit time is constant, and the drug has a single constant elimination half-life. Fortunately, first-order elimination (at least apparent first-order elimination) is typical in drug studies. First-order linear systems application greatly simplifies dosage design, bioavailability assessment, dose-response relationships, prediction of drug distribution and disposition, and virtually all quantitative aspects of pharmacokinetic simulation.

However, drugs most often are *not* eliminated from the body by mechanisms that are truly first-order by nature. Actual first-order elimination applies only to compounds that are eliminated exclusively by mechanisms not involving enzymatic or active transport processes (i.e., processes involving energy).

The reason energy-involved processes are not strictly first-order is that they are generally saturable, or more specifically are *capacity-limited*. At clinical dosages, the majority of drugs do not reach saturation concentrations at the reaction sites and follow first-order linear kinetics. For drugs eliminated by zero-order kinetics or saturated pathways, however, a constant quantity of drug is eliminated per unit of time, and this quantity is drug concentration-independent and the drug does not have a constant, characteristic elimination half-life. The potential impact of saturable, leading to zero-order (versus first-order) elimination, can be profound, and its effects include altered drug concentration profiles, scope and duration of drug activity, distribution and disposition among tissues (Riviere, 2008; Bonate, 2011).

1.1.4 RECENT EXPLORATIVE APPLICATIONS OF PHARMACOKINETICS

1.1.4.1 Non-conventional Routes of Administration

In the last decades, the modern human and veterinary drug therapy has focused on expanding knowledge of alternative routes of administration to the classic routes of oral and parenteral administration (Ansah et al., 1998; Zhang H et al., 2002; Stanley, 2008; Lam et al., 2014; Messenger et al., 2016; Thwala et al., 2017; Aldawsari et al., 2018).

These "new" routes of administration (e.g. oral transmucosal, intranasal, transdermal) are considered noninvasive, painless and generally well tolerated by patients (Stanley, 2008; Lam et al., 2014; Messenger et al., 2016).

Among the main advantages offered by these types of drug delivery, there is the ability of bypass hepatic first-pass metabolism and the avoidance of drug degradation caused by the passage through the gastrointestinal tract (Thwala et al., 2017). Furthermore, alternative parenteral routes ensure an improvement in drug safety by reducing high plasmatic concentration, which often occur with classical parenteral drug administration (e.g. intravenous and intramuscular) (Stanley, 2008).

Finally, it is important to remember that these alternative routes of administration are easy to perform even for people without special technical skills, an important advantage for particular categories of subjects, such as pediatrics or veterinary patients, in which the intervention of the parents or the owner is often required (Lam et al., 2014; Messenger et al., 2016). On the other hand, the main disadvantages of these routes of administration are above all their influencing factors and the incomplete knowledge (especially in veterinary medicine) of their pharmacokinetic and pharmacodynamic characteristics. To interpret drug delivery data in veterinary species, as well as to consider the design constraints in developing specific formulations for animal health, a thorough knowledge of the mechanism of chemical absorption across the skin barrier and the mucosae as well as the criteria for selecting appropriate model systems is needed. In order to reach these goals, the most important tool available is clinical pharmacokinetics.

Transdermal drug delivery

The term transdermal implies use of a topical drug application to achieve systemic pharmacological effects. In human medicine, the transdermal application of medications is not new, as mankind has been applying ointments, creams, lotions and gels (primarily for local effects) for centuries. However, the stratum corneum, which is 15 to 20 cells thick and covers 99% of the body surface area, has proved difficult to penetrate, and systemic delivery using this route has increased only gradually over the past 25 to 30 years. Nonetheless, there are more and more transdermal drugs receiving approval and becoming available to clinicians. A few of interest include scopolamine (for nausea and sedation), nitroglycerine (for angina), a number of oestrogen

preparations (for oestrogen replacement after menopause), clonidine (for hypertension), testosterone (for male hormone augmentation), lidocaine (transdermal lidocaine for relief of pain associated with postherpetic neuralgia), ketoprofen (for mild to moderate pain) and fentanyl (for moderate to severe chronic pain) (Stanley, 2008).

Over the last decades, topical 'pour-on' and 'spot-on' applications of pesticides and antiparasitics (e.g., fenthion, ivermectin, levamisole, fipronil) spread for transdermal delivery in veterinary species.

"Patches" differ from other topical formulations developed for systemic delivery through other routes by virtue of the fact that the patch controls the rate of drug delivery from these systems, rather than the drug permeability through the skin that occurs with topical formulations. In small animal practice, the literature describing the use of these specific devices is not very extensive and results quite circumscribed to the field of analgesia (Riviere and Papich, 2001; Bravo et al., 2018).

Intranasal transmucosal drug delivery

The nasal route has gained importance as a noninvasive, and easily accessible route that offers many advantages for the introduction of drugs into the systemic circulation. Compared to other biological membranes the nasal mucosa is rather porous with a thin endothelial basal membrane. It also has a rapid blood flow, with a highly vascularized epithelial layer and a large absorption area. Due to these characteristics, it offers many advantages such as fast absorption of drugs and bypassing both gastric degradation and hepatic first-pass metabolism (Thwala et al., 2017). In addition, the nasal route may also allow for strategies to by-pass obstacles for blood–brain barrier (BBB), while it has also been considered for the administration of vaccines (Gänger and Schindowski, 2018).

In small animal practice, the intranasal route of administration offers the same benefits as human medicine, but its main clinical applications (e.g. multimodal anaesthesia management, treatment of neurological emergencies, behavioral and immunological therapies) are catching on especially in recent years, particularly in small animal practice (Micieli et al., 2017; Charalambous et al., 2017; Thielke et al., 2017; Ellis et al., 2017).

Oral transmucosal drug delivery

Potential advantages of oral transmucosal drug delivery include less hepatic first-pass metabolism and improved patient comfort, convenience and compliance. In addition, since the oral cavity is rich in blood vessels and lymphatics, drug absorption is fast, and the onset of action is rapid when compared with oral and transdermal routes. The fast onset action enables titration of the drug dosage to specific endpoints of effect. The mouth has three areas for potential transmucosal delivery: beneath the tongue (sublingual), between the gums of the upper molars and the cheek (buccal), and between the gum of the incisors and the upper lip (gingival). Drug permeability appears to be highest in the sublingual area and lowest at the gingival site (Stanley, 2008).

In small animal practice, the main fields of application of this route, often accompanied by studies concerning its pharmacokinetics, are still that of anesthesia and analgesia (Ko et al., 2011; Cohen and Bennett, 2015; Messenger et al., 2016; Aldawsari et al., 2018). Especially with regard to cats, which is considered an animal species less collaborative than the canine one, several clinical and pharmacokinetic researches have been performed on the main analgesic molecules employed in this species by the oral transmucosal route (Wells et al., 2008, Santos et al., 2010; Porters et al., 2014; Pypendop et al., 2014, Doodnaught et al., 2017).
However, although the oral transmucosal is perhaps the most studied in veterinary medicine, it is still necessary to deepen its knowledge for the improvement of animal care.

1.1.4.2 Applications of clinical PK in non-domestic species

Differently from small and large animal practice, the aim of zoological medicine is not only to safeguard the well-being of the single patient and/or public health but also to preserve the single as a part of the whole species. In fact, zoologic veterinarians are responsible for captive breeding and reintroduction programs, exploration of the diversity of life, comparative medicine, studies of disease of conservation concern, health care of sustainability of biodiversity and disease surveillance for the wildlife and its interface with domestic animals and humans (Deem, 2015).

Ideally, treatment regimens for nondomestic species should be based on experimentally derived, species-specific pharmacokinetic, safety, and efficacy data. Differences in drug absorption, distribution, metabolism, and excretion (ADME) for numerous pharmaceutical agents have been well documented for domestic species (Baggot, 2001; Riviere, 2008); however, there is limited information concerning the ADME process in nondomestic species. Nonetheless, zoo and wild animals require treatment, and almost every case reported in the literature has extrapolated dosage, but few discuss how the clinician selected the dosage (Hunter et al., 2008).

Zoological collections represent a large investment, and many individual animals are considered of inestimable value. More importantly, many species are threatened or endangered, which makes every individual valuable for conservation issues. Given the relative lack of pharmacokinetic data, zoological veterinarians working with nondomesticated species are often forced to extrapolate drug schemes for the majority of the animals in their care (Van Bonn, 2015; Wiedner, 2015). Beyond the pharmacological variability throughout species, the difference of animals in their care also ranges in size from very small invertebrates to megavertebrates (e.g. elephants or whales). By necessity, the decisional process for drug dosage establishment starts by evaluating approved drugs for the closest domestic or exotic species, considering factors such as drug availability, pertinence of the commercial formulation, and potential negative adverse effects (Morris, 1999).

The most commonly methods for dosage translation are the linear extrapolation, the metabolic scaling and the allometric scaling (Hunter and Isaza, 2008). The first one consists in the use of a single mg/kg dose established for one species and its application across all species. The advantage of this system is the simplicity of the calculation and standardization of a single dosage for the species. However, problems may arise when this method is applied to other species without regard to species-specific pharmacologic differences or to the weight range, assuming that any differences in species pharmacokinetics-pharmacodynamics are not clinically relevant. Metabolic scaling uses the ratio of a known physiologic process or anatomic feature (e.g. metabolic rate or body-surface area) of two species to estimate a dosage in a species in which there is limited pharmacokinetic data. Briefly, all species are placed in one of five groups (termed Hainsworth's energy groups): passerine birds, non-passerine birds, placental mammals, marsupial mammals, or reptiles. The species group is used to select a predetermined K value to calculate the metabolic rate for the selected

species. A specific minimum energy cost (SMEC) value is calculated for each species, and the ratio of the target species SMEC to the SMEC of a safe, effective dose in a known species is calculated to achieve an appropriate dosage regimen. Despite its common usage in zoologic medicine (Sedgwick and Borkowski, 1996; Jacobson, 1996; Morris, 1999; Mortenson, 2001) this method has not been well validated, and several surveys illustrated specific failures in this method of extrapolation (Page et al., 1991; Jacobson, 1996; Mahmood et al. 2006; Hunter et al., 2008). The third approach, the allometric scaling, is to measure a pharmacokinetic parameter in multiple species and then to derive a new allometric equation that can be used to estimate the pharmacokinetic parameter in an unknown species. This method shares the assumptions that drug differences are clinically negligible between species and that the drug pharmacokinetics has a nonlinear (allometric) relationship to weight. Although not critically evaluated in zoologic medicine, this method may provide a more reliable method for drug-dose estimation (Mahmood et al., 2006; Hunter, 2008; Hunter et al., 2008).

The most common pharmacological categories used in zoo and wild animals are represented by sedative and anesthetic agents, antimicrobial and antiparasitic molecules. The first category is frequently employed not only for surgical or painful maneuvers, but also for more routine procedures (e.g. clinical examination, collection of biological samples or movimentation).

The lack of pharmacokinetics studies in zoo and wildlife medicine derives from several limitations such as the difficulty in having a significant sample number: even with common and widespread species (e.g. lions or tigers) it can take years to have enough specimens for completing a pharmacokinetic study. Moreover, since most of the times, pharmacological restraint is required to even approach the patient (with anesthetic drugs that are not calculated on the animal actual body weight, but only on estimate), that may “pollute” the samples required for the study, hindering all the possibilities to obtain blank samples. Other variables such as hydration status, fasting or concurrent diseases are assessed only after sedation, and the consequence is that many subjects cannot be considered suitable for recruiting as kinetics models.

Starting from these assumptions, it appears evident the necessity to fill the lack of knowledge regarding zoo and wildlife animals’ pharmacokinetics. In fact, with dedicated pharmacokinetic studies, it would be possible to improve the pharmacological therapy in these particular species by making it more targeted and effective, anticipating and trying to avoid the risks linked to possible unintentional overdoses and undesirable side or adverse effects.

1.2 ADVANCES IN PHARMACOKINETICS MODELLING

1.2.1 POPULATION PHARMACOKINETICS

1.2.1.1 Basic principles

Population pharmacokinetics (popPK) is defined as "*the study of the basic features of drug disposition in a population, accounting for the influence of diverse pathophysiological factors on pharmacokinetics, and explicitly estimating the magnitude of the interindividual and intraindividual variability*" (Martín-Jiménez and Riviere, 1998).

In 1980, Sheiner and Beal firstly evaluated and compared several methods for estimating the population pharmacokinetic parameters. These parameters quantify population average kinetics, including relationships between physiology (e.g., renal function) and pharmacokinetics (e.g., clearance), the typical magnitude of interindividual variation in these kinetics and relationships, and the average (across individuals) magnitude of the residual deviations between expected and observed drug levels caused by (intraindividual) kinetic variation and drug level measurement error (Sheiner and Beal, 1980).

Disease states may alter physiological processes which in turn may influence both the kinetics and effects of drugs administered to diseased individuals. Consequently, estimates of the basic characteristics of drug disposition in healthy individuals arising from "average" demographic subsets of the population may, in many cases, only approximate the real characteristics of the disposition in individuals undergoing different disease processes and/or belonging to different demographic subpopulations. The complexity of the physiological processes involved in drug disposition and the dependency of these processes on individual features, precludes pharmacokinetic homogeneity even in healthy subjects. Thus, when a disease state is present and physiological mechanisms are altered, the level of interindividual heterogeneity in drug disposition will be much greater as individual patients respond differently to the disease process. Therefore, designing drug dosage regimens under disease conditions requires estimating the pharmacokinetic parameters relative to the clinical factors that are present in an individual, as well as estimating the precision with which these pharmacokinetic parameters can be characterized (Martín-Jiménez and Riviere, 1998).

Ideally, all methods of data analysis should ideally provide for each population parameter its point estimates and an estimate of the remaining uncertainty in this parameter, that is its confidence intervals (Scheiner and Beal, 1981; Scheiner and Beal, 1983; Scheiner, 1984).

Before the above-mentioned studies, only two standard approaches to estimating population pharmacokinetic parameters from routine data were known. The first, named Naive Pooled Data (NPD), pooled all the data together and analyses them according to simple nonlinear least squares, as though they had all come from one individual. The second approach, called Two-Stages (TS), proceeded in two steps. First, the data from each individual are analysed separately (again using simple nonlinear least squares), and then,

in a second stage, these individual parameter estimates are combined to generate estimates of the population parameters. Scheiner and Beal (1980) proposed a third method, the Nonlinear Mixed Effect Modelling (NONMEM), which pools data, as does the first of the standard methods, but explicitly adjusts for the correlation of data within each individual, whereas the NPD method does not. On the other hand, the TS method does account for intraindividual correlation, but fails to take advantage of pooling. There are certain theoretical problems with either tactic, although NONMEM approach remains the best in order to improve the estimation process (Sheiner, 1980).

The NONMEM method uses a variety of algorithms related to nonlinear regression and matrix algebra to obtain estimates of the fixed - effect parameters (defined covariates, e.g. age, gender, weight, body condition score), the inter and intraindividual random-effect parameters (variances), and the standard errors of all these parameter estimates (Martín-Jiménez and Riviere, 1998).

In a routine clinical setting, carrying out a precise collection of samples necessary for a classical pharmacokinetic study, is hardly compatible. The population pharmacokinetics, thanks to the NONMEM method of parameter estimation characteristics, allows to delineate accurate kinetic profiles relevant for a specific population or sub - population, collecting few samples from a large number of subjects and conducting all data into a cohesive mathematical framework (Dykstra et al., 2015).

The fact that many of the population pharmacokinetic studies that are conducted have a clinical-observational rather than an experimental nature has led to the necessity of establishing appropriate validation methods, to assure that the parameter estimates obtained in a population pharmacokinetics study can be extrapolated to the general population, and that the results are reasonable and independent of the analyst. Validation procedures are intended to assess how well a population model describes a set of data ("validation" set) that has not been used to develop the model itself. Whether validation of the population study is accomplished or not depends on the objective of the analysis. When a population model is developed for dosage recommendation, it must be adequately validated. Alternatively, when population models are developed for explaining variability or for providing some descriptive labelling information, validation may not be required (Martín-Jiménez and Riviere, 1998).

The validation of a population model consists of the assessment of the stability and/or predictive performance of a population model (obtained from a "study" or an "index" population) on a "validation" data set, different from that used to develop the model. Depending on the availability of validation data, is possible to distinguish two types of validation named, respectively, external and internal. In external validation, the validation set consists of an entirely new data set obtained from another study. Alternatively, internal methods use the original data set to derive both the "index" and "validation" data sets or use resampling techniques to validate the developed model. Internal validation techniques include data-splitting (Roecker, 1991), and resampling techniques such as cross-validation (Efron, 1983) and bootstrapping (Ette, 1997).

Since popPK approach does not require many samples from individual subjects, it is also considered to be an attractive method for circumventing ethical problems of frequent and long sampling times in both human and animal research. Although veterinary literature regarding this topic is still quite poor, popPK is viewed as a promising methodology for estimation of drug disposition parameters on a population basis and for tailoring dosage to individual patients on the basis of physiological and pathological status. Finally, studies of population characteristics of pharmacokinetic parameters between animal species may also reveal some fundamental aspects of comparative pharmacology (Kinabo and McKellar, 1989).

1.2.1.2 PopPK applications in veterinary medicine

Several are the possible applications of the popPK approach in veterinary medicine.

Clinical use

Population pharmacokinetics can be applied to the clinical setting manly for two reasons. First, it can be utilized to define dosage regimens for new individual patients or patient-clusters according to their clinical characteristics. Second, population models can be employed to improve the accuracy of the predictions in a patient from which only a small number of samples can be obtained. When a drug is administered to treat a pathologic condition, the first objective is to optimize the dose for the individual patient. This goal is particularly important when the drug that has to be administered has a narrow therapeutic index and/or a large interindividual variability in its disposition or effect. Variability in therapeutic outcome can be influenced by pharmacokinetic and pharmacodynamic components. Consequently, pharmacokinetic and pharmacodynamic variability in a population will dictate how confidently the clinician will be able to administer an average population dose to an individual subject. The magnitude of this variability and the factors which contribute to it represent the main critical issues in dealing with dose individualization. When drugs exhibit a large variability in disposition across individuals, poor correlation between plasma concentrations and dose will exist. The consequence of this will depend on the pharmacodynamic characteristics of the drug for both the therapeutic and the toxic effects. By explaining part of this variability in terms of a series of pathophysiological variables (weight, age, renal function, etc.), dosage regimens can be designed, using this additional clinical data, that correlate well with serum concentrations for each particular subpopulation as the residual variability is greatly reduced. If the inclusion of pathophysiological variables in the model manages to reduce the interindividual variability to a relatively small magnitude and the pharmacodynamic variability is not large, we can design an optimum dose for each of these subpopulations derived from their average pharmacokinetic parameter estimated values. This is especially valuable for subpopulations that are more prone to deviate from the general population values (e.g. infants

or pediatrics, geriatrics, subjects with renal or hepatic impaired functions) (Martín-Jiménez and Riviere, 1998).

Application in food-production animal medicine

The use of population pharmacokinetic approach, combined with pharmacodynamics (pharmacokinetic-pharmacodynamic, PK-PD methods), in food animals will most likely improve the conditions of herd drug usage in the near future. The ability of these methods to obtain valuable information from large populations in which each individual is sparsely sampled seems ideal to study drug therapeutics in food-producing animals. Differences in drug disposition across individuals could be related to disease conditions, management practices, lactation status, or breed. This knowledge, together with a better assessment of the sources and magnitude of variance will allow a more reasonable use of drugs (especially antimicrobials) in these animals. Differences in disposition can be influenced by individual characteristics and also by subpopulation characteristics, such as breed of animals or crop groups in fish. Consequently, population pharmacokinetics in production medicine could be applied both to individual and subgroup therapeutics (Kinabo and McKellar, 1989).

Food animal residues avoidance

In food animal medicine, the importance of accurately describing the disposition of drugs in animals according to clinical or production variables without designing extensive individual pharmacokinetic studies, is fundamental, especially considering the influence that these variables may induce in the deposition of drug residues in those animals tissues or food products (e.g. meat, milk, eggs). The strength of the population approach is that data collected from a wide variety of experimental protocols (efficacy, safety, residues) can be assembled into a single model for the drug. The final objective would be to estimate the probability of violative tissue residue levels in a zootechnical population or subpopulation undergoing drug therapy by considering the concomitant production variables (e.g. weight, daily gain) and screening a reduced number of animals in the production unit. Although there is great potential for popPK approach to address drug tissue disposition and residue avoidance, is important to remember that one of the primary limitations of tissue residue studies is the lack of sufficient tissue samples per individual to characterize tissue depletion kinetics individually. To by-pass these limitations, population pharmacokinetic studies could be conducted according to a multicompartment experimental protocol. Adequate multicompartmental or hybrid physiological-compartmental models could be elaborated in order to define relationships between plasma and tissue concentrations, considering the influence of concomitant pathophysiological or production variables (Martín-Jiménez and Riviere, 1998).

For these reasons and with these objectives, physiologically-based pharmacokinetic (PBPK) modeling, another modern tool in the pharmacokinetic field, is reputed to be more suitable in improving knowledge regarding food animal residues avoidance (Riviere, 2008).

1.2.1.3 PopPK approach for antimicrobials use

Antimicrobial drugs have revolutionized human and veterinary medicine through the provision of effective and inexpensive resources of treating, and in some circumstances preventing, bacterial infectious disease. In veterinary medicine, intensified production methods have led to an increase in the spread of disease where animals are kept in confined spaces. Antimicrobial drug prophylaxis and therapy have permitted to maintain animals in these husbandry systems without the adverse impact on animal health and welfare which bacterial disease would have otherwise inflicted. Furthermore, it has long been recognized that antimicrobial drugs confer growth-promoting effects, even when administered to healthy animals at dosage rates lower than those effective in treating clinical disease. However, three major factors have now encouraged a review of antimicrobial drug use in animals. First, the outspreading selection of bacteria resistant to available drugs in animals and more significantly in man. Moreover, there is a growing evidence that bacteria selected for resistance in animals can be transmitted to human. Furthermore, the acquisition of bacterial resistance has outstripped the ability of pharmaceutical companies to produce new products with mechanisms of action which overcome resistance, as is evidenced by the growing number of virtually untreatable bacterial infections in man and, contemporary, the few truly new agents are reserved for human use in hospitals and it is unlikely that these drugs will be authorized for veterinary use in the years to come. Thus, it is of vital importance to preserve the efficacy of the veterinary antimicrobial products available today (McKellar et al., 2004; Guardabassi and Prescott, 2015).

Antimicrobial drug resistance (AMR) is an important problem that challenges veterinary clinicians to provide effective treatments without further spreading resistance to other animals, people, and the environment (Papich, 2014) and is considered one of the greatest challenges currently facing small animal veterinary medicine. During the past decade, in fact, various multidrug-resistant bacteria (MDR) have emerged and spread among dogs and cats on a worldwide basis (methicillin-resistant *Staphylococcus pseudintermedius* (MRSP), *Escherichia coli* producing extended-spectrum β-lactamase (ESBL), *Pseudomonas aeruginosa* and enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA), carbapenemase-producing *E coli* and *Klebsiella pneumoniae* and MDR *Acinetobacter baumannii*) (Perreten et al., 2010; Endimiani et al., 2011; Papich, 2013; Abraham et al., 2014; Rubin and Pitout, 2014; Vincze et al., 2014). All these MDR bacteria are frequently resistant to all conventional antimicrobials licensed for animal use, and therefore pose a serious threat to animal health by increasing the risk of therapeutic failure and the recourse to euthanasia. For the reasons described, there is an urgent need to mitigate the escalation of AMR in small animal veterinary practice (Guardabassi and Prescott, 2015).

Antimicrobial stewardship programs (ASPs) are a cornerstone of the response to the AMR crisis in human medicine but are still largely underdeveloped in veterinary medicine. The term "*antimicrobial stewardship*" (in which the word *"stewardship"* implies the obligation to preserve something of enormous value for future generations) is used to describe the multifaceted and dynamic approaches required to sustain the clinical efficacy of antimicrobials by optimizing drug use, choice, dosing, duration, and route of administration, while minimizing the emergence of resistance and other adverse effects (Guardabassi and Prescott, 2015; Toutain et al., 2017).

Pharmacokinetics (PK) studies the fate of drugs in the animal whereas pharmacodynamics (PD) studies the action of drugs from its interaction with receptors, to the effect on animal populations. Pharmacokinetic/pharmacodynamic (PK/PD) integration consists of describing and explaining the time course of the drug effect (PD) via the time course of its concentration in the plasma (PK) (Toutain, 2008).

In general, three different models are considered when building a PK/PD model: a PK model transforming the dose into a concentration versus time profile, a link model describing transfer of the drug from the plasma into the biophase, and a PD model relating the biophase concentration to an effect (Holford and Sheiner, 1981).

The PK/PD approach may also be used to determine an optimal dosage regimen for antibiotics. The objectives of rational antibiotic therapy are to optimize clinical efficacy and to minimize the selection and spread of resistant pathogens (Toutain et al., 2002). The poor sensitivity of clinical outcomes in indicating the best dosage regimen in terms of bacteriological cure, has inspired investigation of the value of PK/PD surrogate indexes to establish an optimal dosage for antibiotics. Three PK/PD indexes appear to be sufficient to predict antibiotic effectiveness: the AUC/ MIC ratio, an index used for quinolones, the C_{max}/MIC ratio, an index selected for aminoglycosides, and T > MIC (the time during which plasma concentrations exceed MIC, expressed as a percentage of the dosage interval), an index selected for the so-called time-dependent antibiotics (e.g. β-lactams). All three indexes are surrogate markers of what is ultimately expected, that are clinical recovery and bacterial eradication (Toutain, 2008).

The PK-PD indexes determine exposure relationships. One prominent veterinary pharmacologist stated that ''*It is exposure, and especially exposure to sub-optimal drug concentrations that is the most important single factor in resistance emergence and its subsequent spread*'' (Lees et al., 2008). Therefore, strategies have been developed to administer antimicrobials to animals using regimens to achieve appropriate pharmacokinetic-pharmacodynamic (PK-PD) targets, which are different for different antimicrobial categories (Papich, 2014). The use the PK-PD exposure relationships has been an important tool for regulatory authorities when reviewing antimicrobial applications. The PK–PD indexes are also now used to derive clinical susceptibility breakpoints by the Clinical and Laboratory Standards Institute (CLSI) subcommittee on Veterinary Antimicrobial Susceptibility Testing (VAST) (Papich, 2014).

The CLSI subcommittee for Veterinary Antimicrobial Susceptibility Testing (VAST), uses strict criteria to establish and evaluate clinical susceptibility breakpoints. Sponsors are asked to follow guidelines provided by CLSI and must submit data to support a proposed clinical breakpoint. The data may include pharmacokinetic parameters in the target species, MIC distributions for the pathogens targeted, clinical data from the drug used under field conditions at the approved dose, and pharmacokinetic–pharmacodynamic (PK–PD) analysis, using Monte Carlo Simulations (Ambrose, 2006) to show that at the approved dose the drug attains PK-PD targets for the labeled pathogen (Papich, 2014).

In order to improve the method useful in establishing clinical susceptibility breakpoints, one important advance has been to separate the two main sources (PK and PD) of variability through the use of population PK/PD approaches. In this way, population analysis can explain the variation between animals (or groups of animals) not only in terms of drug exposure but also in terms of drug responsiveness (Toutain, 2008). In fact, the NLME (Nonlinear Mixed Effect or NONMEM) model approach and simulation method can also be applied to determine the pharmacodynamic (PD) cutoffs for dosage regimens especially for antimicrobial agents. For antibiotics, breakpoints are the concentrations at which bacteria are susceptible to successful treatment. Veterinary breakpoints are sometimes unclear and, therefore, need reevaluation. Clinical breakpoints can be evaluated by deterministic or probabilistic approaches. Traditionally, a deterministic approach is used to set the breakpoints. However, this approach fails to consider the interindividual variability and can only provide a possible breakpoint value. The stochastic nature of NLME model and simulation makes it an ideal tool to integrate variability of PK and PD data to establish a breakpoint (Li et al., 2015).

1.2.2 PHYSIOLOGICALLY-BASED PHARMACOKINETICS

Physiologically-based pharmacokinetic (PBPK) modeling is a computational simulation process that describes the *ADME* of environmental chemicals, drugs, or nanomaterials in an organism based on interrelationships among key physiological, biochemical, and physicochemical determinants using mathematical equations (WHO, 2010).

The concept of PBPK modeling dates back to 1930s, when it had been realized that the body regulates drug disposition as an integrative system, that is, pharmacokinetic processes that take place in one organ affect, and are in turn influenced by, processes occurring in other tissues through a central connective circuitry, the vascular system (Teorell, 1937). However, due to the mathematical and computational complexities of PBPK models and the lack of relevant physiological data at the time, the field of PBPK modeling did not advance substantially until the 1960s, when the necessary computational capabilities became available (Rowland et al., 2004).

In the last several decades, PBPK models have been extensively applied in numerous fields, ranging from risk assessment of environmental chemicals on human health and drug pharmacokinetic predictions to aid drug development, to nanomaterial pharmacokinetic simulations and nanotechnology-based drug delivery assessment. In particular, PBPK models have become an important and indispensable tool in the risk assessment of toxicants by regulatory agencies because they offer the only ethical yet scientifically robust method of predicting the systemic exposure to toxic xenobiotics in humans through animal-to-human extrapolation or based on human biomonitoring data (McLanahan et al., 2012).

1.2.2.1 Principles of PBPK modeling

The aim of physiologically-based modeling is to integrate available knowledge on physiological processes with physicochemical attributes of an investigated xenobiotic in order to predict and/or simulate concentrations in various tissues and body fluids under complex biological scenarios. In essence, PBPK models of cells, tissues, organs, and organisms as a whole aid in increasing the mechanistic understanding of how xenobiotics interact with biological systems (Jones and Rowland-Yeo, 2013).

These complex models are composed of three major parts: species-specific physiological parameters, chemical-specific parameters, and the structural model. All components can be described mathematically, obtained experimentally, and estimated by computation algorithms. Physiological parameters include body weight, cardiac output, organ mass or volume, blood flow, vascular space of each organ, tissue composition, and metabolizing enzyme phenotype. Chemical-specific parameters constitute partition and permeability coefficients, metabolic rate constants, protein binding affinity, and enzyme/transporter activity among others. Incorporation of chemical-specific parameters allows the model to assess the impact of changes due to drug- specific properties on the whole-body pharmacokinetics, making PBPK models mechanistic in nature. The structural model comprises the exposure routes (e.g., intravenous, intramuscular, subcutaneous, and

oral) and anatomically correct network of tissues and organs that comprise the whole body. The capability of including various exposure routes provides PBPK models the power to conduct extrapolation across routes. (Lin et al., 2016).

Each tissue/organ (termed “a model compartment”) is perfused by and connected through the circulatory system. The physiological organ-based model feature empowers PBPK models to predict the concentration of a substance in a particular organ of interest at a specific time under a certain exposure scenario. It should be noted that the structural model for a drug is the same across species within a given class of animals (mammals, avian, reptiles, etc.), making it possible to conduct interspecies pharmacokinetic extrapolation by adjusting species-specific physiological parameters (Thiel et al., 2015). This is particularly important in veterinary medicine because pharmacokinetic data for most drugs are available in common species (e.g., cattle and swine), but very limited in minor species (e.g., rabbits, pheasants, quail, domestic game and exotic animals). In theory, PBPK model extrapolation across species/breeds within the same class of animals using species- or breed-specific model parameters enables simulation of drug pharmacokinetics from one species/breed to another in which experimental pharmacokinetic data may not be available. Overall, the mechanistic nature, the ability to predict target organ concentrations, and the great extrapolation power across species and dosing scenarios are the main reasons for the extensive applications of PBPK models in various areas (Li et al., 2016).

1.2.2.2 Current applications in veterinary medicine

Estimation of drug tissue residues and withdrawal times in food-producing animals

In veterinary medicine, the most common application of PBPK modeling relates to the prediction of drug residues depletion and withdrawal times in food-producing animals, since this approach has been reputed as robust to estimate because mechanistic physiological information (e.g., mode of action of the drug, exposures in organs of interest, and effect of the disease on drug disposition) can be incorporated in the model predictions (Li et al., 2016).

The first PBPK model for predicting drug residue depletion and withdrawal times was reported in Canada by Law in 1992: he realized that the legislation on the withdrawal periods of oxytetracycline (OTC)-treated fish did not take into consideration the various doses, dosing schedules, fish weights, and several additional factors, which would significantly affect OTC distribution and residues in fish tissues (Law, 1992). He therefore developed a nine-compartment (flow-limited: blood, gills, liver, gut, kidney, and carcass; membrane-limited: bone, muscle, and skin) PBPK model for OTC disposition in trout and chinook salmon that successfully predicted the measured concentrations of OTC in fish tissues after single or multiple oral dosing (Law, 1992). This model predicted a withdrawal period of 100 days postdosing in a 14-day 100 mg/kg oral exposure paradigm, which was much shorter than the 145 days recommended withdrawal time calculated using a classical pharmacokinetic approach (Brocklebank et al., 1997). Although it proved extremely valuable, this initial model did not consider population variability. In fish, body (water) temperature is a major covariate for withdrawal times. To address this drawback, the model was further optimized using Monte Carlo sampling techniques to account for the interindividual variability across the population (Law, 1999). The authors concluded that the population PBPK model was a more useful tool than the statistical method for withdrawal time determination because treatment specific information, such as fish weight, bioavailability, dose regimen, and water temperature could be incorporated in the simulation. This earlier application of PBPK modeling was used as a basis for all subsequent PBPK study efforts in the later years.

Risk assessment of environmental contaminants in food animals and in wildlife

Food and wildlife animals may be exposed to environmental contaminants, including lipophilic pesticides, through the contamination of their food supply and/or water. This could result in residues in plasma, tissues, milk, or eggs, requiring an appropriate assessment and management of the risks associated with it. For food animals, for example, was developed a PBPK model to simulate the transfer of lipophilic xenobiotics from the feed to lactating dairy cows, and this model was extrapolated to beef cattle, goats, sheep, and pigs (MacLachlan, 2009). A similar model was developed in laying hens and extrapolated to broilers (MacLachlan, 2010). These studies demonstrated that PBPK modeling can be used to assess the risks of environmental

contaminants in the feed supply of food animals by extrapolating within and between species to maximize the use of available experimental data.

In the environment, fish can be exposed to various water contaminants. Franco-Uria and colleagues developed a PBPK model using a set of generic parameters (only the absorption rate was condition-specific) to predict cadmium concentrations in the tissues of diverse fish species under different environmental conditions (Franco-Uria et al., 2010).

These examples suggest that PBPK models could be employed to predict soundly environmental contaminant concentrations in different species in different exposure scenarios to aid risk assessment.

Design of therapeutic regimens in veterinary medicine and translational medicine

Similar to applications in human medicine, PBPK models can also be used in the design of optimal therapeutic regimens for veterinary drugs. Recently, was developed a multiroute PBPK model for OTC in dogs after intravenous, intramuscular and oral administration with traditional or long-acting formulation (Lin et al., 2015). This dog model was then validated using multiple independent datasets and successfully extrapolated to humans. The dog model was applied to predict the 24-h area under the curve of OTC concentrations in the plasma, liver, kidney and muscle under three different therapeutic regimens. These simulations are able to guide the design of optimal therapeutic plans with OTC in veterinary, and potentially, human medicine. In particular, this last application is valuable when human exposure to potentially toxic therapeutics or drug trials to treat lethal human pathogens are not possible, requiring translational animal studies as a substitute (Lin et al., 2016).

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Aim of the Study

The topics addressed in the introduction of the present thesis deal with the evolution of pharmacokinetics, the branch of pharmacology that studies the fate of the drug following its administration, in other words the discipline that describes the so-called ADME process.

From the fundamental theoretical and mathematical concepts of this science, the focus shifted to the importance of clinical PK and its applications, starting from the theory and use of compartmental analysis and at the end arriving to illustrate the more recent notions of this discipline, popPK and PBPK, which are today reputed the most innovative topics in the clinical and translational pharmacology field.

Clinical pharmacology and, particularly, clinical pharmacokinetics, is an ever-expanding scientific field. The classical PK usually, is employed in preliminary investigations concerning the use of drugs. If translated clinically, it is transformed into studies that, although "small", have a significant relevance in understanding ADME process of the drug with its desired, collateral or adverse effects.

Three studies of classical PK have been included in this doctorate thesis.

The first concerns the dexmedetomidine (DEX), a sedative belonging to the class of alpha-2 agonists, administered in the bovine species. The objective of the work was to define the kinetic profile of DEX following intravenous administration in a group of dairy calves, comparing its pharmacological and clinical effects with those induced by another sedative alpha-2 agonist, xylazine, registered for the bovine species.

The second study is related to the simultaneous administration, as preanaesthetics in the canine species, of a mixture of DEX and methadone, an analgesic with a remarkable sedative efficacy belonging to the class of opioid μ-agonists. The aim of the work was to establish the pharmacokinetic profile of this co-administration in dogs by oral transmucosal route and compare it with the intramuscular kinetic profile of the same co-administered drugs. As widely pointed out in the introductive part of this thesis, the oral transmucosal route is an alternative route of administration that in recent years is influencing the medical field and the drug industry for potential applications. Being the first kinetic study of an oral transmucosal drugs combination in dogs, this study represents a scientific novelty regarding this topic.

A further pharmacokinetic work included in this PhD thesis concerns the species *Panthera tigris*. The pharmacology and therapy of zoo animals are extrapolated from the concepts developed for domestic animals (i.e. pets and livestock), whereas poor data are available directly from exotic species. In fact, the scientific literature concerning the PK of these particular animals is lacking and needy of new information. Specifically, the objective of the study was to compare the kinetic profile of a simultaneous administration of DEX and ketamine, an injectable dissociative anaesthetic antagonist of NMDA receptors, following intramuscular administration for induction of deep sedation in two groups of tigers. Since the two groups have undergone different procedures, the aim of the kinetic comparison was to investigate the possible influence of other drugs administration on the kinetic profiles of the two molecules studied.

On the other hand, the future of pharmacokinetics is represented by the improvement of more complex mathematical approaches and their application in various fields, specifically, popPK and PBPK. The time

required for the development of a popPK study applied to the clinical setting is much longer than studies of classical PK. In the present doctorate thesis, one clinical population pharmacokinetic study is reported, which took three years for its execution and conclusion.

This study concerned cefazolin, an injectable beta-lactam antibiotic (belonging to the family of first-generation cephalosporins) used *off-label* in the canine species. This active compound is commonly used in surgery for the prophylaxis of post-operative infections in dogs. At present, the posology for cefazolin in dogs is extrapolated from human medicine but, as there are no consistent and uniform prophylactic and therapeutic schemes, there is no scientific consensus on the variables that may influence the ADME process of this molecule in the canine species.

Moreover, in human medicine as in the veterinary field, given the increase of the antibiotic-resistance problem and the worldwide spread of multi-drug-resistant bacteria strains, the revaluation of the clinical efficacy of antimicrobials is considered mandatory by regulatory agencies.

In recent years, veterinary medicine has become sensitised to these issues, since one of its main missions is the safeguard of public health through the protection of animal health, in full agreement with One Health concepts.

From these premises a study was carried out to define the popPK profile of cefazolin administered for prophylactic purposes in 78 dogs, of different breed, age, weight, sex, body condition scores and health status, undergoing different surgical procedures, and to achieve the determination of Clinical Breakpoints for this antimicrobial administered to the canine patient according to the guidelines of the Veterinary Committee on Antimicrobial Susceptibility Testing (VetCAST).

Finally, during these three years of PhD program, another popPK work has been started. It concerned a GABAergic drug, propofol, which is the most common injectable anaesthetic used in small animal practice for induction and, sometimes, the maintenance of general anaesthesia. The idea originated from the veterinary anaesthesia Operative Unit of the University of Padua, which requested our collaboration for sampling analyses and propofol quantification. The aim of this clinical work, which is still ongoing, is the definition of the kinetic population profile of propofol, administered for the induction and maintenance of general anaesthesia with modern TCI technique, in order to determine a popPK model that, in respect of the criteria imposed by the validation methods, would be applicable to dog anaesthesiology. Until now, propofol quantification has been accomplished, but the final model is still under investigation (so data are not reported in the thesis).

Since this doctorate thesis consists of studies on different topics, but all gathered in the clinical pharmacokinetic field, the purpose of this work was to bring new information into the scientific veterinary pharmacokinetic literature in the most modern fields of its application by the use of different pharmacokinetic modelling approaches.

Research Papers

The papers published were reported keeping the reference style indicated by the guidelines of each Journal.

PHARMACOKINETICS AND SEDATIVE EFFECTS OF DEXMEDETOMIDINE IN DAIRY CALVES

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PHARMACOKINETICS AND SEDATIVE EFFECTS OF DEXMEDETOMIDINE IN DAIRY CALVES

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Conflict of Interest Statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

AIMS: To evaluate the pharmacokinetics of dexmedetomidine (DEX) administered I/V at a dose of 5 µg/kg bodyweight in dairy calves and to compare the sedative effects of anaesthetic protocols involving DEX and xylazine.

METHODS: Nine dairy calves, aged 17–20 days, were treated with 5 µg/kg I/V dexmedetomidine. For pharmacokinetic evaluation, blood samples were collected over 12 hours and serum samples were analysed by high performance liquid chromatography-mass spectrometry. Another nine dairy calves, aged 16–20 days, were treated with 0.2 mg/kg I/V xylazine. After both treatments, heart rate, respiratory rate and rectal temperature were measured for 20 minutes. Sedation quality and recovery times were also assessed.

RESULTS: The kinetics of DEX was best described by a two- compartment model. The distribution and elimination half- lives were 8.7 (SD 5.0) and 83.5 (SD 67.5) minutes, respectively. Mean maximum concentration and body clearance were 12.5 (SD 8.6) ng/mL and 27.9 (SD 13.1) mL/minute/kg, respectively; the mean volume of distribution at steady state was 2,170.8 (SD 1,657.5) mL/kg. A decrease in heart rate was observed after treatments with both DEX and xylazine. No differences in heart or respiration rate, or rectal temperature were observed between the two treatment groups. The onset of sedation occurred after 2.7 (SD 0.67) minutes for calves treated with DEX and 2.8 (SD 0.78) minutes for calves treated with xylazine, and was characterised by a similar degree of deep sedation and ease of handling of the calves. All recoveries were eventless, and no adverse reactions were noted.

CONCLUSIONS AND CLINICAL RELEVANCE: Dexmedetomidine treatment resulted in a reliable and long lasting sedation in calves, a transient decrease in heart rate and no modification in respiratory rate or rectal

temperature. The results were comparable to xylazine, the most popular alpha-2-agonist among bovine practitioners. The use of DEX in dairy calves for rapid procedures such as dehorning or castration could be suggested.

Keywords: Dexmedetomidine, pharmacokinetics, sedative effects, xylazine, dairy calf

Introduction

The sedative effects of xylazine in horses and cattle were first reported in the late 1960s (Clarke and Hall 1969). Since then other alpha-2-adrenoreceptor agonists, such as detomidine, romifidine, medetomidine and dexmedetomidine (DEX) have been introduced in small and large animal practice, gaining wide acceptance. Alpha-2-agonists are dose-dependent sedative agents, used for premedication prior to general anaesthesia, to reduce the required amount of injectable anaesthesia (Büehrer et al. 1994) and decrease minimum alveolar concentration of inhaled anaesthetic agents (Ewing et al. 1993). Further positive activities that influence alpha-2 agonists use are their synergistic action with opioids and analgesic properties (Short 1992). In addition, alpha-2-agonists are relatively safe substances and their effects are reversible by antagonists such as yohimbine and atipamezole (Schwartz and Clark 1998).

Xylazine was the first alpha-2-agonist to be licensed in veterinary medicine. It is commonly used in bovine practice to sedate calves undergoing clinical or surgical practices thanks to its rapid onset, relatively short duration of action, analgesic properties and quality of sedation (Rioja et al. 2008). DEX is structurally related to detomidine and is the pharmacologically active d-enantiomer of the racemic mixture medetomidine. DEX is authorised for use in small animal practice and is one of the most potent alpha-2-agonists commercially available (Marcilla et al. 2012). Compared to medetomidine, DEX has sedative and analgesic effects at equivalent doses, but is twice as potent and has various cardiovascular and analgesic advantages (Kuusela et al. 2000).

Pharmaceutical products authorised for pain management in cattle are quite limited (Hewson et al. 2007). The concern regarding pain management in food animals is increasing and research on this issue in cattle is needed. Calf dehorning and castration are common practices in cattle husbandry and are perceived as painful, though pain management is not always deemed necessary (Stafford and Mellor 2005a, b; Hewson et al. 2007). The need for pain relief during these procedures and the use of analgesia and anaesthesia for castration and dehorning have been proposed. The administration of DEX in dairy calves during dehorning or castration could represent a significant improvement in pain management. Thus, the aims of this study were to evaluate the pharmacokinetics of DEX administered I/V at a dose of 5 μg/kg bodyweight in dairy calves, and to compare the sedative effects of anaesthetic protocols involving DEX and xylazine. The dose of DEX

administered was derived from the authorised dose in dogs and cats, and from the dose used in sheep and horses (Raekallio et al. 2010; Rezende et al. 2015).

Materials and methods

Animals

The protocol for this study was approved by the Institutional Ethical Committee for Animal Care at the University of Milan (Milan, Italy; protocol No. 28/2011).

Eighteen (seven male and 11 female), Italian Holstein Friesian calves, aged 17.7 (SD 1.3) days, weighing 42.7 (SD 6.3) kg, were included in the study. All animals were admitted to the Clinic for Ruminants and Swine of the Veterinary Teaching Hospital of Lodi (University of Milan, Italy) and were judged to be healthy on the basis of physical examinations and haematological and biochemical blood tests.

At arrival at the facility, the calves were weighed and housed separately in 1.8 × 1.2 m single indoor pens with a controlled temperature of 20°C. Pens were separated by solid walls and had straw bedding. During an acclimatisation period of 7 days, each calf had unlimited access to water, grass, hay and pellets, and was fed three times/day with 2 L milk replacer, at 7:00, 13:00 and 19:00.

Calves were randomly assigned to two groups. Calves in Group 1 were aged 17–20 days, weighed 32–50 kg and comprised five females and four males, and those in Group 2 were aged 16–20 days, weighed 30–52 kg and comprised six females and three males. For the comparison of sedation, calves in Group 1 were treated with 5 µg/kg bodyweight I/V dexmedetomidine (Dexdomitor, Orion Corporation, Espoo, Finland) diluted in 0.9% NaCl saline solution to a volume of 5 mL, and calves in Group 2 were treated with 0.2 mg/ kg bodyweight I/V xylazine (Rompun, Bayer AG, Leverkusen, Germany) diluted in 0.9% NaCl saline solution to a volume of 5 mL. Both groups were injected using the right jugular vein.

Sample collection

The left jugular vein of calves from Group 1 was catheterised aseptically approximately 48 hours before the study start. Catheter patency was maintained using 5 mL heparinised saline flush solution (5 IU of heparin sodium/mL of 0.9% NaCl saline solution) administered three times/day.

Blood samples for the pharmacokinetic analysis were collected via the catheter, starting 30 minutes prior to DEX administration and then at 5, 15, 30, 45, 60, 90 minutes and at 2, 3, 4, 6, 8 and 12 hours after DEX administration. Blood was immediately centrifuged for 15 minutes at 1,500 g, serum was harvested and divided into aliquots which were immediately stored at −80°C until analysis.

Liquid chromatography-mass spectrometry analysis

Dexmedetomidine was extracted from the serum samples according to the method described by Li et al. (2009) which was modified and validated in our laboratory. Liquid–liquid extraction was chosen for the sample preparation. The serum sample (500 µL) was extracted with 5 mL of acetonitrile after addition of 10 µL internal standard solution (6 µg/mL tolazolin in methanol) and 50 µL saturated Na_2CO_3 solution. The mixture was vortexed for 10 minutes, and then centrifuged at 3,000 g for 10 minutes. The upper organic layer was transferred and evaporated to dryness under an air stream at 50°C using a TurboVap evaporator (Zymarck, Hopkinton, MA, USA). The dry residue was re- dissolved in 200 µL mobile phase and filtered on Phenex-RC (Regenerated Cellulose) 0.22 µm syringe filters (Phenomenex, Torrance, CA, USA) and 20 µL was used for high performance liquid chromatography (HPLC)-mass spectrometry.

An Accela 600 HPLC pump with a CTC automatic injector was used (Thermo Fischer Scientific, San Jose, CA, USA). Chromatographic separation was achieved using a C-18 Kinetex column (100 × 2.1 mm, 2.6 µm, Phenomenex, Torrance, CA, USA) with guard column. Samples were eluted with a mobile phase consisting of 5 mM ammonium acetate solution with 0.1% formic acid (A) and methanol/acetonitrile (50:50, v:v) with 0.1% formic acid (B). The flow rate was set at 200 µL/minute and the sample tray was maintained at 4°C.

Mass spectrometric analysis was performed using an LTQ XL ion trap (Thermo Fisher Scientific) equipped with a heated electrospray ionisation probe operating in the positive-ion mode. The mass transitions were: DEX, m/z 201→ 95 and internal standard m/z 161→77 (Ji et al. 2004). The Xcalibur (version 2.1) data acquisition software from Thermo Fisher Scientific was used.

Calibration curves were constructed using pooled calf serum obtained from untreated animals. The blank serum was spiked with 10 µL of internal standard (6 µg/mL tolazolin in methanol) and with DEX to obtain a concentration range of 0.025 – 20ng/mL. DEX (>99% pure) was purchased from Tocris (Milan, Italy) and tolazoline (>99% pure) from Sigma-Aldrich (St. Louis, MO, USA). Other reagents and solvents were purchased from Carlo Erba–Reagenti (Milan, Italy).

The linearity of the method was evaluated through the preparation of six different calibration curves on six different days by spiking serum samples with different DEX concentrations in the range 0.025 – 20 ng/mL. To verify the specificity of the method, 20 blank serum samples were analysed to check for the absence of potential interfering peaks from the matrix at the retention times of the DEX.

The within-day precision and accuracy were determined by analysing blank samples (six for each concentration) spiked with DEX at 0.05, 0.5 or 5 ng/mL on the same day. The between- day precision and accuracy were calculated using replicate determinations (n=9) of each concentration (0.05, 0.5, 5 ng/mL) made on three separate days. The precision was determined using the CV (%) and the accuracies were expressed as the percentage difference between the measured concentration and the nominal concentration. The extraction efficiency of DEX (recovery) from serum was determined by comparing the peak areas of DEX added into blank serum before the extraction procedure with those obtained for un-

extracted standard added with the same concentrations to the blank extracts. The limit of detection (LOD) and limit of quantification (LOQ) were estimated as the concentration corresponding to the mean signal-to-noise ratio plus three times (LOD), and 10 times (LOQ) its SD in 20 blank samples, respectively.

Pharmacokinetic analysis

Pharmacokinetic parameters of DEX were determined from serum concentration data using the WinNonLin 6.3 Prof software (Pharsight Corporation, Mountain View, California, USA) which enables compartmental and non-compartmental analyses of the experimental data. Visual inspection of the curve, residual analysis and minimum Akaike's information criterion estimation (Yamaoka et al. 1978) were used to choose the model best fitting the data. All data points were weighted by the inverse square of the fitted value. The disposition of DEX following I/V administration was described by a standard two-compartment model (Gibaldi and Perrier 1982).

Sedative effects

Heart and respiration rates of calves in both treatment groups were measured using a Mindray PM5000 (Shenzhen, China) multiparametric monitor 30 minutes before treatment and then at 5, 10, 15 and 20 minutes after treatment. Rectal temperature was also recorded at the same time points. The quality of sedation was evaluated in all calves 10 minutes after drug administrations using the classification shown in Table 1, by an evaluator unaware of the drug administered to the groups.

Degree/Level	Quality of sedation
0	The animal is slightly sedated, quadrupedal standing.
1	The animal is uncoordinated in lying down, falling to the ground
2	The animal shows ataxia and in the act of lying down tries to stand up
3	The animal lies down in a coordinated way, pushing on the carpus and lowering the back, taking a sternal position with the head turned toward the flank

Table 1 - Classification of the quality of sedation used in dairy calves following treatment with dexmedetomidine or xylazine.

The induction time of sedation was identified for each animal as the time between drug administration, reduction in reactivity to environmental stimuli, and acquisition of sternal recumbency. Recovery time was defined as the time between drug administration and the return to the quadrupedal position.

Statistical analysis

Pharmacokinetic parameters and intra-operative variables were reported as means and SD; harmonic means with pseudo-standard deviations were calculated for half-lives using a jack-knife technique (Lam et al. 1985). Repeated measures ANOVA with the Bonferroni post-test were used to compare clinical variables between treatment groups. The differences in sedation quality scores between groups were compared using a U-Mann Whitney test. All analyses were carried out using GraphPad InStat Software version 3.10 (La Jolla, California, USA).

Materials and methods

Dexmedetomidine concentrations and pharmacokinetic analysis

The HPLC method was shown to be linear with the correlation coefficient being >0.99, for each of the calibration curves from the six different days. At the retention times of the DEX peak, no significant endogenous interfering molecules were observed in the blank samples tested. Results for within-day and inter- day precision gave CV% <15%, and accuracies were within ±15% of the theoretical value. The mean extraction recovery of DEX from serum was 83.2 (SD 11.8)%. An LOQ of 0.023 ng/mL and an LOD of 0.006 ng/mL were obtained.

The change in mean concentrations of DEX in serum of nine calves is shown in Figure 1.

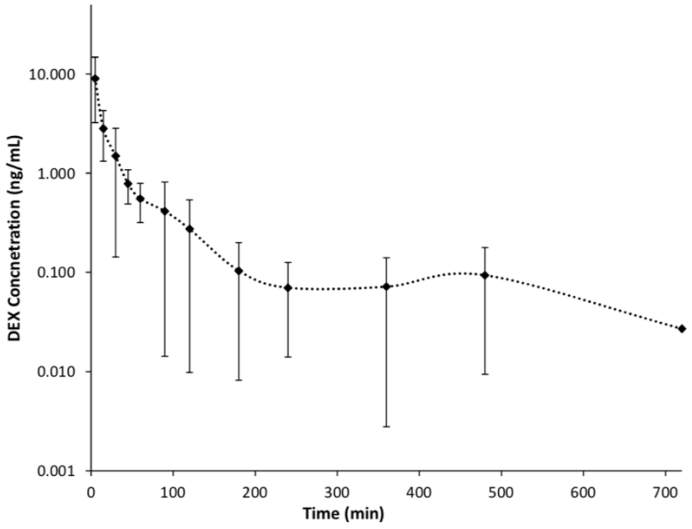

Figure 1 - Mean (±SD) concentration of dexmedetomidine in serum of nine dairy calves following I/V administration of 5 µg/kg dexmedetomidine. Note logarithmic scale on y-axis.

The mean concentration at 5 minutes after administration was 9.01 (SD 5.78) ng/mL. There was then a rapid decrease in concentration and at 45 minutes the mean value was 0.78 (SD 0.29) ng/mL. Subsequently, concentrations decreased progressively and DEX was detectable in all calves up to 120 minutes, when the mean concentration was 0.3 (SD 0.26) ng/mL. At 180 and 240 minutes the concentration of DEX was below the LOQ (0.023 ng/mL) in two calves, and at 360 and 480 minutes was below the LOQ in five calves. At 720 minutes DEX was detected in only one calf, with a value of 0.027 ng/mL, close to the LOQ.

Results from all subjects were best fitted by a bi-compartmental model and the results of the pharmacokinetic analysis are presented in Table 2.

Parameter	Mean±SD	Min, Max
Distribution half life (minutes)	8.7±5.0 [a]	3.6, 18.3
Elimination half life (minutes)	83.5±67.5 [a]	31, 317.3
Mean residence time (minutes)	87.9±69.5	30.5, 242.9
Body clearance (mL/minute/kg)	27.9±13.1	8.56, 42.5
$AUC_{(0-\infty)}$ (minute*ng/mL)	238.0±56.2	117.6, 584.4
$AUMC_{(0\rightarrow)}$ (minute*minute*ng/mL)	23,494.3±25,747.8	3,680.4, 73,593.6
K_{10} (per minute)	0.05±0.02	0.03, 0.08
K_{12} (per minute)	0.03±0.02	0.01, 0.08
K_{21} (per minute)	0.02±0.02	0.004, 0.05
$t½_{K10}$ (minutes)	15.0±7.4 [a]	7.1, 26.3
V_1 (mL/kg)	643.3±454.0	167.6, 1615.1
V_{dss} (mL/kg)	2,170.8±1,657.5	287.6, 4,468.1
V_2 (mL/kg)	1,527.5±1,426.0	119.9, 2,853.1
V_{dz} (mL/minute/kg)	5,954.6±4,236.4	881.9, 9,121.2

[a] Harmonic mean±pseudo-SD

AUC=area under serum concentration-time curve ; AUMC=area under moment curve; K_{10}=the rate at which the drug leaves the system from the central compartment; K_{12}=the rate at which the drug passes from central to peripheral compartment; K_{21}=the rate at which the drug passes from peripheral to central compartment; $t½_{K10}$=the half-life associated with the rate constant K_{10} ; V_1=volume of distribution in the central compartment; V_{dss}=volume of distribution at steady-state curve; V_2=volume of distribution in the peripheral compartment; V_{dz}=volume of distribution based on the terminal phase

Table 2 - Mean (±SD) and range of pharmacokinetic parameters determined using a two-compartment model following I/V administration of 5 µg/kg dexmedetomidine to nine dairy calves.

Sedative effects

The changes in mean heart rate and respiration rate in calves treated with DEX or xylazine are shown in Figure 2.

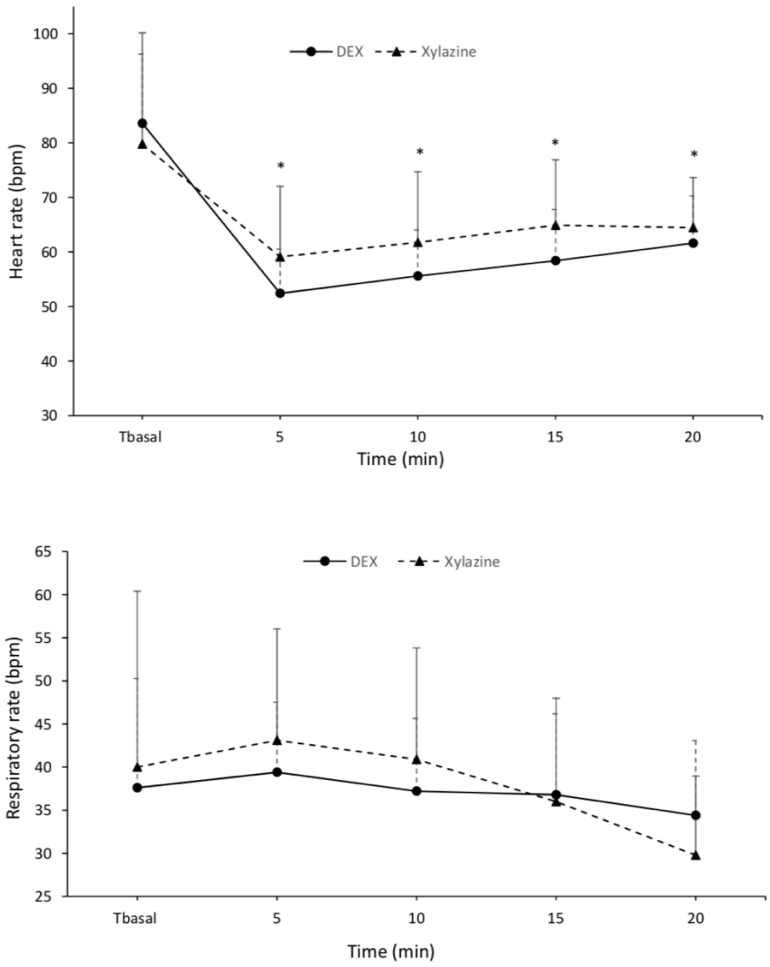

Figure 2 - Mean±SD (a) heart rate and (b) respiratory rate of dairy calves 30 minutes before and following I/V treatment with 5 μg/kg dexmedeto- midine (●; n=9) or 0.2 mg/kg xylazine (▲; n=9).

*indicates mean differs from value at −30 minutes ($p<0.05$).

A decrease in heart rate was observed after treatment with both DEX and xylazine ($p<0.05$). Temperature did not differ compared with the initial temperature (38.4 – 39.4°C and 38.2 – 39°C for the xylazine and DEX calves, respectively) following treatment in either group. There were no differences in heart rate, respiration rate or temperature between the two groups ($p>0.05$).

The quality of sedation, and intervals to induction and recovery did not differ between the two groups ($p>0.05$; Table 3). All recoveries were eventless, and no adverse reactions were noted in any of the animals. All calves were able to stand up and walk at the end of the observation period.

	Dexmedetomidine	Xylazine
Sedation quality	3 (2–3)	3 (2–3)
Induction time (minutes)	2.7±0.67	2.8±0.78
Recovery time (minutes)	80.5±30.7	88±28.7

Table 3 - Median (min, max) quality of sedation, and mean (±SD) interval to induction of and recovery from sedation in dairy calves treated I/V with 5 µg/kg dexmedetomidine (n=9) or 0.2 mg/kg xylazine (n=9).

Discussion

The welfare of livestock and the limited number of drugs available for pain relief in calves have instigated this study to determine the pharmacokinetic parameters of DEX administered I/V in dairy calves and to evaluate its sedative effect compared to xylazine. The pharmacokinetic profile of DEX in calves was characterised by a fast distribution half life (8.7 (SD 5.0) minutes) and relatively short elimination half life (83.5 (SD 67.5) minutes). Despite the young age of the calves (approximately 20 days) in the present study, and the presumable immature metabolic pool, the volume of distribution at steady state and body clearance values were reasonably homogenous among individuals, with values of 2,170.8 (SD 1,657.5) mL/kg and 27.9 (SD 13.1) mL/kg/minute, respectively.

The mean clearance value reported in this study is comparable to the hepatic blood flow calculated for calves (26.5 mL/kg/minute; Toutain and Bousquet-Melou 2004), which suggests that hepatic metabolism plays a primary role in the DEX metabolic pathway in young calves. The distribution volume at steady state (2,170.8 (SD 1,657.5) mL/kg) was approximately 42% of the volume of distribution based on the terminal phase (5,954.6 (SD 4,236.4) mL/kg). Therefore, in our calves limited amounts of DEX were eliminated during the distribution phase.

The two groups of calves treated with DEX or xylazine were very similar in terms of age, bodyweight and gender. DEX is a more potent and selective alpha-2-agonist than xylazine (Rioja et al 2008), but surprisingly no differences were observed in respiration or heart rate between the two groups after treatment. The typical bradycardia induced by alpha-2-agonist developed soon after DEX and xylazine administration and lasted for the whole observation period of 20 minutes. This is due to the development of peripheral vasoconstriction and probable reflex and centrally mediated decrease in heart rate (Rezende et al. 2015). In calves treated with xylazine, similar cardiovascular effects were detected and the decrease in heart rate lasted for 35 minutes (Rioja et al. 2008). In our calves heart rate measurements were carried out for 20 minutes and the time course of bradycardia could not be monitored for longer periods.

The development of hypoxaemia has been shown with xylazine, detomidine, romifidine, and medetomidine in sheep (Celly et al. 1997). In our study, respiration rate was highly variable and not influenced by the

treatments, however respiratory rate alone is probably not a reliable clinical indicator to detect and explain hypoxaemia in ruminants injected with an alpha-2-agonist.

The effect of alpha-2-agonists on the body temperature of ruminants is variable. We did not observe any influence of DEX or xylazine administration on the rectal temperature of our calves. Some alpha-2-agonists have been reported to cause hypothermia or hyperthermia in cattle, but the mechanism by which this is produced seems to be drug- and species-specific (Young 1979; Hall and Clarke 1991; Ranheim et al. 1999).

Induction and recovery times were comparable between the two groups. The onset of sedation was very rapid with both drugs and the clinical effects associated with DEX and xylazine were characterised by a similar degree of deep sedation and ease of hand- ling of the animals. The long and comparable duration of the sedative effect was not completely expected. Ruminants are considered to be extremely sensitive to xylazine compared to horses or dogs and cats (Plumb 2011). The young age of our calves could have influenced the metabolism and elimination of both DEX and xylazine and could explain the long sedative effect of both drugs. The recovery time (return to quadrupedal position) for calves administered medetomidine I/V at a dose of 0.03 mg/kg was reported as 242.11 (SD 108.67) minutes (Rioja et al. 2008), which was longer than we observed after I/V injection of DEX (80.5 (SD 30.7) minutes). For xylazine, the recovery time of 88 (SD 28.7) minutes in this study was comparable to 128.12 (SD 84.83) minutes that was reported previously (Rioja et al. 2008).

Induction quality was evaluated with a specific scale developed for this study. We observed a good quality of sedation with both drugs. In our opinion the scale was easy to use, specific and appropriate to determine induction quality in calves, therefore we propose its use in future studies on sedation quality in this species.

In order to obtain concentrations of DEX at the recovery time (approximately 80 minutes), these were extrapolated from the curve of the predicted concentrations and ranged between 0.16–1.09 ng/mL. However, for high lipophilic drugs, such as alpha-2-agonists, concentrations of drugs in serum do not necessarily represent concentrations at the effector site. A remote agonist-receptor interaction may occur with a serum concentration below the limit of detection (Kästner et al. 2003).

In conclusion, DEX induced a safe, reliable and long lasting sedation in our calves, leading to a transient decrease in heart rate and no modification in respiration rate or temperature. The results were comparable to xylazine, the most popular alpha-2-agonist among bovine practitioners. The lack of specific maximum residue levels for DEX limits its use in animals destined for human consumption, but the low dose administered and the short tissue and milk withdrawal times of all alpha-2-agonists are positive aspects which should stimulate further residual studies in cattle. In addition, the higher selectivity and potency of DEX compared to other alpha-2-agonists are further positive pharmaco- logical aspects which could indicate the selection of this drug in the therapeutic armamentarium of calves. It should be underlined that pain management is still underestimated in calves and that there is an increasing need for new and safe analgesic

molecules for this species. Thus, DEX could be used in calves for rapid procedures such as dehorning or castration, as it possesses a specific antagonist and was shown to be safe for young healthy calves.

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CLINICAL PHARMACOKINETICS OF DEXMEDETOMIDINE-METHADONE COMBINATION IN DOGS AFTER BUCCAL OR INTRAMUSCULAR ADMINISTRATION

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(Although it has been almost completed, the manuscript reported here is still in preparation)

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Conflict of Interest Statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

The study aimed to define and compare the pharmacokinetic profiles of dexmedetomidine and methadone administered simultaneously in dogs by oral-transmucosal or intramuscular route and to determine their relative bioavailability by oral-transmucosal route and the applicability of this administration route in dogs. Fourteen healthy client-owned dogs, scheduled for diagnostic procedures, were treated with a combination of dexmedetomidine hydrochloride (10 μg/kg) and methadone hydrochloride (0.4 mg/kg) by oral-transmucosal route in the oral pouch or intramuscularly in epaxial muscles. Blood samples were collected between 0 and 240 min after administrations. On drugs concentration non-compartmental analysis was carried out. Oral transmucosal administration caused ptyalism in most subjects and intramuscular administration a transient peripheral vasoconstriction. The results showed a low and delayed absorption of both dexmedetomidine and methadone by oral-transmucosal route, with median (range) C_{max} values of 0.82 (0.42 – 1.49) ng/mL and 13.22 (2.80 – 52.30) ng/mL, respectively. Relative bioavailability was low, 16.34% (dexmedetomidine) and 15.5% (methadone). By intramuscular route a more efficient absorption profile was observed, with approximately ten times higher AUC and C_{max} values for both drugs. Dexmedetomidine and methadone simultaneously administered by oral-transmucosal route with injectable formulations are not readily absorbed through the oral mucosa in dogs.

Keywords: Pharmacokinetics, Dexmedetomidine, Methadone, Oral trans-mucosal, Intramuscular; Dogs

Introduction

Alpha-2 adrenergic agonists are widely used in veterinary medicine, where medetomidine and dexmedetomidine play a primary role in small animal practice. They activate α-2 presynaptic receptors in the central nervous system producing inhibitory effects on epinephrine release and stimulate postsynaptic G-protein-coupled α-2 receptors, resulting in peripheral vasoconstriction, analgesia and other effects (Muir and Mason, 1996). These drugs induce deep sedation, muscle relaxation and analgesia, and allow a reduction in the dose of induction agents required for general anaesthesia (Murrell and Hellebrekers, 2005). Dexmedetomidine (DEX), the pharmacologically active d-enantiomer of medetomidine, is the most potent and selective α-2 adrenergic receptor agonist and is approved for use in canine patients via intravenous (IV) and intramuscular (IM) route as sedative/analgesic or as preanesthetic and as gel formulation via buccal administration for the treatment of noise aversion (Granholm et al., 2007; Plumb, 2018).

In veterinary medicine, α-2 receptor agonists are usually combined with opioids, such as methadone, to potentiate their analgesic and sedative effects, thanks to a synergistic effect, without important alterations in cardiorespiratory variables (Monteiro et al., 2008; Cardoso et al., 2014; Puighibet et al., 2015). In fact, synergism of action between α−2agonists and opioids is well recognized. At the base, there would be the same signal transduction system (G protein activation) and a partly overlapping central receptor localization (Murrell & Hellebrekers, 2005).

Methadone (MET) is a pure μ-agonist synthetic opioid with similar characteristics to morphine. It works at μ and κ receptor level, activating the second messenger (G-protein), inhibiting adenylate cyclase and causing modification of ion channel activity. Methadone, in addition, acts as a non-competitive antagonist for N-Methyl-D-Aspartate (NMDA) for glutamate (Gorman et al., 1997) and is able to inhibit the serotonin and norepinephrine reuptake and to promote the block of nicotinic receptors that contribute to analgesic activity (Codd et al., 1995). It provides good analgesia and causes dose-dependent sedation in dogs, moreover, it is considered to be as powerful as morphine, but with fewer side effects, i.e. nausea, vomiting, and dysphoria (Monteiro et al., 2008; Trimble et al., 2018).

Oral-transmucosal (OTM) drug administration is attractive to veterinarians for several reasons. It is noninvasive and it does not cause pain or distress to the patient, thus it is even more attractive for patients needing sedation that are difficult to inject or fearful when restrained (Ansah et al., 1998; Slingsby et al., 2009; Cohen and Bennett, 2015). Oral trans-mucosal route requires minimal restraint of the patient or technical skill of the clinician, if compared with other routes of administration (e.g. intramuscular). In addition, the first-pass metabolic effect of orally administered drugs is avoided with OTM administration, and the rich blood supply to the oral mucosa allows reaching systemic therapeutic concentrations (Zhang H et al., 2002; Sattar et al., 2014; Messenger et al., 2016). In cats it was extensively evaluated in several trials (Wells et al., 2008; Santos et al., 2010; Ferreira et al., 2011; Ko et al., 2011; Bortolami et al., 2012) and in particular it was successfully used in a study comparing IM and OTM administrations of DEX and

buprenorphine (Porters et al., 2014). In dogs some studies have been published on clinical aspects or pharmacokinetics of OTM administration, some of these evaluated sedatives as α-2 agonists (Cohen and Bennett, 2015; Messenger et al., 2016) and benzodiazepines (Zhang J et al., 2002; Aldawsari et al., 2018) or opioids (Streisand et al., 2002; Abbo et al., 2008; Ko et al., 2011), but none investigated the pharmacokinetic behaviour of a drugs combination.

The primary aim of the present study was to define the pharmacokinetic profiles of DEX and MET administered simultaneously in dogs by OTM or IM route. The secondary aims were to compare the pharmacokinetic profiles by both routes of administration, determining the relative bioavailability of DEX and MET by OTM route and the applicability of this route of administration in dogs.

Materials and Methods

Animals — Fourteen healthy adult client-owned dogs (7 neutered males and 7 spayed females) aged from 1 to 11 years, weighting from 20 to 60 kg (mean ± SD weight of 31.0 ± 8.5 kg), were enrolled in the study. All animals were scheduled for different diagnostic procedures (X-ray exams) at the Veterinary Hospital of the University of Milan (Lodi, Italy) and were judged to be healthy (ASA status I) on the basis of physical examination findings and results of routine blood tests. The protocol of the study was approved by the Institutional Ethical Committee for Animal Care at the University of Milan (OPBA_19_2016) and all dogs were enrolled after obtaining written consent by the owners. None of the dogs had received any medications in the 30 days before the study.

Study design—A parallel-group clinical trial was performed by randomly assigning (coin flip) dogs to either the OTM or IM group; group OTM consisted of 8 dogs, whereas IM group consisted of 6 dogs. Prior to treatment, a 20-gauge, 32-mm-long angiovenous catheter (Surflo® I.V. Catheter, Terumo) was inserted into a cephalic vein and secured.

Dogs were treated with a mixture of DEX hydrochloride (Dexdomitor® 0.5 mg/mL, Pfizer) at 10 μg/kg and MET hydrochloride (Semfortan® 10 mg/mL, Dechra) at 0.4 mg/kg by OTM or IM. Oral pH was determined before and every 10 min up to 30 min after OTM administration using commercially available pH-indicator strips (MQuant® pH 6.5-10, Merck Millipore). Oral transmucosal administration of the drugs mixture was performed with a 2.5 mL syringe (without needle) inserted into the buccal pouch. For IM group, the drugs mixture was administered through a single intramuscular injection into the epaxial muscles. Thirty minutes after DEX and MET treatment administration all dogs were induced with titrate-to-effect propofol to achieve orotracheal intubation and maintained under general anaesthesia with isolurane 100% in oxygen. After recovery from anaesthesia, all dogs were discharged.

Blood sample collection—Blood samples (2.5 mL) were collected via a peripheric venous catheter prior to drug administration (time 0) and at 10, 20, 30, 40, 50, 60, 90, 120, and 240 minutes after OTM and 10, 30, 45, 60, 90, 120, 180 and 240 minutes after IM administration. For each dog, a volume of sterile saline (0.9% NaCl) solution equal to the volume of blood taken was administered IV through the cephalic catheter at the end of blood sample collection. Blood samples were immediately transferred into a glass tube containing a clot activator and centrifuged for collection of serum (for DEX quantification) or into heparinized glass tubes and centrifuged for collection of plasma (for MET quantification). Serum and plasma were stored at –80°C until dexmedetomidine and methadone concentrations were measured.

Dexmedetomidine or methadone analysis and validation— Dexmedetomidine was extracted from the serum samples according to the method described by Cagnardi et al. (2017) with slight modifications due to different volume of blood samples. Thus, the serum samples (300 µL) were added with 10 µL of internal standard solution (4.5 µg/mL tolazolin in methanol) and then extracted with 3 mL of acetonitrile after addition of 50 µL saturated Na_2CO_3 solution. The mixture was vortexed for 10 minutes, and then centrifuged at 3000 g for 10 minutes. The rest of the extraction procedure and the mass spectrometry analysis by LC-MS/MS was carried out as described by Cagnardi et al. 2017.

For methadone extraction from canine plasma, 200 µL of sample were added with 1.8 mL of deionized water, 25 µL of internal standard (300 ng/mL codeine D3) and 75 µL of H_3PO_4 solution (20 %). The mixture was vortexed for 10 min and then 4 mL of KH_2PO_4 (1 M pH 5.8) were added. Then, the sample was centrifuged at 3700 g for 30 minutes. Samples were purified by solid phase extraction using Bond Elute C8 cartridges (500 mg/3mL, Agilent Technologies, Milan, Italy) activated with 2 mL of methanol, 2 mL of deionized water and 2 mL KH_2PO_4 buffer (0.1 M pH 6) and then washed with 2 mL of acetic acid (1 M) and 2 mL of methanol. At the end samples were eluted with 6 mL of elution solution (ammonium hydroxide 80 %, isopropanol 18%, dichloromethane 2%). The eluate was evaporated to dryness on an electric stove set at 60 $\pm$ 5°C, then 400 µL of methanol were added, and the sample was vortexed. The mixture was transferred in a vial and again evaporated to dryness on an electric stove at 60 $\pm$ 5°C. Afterwards the sample was reconstituted with 50 µL of mobile phase, vortexed and injected into the column.

The mobile phase consisted of a 0.1% formic acid in deionized water (80%) and methanol (20%). Separation was achieved with a 150 mm x 2 mm, 4 µm C-18 column (Synergi Polar®, Phenomenex Inc., Torrance, CA, USA). Injection volume was 10 µL, separation was achieved at a flow rate of 0.3 mL/min. The HPLC gradient was (A:B, v/v) 80:20 for 2 min; 2:98 for 6 min and 80:20 for 5 min to re-equilibrate the system.

Mass spectrometric analysis was performed using a triple quadrupole LC/MS system (Agilent 6410, Agilent Technologies, Inc., Santa Clara, California, USA) equipped with a heated electro - spray ionization probe

operating in the positive-ion mode. The mass transitions were: MET m/z 310→ 265 and internal standard codein D3 m/z 303→165. The MassHunter Quantitative Analysis software (version B.06.00; Agilent Technologies, Inc., Santa Clara, California, USA) was used.

All analytical methods were subject to intra-laboratory validation in compliance with the recommendations defined by the European Community (Commission decision 2002/657/EC) and with the international guidelines (EMA, 2011 - VICH GL49). Validation data for DEX and MET are reported in Table 1. The calibration curves were prepared with 6 spiked solutions obtained diluting the original stock solution of DEX (1 mg/mL + internal standard, 4.5 µg/mL tolazolin) or MET (1 mg/mL + internal standard 300 ng/mL codeine D3) in canine blank serum or plasma to achieve concentrations ranging from 0.025 to 10 ng/mL for DEX or 0.5-100 ng/mL for MET. Dexmedetomidine (>99% pure) was purchased from Tocris (Milan, Italy) and tolazoline (>99% pure) from Sigma-Aldrich (Milan, Italy). Methadone (>99% pure) and codeine D3 (>99% pure) were purchased from Cerillant (Sigma Aldrich, Milan, Italy). All salts and solvents were of LC-MS quality grade (Sigma Aldrich, Milan, Italy or Carlo Erba–Reagenti, Milan, Italy). There was a linear relationship (r^2 value > 0.98) between drugs' concentrations and peaks' area over the range investigated. The intraday repeatability was measured as coefficient of variation (CV%) on 6 replicates of 3 concentrations, whereas the trueness (%) was measured as closeness to the concentration added on the same replicates. The results fell within the accepted ranges for precision and trueness (Table 1). For DEX a LOQ value of 0.025 ng/mL and a LOD of 0.006 ng/mL value were set. For MET, LOQ and LOD were 0.5 ng/mL and 0.003 ng/mL, respectively. The specificity of the methods was demonstrated by the absence of interference in 20 blank serum or plasma samples at the DEX or MET retention times.

Parameter (units)	DEX	MET
LOQ (ng/mL)	0.025	0.5
LOD (ng/mL)	0.006	0.003
Trueness (%)	95.6 - 104.7	95.5 – 104.2
Intra-day repeatability (CV%)	4.0 – 6.6	9.8 – 14.6
Recovery (%)	98.3 ± 6.6	76 ± 6
Trueness, Intra-day repeatability and intra-laboratory reproducibility reported as range values; CV= coefficient of variation; Recovery is reported as mean ± SD (n=18);		

Table 1 - Intra-laboratory validation of analytical methods for dexmedetomidine and methadone in serum or plasma samples, respectively.

Pharmacokinetic and statistical analysis— Pharmacokinetic parameters were determined from serum/plasma concentration – time data using the Phoenix WinNonLin 8.0 software (Pharsight Corporation, USA), which allows compartmental and noncompartmental analyses of the experimental data. Visual inspection of the curve, residual analysis and minimum Akaike's information criterion estimates (MAICE; Yamaoka et al., 1978) were used to choose the model best fitting the data. All data points were weighted by the inverse square of the fitted value. The dispositions of DEX and MET following OTM or IM administration were described by standard noncompartmental analysis (NCA).

The elimination half-life ($t_{1/2\lambda z}$) was calculated as $\ln 2/\lambda z$. The area under the concentration-time curve from administration to last measurable concentration ($AUC_{0\text{-last}}$) and area under the first moment curve ($AUMC_{0\text{-last}}$) were calculated using the trapezoidal method. Mean residence time ($MRT_{0\text{-last}}$) was determined from the following equation (Gibaldi and Perrier 1982):

$MRT_{0\text{-last}} = AUMC_{0\text{-last}} / AUC_{0\text{-last}}$.

The peak concentrations, C_{max}, and time to peak T_{max} were obtained from the experimentally observed data. The relative bioavailability of DEX and MET after OTM administration was calculated as the ratio of $AUC_{0\text{-last}}$ after OTM and IM administration:

$F_{rel}\% = (AUC_{OTM}/AUC_{IM} \times 100)$.

Pharmacokinetic parameters were reported as median and range. A normality test (Kolmogorov_Smirnov test) was performed on pharmacokinetic parameters. The principal kinetic parameters obtained after DEX and MET analysis were compared after OTM and IM administration using unpaired t tests with Welch corrections (variances unequal) (InStat 3.0, GraphPad Software). Differences with $P < 0.05$ were considered significant.

Results

Oral transmucosal administration was well tolerated by all dogs, however in 6 out of 8 subjects severe ptyalism occurred at the end of drugs co-administration. Vomiting was also reported in 2 subjects of OTM group 10 minutes after co-administration. Oral pH was stable for all measurements in all animals ranging from 8.9 ± 0.6 at time 0 to 8.7 ± 0.5 after treatment. Two dogs in the IM group did not complete the sampling phase due to unrelated medical problems and were excluded from the study.

The doses administered by IM were relatively high from a clinical point of view and were selected for an adequate comparison of the pharmacokinetics with the OTM route. After IM administration a transient peripheral vasoconstriction was observed in all subjects for the first 15-20 min, in some animals the level was so severe to preclude blood sampling at first time-points. Transient bradypnea and bradycardia were observed by both routes, although more evident by IM route.

Mean serum or plasma concentrations ± SD of DEX or MET after OTM and IM administration are shown in Fig. 1 and 2, respectively. For DEX after OTM administration the mean concentration in serum was 0.39 ± 0.22 ng/mL at the first sampling (10 min), then increased to 0.75 ± 0.33 ng/mL at 30 min and reached 0.2 ± 0.03 ng/mL at 240 min. After IM very variable results were obtained at the first sampling times, due to peripheral vasoconstriction. In 1 dog the first sample was taken at 10 min with a concentration of 7.94 ng/mL, in another dog was taken at 15 min with a concentration of 11.26 ng/mL, in the rest 2 dogs, first samples were at 20 and 25 min with concentrations of 2.66 and 8.78 ng/mL, respectively. Within each subject, concentrations progressively decayed with time and reached a mean concentration of 0.76 ± 0.29 ng/mL at 240 min.

For MET after OTM administration the mean concentration in plasma was 4.95 ± 3.53 ng/mL at the first sampling (10 min), then increased to 10.91 ± 7.04 ng/mL at 30 min and then concentrations were fluctuating to reach 14.03 ± 16.4 ng/mL at 60 min and 4.56 ± 3.35 ng/mL at 240 min. After IM more variable results were obtained at the first sampling times. In 1 dog the first sample was taken at 10 min with a concentration of 27.55 ng/mL, in another dog was taken at 15 min with a concentration of 127 ng/mL, in the rest 2 dogs, first samples were at 20 and 25 min with concentrations of 46.38 and 465 ng/mL, respectively. Within each subject, concentrations were then progressively decreasing to a mean concentration of 22.46 ± 18.1 ng/mL at 240 min.

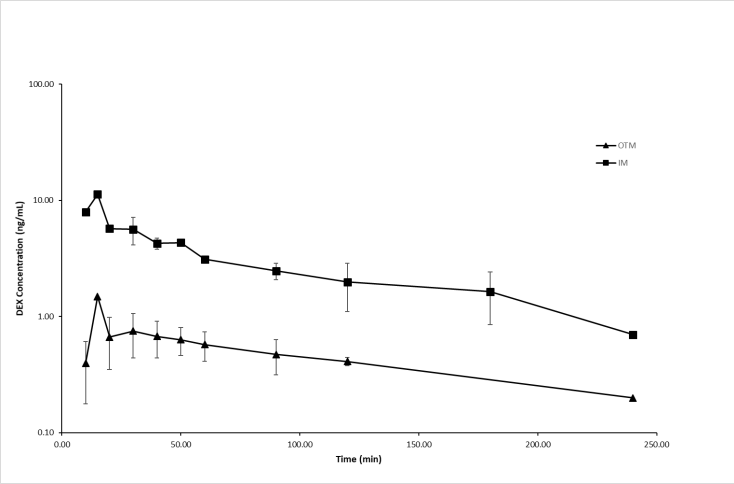

Figure 1 - Mean dexmedetomidine concentration (ng/mL) ± S.D. in dogs by OTM (n = 8) or IM (n = 4) administration of the mixture of DEX (10 μg/kg) and MET (0.4 mg/kg).

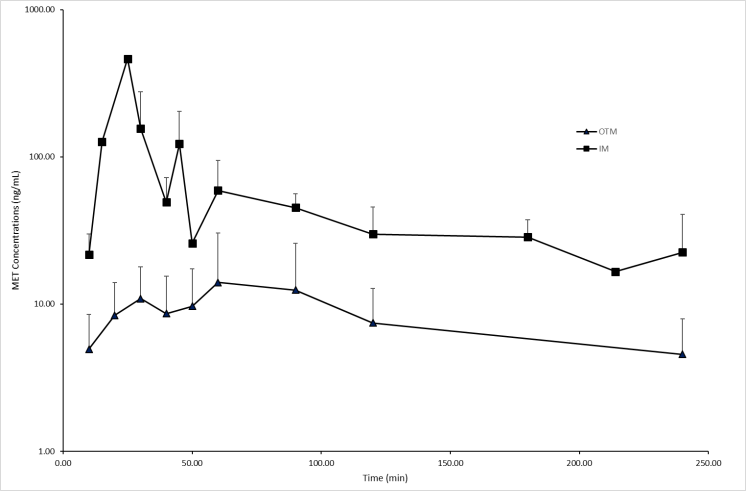

Figure 2 - Mean methadone concentration (ng/mL) ± S.D. in dogs by OTM (n = 8) or IM (n = 4) after administration of the mixture of DEX (10 μg/kg) and MET (0.4 mg/kg)

Pharmacokinetic parameters (median and range) for DEX and MET after OTM and IM co-administration are reported in Table 2.

Parameter	Unit	OTM (n. 8)	IM (n.4)
		Dexmedetomidine	
C_{max}	ng/mL	0.82* (0.42-1.49)	8.36* (4.40-11.26)
T_{max}	min	35 (15-120)	20 (10-40)
$t_{½ \lambda z}$	min	130.15 (89.79-397.57)	67.53 (44.33-135.15)
$AUC_{0\text{-last}}$	min*ng/mL	97.90* (62.40-132.88)	593.48* (486.70-650.25)
$AUMC_{0\text{-last}}$	min*min*ng/mL	9280.50* (4901-12763)	47248.70* (34771.50-53085.75)
$MRT_{0\text{-last}}$	min	94.78 (57.22-114.78)	81.08 (60.87-92.70)
F_{rel}	%	16.4	
		Methadone	
C_{max}	ng/mL	13.22* (2.80-52.30)	118.77* (46.38-465.00)
T_{max}	min	40 (10-90)	30 (25-45)
$t_{½ \lambda z}$	min	178.99 (45.99-290.34)	129.63 (98.37-220.34)
$AUC_{0\text{-last}}$	min*ng/mL	1313.80* (416.30-3755.90)	8862.86* (4735.40-22065.23)
$AUMC_{0\text{-last}}$	min*min*ng/mL	141204.50* (50885-361777)	666428.53*(443093.50-1601788.50)
$MRT_{0\text{-last}}$	min	110.35 (53.30-122.23)	75.16 (72.59-93.57)
F_{rel}	%	15.5	

*C_{max} = maximum concentration observed; T_{max} = observed time for C_{max}; $t_{½\lambda z}$ = elimination half-time; AUC_{0-last} = area under serum concentration–time curve from 0 to last concentration; AUMC = area under moment curve; MRT = mean residence time; F_{rel}= relative bioavailability. *Significantly different (P < 0.05).*

Table 2 - Median and range of pharmacokinetic parameters calculated with non-compartmental analysis after OTM or IM simultaneous administration of the mixture of DEX (10 μg/kg) and MET (0.4 mg/kg) in 12 dogs.

Discussion

Unconventional systemic routes of administration, i.e. buccal or intranasal are gaining higher importance in veterinary practice thanks to the advantages they offer over oral or IM-IV administration for systemic drug delivery. The major advantages are the easy practicability, the lack of pain during administration, the high blood supply of the mucosa and the avoidance of hepatic first pass effect or gastrointestinal degradation (Wells et al., 2008). Thus, oral-transmucosal route results particularly attractive for veterinary patients needing sedation that are difficult to inject, or for drugs with high rates of pre-systemic metabolism (Cohen and Bennett, 2015; Messenger et al., 2016).

The aims of the present study were to define the pharmacokinetic profiles of DEX and MET simultaneously administered in dogs by OTM or IM route, the relative bioavailability of both drugs by OTM route and the clinical applicability in dogs.

So far, no other studies have been published in dogs on the pharmacokinetics of these drugs concomitantly administered by OTM or IM routes. It has to be highlighted that this is a clinical study, thus the combination of DEX hydrochloride (10 μg/kg) and MET hydrochloride (0.4 mg/kg) by OTM and IM route was administered as preanaesthetic protocol to obtain a good sedation prior to general anaesthesia induction.

Synergism of action between α-2 agonists and opioids is widely recognized and employed in veterinary medicine with the aim of increasing the desired effects (sedation and analgesia), lowering the dosages and limiting the side effects (Cardoso et al., 2014; Puighibet et al., 2015).

Dexmedetomidine and MET in their hydrochloride form present a pKa of 7.1 and 8.94, respectively (Plumb, 2011). As reported by Riviere and Papich (2008), drugs pKa, their lipophilicity and molecular dimension are the factors that mainly influence the ability of a drug to cross the cellular membranes. Regarding OTM administration, these characteristics are particularly essential together with the volume of drug administered, the regional perfusion and the local pH of the oral cavity (Zhang H et al., 2002; Sattar et al., 2014). Several pharmacokinetic studies report that drugs with weak base characteristics, such as opioids and α-2 agonists, are easily absorbed through the oral mucosa of cats and dogs (Robertson et al., 2005; Abbo et al., 2008; Bortolami et al., 2012; Pypendop et al., 2014; Messenger et al., 2016). In particular, in canine and feline patients, the alkalinity of the oral cavity would favour the presence of non-ionized form for drugs with weak base characteristics and increase this drugs absorption (Ko et al. al., 2011; Pypendop et al., 2014).

Given the above, unexpectedly, the results reported in the present study showed a low absorption of both DEX and MET following co-administration by OTM, with C_{max} values of 0.82 ng/mL and 13.22 ng/mL and a low relative bioavailability 16.34% (DEX) and 15.5% (MET). These results might be related to the formulations used. In fact, the available injectable formulation of both DEX and MET were used, although the gel formulation of DEX is commercially available. This choice was obligated, because there are no commercially available MET gel formulations, and the simultaneous administration of two pharmaceutical specialties with different physical-chemical characteristics could cause a mutual incompatibility to the co-administration itself. Moreover, the mean volume of mixture administered was rather high (2.0 $\pm$ 0.7 mL), due to the concentrations of the injectable formulations employed of DEX (0.5 mg/mL) and MET (10 mg/mL). Such a high administration volume, probably, might have hindered absorption: in fact, according to another study results, during OTM administration, a high volume of drug leads to partial losses during administration and to the swallowing of a drug volume proportional to the amount of drug administered (Porters et al., 2014).

Furthermore, in the present work, 6 out of 8 dogs showed a strong salivation increase (ptyalism) few seconds after OTM administration. Given its severity, the ptyalism might have played an important role in decreasing the absorption of DEX and MET co-administered by OTM route. As reported by other studies, in fact, the salivation increase would induce a dilution effect of the administered drug and would stimulate, at the same time, the reflex of swallowing that, at the end, would lead to a decrease in OTM absorption (Santos et al., 2010; Ferreira et al., 2011; Bortolami et al., 2012; Porters et al., 2014). According to the literature, the ptyalism would be due, primarily, to the unpleasant taste of injectable formulations (Abbo et al., 2008; Santos et al., 2010; Bortolami et al., 2012).

Finally, another important factor, that could have hindered the systemic absorption of DEX and MET, is the peripheric vasoconstriction produced by DEX itself due to the interaction with the pre-capillary sphincters α-

2B receptors of the peripheral vascular beds (Murrell and Hellebrekers, 2005), which leads to a reduction in peripheric drugs absorption (Porters et al., 2014; Kallio-Kujala et al., 2018).

To the authors' opinion, all these factors might have contributed in reducing the systemic absorption of DEX and MET co-administration by OTM route. The results of the present work are in contrast with other studies where injectable formulations of opioids showed a good absorption profile by OTM with high bioavailability values (Robertson et al., 2005; Abbo et al., 2008; Bortolami et al., 2012; Pypendop et al., 2014). However, in these works the drugs were not administered in combination with other molecules, thus were not influenced by possible physical-chemical interferences of formulation or pharmacological effect. Only one study (Porters et al., 2014) reported the pharmacokinetic profile of DEX combined with buprenorphine in cats following OTM or IM administration. Porters and colleagues (2014) obtained similar results regarding lower drugs absorption by OTM compared to IM route and high variability in pharmacokinetic parameters, such as Cmax, Tmax and AUC. Those results were explained with the same hypotheses, i.e. high administration volume, ptyalism and peripheric vasoconstriction. In addition, DEX-induced peripheric vasoconstriction was observed also by other authors by both OTM and IM routes (Pypendop et al., 2017; Kallio-Kujala et al., 2018).

Intramuscular administration route was used in this study in comparison to OTM, because the first represents a more common route of administration and allows for more direct clinical application of this study data. The small IM sample size used (reduced by unrelated medical problems) may be appropriate for pharmacokinetic modeling, although a larger sample of the canine population would have provided more powerful results, lowering the inter-subject variability. Additionally, the reduced number of animals in the IM group together with the difficulties in blood sampling at the first time-points precludes the possibility of exhaustive conclusion on the pharmacokinetic profile of both drugs by this route.

The pharmacokinetic comparison of both drugs after OTM or IM co-administration showed a more efficient absorption profile for the IM route, with higher values for AUC and on average presented C_{max} values approximately ten times higher for both molecules. These results agree with the studies reported by Wells and colleagues (2008) and Porters and colleagues (2014). Moreover, the IM route showed a tendency of a longer T_{max} ($P>0.05$) for MET than DEX (30 vs 20 min, respectively), resulting in agreement with data reported by Kallio-Kujala and colleagues (2018).

One limitation of the study is lack of data on the OTM route with DEX and MET singularly administered. On the other hand, for sedative purposes, the importance of this synergism is well known, and thus our interest was more focused on their use in combination. A more favorable kinetic profile of DEX and MET cannot be excluded, when separately administered, as observed in other studies for OTM (Robertson et al., 2005; Abbo et al., 2008; Bortolami et al., 2012; Pypendop et al., 2014; Gulledge et al., 2018).

Furthermore, another limitation is the use of injectable formulations not specific for the OTM route. Other studies have shown an increase in bioavailability with the use of gel formulations, developed specifically for the OTM administration (Messenger et al., 2016; Aldawsari et al., 2018).

Although not analysed in the present study (unpublished data), the sedative effects of the DEX-MET combination administered by OTM or IM. In general, higher sedation levels were reached with the IM route, in a much shorter time (approximately 10 min) compared with the OTM administration (approximately 25 min). However, in spite of a delayed onset, the OTM route was anyway considered effective in obtaining a satisfactory level of sedation.

Conclusions

In conclusion, from the results of this study is possible to state that DEX (10 μg/kg) and MET (0.4 mg/kg) simultaneously administered by OTM route with injectable formulations are not readily absorbed through the oral mucosa in dogs. The increase in salivation (ptyalism) and the possible consequent swallowing of the drugs, may have played a major role in this process. In fact, the blood concentrations of DEX and MET following OTM co-administration if compared to the IM route are not sufficient to recommend this administration in the normal routine of surgical preanaesthesia.

Despite these considerations, the preliminary evaluation of clinical effects was satisfactory although with a long onset and resulted probably from the synergistic action of the two molecules. Thus, this type of sedative protocol needs more extensive studies, i.e. with different formulations or in larger groups of animals, before being suggested. As anyway, it might be useful in those non-collaborative canine patients that excessively react to intramuscular administration or that can be safely approached only by the owners to perform complete clinical visits.

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CLINICAL PHARMACOKINETICS OF DEXMEDETOMIDINE AND KETAMINE FOLLOWING INTRAMUSCULAR SIMULTANEOUS ADMINISTRATION IN TIGERS (*Panthera tigris*)

Remote delivery of injectable drugs for chemical immobilisation is frequently required in captive large felids (e.g. tigers) to perform physical examination, biological sample collection, vaccination, drugs administration, diagnostics and minor surgical procedures. Since it is often impossible to assess animals health status prior to chemical restraint, it is necessary that the active compounds used are safe and have predictable sedative effects (Isaza, 2014).

Ketamine (KET), a dissociative anaesthetic NMDA-receptors antagonist frequently employed in veterinary medicine, in tigers can cause salivation, muscle rigidity, ataxia and seizures (Bennett et al., 1971; Gunkel and Lafortune, 2007; Laricchiuta et al., 2015). Sedative drugs belonging to the α-2 agonist class are reported to induce cardiovascular changes in tigers including bradycardia, ventricular arrhythmias, hypertension and hypotension (Miller et al., 2003; Larsson et al., 2008; Sontakke et al., 2009). However, is well recognized the synergistic sedation and anaesthesia effect of KET and DEX combination mediated by the α-2 adrenergic receptors (Song et al., 2015). Furthermore, is reported that DEX can regulate the activity of the NMDA receptor in spinal dorsal horn through inhibiting tyrosine phosphorylation of NR2B subunit, modulating KET clinical effects (Zheng J et al., 2012).

Thus, for tigers immobilisation, the most modern protocols report the use of ketamine in combination with the class of α-2 agonists, such as medetomidine and dexmedetomidine (DEX) (Forsyth et al., 1999; Curro et al., 2004; Reilly et al., 2014; Clark-Price et al., 2015).

In zoo and wildlife medicine drug therapy is often extrapolated from domestic species data (Hunter et al., 2008) and the most common methods for dosage translation are represented by the linear extrapolation, the metabolic scaling and the allometric scaling (Hunter and Isaza, 2008).

To the authors' knowledge, the literature concerning pharmacokinetic studies in tigers is very lacking, with only two manuscripts published, one about tiletamine and zolazepam and one about cefovecin (Lewis et al., 2014; Cushing et al., 2017). Thus, there are no data regarding DEX and KET pharmacokinetics in the species. Consequently, the aim of the present study was to determine the pharmacokinetic profile of DEX, KET and its active metabolite (norketamine, NORKET) in captive tigers administered with different sedative protocols, based on each animal needs, and thus investigating also the possible influence of other compounds simultaneously administered on the disposition of these drugs.

Eighteen adult captive tigers, scheduled for periodic physical examination or diagnostic procedures, were administered with a combination of DEX at 10 μg/kg and KET at 2 mg/kg, given by remote intramuscular (IM) injection through two consecutive blowpipe darts. If needed, tigers were administered with other variable doses of DEX and KET and/or other drugs (Table 1). When animals reached a satisfactory level of sedation, as ascertained by the disappearance of the ear twitch reflex, a venous peripheric catheter was inserted into

the saphenous vein in order to allow blood samples collection. On the basis of the sedative protocols, nine animals were assigned to Standard Protocol group, i.e (DEX 10 μg/kg and KET 2 mg/kg; SP group) and nine animals were allocated in the Non-Standard Protocol group, i.e. administered with different doses of DEX 10-25 μg/kg and KET, 2-4 mg/kg, or with any other necessary drug, such as titrate-to-effect propofol and isoflurane, respectively for anaesthesia induction and maintenance (NSP group). Any adverse effects occurred during the procedures were recorded.

During the time tigers were safely approachable, blood samples were collected every 5-10 min and put into tubes containing clot activator or into heparinized tubes for DEX and KET, respectively. Then samples were centrifuged, and serum and plasma were stored at -80°C pending analyses of DEX, KET and NORKET, respectively.

Table 1 - Animal details, anaesthetic protocols and sampling times in eighteen tigers enrolled in the study divided into the two groups, Standard protocol (SP) and Non standard protocol (NSP).

Sub-species	Sex	Age (years)	Estimated Weight (kg)	Indication for Anaesthesia	KET dose (mg/kg)	DEX dose (μg/kg)	Other Drugs (mg/kg)	Sample Times (minutes)
Standard protocol (SP)								
Siberian	F	9	110	Routine Examination	2	10	/	27, 32, 47, 52, 58, 64, 69, 73, 78, 83*
Siberian	F	8	120	Routine Examination	2	10	/	20, 35*, 39, 55*, 58, 63, 69
Hybrid	M	4	120	Routine Examination	2	10	/	12**, 13*, 17*, 22, 27, 32, 37, 41*, 44**
Hybrid	F	5	120	Routine Examination	2	10	/	22*, 28, 33, 38, 43, 47
Bengal	F	5	100	Routine Examination	2	10	/	19, 24, 29, 34, 39, 44
Hybrid	M	17	150	Routine Examination	2	10	/	21, 26, 31, 36, 41, 46, 51, 56*
Hybrid	F	17	110	Routine Examination	2	10	/	19, 24*, 30, 34, 39, 44, 49, 55
Bengal	F	17	140	Routine Examination	2	10	/	17, 22, 27, 35, 39, 45, 50, 55
Bengal	F	16	140	Routine Examination	2	10	/	18, 23, 28, 33, 38, 44, 49, 54
Non standard protocol (NSP)								
Bengal	F	2	80	Echocardiography	2	10	BTF 0.1 PPF 0.5	20, 23*, 29,35*, 40*, 45, 56, 71, 87, 106
Hybrid	M	8	140	Routine Examination	2	10	PPF 1	28*, 33, 38*, 43, 48
Hybrid	M	9	150	Routine Examination	4	20	/	43, 48, 50, 58, 63, 68, 73, 78, 83, 88, 94, 99
Siberian	F	9	115	Routine Examination	2.5	25	/	74, 79, 84, 89, 94, 99, 104
Hybrid	M	5	135	Routine Examination	2.4	12	PPF 0.4	33, 38, 50, 65, 75, 80
Hybrid	M	5	150	Routine Examination	2	10	BTF 0.05 g	21, 26, 31, 36, 41, 46
Bengal	M	17	150	Computed Tomography	2	10	PPF 1 ISO to effect	17, 34, 39, 44*, 54, 63, 69, 74, 79, 84, 95
Siberian	F	3	120	Routine Examination	3	15	/	24, 29, 34, 44, 49, 54, 59
Siberian	F	18	100	Computed Tomography	2	10	PPF 0.5	24, 32, 41, 50, 60, 72, 82, 92

SP = Standard Protocol; NSP = Non Standard Protocol;
F = Female; M = Male;
KET = Ketamine; DEX = Dexmedetomidine; BTF = Butorphanol; PPF = Propofol; ISO = Isoflurane;
* = blood sufficient only for DEX quantification; ** = blood sufficient only for KET and NORKET quantification

For drugs quantification, DEX was extracted from tiger serum according to an intra-laboratory validated HPLC-MS method (Cagnardi et al., 2017), while KET and NORKET were extracted from tiger plasma according to a validated HPLC-UV method (Zonca et al., 2012). Both methods were employed in the present study with slight modifications and were subject to intra-laboratory validation in compliance with the recommendations defined by the European Community (Commission decision 2002/657/EC) and with the international guidelines (EMA, 2011 - VICH GL49). Pharmacokinetic parameters were determined from serum/plasma concentration – time data using the Phoenix WinNonLin 8.0 software (Pharsight Corporation, USA), which allows compartmental and noncompartmental analyses of the experimental data. Visual inspection of the curve, residual analysis and minimum Akaike's information criterion estimates (MAICE; Yamaoka et al., 1978) were used to choose the model best fitting the data. All data points were weighted by the inverse square of the fitted value. The dispositions of DEX, KET and NORKET following remote IM administration in tigers were described by standard noncompartmental analysis (NCA). Pharmacokinetic parameters were reported as mean and standard deviation. A normality test (Shapiro-Wilk test) was performed on pharmacokinetic parameters. The principal kinetic parameters obtained after DEX, KET and NORKET analyses, together with the KET metabolization rate (expressed as ratio between NORKET and KET Area Under the Curves, AUC) were compared using unpaired *t* test or Mann-Whitney U test for normal and non-normal data, respectively (SPSS 25.0, IBM SPSS Statistics). Differences with $P < 0.05$ were considered significant.

This is the first study that evaluates the pharmacokinetic of DEX, KET and NORKET in tigers, thus it is difficult to compare this data with other results for the same compounds or for other compounds of the same pharmacological classes, since the lack of data for this species.

Due to the harmful attitude of these animals, samples collection was limited to the period of sedation, which lasted variably among the animals, this limitation in sampling is typical for this species, as also observed in another study (Lewis et al., 2014).

During the entire observation period, no adverse effects were recorded. All inductions by IM remote drug delivery were smooth, the level of sedation was satisfactory for the respective procedures and all animals recovered well from anaesthesia.

Table 2 reports the results of pharmacokinetic parameters for DEX, KET and NORKET, respectively in serum and plasma of the eighteen captive tigers.

Table 2 - Mean ± s.d. of noncompartmental parameters from serum and plasma concentrations of DEX, KET and NORKET in 18 captive tigers following IM administration of different DEX and KET dosages in standard protocol group and non standard protocol group

PK Parameters	Units	Standard Protocol (n =9)	Non-Standard protocol (n=9mean ± s.d.)
Dexmedetomidine			
$t_{1/2\lambda z}$	min	52.49 ± 40.60	39.41 ± 20.59
T_{max}	min	21.22 ± 3.87*	34.75 ± 18.16*
C_{max}	ng/mL	6.18 ± 2.01	6.33 ± 2.00
$AUC_{0\text{-}last}$	min*ng/mL	208.53 ± 81.41	290.88 ± 98.90
$AUMC_{0\text{-}last}$	min*min*ng/mL	6563.46 ± 2805.14*	14120.41 ± 7074.39*
$MRT_{0\text{-}last}$	min	31.12 ± 5.18*	46.76 ± 16.64*
Ketamine			
$t_{1/2\lambda z}$	min	77.62 ± 54.50	77.96 ± 71.73
T_{max}	min	27.78 ± 7.90	45.89 ± 28.72
C_{max}	µg/mL	0.63 ± 0.17	0.69 ± 0.17
$AUC_{0\text{-}last}$	min*µg/mL	23.84 ±6.40	36.63 ± 13.44
$AUMC_{0\text{-}last}$	min*min*µg/mL	802.24 ± 331.03	1981.38 ± 1052.12
$MRT_{0\text{-}last}$	min	32.88 ± 5.71*	50.35 ± 15.95*
Norketamine			
T_{max}	min	51.89 ± 8.95*	73.22 ± 22.39*
C_{max}	µg/mL	0.24 ± 0.07	0.24 ± 0.09
$AUC_{0\text{-}last}$	min*µg/mL	7.30 ± 3.98	11.51 ± 5.60
$AUMC_{0\text{-}last}$	min*min*µg/mL	291.94 ± 227.01	706.81 ± 450.27
$MRT_{0\text{-}last}$	min	36.95 ± 7.32*	54.96 ± 16.68*

$t_{1/2\lambda z}$ = Elimination Half-Life; Tmax = Time to Maximum concentration; Cmax = Maximum Concentration; $AUC_{0\text{-}last}$ = Area Under the Curve from 0 to the last concentration; $AUMC_{0\text{-}last}$ = Area under the first Moment Curve from 0 to the last concentration ; $MRT_{0\text{-}last}$ = Mean Residence Time from 0 to the last concentration
*P<0.05

Assuming that the main goal in zoo large felids medicine is to reach a satisfying level of sedation or anaesthesia to perform the required procedure, it is normal to use sedative protocols adapted to the single animal. Moreover, being a necessity (routine or extraordinary) to sedate these animals in order to carry out a complete clinical examination or collateral exams (e.g. diagnostic procedures), the possible influence on drugs disposition induced by protocols variations must be indagated during pharmacokinetic parameters interpretation, as a source of possible variability.

Given the above, it is important to explore the data not only in terms of statistical significance, but also in terms of interindividual and interprotocol pharmacokinetic variations.

In NSP group the dose of DEX administered to the animals was, in most cases, higher than in the SP group, because additional DEX was administered later. Statistically significant differences between groups in T_{max}, $AUMC_{0\text{-last}}$ and $MRT_{0\text{-last}}$ were observed. Surprisingly, C_{max} and $AUC_{0\text{-last}}$ did not show differences between groups. Despite this, especially for $AUC_{0\text{-last}}$, the standard deviation showed a high interindividual variability. Ketamine showed a statistically significant difference between groups only for $MRT_{0\text{-last}}$. This result, as for DEX, was expected but, surprisingly, none of the other pharmacokinetic parameters differed between groups. Actually, observing the high standard deviations in group NSP for mean T_{max}, $AUC_{0\text{-last}}$ and $AUMC_{0\text{-last}}$, it is not possible to exclude that the wide data distribution had hindered the possibility in detecting the differences. Moreover, it is possible that with the simultaneous administration of the two drugs, DEX might have influenced at least T_{max} values, due to a peripheral effect of vasoconstriction, as also reported by other authors (Pypendop et al., 2017; Kallio-Kujala et al., 2018).

Norketamine concentrations were increasing for all observation period, as the metabolite production lasted longer than our rather short observation period. In both groups, main pharmacokinetic parameters were estimated (T_{max}, C_{max}, $AUC_{0\text{-last}}$, $AUMC_{0\text{-last}}$ and $MRT_{0\text{-last}}$ except for elimination half-life ($t_{1/2\,\lambda z}$)), maybe due to the limited sampling time that did not allow to have samples during the elimination phase of the drugs. In particular, statistically significant differences between group SP and group NSP were reported for T_{max} and $MRT_{0\text{-last}}$, with higher means in the latter group, which is not surprising since KET was additionally administered in this group to reach the desired level of sedation. Anyway, the ratio between NORKET and KET $AUC_{0\text{-last}}$ (i.e. the KET metabolization rate), was 0.30 ± 0.09 and 0.30 ± 0.08 for group SP and group NSP, respectively and did not show any significant difference between protocols, this result indicates that all animals from both groups were able to metabolise KET at the same rate.

However, regarding all drugs a higher inter-individual variability was observed in the NSP group, this was not unexpected considering the differences in the protocols and the possible influences of other drugs administered on DEX, KET and NORKET pharmacokinetics.

In conclusion, despite the limited period of blood sampling, quite short for a complete pharmacokinetic evaluation, a favourable kinetic profile of DEX, KET and NORKET in tigers were observed. Moreover, the results showed that the concurrent administration of other drugs seems not to affect the disposition of DEX and KET in this species. Finally, from a clinical point of view, all animals showed no adverse effects and a satisfactory level of sedation, suitable for the respective procedures, with smooth inductions and good recoveries from anaesthesia.

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POPULATION PHARMACOKINETIC STUDY OF CEFAZOLIN USED PROPHYLACTICALLY IN CANINE SURGERY FOR SUSCEPTIBILITY TESTING BREAKPOINT DETERMINATION

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POPULATION PHARMACOKINETIC STUDY OF CEFAZOLIN USED PROPHYLACTICALLY IN CANINE SURGERY FOR SUSCEPTIBILITY TESTING BREAKPOINT DETERMINATION

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Conflict of Interest Statement
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Abstract

This study aimed to determine the population pharmacokinetic (Pop PK) parameters of cefazolin administered prophylactically at 25 mg/kg intravenously (IV) 30 min before surgery in a canine population of 78 dogs and assess whether covariates, such as sex, age, body weight, breed, health status, creatinine level and surgery time, have an influence on cefazolin disposition. The ultimate goal was to compute PK/PD cut off values and subsequently establish a specific clinical breakpoint for the development of an antimicrobial susceptibility test of cefazolin in dogs according to the VetCAST approach. Two to 11 blood samples were collected from each dog from 5 to 480 min after cefazolin administration. A two-compartment model was selected, and parameterization was in terms of serum clearance (CL), intercompartmental clearance(s) (Q) and volume(s) of distribution (V). The percentage of cefazolin binding to serum protein was 36.2 ± 5.3%. Population primary parameter estimates V1, V2, CL and Q were (typical value ± SE) 0.116 ± 0.013 L/kg, 0.177 ± 0.011 L/kg, 0.0037 ± 0.0002 L/kg/min and 0.0103 ± 0.0013 L/kg/min, respectively. Cefazolin presented rapid distribution and elimination half-lives (mean ± SE) 4.17 ± 0.77 min and 57.93 ± 3.11 min, respectively. The overall between-subject variability for estimated primary parameters ranged from 36 to 42%, and none of the seven explored covariates were able to reduce this variability by an amplitude clinically relevant. By Monte Carlo simulation, the probability of a PK/PD target attainment (here to achieve a free serum concentration exceeding the MIC for 50% of the dosing interval in 90% of dogs) was computed with a dosage of 25 mg/kg administered IV every 6 h for 4 administrations in 24 h. The computed PK/PD cut off value was 2 mg/L. In conclusion, cefazolin administered prophylactically in surgical dogs at 25 mg/kg IV every 6 h was deemed effective against pathogens with a MIC value ≤ 2 mg/L and from a PK/PD perspective, can be

recommended in a wide range of canine patient populations with no necessary dose adjustment for special dog subpopulations.

Keywords: Cefazolin, dog, prophylactic administration, surgery, population pharmacokinetics, PK/PD cut off value

Introduction

In animals as well as in human medicine, surgical site infection (SSI) represents a dangerous complication that can easily lead to an extension of hospital stay and an increase in medical costs (Whittem et al., 1999; Hauser et al., 2006; Xu et al., 2015). The perioperative administration of antimicrobial drugs (AMDs) can decrease the incidence of SSIs (Brown et al., 1997; Prospero et al., 2011; Xu et al., 2015); however, to avoid serious consequences, such as the risk of hospital-acquired infection and the selection of antimicrobial-resistant bacterial strains, administration has to be carried out carefully and appropriately (Song et al., 1998; Knights et al., 2012). In veterinary surgery, depending on the surgery site, pathogens, such as *Staphylococcus* spp, *Streptococcus* spp, *Enterobacteriaceae* and *Pasteurella*, are commonly encountered (Boothe and Boothe, 2015). Cefazolin is a first-generation cephalosporin with good activity against gram-positive cocci (*Staphylococcus* spp, including beta-lactamase-producing strains, and *Streptococcus* spp), many *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp., *Proteus mirabilis*), *Pasteurella* spp and anaerobes (Papich and Riviere, 2009). Thus, cefazolin has become one of the most commonly AMD used perioperatively, and it has been recommended as the ideal prophylactic AMD for dogs undergoing surgery based on its spectrum, low toxicity and cost (Rosin et al., 1993; Marcellin-Little et al., 1996; Gonzalez et al., 2017). Cephalosporins like all beta-lactams have time-dependent killing activity; therefore, antibiotic plasma concentrations should be kept above the minimum inhibitory concentration (MIC) as long as possible during therapy. This index is defined as T > MIC, and it has been established that a T > MIC of 40-50% of the dosing interval is adequate for a successful outcome. More precisely, as only unbound drug concentration is available for antimicrobial activity, the index should be measured as the duration of free plasma concentration exceeding the MIC and is reported as *f*T > MIC (Turnidge, 1998; Toutain et al., 2002; Papich, 2014). Recently for cefazolin, a breakpoint of efficacy against bacteria isolated from animals was set at ≤ 2 mg/L (Papich, 2014; CLSI 2015). Cefazolin pharmacokinetics (PK) has been extensively studied in dogs with a classical approach (Richardson et al., 1992; Rosin et al., 1993; Petersen and Rosin, 1995; Marcellin-Little et al., 1996; Singh et al., 1998; Harika et al., 2001; Gonzalez et al., 2017). Following this, it has been recommended that time-dependent AMD should be readministered every 2 half-lives during surgery to maintain targeted plasma concentrations (Marcellin-Little et al., 1996; Plumb 2011; Gonzalez et al., 2017).

Population pharmacokinetics (Pop PK) is widely applied to define the sources of PK variability in target patient populations, thus identifying and assessing demographic, pathophysiological, environmental and drug-related factors that can influence drug disposition (Ette and Williams, 2004; Kiang et al., 2012). Population modeling is also used in clinical trials, where the participants are representative of the real treated population, in contrast to healthy subjects or highly selected patients in traditional PK studies (Riviere, 2009; Bon et al. 2017). Moreover, through a Pop PK approach with a sufficient knowledge of covariates, it is possible to predict a typical PK profile for any given patient, allowing the definition of the correctness of the treatment (Concordet et al., 2004). In veterinary medicine, over the last decades, many Pop PK studies regarding AMDs have been performed in dogs (Regnier et al., 2003; Zhao et al. 2012; Prados et al., 2014; Hnot et al. 2015; Papich, 2017), but no study has been carried out with cefazolin. Starting from these assumptions, the aim of the study was to determine the Pop PK profile of cefazolin administered prophylactically at 25 mg/kg by intravenou (IV) bolus 30 min before surgery in a representative canine population to identify whether covariates such as sex, age, body weight, breed, health status, creatinine level and surgery time, have an influence on cefazolin disposition. Furthermore, the ultimate goal was to compute PK/PD cut off values to establish a specific clinical breakpoint (CBP) for the development of an antimicrobial susceptibility test (AST) of cefazolin in dogs, according to the VetCAST approach (Toutain et al., 2017).

Material and methods

Animals

With ethical approval (Organismo Preposto al Benessere Animale, OPBA_23_2016) and after obtaining the owners' written consent, 78 client-owned dogs were enrolled for the study. Dogs were of different breeds, sexes, ages, body weights and healthy or presenting concomitant diseases, all scheduled at the University Veterinary Hospital of Milan for any type of surgical procedure. In all subjects, type of surgery, duration of the procedure, anesthetic and analgesic perioperative protocol and medical history were recorded. For each dog, the blood count and biochemical profile were evaluated.

Sample collection and analysis

Before drugs administration, 1 mL of venous blood was collected through an angio-venous catheter (Surflo®, 20 G, 32 mm IV catheter Terumo, Vetefarma Srl, Cuneo, Italy) previously placed into a peripheral vein. For surgery, each dog was premedicated, generally with sedatives (i.e., α2-agonists) and opioids (i.e., methadone) and put under general anesthesia, induced by propofol and maintained with isoflurane in 100% oxygen. Thirty min after the surgical procedures, meloxicam (0.2 mg/kg, Metacam, Boerhinger Italia, Milan, Italy) was administered subcutaneously as an analgesic drug. Cefazolin (Cefazolina Teva, Teva Italia S.r.l., Milan, Italy) was administered via IV bolus to all dogs at 25 mg/kg 30 min before surgery. For cefazolin quantification, venous blood samples (1 mL each) were collected from each subject at prefixed times (5, 10,

15, 30, 45, 60, 90, 120, 150, 180, 240, 360, 480 min). Then, all samples were centrifuged (4000 *g*, 10 min) to obtain serum and frozen at -20°C pending analysis.

Cefazolin was extracted and quantified by HPLC-UV from canine serum samples according to the published method by Kunicki and Was (2012) with slight modifications (see supplementary file S1 for details). The analytical method was validated in our hands in compliance with the recommendations defined by the European Community (Commission decision 2002/657/EC) and with the international guidelines (EMA, 2011 - VICH GL49). The calibration curves were prepared with 6 spiked solutions obtained diluting the original stock solution of cefazolin (1 mg/mL) in canine blank serum to achieve concentrations ranging from 0.2 to 20 μg/mL. The correlation coefficients (r) resulted > 0.99 for 2 replicates. The precision (repeatability) and accuracy were determined by analyzing blank samples (n = 6 for each concentration) that were spiked with 0.2, 2 or 20 μg/mL of cefazolin. The results fell within the accepted ranges for precision (6.75%, 13.9% and 13.5% for 0.2 μg/mL, 2 μg/mL and 20 μg/mL, respectively) and accuracy (2.5%, -3.42% and 2.66% for 0.2 μg/mL, 2 μg/mL and 20 μg/mL, respectively). A LOQ value of 0.2 μg/mL was set. The LOD was 0.00024 μg/mL. The specificity of the method was demonstrated by the absence of interference in 20 blank serum samples at the cefazolin retention time. In the case of samples above the upper limit of quantification (20 μg/mL), these samples were quantified upon dilution.

The percentage of cefazolin serum protein binding was determined *in vitro* with ultrafiltration units (Amicon Ultrafree MC-0.5 mL, Centrifugal Filter Unit 30 K, Merck Millipore, Milan, Italy) according to Villa et al. (1997). Spiked blood samples with cefazolin concentrations from 5 to 100 μg/mL were incubated for 30 min at 37°C before ultrafiltration. Subsequently, the sera were centrifuged (5000 *g*, 20 min) with 30000 Nominal Molecular Weight Limit (NMWL) cut-off ultrafiltration units and injected into a HPLC system. The binding percentage was calculated by serum (unbinding drug) and water (total cefazolin) peak area ratio.

Population pharmacokinetics and Monte Carlo simulation

Pharmacokinetic modeling was carried out using commercially available software (Phoenix NLME version 7.0, Certara, St. Louis MO, United States). A nonlinear mixed effects (NLME) approach was used to generate Pop PK parameter estimates. Two- and three-compartment models were evaluated to identify the model that best described the dataset. After visual inspection of plots for all dogs showing a polyphasic decay of plasma concentration vs. time, data were fitted with a two or three-compartment models. The two concurrent models were then compared using the likelihood ratio test (LRT) that is appropriate when models are nested (one model is a subset of another) and have different numbers of parameters. The critical value of the $\chi 2$ distribution to consider was obtained using Excel (Microsoft Office 2016) to estimate the risk of type one. Finally, a two-compartmental model was selected. Parameterization was in terms of serum clearance (CL),

intercompartmental clearance(s) (Q) and volume(s) of distribution (V) with V1, V2, CL and Q being the primary estimated parameters. The following parameters were computed as secondary parameters.

The terminal slope *Beta* was obtained with Eq: 1;

$$Beta = 0.5 \times \left[\frac{Q}{V1} + \frac{Q}{V2} + \frac{CL}{V1} - \left[\left(\frac{Q}{V1} + \frac{Q}{V2} + \frac{CL}{V1}\right)^2 - 4\frac{Q}{V2} \times \frac{CL}{V1}\right]^{0.5}\right] \quad \text{Eq: 1}$$

The initial slope of distribution, *Alpha*, was obtained with Eq: 2;

$$Alpha = \frac{Q}{V2} \times \frac{CL}{V1} / Beta \quad \text{Eq: 2}$$

and the elimination (HL_{Beta}) and distribution (HL_{Alpha}) half-lives were obtained with classical equations. The area under the curve (AUC), the steady-state volume of distribution (V_{ss}) and the volume of distribution associated with the terminal phase (V_z) were also computed as secondary parameters with classical equations.

The between-subject variability (BSV) was modeled using an exponential model, and hence the clearance for the i^{th} subject was written as:

$$Cl_i = \theta_{median} \times Exp(\eta_i) \quad \text{Eq: 3}$$

where Cl_i is the clearance in the i^{th} animal, θ_{median} is the population median clearance (typical value of clearance) and η_i the deviation (noted ETA) associated with the i^{th} animal from the corresponding θ_{median} population value. Others individual parameters (i.e., V1, V2, and Q) were modeled using equations of the same form. The distribution of the ETAs was assumed to be normal with a mean of 0 and a variance (ω_x^2). In addition, the individual parameters and consequently their corresponding ETAs can be correlated. All these correlations were estimated and the corresponding covariances were stored in the variance-covariance omega (Ω) matrix. The following equation 4 was used to convert the variance ($\omega^2_{clearance}$) of the log-transformed clearances into a coefficient of variation (CV %) in the original scale:

$$\text{CV}_{\text{clearance}}(\%) = 100 \times \sqrt{\exp(\omega^2_{clearance}) - 1} \quad \text{Eq: 4.}$$

The shrinkage of random effects toward the means was calculated for the ETAs (Karlsson and Savic, 2007) with equation 5:

$$shrinkage = 1 - \frac{SD(EBE_\eta)}{\omega} \quad \text{Eq: 5}$$

where ω is the estimated variability for the population and SD is the standard deviation of the individual values of the empirical Bayesian estimates (EBE) of η.

The residual model was an additive plus a multiplicative (proportional) model of the form:

$$\mathrm{C(t)} = f(\theta, Time) \times (1 + \varepsilon_1) + \varepsilon_2 \qquad \text{Eq: 6}$$

with ε1 and ε2, the multiplicative and additive error terms having a mean of 0 and a variance noted σ1 or σ2, respectively. In Phoenix, when this error model is used, the additive sigma is reported as its standard deviation, noted stdev, with the same units as serum concentration (µg/mL) and the multiplicative sigma is called multStdev and the 100* multStdev is the corresponding coefficient of variation.

Parameter estimation was based on minimizing an objective function value (OFV), using maximum likelihood estimation (i.e., minus twice the log of the likelihood) given for each model. The first order conditional estimation extended least squares (FOCE ELS) engine was used for analyses approximating the marginal likelihood while searching for the maximum likelihood. There was no censored data. A bootstrap approach was used to estimate typical values of parameters and precision of estimates that are reported as SE, CV % and by their 95% confidence intervals. To evaluate the overall performance of the final model, a visual predictive check was plotted to compare actual observations with simulated replicates from the model (500 replicates per investigated dogs). The 90% prediction intervals were constructed and plotted together with the observed data allowing for a visual assessment of the agreement between simulation and observation. Diagnostic plots, the distribution of errors, and the precision of the parameter estimates were used as tools to evaluate the goodness of fit and to compare models.

The likelihood ratio test was used to examine different models for testing the residual variability and the covariate effect on each PK parameter. An analysis of each covariate in all PK parameter was carried out to evaluate the significance on the model. The categorical covariates considered were the health status with two levels (healthy, diseased), the sex with three levels (male, female and female neutered) and the breed with two levels (mongrel and other breeds) (Eq: 7):

$$Param = \theta_{median} \times (1 + \theta_1 X_1) \qquad \text{Eq: 7}$$

where *Param* is one of the structural parameter of the disposition model (V1, V2, CL, Q), X_1 is an indicator variable with a value of 0 for control condition (the healthy condition for the health status, mongrel dog for breed), and of 1 for the non-mongrel breed and disease status. For example for V1, the model was given either by Eq 8 for the healthy condition, or Eq 9 for the diseased condition:

$$V1 = \theta_{V1median} \times EXP(\eta V1) \quad \text{Eq: 8}$$

$$V1 = \theta_{V1median} \times (1 + \theta_1) \times EXP(\eta V1) \qquad \text{Eq: 9}$$

where $\theta_{\mathrm{V1median}}$ is the typical value of V1, ηV1 is the ETAs associated with V1 and θ_1, the fixed effect of the covariate for the diseased condition. If θ_1 is significantly different from 0, it provides evidence that a difference exists between the healthy status and the disease condition for V1.

Age, body weight (BW), creatinine level and surgery time were considered as continuous covariates and their influence were modeled using a classical regression equation with a power model and an appropriate scaling factor for each covariate; for example serum clearance was modeled with the following general equation 10:

$$CL = \theta_1 \times \left[\frac{Age}{8}\right]^{\theta_2} \times \left[\frac{BW}{20}\right]^{\theta_3} \times \left[\frac{CREAT}{0.9}\right]^{\theta_4} \times \left[\frac{Surgery\_time}{80}\right]^{\theta_4} \text{ Eq: 10}$$

where θ_1 is the typical value of clearance for a 8 years old dog, weighting 20 kg, having a creatinine level of 0.9 mg/dL and for a surgery time of 80 min. The stepwise covariate search mode was used to define the statistically significant covariates for each of the structural parameters of the model. This run mode performs a stepwise forward or backward addition or deletion of covariates effects (by adding one at a time) to determine the improvement of the final model based on the Bayesian information criterion (BIC). For the present analysis, we selected a BIC value of 6.635 for adding a covariate and a value of 10.823 for deleting a covariate, as these values are equivalent of P<0.01 and P<0.001 for the minus twice the log-likelihood (2-LL) criterion when using the LRT test (see supplementary file S2 for further details).

Using the previously developed Pop PK model and estimated parameters, 2500 curves of the cefazolin disposition were generated by the Monte Carlo simulation. The simulated dosage regimen was of 25 mg/kg IV at 6 h intervals over 24 h (i.e., four administrations of cefazolin at 0, 360, 720 and 1080 min). The 2500 curves were analyzed with the non-compartmental tool of Phoenix. The duration for which free plasma concentrations were above the selected MICs (from 0.25 to 8 mg/L) was computed using the statistical tool of Phoenix. The quantiles 90 and 95% of the distributions of these different times above MIC were computed to give the corresponding probability of target attainment (PTA) of the selected index (*f*T>MIC for 50% of the dosing interval). The PTAs were computed without their confidence intervals.

Results

Animals and cefazolin concentrations

Seventy-eight dogs were enrolled in the study; animal characteristics are reported in Table 1 together with the covariates and coding used for Pop PK modeling. Most dogs had some conditions and were undergoing different types of surgery from oncologic to ophthalmic; only 19 dogs were healthy and undergoing gynecological or andrological surgery. The dogs of the study represented many different breeds (n = 26), from Jack Russell Terriers to Pyrenean Sheepdog; among these, 12 breeds were represented by more than 1 dog and 14 were represented by only one dog.

Table 1 *- Animal characteristics, covariates and coding used in Pop PK analysis*

Continuous covariates			
	Mean ± S.D.	**Range (median)**	
Age (y)	7.22 ± 4.11	0.66-14 (8)	
Body weight (kg)	26.13 ± 0.88	4.5-56 (27)	
Creatinine level (mg/dL)	0.91 ± 0.32	0.3-1.88 (0.9)	
Surgery Time (min)	87.63 ± 58.09	20-260 (80)	
Categorical covariates			
	Type and number of subjects (Code)		
Health status	Healthy n = 19 (Code 0)	Diseased n = 59 (Code 1)	
Breed	Mongrel n = 27 (Code 0)	Other breeds n = 51 (Code 1)	
Sex	Male n = 32 (Code 0)	Female n = 23 (Code 1)	Female neutered n = 23 (Code 2)

Two to 11 (median 9) blood samples were collected from each dog from 5 to 480 min for 14 different sampling times for a total of 629 collected samples. Cefazolin serum concentrations obtained in all animals are reported in Figure 1 and a biexponential decay over time is shown. The percentage of cefazolin binding to serum protein, calculated with the ultrafiltration method, resulted in 36.2 ± 5.3%.

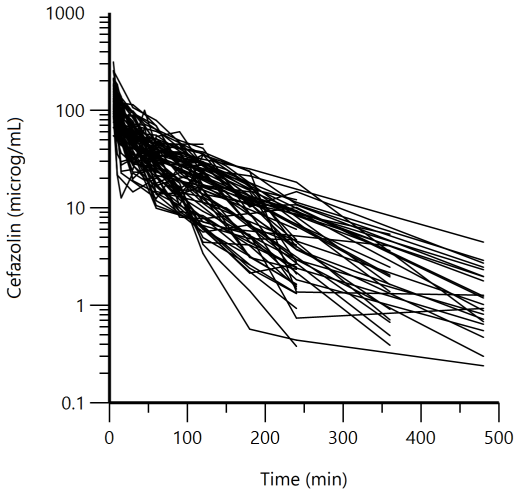

Figure 1 *- Semi-logarithmic spaghetti plots of the disposition curves of cefazolin over 480 min after a single IV bolus administration (25 mg/kg) in 78 dogs.*

Population pharmacokinetics and Monte Carlo simulation

The two-compartment model was adequate to describe cefazolin disposition in our dogs, as shown in the visual predictive check plot (Figure 2) and in the plots of the observed cefazolin concentration versus population predicted concentration (PRED) or versus individual predicted concentration (IPRED) (Figure 3 A-B-C-D).

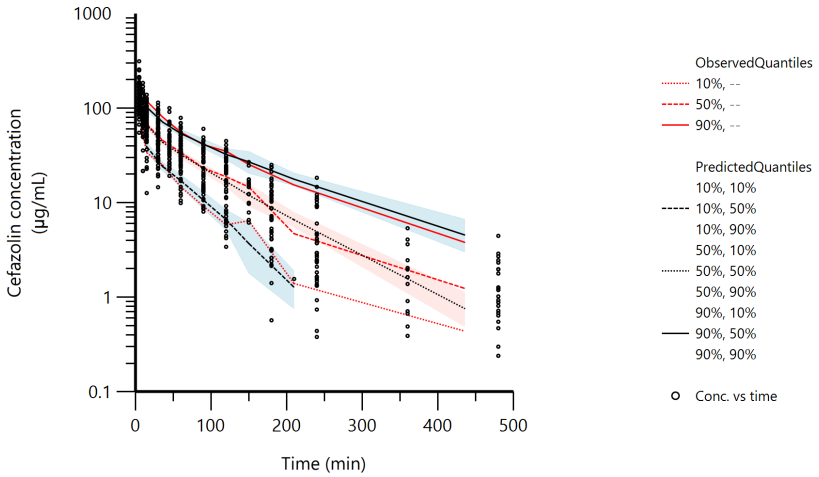

Figure 2 *- Visual Predictive Check (VPC) plot obtained with 500 replicates of each animal (314500 simulated data). Red lines: observed quantiles; Black lines: predicted quantiles; Black circles: observed data. The shaded areas represent the 90% confidence intervals around the 10th, 50th, and 90th percentiles of the simulated data.*

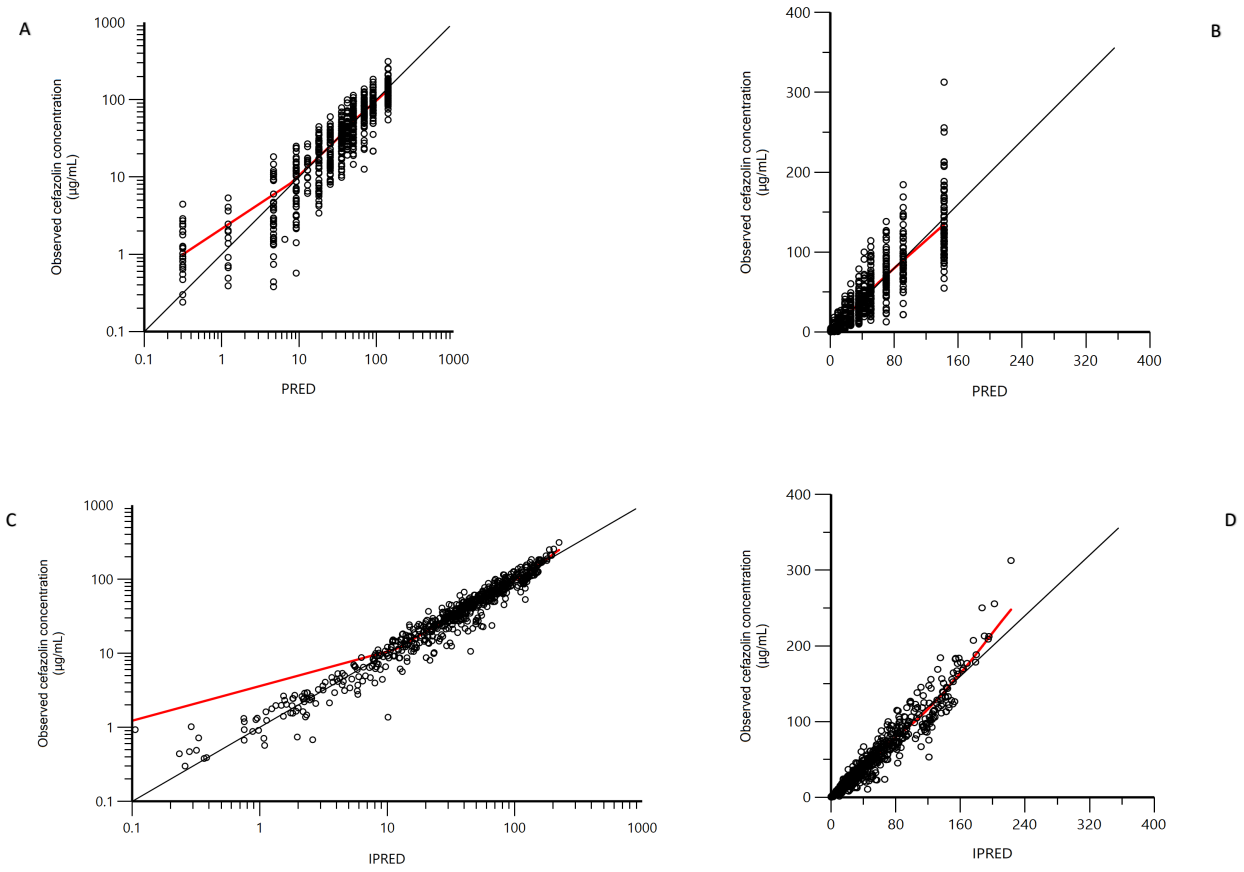

***Figure 3**: Plots of the observed cefazolin concentration (µg/mL) versus population predicted concentration (PRED; µg/mL - **A** in logarithmic scale: **B** in arithmetic scale) and versus individual predicted concentration (IPRED; µg/mL - **C** in logarithmic scale; **D** in arithmetic scale.*

The model adequacy was further supported by the inspection of different goodness-of-fit plots (see supplementary Figures S3-S6).

Typical values of the primary structural parameters of the model, secondary parameters, their associated standard error (SE) and the standard deviation (SD) of the residual for the basic model are given in Table 2.

Table 2 *- Population primary and secondary parameters of cefazolin in dogs obtained with a two-compartment model (bootstrap estimates of mean, median, SE, CV%, 2.5% and 97.5% percentiles)*

Population primary parameters							
Parameters	**Units**	**Mean**	**SE**	**CV%**	**Median**	**2.50%**	**97.50%**
tvV1	L/kg	0.116	0.013	11.36	0.115	0.084	0.137
tvV2	L/kg	0.177	0.011	6.01	0.176	0.158	0.194
tvCL	L/kg/min	0.0037	0.0002	4.26	0.0036	0.0034	0.0040
tvQ	L/kg/min	0.0103	0.0013	12.82	0.0105	0.0073	0.0123
tvCMultStdev		0.257	0.016	6.08	0.256	0.226	0.285
stdev (sigma)	µg/mL	0.564	0.166	29.42	0.543	0.314	0.943
Secondary parameters							
AUC	µg*min/mL	6790	286	4.21	6810	6189	7391
Beta	1/min	0.0111	0.0006	5.29	0.0111	0.0100	0.0122
Beta half-life	min	57.93	3.11	5.38	57.72	52.45	63.84
Alpha	1/min	0.172	0.031	18.21	0.171	0.115	0.241
Alpha half-life	min	4.17	0.77	18.45	4.06	2.88	6.04
Vss	L/kg	0.292	0.013	4.33	0.293	0.266	0.316
MRT	min	79.34	4.25	5.36	79.25	71.74	86.12
Vz	L/kg	0.334	0.016	4.71	0.335	0.305	0.365

Typical values (tv); V1: volume of the central compartment; V2: volume of the peripheral compartment; CL: serum clearance; Q: intercompartmental clearance; CmultStdev: multiplicative component of the residual that can be read as a coefficient of variation of 25.7%; stdev: standard deviation of the additive component of the residual; AUC: area under the curve; Beta: slope of the terminal phase; Beta half-life: half-life of elimination; Alpha: slope of the distribution phase; Alpha half-life: half-life of distribution; MRT: Mean residence time; Vz: volume of distribution associated with the terminal phase. SE: standard error of estimate.

For BSV, an exponential model was selected, because the estimated thetas parameters must be positive and their distribution is generally right-skewed. The estimates of the random effects variance-covariance matrix, correlation matrix and shrinkage are reported in Table 3.

Table 3 *- Estimates of the random effects variance-covariance matrix, correlation matrix and shrinkage. Variance (diagonal) is in bold.*

Omega				
nV1	**0.092598**			
nV2	0.099641	**0.135063**		
nCL	0.031062	0.073881	**0.130511**	
nQ	0.00518	0.062008	0.068201	**0.196614**
Correlations between ETAs and Shrinkage				
nV1	**1**			
nV2	0.89	**1**		
nCL	0.28	0.56	**1**	
nQ	0.04	0.38	0.43	**1**
Shrinkage	0.15	0.11	0.05	0.34

nV1, nV2, nCL and nQ are the random component of the model (ETA). Corresponding BSV expressed as a CV % are 31.15, 38.03, 37.34 and 46.61% for nV1, nV2, nCl and nQ, respectively (see equation 4).

The full variance-covariance omega (Ω) matrix was selected, because in a mixed effect model, inclusion of covariance terms prevented the risk of biased estimation of the variance terms (the diagonal). It also allowed for checking possible correlations between the ETAs that would suggest some over parameterization of the model (statistical collinearity). Inspection of Table 3 showed no spurious correlation between ETA and low values for ETA shrinkage indicating that data were rich enough to properly estimate the random component of the model.

The bootstrap estimate of the BSV for the 4 primary parameters and precision of their estimate (expressed as CV %) were also calculated and are reported in Table 4.

Table 4 *- The bootstrap estimates of the Between Subject Variability (BSV) for the 4 primary parameters and precision of their estimate (expressed as CV%)*

	BSV (%)	**Precision (CV%)**
nV1	31.42	24.00
nV2	42.70	22.34
nCL	36.83	12.80
nQ	46.12	26.48
nV1, nV2, nCL and nQ are the random component of the model (ETA). Corresponding BSV is expressed as a CV%, as well as precision of the estimate.		

Inspection of Table 4 indicates that bootstrap estimates of the BSV were consistent with those obtained by a single run of all the data set (Table 3) and that the precision of the BSV estimates were robust (low CV%). The BSV for CL (the parameter controlling the overall cefazolin exposure) was rather high (CV = 36.83%) and prompted us to explore the influence of different clinically relevant covariates to explain the variability in the observed, large clinical population.

The significant influence of each covariate (sex, age, breed, BW, health status, creatinine level, surgery time) on the model was explored with a stepwise covariate search mode. The diseased condition was defined based on clinical exam, anamnesis and haematological and biochemical blood tests. As cefazolin is mainly excreted by the kidney (approximately 80%, Nisida et al., 1970) the creatinine level was used as a covariate to assess individual kidney conditions. Continuous covariates were scaled to avoid instability of the optimization process and to provide parameter estimates that were more reflective of the average subject; the scaling values were BW = 20 kg, age = 8 years, creatinine level = 0.9 mg/dL and surgery time = 80 min. The scatter plot matrix for the continuous covariates (age, BW, creatinine level and surgery time) is reported in supplementary Figure S7. The visual inspection of the figure shows no obvious relationship, but there was a trend between creatinine level and age and BW. The scatter plot matrices for the continuous covariates (age, BW, creatinine level and surgery time) *per* health status level (0 = healthy, 1 = disease) are reported in

supplementary Figure S8A and B. Moreover, in this case, the visual inspection of the figure shows no obvious relationship.

The Phoenix stepwise search exploratory tool returned 27 combinations (scenarios) of covariates, ensuring a statistically significant (P<0.01) reduction of the BIC criterion. The most significant scenarios were those related to V1 and for 16 scenarios that included two covariates, the BW was selected as the covariate. Among these scenarios, the most relevant to perform subsequent simple runs were chosen and fitted to estimate the magnitude of the effect. Finally, to assess whether these statistically significant covariates had clinical relevance and merit for future recommendation or warning, the influence of covariates on PK parameters (V1, V2, CL, Q) was explored by computing the multiplicative/dividing factor when the covariate increased or decreased by 50%.

For example for BW as a covariate, we computed the values of PK parameters for a typical dog of 20 kg BW (see equation 10). When only the BW influenced the clearance, the typical value of the fixed effect (θ_2 in equation 10) was of -0.2368. This means that for dogs of 10 and 30 kg BW, i.e. for dogs having a BW of plus or minus 50 % of the scaled typical value, the typical value of clearance was increased of time folds a factor of 1.178 for a dog of 10 kg BW and was multiplied by 0.908 in a dog of 30 kg BW (or equivalently divided by 1.10). Such a difference can be considered as not relevant from a clinical point of view (see supplementary file S2 for further details).

For age, BW, creatinine level and surgery time, the influence was not clinically relevant. For the health status, all PK parameters were influenced, but the largest effect was observed for the Q (-0.267), meaning that in diseased dogs, the intercompartmental clearance that likely reflects tissular blood flow, was decreased by 26.7% compared with that in control dogs. Other influences of disease were clinically irrelevant. For breed and sex, the magnitude of the effects was also clinically irrelevant despite their statistical significance. It has to be said that, statistical significance does not always mean clinical relevance, as especially in this trial where rich and robust data were analyzed, allowing to easily detect statistically significant differences but having no clinical impact.

By Monte Carlo simulation, 2500 curves were generated using this Pop PK model to compute the PTA corresponding to the selected possible MIC in order to propose a PK/PD cutoff, considering the average percentage of unbound drug calculated (i.e., 0.64; percentage of cefazolin binding 36 ± 0.53%). Table 5 reports the results of $fT > MIC$ for the 2500 curves simulated for a dosing regimen of 25 mg/kg at 6 h interval over 24 h.

Table 5 *- Time (min) above possible MICs ranging from 0.25 to 8 mg/L corresponding to total serum concentration ranging from 0.39 to 12.5 mg/L for the quantiles (Q) 90 and 95% and corresponding value of the T>MIC in % of 24 h.*

MIC (mg/L)	Total serum concentration (MIC/unbound fraction)	Time min (Q90%)	T>MIC (%)	Time min (Q95%)	T>MIC (%)
0.25	0.39	1345	93.4	1197	83.1
0.5	0.78	1169	81.2	1062	73.7
1	1.56	882	61.2	787	54.6
2	**3.12**	**821**	**57.0**	**735**	**51.1**
4	6.25	530	36.8	468	32.5
8	12.5	463	32.1	407	28.3

Q90%: quantile 90%; Q95%: quantile 95%; for a MIC of 2 mg/L corresponding to a total serum concentration of 3.12 mg/L, 90% of the simulated curves were equal or above a free plasma concentration for 821 min, i.e*., for 57% of the considered dosing interval.*

In Table 5 it is shown that for a MIC of 2 mg/L (i.e., a total serum concentration of 3.12 mg/L, corrected for the unbound fraction of the drug), 90% of dogs had a *f*T>MIC of 57% over the dosage interval (24 h). Thus, the PK/PD cutoff for a *f*T>MIC target of 50% of the dosage interval and a 90% quantile (or 95%) is set at 2 mg/L.

Discussion

Cefazolin is very commonly used perioperatively and has been recommended as an appropriate prophylactic antimicrobial for dogs undergoing surgery. Currently, its use is based on not recent studies with classical PK investigation that do not consider the possible large inter-animal variability encountered in clinical practice nor the most recent PK/PD paradigms allowing to support the prudent use of the AMD at a population level. Thus, by developing a Pop PK model, the aim of this study was to estimate typical PK parameters of cefazolin, their BSVs and to identify whether covariates such as sex, age, BW, breed, health status, creatinine level and surgery time, have an influence on these parameters and in turn, to explain the BSV of cefazolin disposition. Moreover, to promote a responsible use of cefazolin in dogs, the study aimed to compute a PK/PD cutoff value for the subsequent determination of a specific CBP for the development of an AST, using the VetCAST approach (Toutain et al., 2017).

A large number of dogs were enrolled in the study (n = 78) with variable characteristics reflecting the target clinical population of dogs undergoing surgery. Only 19 dogs were healthy, and among the diseased dogs, 60% were oncologic patients. Many different breeds were represented in the study together with a large number of mongrels, accounting for the wide variability encountered in clinical practice. Many blood samples were taken from each animal; thus, the large availability of data (n = 629 samples) made its analysis reliable,

especially for the estimation of the BSV that require a minimal number of samples per animal to avoid an ETA-shrinkage, i.e., the individual parameter estimates "shrink" back toward the population parameter estimate (Karlsson and Savic, 2007). In the present trial, all ETA-shrinkages were rather low giving confidence in the value of the individual EBE (individual ETA) and post hoc computations.

After cefazolin administration, a biexponential decay was observed in our samples, as also reported by other authors (Rosin et al. 1993; Marcellin-Little et al., 1995; Singh et al., 1998; Harika et al., 2001), and the two-compartment model was the most adequate to describe cefazolin disposition in dogs. The percentage of cefazolin binding to protein was 36.2 ± 5.3%. This value was in agreement with a previous study reporting 38.8 ± 2.51% from a bioassay and 35.8 ± 2.64% from isotopic methods (Daly et al., 1982).

The primary and secondary Pop PK estimates were in agreement with the results obtained with classical PK modeling reported by other authors, although all were obtained with different analytical techniques (microbiological assay vs. HPLC) or with different doses (Rosin et al., 1993; Marcellin-Little et al., 1996; Singh et al., 1998; Harika et al., 2001). For example, elimination half-life was (typical value ± SE) 57.93 ± 3.11 min in our study vs. 55.08 ± 7.92 min (mean ± SD) in the study by Rosin et al. (1993) or 52.3 min by Marcellin-Little et al. (1995) when administered via IV at 20 or 22 mg/kg, respectively. In contrast, only a population investigation allows for proper estimation of a BSV reflecting altogether the main sources of variability encountered in all-coming dogs. For example, the BSV as it can be roughly estimated from the mean and SD reported by Rosin et al. (1993) is of approximately 14.4%, while from our 78 dogs, considering the post hoc estimates of individuals ETAs and solving equation 1 to compute the terminal half-life, its BSV was estimated at 31.2%. This point should be highlighted when computing the PK/PD cut off to establish a CBP for an AST using the VetCAST approach, because AST should *a priori* cover most individuals within the targeted population, not only a limited number of experimental dogs. We were also in position to investigate the influence of the different measured covariates. This is of relevance when establishing a CBP, because the identification of a subpopulation could lead to some specific comments to assist clinical microbiologists in the routine interpretation of AST data and in suggesting the most appropriate actions to be taken in response to AST results. In addition, identification of a subpopulation could lead to some specific recommendation in terms of dosing regimen.

The influences of age, BW, creatinine level and surgery time on the exposure of cefazolin were not clinically relevant. Health status statistically influenced all primary PK parameters, with the most evident effect being on the decrease in the intercompartmental clearance in diseased dogs; nevertheless, this influence can be considered too low a magnitude to be clinically relevant. Overall, the Pop PK analysis performed showed that the 25 mg/kg dosage provides consistent cefazolin exposure in a wide range of canine patients and no adjustment of dose for special dog populations seems necessary.

Dosage recommendations for surgical prophylaxis administration of cefazolin to dogs varied from 20 to 25 mg/kg IV at beginning of surgery or 30 min before, followed by 20 mg/kg IV every 60 or 90 min until wound closure or 20 mg/kg SC at 6 h (Rosin et al. 1993, Whittem et al 1999; Plumb 2011). The practice of frequent repetition of cefazolin administration during surgery has been suggested to produce very high serum concentrations (10 x MIC; i.e., 20 mg/L) to prevent infection from skin contaminants (Marcellin-Little et al., 1996). Nevertheless, for a time-dependent AMD, like cefazolin, these very high concentrations may be unnecessary, and a standard PK/PD target is to maintain plasma concentration above the MIC_{90} of putative pathogens for a least 50% of a dosing interval (Turnidge, 1998; Toutain et al., 2002; Papich, 2014). The bacteria most commonly involved in SSIs of dogs are commensal organisms on the skin such as *Staphylococcus pseudintermedius*, a gram-positive bacterium for which a CBP of 2 mg/L has been proposed by the Clinical Laboratory Standards Institute (CLSI) for skin and soft tissue infections with a dosage regimen of 25 mg/kg administered every 6 h (CLSI, 2015). The present trial is consistent with such a CBP. By using our population model and Monte Carlo simulations, we established a PK/PD cut off of 2 mg/L for a dosage of 25 mg/kg administered every 6 h (4 administration in 24 h) and a target *f*T>MIC set at 50% of the dosing interval to be achieved in at least 90% of a representative dog population.

In conclusion, from a PK/PD perspective, the present population investigation supports cefazolin use for empirical prophylactic administration to dogs 30 min before surgery with possible readministration at 6 h interval for pathogens with a MIC ≤ 2 mg/L.

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SUPPLEMENTARY MATERIAL S1

Materials and Method

SUPPLEMENTARY MATERIAL ON CEFAZOLIN ANALYTICAL METHOD FOR EXTRACTION AND QUANTIFICATION

Two hundred µL of canine serum were transferred to a 1.5 mL standard Eppendorf tube, then mixed with 400 µL of acetonitrile to precipitate proteins and the sample was vortexed for 30 s. After centrifugation (12,000 g, 10 min), the supernatant was transferred to a 10 mL Pyrex conical glass tube and evaporated to dryness by a centrifugal evaporator at 30° C. Then the dried extract was reconstituted in 100 µL of mobile phase and 20 µL were injected into the column. The cefazolin serum quantification was performed by an HPLC system that included a binary pump, an autosampler, a Peltier column oven set at 20°C and an UV/Visible detector (Series 200, Perkin Elmer, Milan, Italy) set at 272 nm of wavelength.

The drug separation was achieved by Accucore XL column C18 (250x4.6, 4 µm, Thermo Scientific, Milan, Italy) with adequate pre-column. The mobile phase consisted of a mixture of acetonitrile (A), water (B) and potassium dihydrogen phosphate KH_2PO_4 (0.5 M) (C) (A:B:C, 110:884:6, v/v) with a flow rate of 0.8 mL/min.

The analytical standard of cefazolin sodium salt (purity grade 96.8%) was provided by Sigma Aldrich (Milan, Italy). All reagents and solvents were purchased from VWR (Milan, Italy).

SUPPLEMENTARY MATERIAL S2

Materials and Method

SUPPLEMENTARY MATERIAL ON POPULATION PHARMACOKINETIC ANALYSIS

The Phoenix Cov. Srch. Stepwise (stepwise covariate search) run mode was used to search, without a priori, what are the statistically significant covariates for each of the structural parameter of the model. This run mode performs an automatic stepwise forward or backward addition or deletion of covariates effects by adding one at a time to determine if they make a sufficient threshold improvement based on the specified criterion options. The Bayesian Information Criterion (BIC) was the criterion chosen:

$$BIC = OBJ + n_p LN(N)$$

where np is the total number of parameters in the model, and N is the number of data observations. BIC penalizes the OBJ for model complexity more than the classical AIC, and may be preferable when data are limited. Kass and Raftery (Kass & Raftery. Bayes Factors, Journal of the American Statistical Association, 1995, 90:430, 773-795), categorized differences in BIC between models of >10 as “very strong” evidence in favor of the model with the lower BIC; 6 - 10 as “strong” evidence; 2 - 6 as “positive” evidence; and 0 - 2 as “weak” evidence. For the present analysis we selected a value of 6.635 for adding a covariate and a value of 10.823

for deleting a covariate. These two threshold values are equivalent of P<0.01 and P<0.001 for the minus twice the log-likelihood (2-LL) criterion when using the LRT. The structural model is used as a baseline and the covariate model is made increasingly complex. After each model estimation, the covariates are evaluated to see which one has the greatest improvement in the goodness-of-fit statistic selected greater than the BIC specified threshold. That covariate is added to the regression model for the structural parameter and the model is estimated. This process is repeated until all significant effects are accounted for. Then the process works in reverse to eliminate covariates on parameters whose removal produces the smallest reduction in goodness-of-fit less than the specified BIC threshold.

Results

SUPPLEMENTARY MATERIAL ON POPULATION PHARMACOKINETIC ANALYSIS

To assess the influence of a quantitative covariate (BW, age, creatinine and surgery time), we computed the multiplicative/dividing factor when the covariate is increase or decrease of 50 % respectively.

For example for BW, we computed the values of PK parameters influenced by BW having an estimated typical value (tv) of θ1, this tv being for a typical dog of 20 kg BW because it is our scaling BW value in the model. Thus, for dogs of 10 and 30 kg BW, i.e. for dogs having a BW of plus or minus 50% of the scaled 20 kg BW dog, the two next equations give for the volume of distribution the typical value for this parameter:

$$V1 = tv\theta_1 \times \left[\frac{10}{20}\right]^{-0.21} = \theta_1 \times 1.156$$

$$V1 = tv\theta_1 \times \left[\frac{30}{20}\right]^{-0.21} = \theta_1 \times 0.8645$$

For the scenario where only the BW influenced the **Clearance** (CL), the typical value of the fixed effect was of -0.2368 meaning that the tv of CL is increased of time folds a factor of 1.178 for a dog of 10 Kg BW and was multiplied by 0.908 in a dog of 30 Kg BW (or equivalently divided by 1.10). Such a difference can be considered as not relevant from a clinical point of view.

For three of the tested scenarios, **creatinine** actually influenced the plasma clearance value with a typical value of the exponent ranging from -0.18 to -0.23 meaning that creatinine, as BW, has a non-relevant influence on plasma clearance (multiplicative or dividing factor ranging from 1.13 to 1.17).

For **age** tv of the exponent ranged from -0.092 to -0.035 meaning multiplicative/dividing factor from 1.02 to 1.06 i.e. not relevant.

For the surgery time, exponents were of 0.0011 and 0.093 meaning multiplicative /dividing factor of 0.999 and 0.93 respectively without practical consequences.

SUPPLEMENTARY MATERIAL – Figures S3-S8

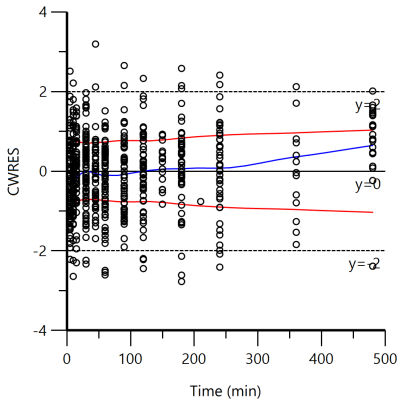


Figure S3: Plot of conditional weighted residuals (CWRES) against time.

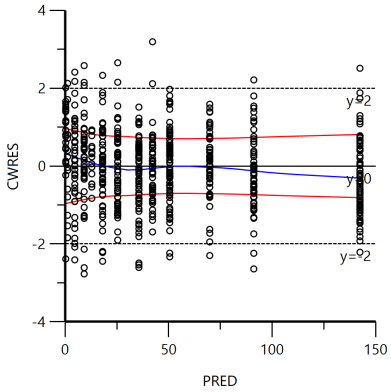


Figure S4. Plot of conditional weighted residuals (CWRES), against population prediction of cefazolin concentrations (PRED).

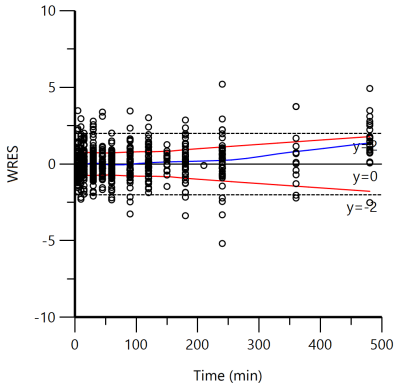


Figure S5: Plot of weighted residuals (WRES) against time.

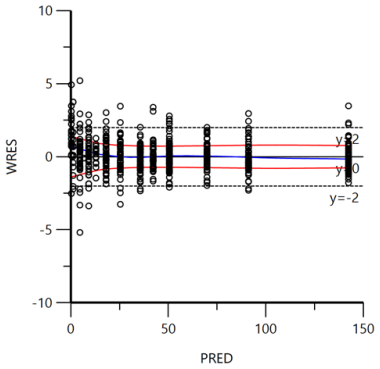


Figure S6: Plot of weighted residuals (CWRES), against population prediction of cefazolin concentrations (PRED).

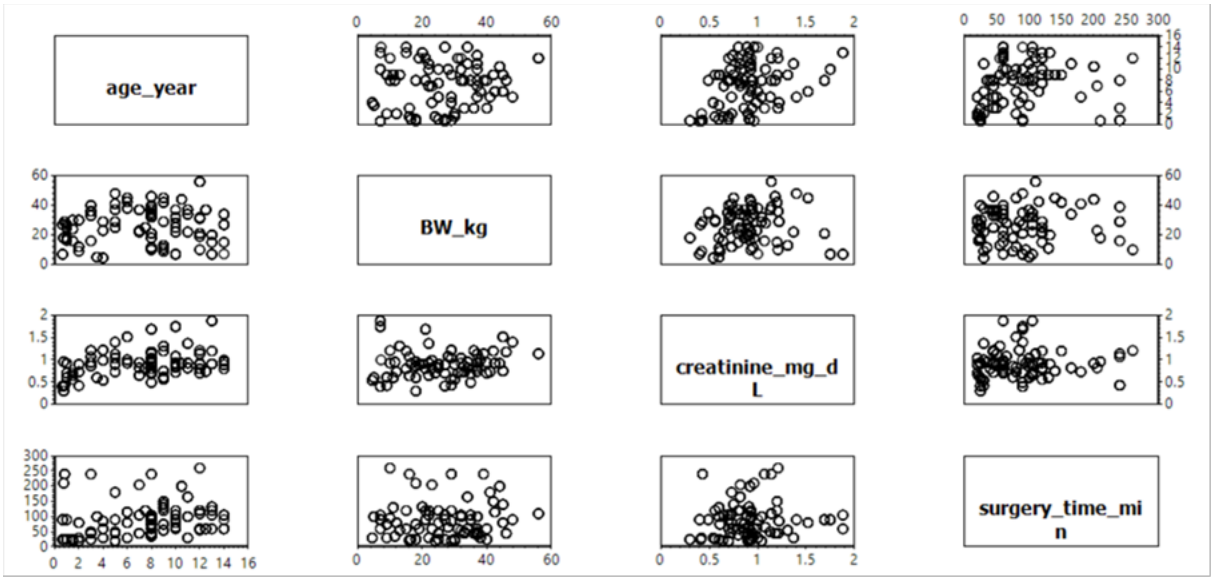
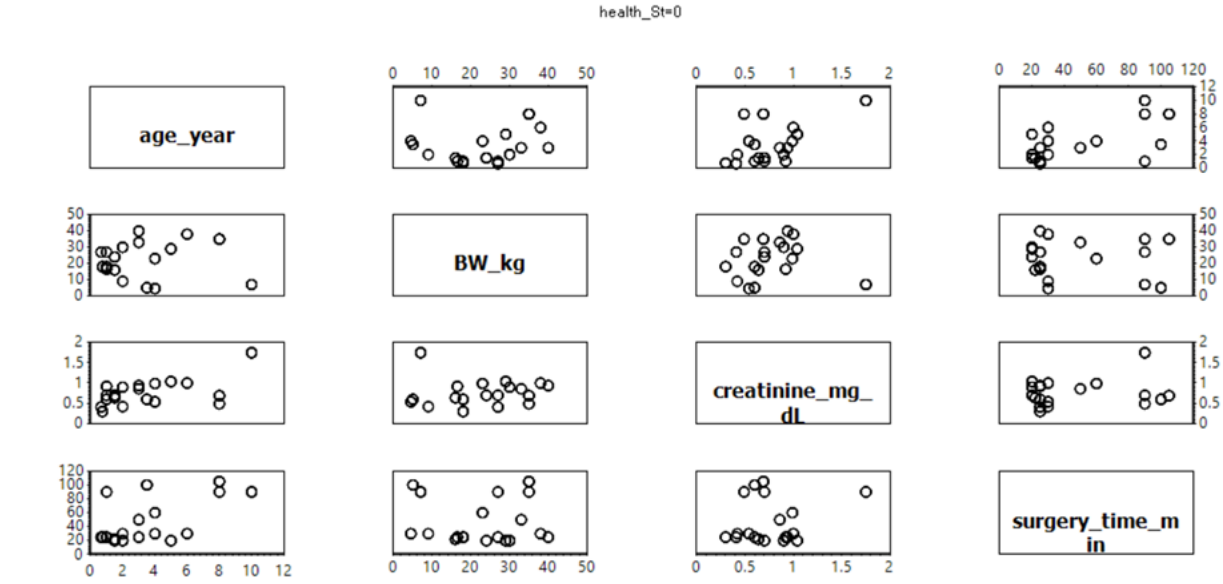
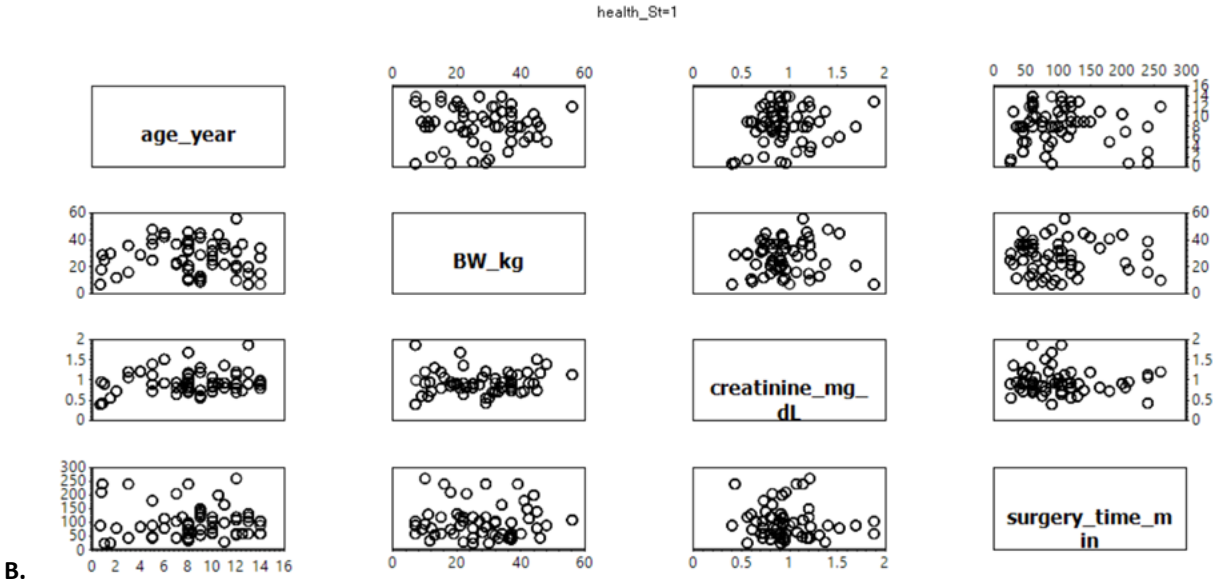


Figure S7: Scatter plot matrix for the continuous covariates, age, BW, creatinine level and surgery time.



A



B.

Figure S8 A and B the scatter plot matrices for the continuous covariates (age, BW, creatinine level and surgery time) per health status level (0 = healthy, 1 = disease).

General Discussion

This doctorate thesis dealt with different aspects and topics in the field of pharmacokinetics (PK), the branch of pharmacology that studies the processes of absorption, distribution, metabolism and elimination of drugs, in other words the fate of a drug following its administration.

The analysis of human and veterinary literature started from the basic PK principles moving to the more difficult mathematical modeling, i.e. from the "simple" experimental and clinical applications of classical PK, to the "complex" analyses concerning the population PK and physiologically-based PK, with the final aim of showing the multivariate features of this discipline.

The classical pharmacokinetic approach pays specific attention to an active compound fate following its administration, with the final aim of optimizing the route of administration and the dosage scheme, trying to reduce the influence of intraindividual and interindividual variabilities. The direct implications of these needs are translated into the attempt to standardize as much as possible the enrollment criteria and the study conditions. Regarding classical PK experimental studies, (e.g. preclinical studies on new drugs) healthy subjects of the same species, breed, presenting comparable age and weight category and of both sexes are enrolled. In these studies, the experimental environment is controlled and also the operators are trained to accomplish the procedures in a standard way. With the classical PK approach, biological samples collection is performed at prefixed times, samples number is generally high, and the sampling period should cover the expected drug elimination phase.

Clinical PK studies (e.g. the use of a molecule in a specific clinical situation) present some differences. First of all, the subjects enrolled are not completely standardised, since they represent the clinical case where drugs are needed and used, and this involves a certain degree of variability. Thus, in these studies groups homogeneity is more necessary than subjects standardization. The other important difference compared to experimental studies is that, clinical PK gives drugs indications with a strong practical utility in direct medical application.

On the opposite, the population pharmacokinetics (popPK) approach aims to investigate the influence of the interindividual variability in a target population of subjects. Therefore, it is no longer the molecule fate that has to be investigated, but the population and its characteristics that influence the fate of the molecule after administration.

The peculiarity of popPK is that it extends the field of investigation, increasing the number of subjects, with the final aim of restricting it to an individual. In fact, popPK evaluating the main factors that influence the ADME process of a specific drug, indirectly catalogue every single subject in which the drug can be administered, ideally arriving to plan an *ad hoc* therapeutic scheme for each member of the target population. Given these assumptions, the population approach seems to have been created for clinical application in refining drugs posology in specific target patients.

One of the main features of a population study is that data can be collected directly, either by samples collection from real patients or indirectly, through meta-analysis. Commonly, for every subject that belongs

to the population, the number of samples collected is low, and their execution is therefore not invasive for patients, time-sparing and inexpensive for the analysis. Given the relatively recent application of popPK in veterinary medicine, the literature does not yet have enough data to allow real meta-analyses to take place. Despite this, given the applicability of this approach in the clinical setting, popPK is spreading in veterinary pharmacology field.

Both classical and population approaches have pros and cons, however the choice of one approach is related to the objective of each specific study more that to its merits and defects.

As previously mentioned, the present doctorate thesis contains several researches, in which different pharmacokinetic approaches have been used.

The first study reported a clinical PK study concerning the determination of the kinetic profile of dexmedetomidine (DEX) in calves after intravenous administration and the comparison of the sedative effects of anaesthetic protocols involving DEX and xylazine. Since no data on DEX use in the bovine species were available in literature, a classical PK approach was employed for PK determination on the DEX use in calves undergoing minor surgical procedures. The clinical effects evaluation was carried out through a comparison with a sedative of the same class, xylazine, normally used in calves for sedation during minor surgical procedures (e.g. castration and dehorning). The subjects enrollment focused on the homogeneity of the study groups in terms of breed, sex, age and weight category. As mentioned above, the objective was to explore the fate of a molecule, with a given dosage and route of administration, in a species in which no data were present.

The objective of the second work illustrated in this thesis was to determine the pharmacokinetic profile of a simultaneous administration of dexmedetomidine and methadone via buccal (i.e. oral transmucosal) and intramuscular route in dogs, then comparing the pharmacokinetic parameters obtained between the two routes.

So far, the oral transmucosal route has not been much explored in dogs and, for this reason, it has been decided to study DEX and methadone (MET) (a drug combination already used in dogs, but not by this route of administration) in this species. In fact, although dogs are generally collaborative during physical examinations and preanaesthetic procedures, sometimes they can be fearful and suspicious and, in some cases, even aggressive towards the clinician. For this reason, to explore the kinetics of this route and the feasibility of this protocol in dogs could be useful to manage those patients who are reluctant to physical restraint or IM drugs administration. Moreover, OTM is considered an easy-to-perform route of administration, thus its employment by the dog's owner (under the veterinarian supervision) could be suitable for more aggressive dogs.

The comparison between OTM and IM administration of a DEX and MET combination showed that, in the canine species, absorption of these drugs by OTM is low and delayed and leads to moderate sedative effects. This route of administration is easy to use and well tolerated by patients for its noninvasiveness and for the

total absence of pain during administration. For these reasons, although it cannot be suggested in the preanesthetic routine, the co-administration of DEX and MET by oral transmucosal route should be more explored and may be used in uncooperative patients, reluctant to physical restraint and easily manageable by their owners.

The third work involved the kinetic profile determination of a DEX and ketamine (KET) combination simultaneously administered IM in eighteen captive tigers *(Panthera tigris)*. Normally, in zoo medicine, due to their harmful attitude, it is necessary to employ chemical restraint of these patients in order to allow performing routine medical practices. Drugs dosages are extrapolated from domestic species, the weight of animals is estimated, and their health status cannot be judged before sedation. For these reasons, it is important to perform dedicated PK studies in the nondomestic species in a clinical setting. The first aim was to determine DEX, KET and NORKET kinetic profile following their simultaneous IM administration by remote delivery; moreover, another goal was to investigate the possible influences induced by sedative and anaesthetic protocol variations on KET metabolization rate.

Tiger were divided in two groups: standard protocol group SP, administered with a fixed dose of DEX and KET and the non standard protocol NSP group, administered with variable doses of DEX and KET, in association or not with other sedative (e.g. butorphanol) or anesthetic drugs for general anesthesia induction and maintenance (e.g. propofol and isoflurane). It was not easy to compare DEX, KET and NORKET kinetic profiles in the two study groups: the SP group, presented a variability probably due to the interindividual differences; the NSP group, showed a variability that probably resulted from the sum of interindividual differences and the variations in the applied protocols themselves. This classical PK study applied to zoo animal medicine has primarily provided new data concerning the determination of the kinetic profile of DEX and KET in this species *Panthera tigris* that represents an absolute novelty. Furthermore, the results provided important clinical information on the lack of influence of the protocol type on the disposition of these two drugs.

This thesis also contains one clinical population pharmacokinetic (popPK) study.

It pointed out the determination of cefazolin kinetic profile used *off label* in a population of 78 dogs undergoing surgery for prophylaxis of surgical site infections. Three years were required for the entire study, for a total of 629 canine serum samples analysed for cefazolin quantification. In this study, the influence of some interindividual factors i.e., breed, age, sex, weight, body condition score, surgery time and health status on the main kinetic parameters of this drug were investigated. Although the choice of employing a popPK approach, the sampling scheme was characterized by a high number of samples collected from each subject, with the ultimate goal of expanding the reliability of the mathematical model. For a popPK monocentric clinical study in veterinary medicine, the number of subjects recruited, and the number of samples quantified is considered remarkable. The applicability of this work, however, lies in the second purpose: once the kinetic model was determined and validated, it was possible to evaluate the efficacy of cefazolin administered with a specific dosage in canine species by the use of Monte Carlo simulation and

pharmacokinetics/pharmacodynamics correlation. In this way, it was possible to determine a Clinical Breakpoint for cefazolin in dogs, according to the principles described by VetCAST, the committee responsible for antimicrobial susceptibility testing of bacterial pathogens of animal origin and animal bacteria with zoonotic potential. This second practical aspect of the study is very important since, to date, the veterinary literature data on this issue is still very poor.

Conclusions

The present doctorate thesis includes studies of different nature, connected to each other by the use of clinical pharmacokinetics for their development.

Despite every single research had its own objective, the global aim of this thesis was, using classical and population pharmacokinetics modelling, to provide the veterinary pharmacological literature with innovative data with wide clinical implications. In all the works reported, clinical pharmacokinetics has proved to be a fundamental tool for achieving the objectives set for each specific study.

The use of clinical classical pharmacokinetics has been useful to explore the kinetic profile of well-known drugs in different species, with different combinations or administered by modern routes of administration. As expected, the classical approach confirmed to be of simple employment but very helpful in determining and comparing kinetic drug profiles, leading to good results for clinical application. On the other hand, despite the use of population pharmacokinetics modelling confirmed to be more difficult to perform and time-expensive, it is important to state that this approach allows to obtain very robust results, realistically describing the drugs use scenario that takes into consideration all the variability of the population. All of this provides very important practical implications on safety and effective drugs use. In particular, this type of approach could be useful in the future for the application in the food-producing animal species, in the responsible use of antibiotics and food safety context, which, thanks to the will to redefine the drug legislation, will be amongst the main topics of veterinary interest in the next years.

In conclusion, considering what has been reported so far, it is possible to affirm that the use of appropriate pharmacokinetic modelling approach was important to perform different types of pharmacological studies, which helped to provide the veterinary pharmacology literature with innovative data with wide clinical implications.