

**Study into selected antimicrobial drugs for koalas
(*Phascolarctos cinereus*), incorporating consideration of
koalas' endogenous plasma and serum antibacterial activity**

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Declaration

This thesis is submitted to The University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy.

The work presented in this thesis, to the best of my knowledge, is the product of my own work with some contributions from other researchers. All the assistance received in preparing this thesis and sources have been acknowledged.

I, hereby, declare that I have not submitted this material, in either full or in part, for a degree at this or any other institution.

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Publications and conference abstracts arising from this thesis

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Conference abstracts

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- 3- Gharibi, S., Kimble, B. and Govendir, M. *In-vitro* plasma protein binding of cefovecin in the koala (*Phascolarctos cinereus*) vs the horse (*Equus caballus*). ANZCVS Science Week, 7-9 Jul 2016, Gold Coast, Australia.
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Author contribution statement

Re the publication entitled:

Pharmacokinetics of posaconazole in koalas (*Phascolarctos cinereus*) after intravenous and oral administration

I, Soraya Gharibi (PhD Candidate), was the chief investigator of the research documented in this publication. I performed all the sample analyses for the p.o. study and undertook the data analyses for both the p.o. and i.v. studies. I was the primary author of all drafts of the manuscript and the chief respondent to the journal referees' comments.

Dr Merran Govendir assisted the project as supervisor and finalising the manuscript for publication. Dr Larry Vogelnest (Taronga Zoo, NSW) administered the posaconazole and collected the blood samples at Taronga zoo. Dr Vogelnest reviewed all versions of the manuscript. Drs Cindy Stadler and Julie Barnes administered the posaconazole and collected the samples at Los Angeles Zoo (CA, US). Drs Stadler and Barnes reviewed all versions of the manuscript. Dr Benjamin Kimble assisted the project on HPLC method development. Dr Kimble reviewed all versions of the manuscript.

Soraya Gharibi 27/09/2017

I, as co-author, endorse that this level of contribution by myself and the candidate indicated above is appropriate:

Merran Govendir 27/09/2017

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Summary of the thesis

Studies on pharmacokinetic profiles on some first-line drugs for koalas argue that traditional *ad-hoc* extrapolations of dosages from carnivorous species such as dogs and cats to the koala, a folivore, are inappropriate. This research describes changes in plasma concentrations of amoxicillin and cefovecin, currently administered by some veterinarians to koalas. A third drug, posaconazole, was also investigated as its broad-spectrum antifungal activity might be efficacious against cryptococcal infections in koalas. HPLC methods to determine plasma concentrations of these antimicrobials were developed and validated. Posaconazole was administered at 3 mg/kg to two koalas intravenously and 6 mg/kg to another six koalas orally. Based on plasma concentrations, posaconazole is predicted to be efficacious for the treatment of cryptococcosis in koalas. An *in-vitro* study to determine cefovecin binding to plasma proteins of koalas and other selected Australian marsupials demonstrated the proportion of binding between 12 to 40 %, suggesting that the elimination half-life of cefovecin in these species is likely to be shorter than those in the dog and cat. Cefovecin was administered as a single bolus (8 mg/kg) to six koalas subcutaneously. Cefovecin plasma concentrations at all time points (0 to 96 h) in all animals were below 1 µg/mL, indicating cefovecin has a short duration of action in koalas. Amoxicillin was administered to another six koalas at 10 mg/kg subcutaneously. Low concentrations of amoxicillin were detected; however, drug instability might have contributed towards these findings. Bioassays (agar disc diffusion and broth microdilution inhibition assays) were undertaken to confirm amoxicillin and cefovecin HPLC results. The bioassay demonstrated variable plasma antibacterial activities at $t = 0$ h (before koalas were medicated). Consequently, the endogenous antibacterial activity of koala blood matrices (plasma, serum, etc.) to inhibit *E. coli* and *S. aureus* were evaluated. Koala blood matrices demonstrated significant variations in inhibiting both pathogens' growth compared to other species studied. Reasons for such variations were unclear but opened a new area for

investigation into koalas' endogenous antimicrobial activity and how it might be utilised to assist this 'vulnerable' species control infectious diseases.

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List of abbreviations

| | |
|--------------------|--|
| AAN | Australian approved name |
| ACT | Australian Capital Territory |
| AEC | animal ethics committee |
| AMPs | antimicrobial peptides |
| ATCC | American Type Culture Collection |
| AUC | area under the plasma concentration vs time curve |
| AUC _{0-t} | area under the curve between zero time point to last sampling time point (t) |
| AUC _{0-∞} | area under the plasma concentration vs time curve from time zero to infinity |
| AUC ₂₄ | AUC from 0 to 24 h |
| AUMC | area under the first moment curve |
| AZWH | Australia Zoo Wildlife Hospital |
| BAN | British approved name |
| BMS | broth microdilution susceptibility |
| b.w. | body weight |
| CFU | colony-forming units |
| Cl | body clearance |
| Cl _t | total body clearance |
| C _{max} | maximum concentration |
| CV | coefficient of variation |
| DCS | delayed-coagulation serum |
| DM | dry matter |
| <i>E. coli</i> | <i>Escherichia coli</i> |
| ED | equilibrium dialysis |
| EOs | essential oils |

| | |
|-----------------|--|
| F | Bioavailability |
| HS | heated serum |
| HLB | hydrophilic-lipophilic balance |
| i.m. | intramuscular |
| INN | international non-proprietary name |
| IQR | interquartile range |
| IS | internal standard |
| i.v. | intravenous |
| K _{el} | elimination rate constant |
| QC | quality control |
| LC | liquid chromatography |
| LC/MS | liquid chromatography / mass spectrometry |
| LLE | liquid-liquid extraction |
| LLOD | lowest limit of detection |
| LLOQ | lowest limit of quantification |
| MCX | mixed-mode reversed-phase/strong cation-exchange |
| MHA | Mueller-Hinton agar |
| MHB | Mueller-Hinton broth |
| MIC | minimum inhibitory concentration |
| MRT | mean residence time |
| MS | mass spectrometer |
| ng | nanogram |
| NSAID | non-steroidal anti-inflammatory drug |
| NSW | New South Wales |
| OD | optical density |

| | |
|--------------------|--|
| OD _{zero} | reference optical density caused by the medium (or any other fluids) that is subtracted from the observed OD to determine the OD caused by the cells |
| OD ₆₂₀ | optical density at the wavelength of 620 nm |
| PD | pharmacodynamic(s) |
| PDA | photo diode array |
| pg | picogram |
| PK | pharmacokinetic(s) |
| pK _a | acid dissociation constants |
| p.o. | oral administration |
| PBS | phosphate buffered saline |
| PPB | plasma protein binding |
| PPE | protein precipitating extraction |
| PSMs | plant secondary metabolites |
| QLD | Queensland |
| R ² | correlation coefficient value |
| SA | South Australia |
| s.c. | subcutaneous |
| SD | standard deviation |
| SE | standard error of the mean |
| SPE | solid phase extraction |
| spp. | Latin abbreviation “species pluralis” standing for multiple species |
| SSVS | Sydney School of Veterinary Science |
| <i>S. aureus</i> | <i>Staphylococcus aureus</i> |
| TBME | tert-butyl methyl ether |
| t _{1/2} | half-life |

| | |
|--------------|---------------------------------------|
| T_{\max} | time to maximum concentration |
| TPP | total plasma protein |
| UF | ultrafiltration |
| UV-Vis | ultraviolet visible |
| V_d | the volume of distribution |
| $V_{d_{ss}}$ | steady state volume of distribution |
| Vic. | Victoria |
| VPB | Veterinary Pathology and Bacteriology |
| vs | versus |

Chemicals and Materials

The following items were purchased from Sigma-Aldrich (Castle Hill, NSW): posaconazole, itraconazole, sulfamethoxazole, amoxicillin trihydrate, cefadroxil, potassium clavulanate, allopurinol, potassium dihydrogen phosphate (KH_2PO_4), dipotassium hydrogen phosphate (K_2HPO_4), sodium dihydrogen phosphate (NaH_2PO_4), acetic acid, phosphoric acid (H_3PO_4), tert-butyl methyl ether (TBME), chloroform, ethyl acetate, heptane and hexane.

Acetonitrile (HPLC-grade), methanol (HPLC-grade), formic acid and a Milli-Q water system (ultrapure water) were obtained from Thermo Fisher Scientific (Scoresby, Vic.)

An analytical grade of cefovecin was provided *gratis* by Zoetis Animal Health (West Ryde, NSW).

Chapter 1

**General introduction, literature review and
aims of this research**

1.1 General introduction

The first pharmacokinetic (PK) study of any therapeutic drug administered to the koala was that of enrofloxacin (and marbofloxacin) published by Griffith et al. (2010). Since then, results of this and other studies have revealed some significant differences in the PK profiles of first-line drugs used to treat diseased and injured koalas. Such studies have raised doubts about the appropriateness of extrapolating drug dosages from other species such as dogs and cats to koalas. For instance, the clearance and half-life of the antifungal drug fluconazole, which is reported to scale allometrically with the body weight of most species (Jezequel, 1994), does not scale to body weight in koalas (Black et al., 2014). Furthermore, the non-steroidal anti-inflammatory drug (NSAID) meloxicam has a half-life of 24 h in dogs (Busch et al., 1998), approximately 13 h in humans (Turck et al., 1996) and 1.19 h in koalas (Kimble et al., 2013a). This research project continues the investigation of the PK profile of some antimicrobial drugs administered to koalas. Although not a first-line drug as yet for koalas, the opportunity arose to investigate the PK profile of the antifungal posaconazole for this species. Therefore, the first research chapter of this thesis investigated the *in-vivo* PK of posaconazole in clinically normal koalas to predict its efficacy for the treatment of cryptococcosis. The next three chapters arose in response to requests from wildlife veterinarians for information on the PK profiles of the first-line veterinary-registered, antibacterial drugs cefovecin and amoxicillin. Hence, the *in-vitro* plasma protein binding (PPB) of cefovecin in koalas and some other Australian marsupials was investigated. Subsequently, the *in-vivo* administration of cefovecin and amoxicillin in clinically normal koalas was performed to document changes in plasma concentration over time. For the determination of plasma concentrations of posaconazole, cefovecin and amoxicillin, individual assays, using high-performance liquid chromatography (HPLC) were substantially modified from published methods and validated with koala plasma. However, modification and validation of both the cefovecin and amoxicillin assays were

challenging. Consequently, in order to confirm the HPLC results, bioassays (agar disc diffusion and broth microdilution inhibition assays) were also undertaken. However, the broth microdilution assay, which had a greater sensitivity than the agar disc diffusion assay, revealed substantial variation in plasma antibacterial activity over time in each individual koala and between koalas. Therefore, the last research chapter describes a study comparing antibacterial activity of different blood matrices (plasma, serum, etc.) collected from unmedicated koalas and other species, resulting in expanding directions for future research into controlling infectious disease in koalas and other species.

The literature review, **Chapter 1**, commences by providing an overview of the fundamentals of antimicrobial drug PKs, followed by a brief description of drug assay development and validation, and sample preparation for the HPLC. Then, a summary of relevant koala anatomy and physiology is provided for readers unfamiliar with this species along with a discussion of issues facing the existence of wild koalas in northern New South Wales (NSW) and south-eastern Queensland (QLD) (two states on the eastern coast of Australia). Then, information is provided on the constituents of the koala's almost exclusive *Eucalyptus* spp. foliage diet and the known biological activities of some of these constituents. A summary of the relevance of PK studies undertaken in this species to date, is also provided. A literature review on posaconazole, cefovecin and amoxicillin with an emphasis on their use in veterinary practice follows. This chapter concludes with the specific aims of this research.

Chapter 2 describes the development and validation of an HPLC method for determining posaconazole concentrations, followed by a description of the PK profile of posaconazole when administered at either 3 mg/kg intravenously (i.v.) or 6 mg/kg orally (p.o.) to clinically normal koalas.

Chapter 3 describes the development and validation of an HPLC method for quantitating cefovecin concentrations. This chapter also describes an *in-vitro* study to determine the

proportion of cefovecin that binds to the plasma proteins of selected Australian marsupials as well as to those of the horse (used as a comparative species).

Chapter 4 describes the development and validation of an HPLC method for determining cefovecin concentrations *in vivo* and reports on the plasma cefovecin concentrations when administered as a single subcutaneous (s.c.) bolus at 8 mg/kg to clinically normal koalas.

Chapter 5 describes the development and validation of an HPLC method for determination of amoxicillin concentrations and then reports on the plasma concentrations of amoxicillin when administered as a single s.c. bolus at 10 mg/kg to clinically normal koalas.

As Chapters 3 and 4 demonstrate that the HPLC analyses of both cefovecin and amoxicillin concentrations in koala plasma were problematic, **Chapter 6** describes attempts to utilise microbiological assays to confirm the concentration of cefovecin and amoxicillin (as determined by HPLC) in koala plasma.

Chapter 7 investigates the endogenous antibacterial activity of plasma, serum, delayed-coagulation serum and heated serum of koalas compared to other species (cows, horses and sheep).

Finally, **Chapter 8** reviews the major findings and limitations of this research, and then, future directions are proposed.

1.2 Literature review

1.2.1 Pharmacokinetics and pharmacodynamics of antimicrobial drugs

Four key physiological processes, absorption, distribution, metabolism and excretion (ADME), govern the time course of therapeutic drugs in the body. Pharmacokinetics describes concentration changes of a drug and its metabolites in the body over time (Riviere, 2009a). The area under the drug concentration vs. time curve (AUC) as an important PK construct represents the body's exposure to the drug and the change in drug concentration in the sampling

compartment over time (Baggot, 1978). The AUC can be estimated by the trapezoidal rule, whereby the area of trapezoids between time points from the first sampling time point after drug administration to infinity are totalled to approximate the total AUC (Gabrielsson and Weiner, 2012). Although samples can be collected from any biological fluid or tissue after drug administration, blood, usually serum or plasma, is more commonly used to measure drug concentration changes over time (Tozer, 1981). The AUC is frequently used as the PK component for the pharmacokinetic/pharmacodynamic (PK/PD) relationship which predicts antimicrobial efficacy, particularly that of concentration-dependant antimicrobial drugs (as described below).

Pharmacodynamics (PD) describes the drug's response (the effect of the drug) and correlates the dose of the drug with its clinical or pharmacological effects (Quintiliani, 2012, Martinez et al., 2013). For antimicrobials the 'response' is typically either the bacteriostatic (growth inhibition) or bactericidal (killing) ability of the drug (Quintiliani, 2012).

Pharmacokinetic/pharmacodynamic integration, especially well-defined for antimicrobial drugs, describes the dose-concentration-response correlation of a drug and its corresponding clinical effects. This PK/PD relationship is used to predict the efficacy of the administered drug dosage required to achieve the desired clinical outcome (Toutain, 2002). The target plasma concentration used to calculate drug dosage for an antimicrobial agent is usually based on its minimum inhibitory concentration (MIC) which is defined as the lowest concentration of a specified antimicrobial that inhibits growth of a specific pathogen (Baggot and Giguère, 2013).

Antimicrobials can be described as having either time-dependent or concentration-dependent activity (Craig, 1995, Jacobs, 2001). Beta-lactam antibacterials that include penicillins such as amoxicillin, and cephalosporins such as cefovecin have a time-dependent bactericidal pattern, i.e. the duration of the exposure is the major determinant of the extent of killing, so the goal of dosing for these drugs is to optimise the duration of exposure of the pathogen to the

antimicrobial (Craig, 1995, Jacobs, 2001). The duration of the time that unbound plasma concentrations of the drug exceed the MIC ($T \% > \text{MIC}$) is important for determining the therapeutic efficacy of beta-lactam antibacterials (this relationship is illustrated in Figure 1-1). Successful therapy for amoxicillin is predicted if the effective concentration during the dosage interval is maintained above 40 to 50 % MIC for both susceptible gram-positive and gram-negative pathogens (Jacobs, 2001, MacVane et al., 2014).

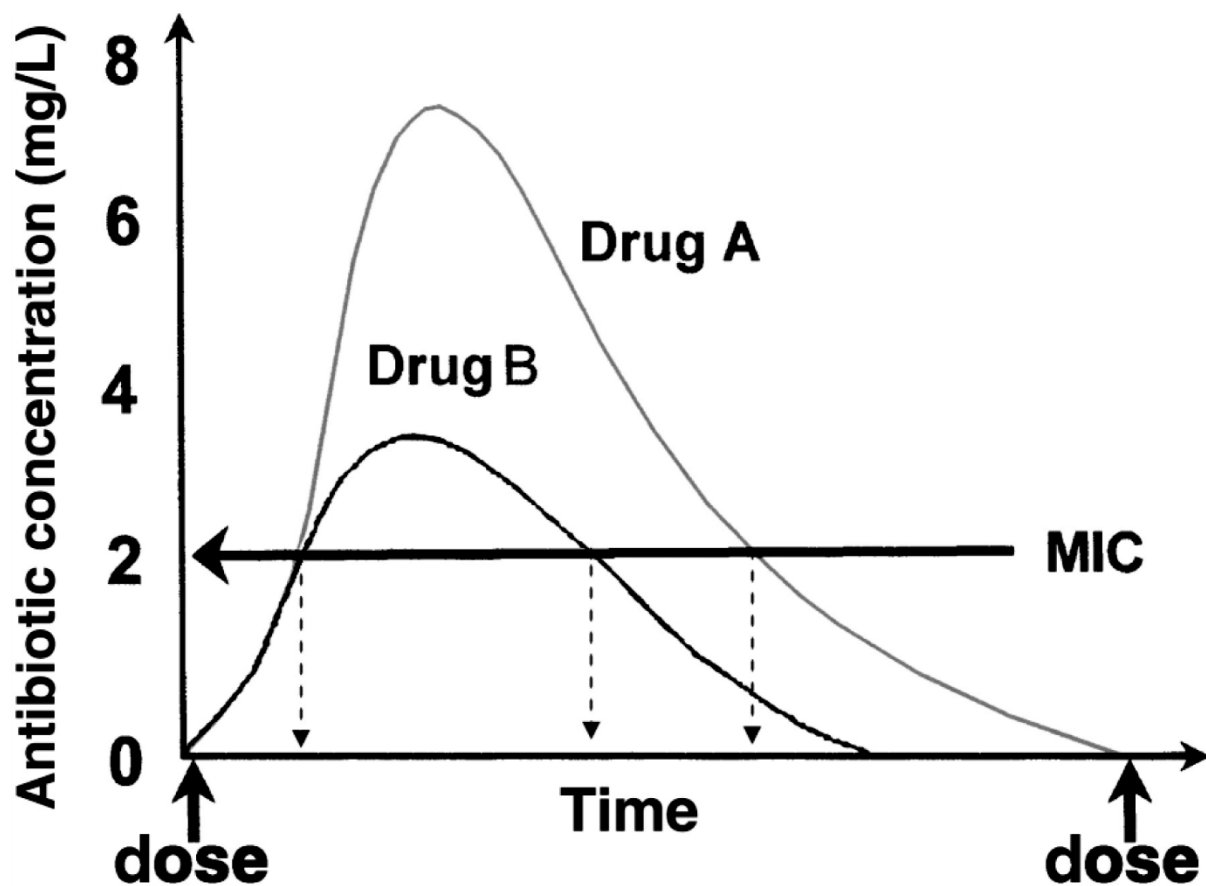


Figure 1-1 Time-dependent killing of antibacterials such as beta-lactams (amoxicillin and cefovecin), the curve shows the association between serum PK with MIC of a pathogen. Drug A presents a concentration of 2 mg/L (2 $\mu\text{g/mL}$) for 50 % of the dosing interval. Drug B presents a concentration of 2 mg/L (2 $\mu\text{g/mL}$) for 30 % of the dosing interval. The Figure is adapted from 'Optimisation of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters' by Jacobs (2001) in *Clinical Microbiology and Infection*, 7, 11, p: 590.

Some antimicrobials, like aminoglycosides and fluoroquinolones, have a concentration-dependent bactericidal effect and the goal is to achieve a high concentration of the

antimicrobial at the site of infection to eradicate the pathogen(s) (Jacobs, 2001). The main PK/PD parameters used to predict clinical efficacy of concentration-dependent antimicrobials are the ratio of AUC_{24} (the AUC from 0 to 24 h) to MIC; and / or the C_{max} (the maximal or peak concentration) to MIC, based on the free plasma concentration of the drug (illustrated in Figure 1-2) (Jacobs, 2001).

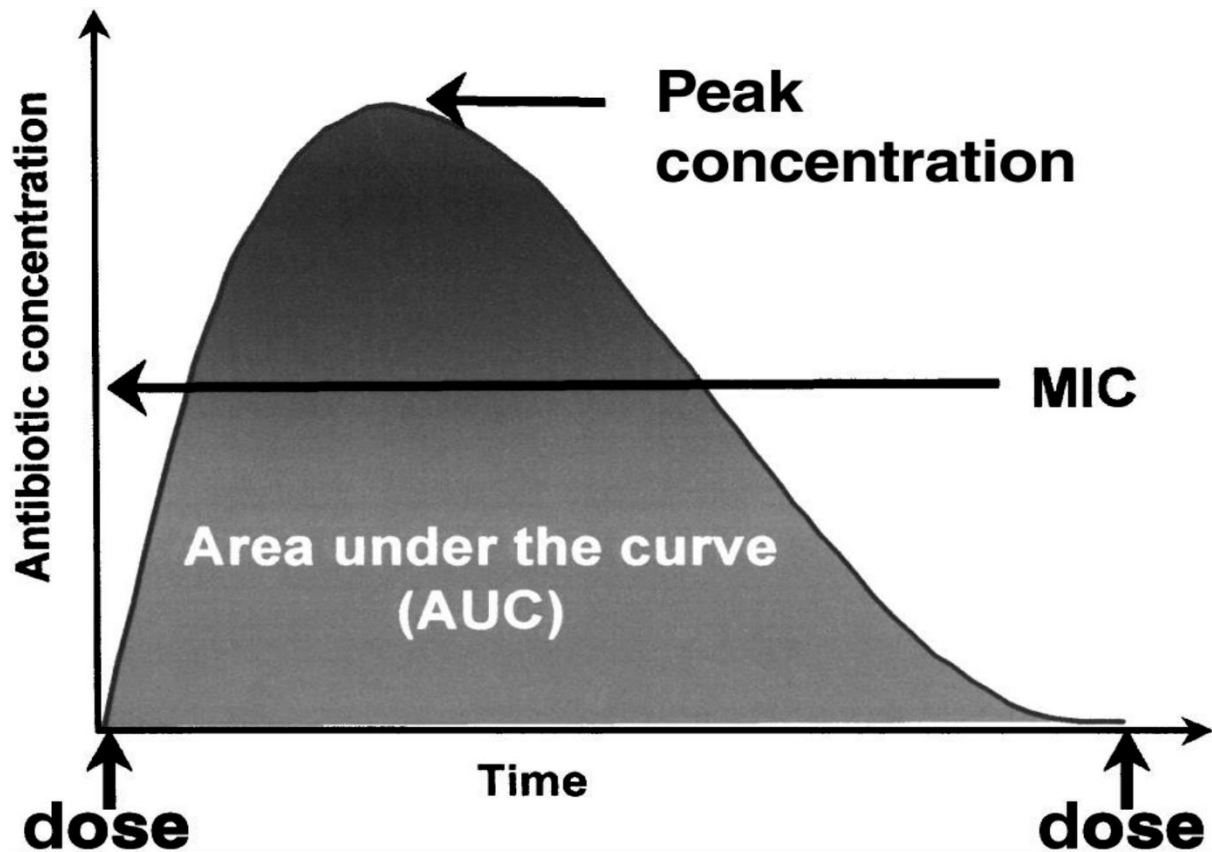


Figure 1-2 Concentration-dependent killing (AUC/MIC and C_{max}/MIC ratios), the curve shows the association between serum PK with MIC of a pathogen. The Figure is adapted from 'Optimisation of antimicrobial therapy using pharmacokinetic and pharmacodynamic parameters' by Jacobs (2001) in *Clinical Microbiology and Infection*, 7, 11, p: 592.

1.2.2 Pharmacokinetic parameters and indices

1.2.2.1 Absorption and bioavailability

Absorption is the transfer of drug molecules from the administration site to the bloodstream (Urso et al., 2002). The extent and rate of drug absorption depends upon the route of administration, the chemical properties of the drug molecules, the drug formulation and the physiological factors such as presence of metabolic enzymes around the site of administration (Sakai, 2009). The extent and rate of the drug's absorption are referred to as the drug bioavailability (F) (Riviere, 2009a). For intravenous administration, as the drug is transferred directly into the bloodstream, absorption is not required, and $F_{i.v.}$ equals 1.00. Drug administration by extravascular routes (p.o., i.m. and s.c.) may result in lower F due to incomplete absorption (Sakai, 2009). The bioavailability of extravascular routes is expressed as a proportion of the absolute bioavailability of the i.v. route and is expressed in the following equation:

$$F = \frac{dose_{i.v.} \times AUC_{extravasular\ route}}{dose_{extravasular\ route} \times AUC_{i.v.}}$$

1.2.2.2 Drug distribution, volume of distribution and plasma protein binding

Once a drug enters the bloodstream, it is distributed throughout the body. The delivery of a drug from the bloodstream to the site of action is governed by blood flow, capillary permeability, drug ability to penetrate the cellular barriers, the physicochemical properties of the drug molecule (lipophilic drugs vs polar and ionised drugs) and the degree of binding of the drug to blood and tissue proteins, and blood brain barriers transporters.

The volume of distribution (Vd) is an important indicator of the apparent dilution of the drug in body compartments after dosing (DiPiro et al., 2010). Most polar drugs or drugs that are ionised in the plasma, such as amoxicillin and cefovecin, have a limited Vd (150 – 300 mL/kg) while lipophilic molecules are distributed extensively throughout body compartments, fluids

and tissues (Vd: 1 – 3 L/kg) (Baggot and Giguère, 2013). The Vd is a measure of the proportion of the total amount of the drug (A) in the body at time t compared to the concentration of the drug in the plasma at time t (C_p) (Kwon, 2001, Toutain and Bousquet-Mélou, 2004b):

$$Vd_t = \frac{A_t}{C_{p(t)}}$$

The Vd is an important parameter required for calculating the dose to provide a therapeutic plasma concentration of the drug (Baggot and Giguère, 2013). As the Vd can change with the state of drug disposition, there are several volumes of distribution in the scientific literature. Using a non-compartmental model, the Vd_{area} is that obtained during pseudoequilibrium when the net drug distribution between the plasma and tissues reaches equilibrium:

$$Vd_{area} = \frac{dose}{K_{el} \times AUC}$$

where K_{el} is the elimination rate constant of the drug that is determined by least squares regression analysis of the terminal four to six concentration vs time data points (Baggot and Giguère, 2013). In other words, the K_{el} is the slope of the elimination phase.

The Vd_{ss} occurs when the drug is at steady state and is the most robust Vd as it is independent of any elimination process but can only be calculated when the drug is administered as an i.v. bolus dose or i.v. infusion (Toutain and Bousquet-Mélou, 2004b; Riviere, 2009b; Baggot and Giguère, 2013):

$$Vd_{ss} = \frac{dose_{i.v.} \times AUMC}{(AUC)^2}$$

where the AUMC is the area under the first moment of the plasma concentration vs time curve or the area under the curve of the product of plasma concentration and time [$(C_p(t) \times t)$ vs time on a linear scale (Baggot and Giguère, 2013).

Once distributed into the bloodstream, most drugs bind to plasma proteins in different proportions, generally reversibly. The bound fraction acts as a reservoir from which the drug dissociates to maintain an unbound to bound equilibrium (Lindup and Orme, 1981). The free drug fraction crosses membrane barriers to be distributed to tissues, undergo metabolism, be excreted and exhibit pharmacological effects (Vuignier et al., 2010). Therefore, the percentage of drug bound to plasma proteins (PPB) can significantly affect the drug PK (Vuignier et al., 2010). Drugs highly bound to plasma proteins ($\geq 80\%$) are often confined to intravascular spaces and cannot undergo immediate distribution to extravascular spaces (Sakai, 2009, Baggot and Giguère, 2013). Furthermore, the protein-bound fraction of the drug is not filtered by the glomerulus nor readily undergoes renal tubular secretion; therefore, filtration and / or secretion by the kidneys is limited to the free drug fraction in plasma (Taft, 2009).

Both the PPB and dissociation rate of the drug from proteins can be of great importance in the PK profile of the drug. Drugs with high PPB and a slow dissociation rate can result in the drug circulating only in the plasma and a reduced Vd. Furthermore, the rate of any metabolism might be reduced, and clearance can be prolonged (Li et al., 2015); however, if the drug's affinity for the metabolising enzymes is greater than plasma proteins (such as buprenorphine and fentanyl), metabolism might not be affected.

Many methods are available for measuring PPB including equilibrium dialysis (ED) and ultrafiltration (UF) (Trainor, 2007a, Trainor, 2007b). Equilibrium dialysis is the most widely used method to quantify drug-protein interactions and is usually regarded as the reference method; however, a standardised method for PPB measurement has not been prescribed (Beer et al., 2009). Both methods are based on separation of the unbound drug from the bound based on differences in the molecular size and / or weight. With ED, the free drug diffuses through a semipermeable membrane from the plasma chamber into the protein-free buffer chamber (Beer et al., 2009, Vuignier et al., 2010). After incubation at body temperature for a specified time,

equilibrium is assumed, so the drug concentration is measured in both compartments and the C_B (bound drug concentration) can be determined (Beer et al., 2009, Vuignier et al., 2010):

$$C_B = C_T - C_U$$

where the C_T is total plasma concentration of the drug and C_U is the free drug concentration of the buffer chamber (Beer et al., 2009).

Using the drug concentration in both chambers after equilibrium (the instruction of Rapid ED, Thermo Fisher Scientific, Scoresby, NSW), the percentage of PPB can be determined via:

$$\text{PPB (\%)} = 100 \% - \left(\left[\frac{\text{drug concentration at buffer chamber}}{\text{drug concentration at plasma chamber}} \right] \times 100 \% \right)$$

Equilibrium dialysis has the advantages of being simple, reliable and cost-effective (Beer et al., 2009). The major drawback of the ED method is its long duration of equilibrium (generally 12 – 48 h) (Vuignier et al., 2010). However, smaller commercial ‘Rapid ED’ devices such as those from Thermo Fisher Scientific (Scoresby, NSW) are now available that require approximately 4 h to reach equilibrium. Subtle shifts in oncotic pressure during dialysis can cause water flux from the buffer chamber to the plasma chamber (Huang, 1983, Vuignier et al., 2010), thereby, diluting the drug-protein complex compartment and an overestimation of the unbound drug concentration (Huang, 1983). Nonspecific membrane adsorption of the drug can also occur depending on the membrane material, the drug concentration and the degree of drug ionisation (Beer et al., 2009) which can lead to further error.

The UF method uses a device consisting of two reservoirs separated by a semipermeable filter which is available in various molecular cut-offs (Vuignier et al., 2010). After a short centrifugation time (average 10 – 15 min), centrifugal force pushes molecules through the pores of the semipermeable filter into the ultrafiltrate while proteins and drug-protein complexes are retained behind the membrane (Beer et al., 2009, Vuignier et al., 2010). The UF method can

be affected by temperature, plasma pH, nonspecific binding of the drug to the membrane and protein leakage (Vuignier et al., 2010). The ultrafiltrate volumes should not exceed 40 % of the initial plasma volume due to alteration of the protein concentration (Beer et al., 2009). Advantages of the UF method are its simplicity and rapidity of the procedure (Beer et al., 2009), resulting in this method being used in drug discovery, drug monitoring and PK/PD studies (Vuignier et al., 2010).

1.2.2.3 Elimination, metabolism and excretion, clearance and elimination half-life

Elimination is the removal of the drug from the body and incorporates the processes of metabolism and / or excretion. Metabolism is defined as the drug molecule and / or its metabolites undergoing a chemical change. Excretion is the elimination of the unchanged parent drug and / or its water-soluble metabolites into the urine (i.e. renal excretion being the most important pathway for most drugs) and the biliary-intestinal circulation to be either recirculated and / or eliminated into the faeces (Sakai, 2009, Riviere, 2009a, Baggot and Giguère, 2013). Lipophilic drugs are mainly eliminated by hepatic metabolism that converts them into more hydrophilic metabolites to facilitate their excretion (Riviere, 2009a, Baggot and Giguère, 2013). Hepatic metabolism is comprised of two phases: Phase I and Phase II. In Phase I, a functional group is added to the parent drug via oxidation, reduction, hydrolysis, hydration, dechloacetylation and isomerisation (Riviere, 2009a). In Phase II, the products of Phase I metabolism are conjugated to make the metabolites more water soluble (Riviere, 2009a). During metabolism the parent drug can bypass either phase.

The PK parameters that describe drug disposition (distribution and elimination together) are obtained by monitoring the changes in the drug concentration over time, after administration of an i.v. bolus. Therefore, these parameters are independent from the drug dosage characteristics that will influence the C_{\max} , T_{\max} and AUC. The exception, however, is zero order elimination and distribution that is dose dependent and saturable. Primary PK parameters

include systemic body clearance (Cl), Vd and half-life ($t_{1/2}$) which is a hybrid parameter that reflects changes in Cl and Vd (Benet, 1983, Van De Waterbeemd et al., 2001). Systemic clearance is probably the most important PK parameter to consider in defining a dosage regimen for maintaining the required therapeutic plasma concentration of the drug (Toutain and Bousquet-Mélou, 2004a). Total body clearance (Cl_t) describes the ability of the body to eliminate a drug and represents the overall rate of the drug elimination from the body (Hollinger, 2007). As the overall elimination of most drugs obeys a time-dependent manner (i.e. first order kinetics whereby a constant proportion of the drug concentration is eliminated from the body per unit time), the systemic clearance of most drugs is constant over the therapeutic plasma concentration range. That is why clearance is extremely important in clinical PK and in defining the drug dosage (Baggot and Giguère, 2013). The Cl is calculated by:

$$Cl_t = \frac{\text{rate of drug removal } (\frac{mg}{min})}{\text{plasma concentration of drug } (\frac{mg}{ml})}$$

As the total amount of the drug administered i.v. is assumed to be finally eliminated, the total amount of the drug administered and eliminated is equal (Jang et al., 2001). Thus,

$$Cl = \frac{dose_{i.v.}}{AUC_{i.v.}}$$

The half-life of a drug defines the time that is required for the drug plasma concentration to fall by one half through the elimination process (Baggot and Giguère, 2013). The plasma elimination half-life is governed by Cl and Vd (Van De Waterbeemd et al., 2001) and can be determined via:

$$t_{\frac{1}{2}} = \frac{0.693 \times Vd}{Cl}$$

where 0.693 is the natural log of 2 ($\ln 2$). As can be seen in the equation, the half-life can be prolonged by increasing the V_d and decreasing the Cl (Van De Waterbeemd et al., 2001).

As it is assumed that the overall elimination of antimicrobials obeys first order kinetics, their half-lives are independent of the administered dose (Baggot and Giguère, 2013). The half-life is also calculated by:

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

where K_{el} is the elimination rate constant of the drug (Baggot and Giguère, 2013).

1.2.2.4 Mean residence time

The mean residence time (MRT) expresses the average total time that molecules of a single dose of a drug spend in the body (Baggot and Giguère, 2013). It is the statistical moment analogy to $t_{1/2}$ and can vary with the route of administration. The MRT is calculated by the derived AUMC (defined in Section 1.2.2.2) divided by the AUC (Baggot and Giguère, 2013).

1.2.2.5 The peak concentration and the peak time

The peak concentration (C_{max}) is related to rate of absorption, rate of elimination, dose and fraction of the dose absorbed. The time to reach C_{max} (T_{max}) occurs when rate of absorption equals rate of elimination. The C_{max} and T_{max} are determined either visually or mathematically from the change in drug concentration over time (Urso et al., 2002).

1.2.2.6 Pharmacokinetic analysis

To transform the plasma drug concentration vs time profile into primary PK parameters (e.g. Cl , V_d and $t_{1/2}$) and other secondary PK parameters (or indices) (e.g. C_{max} , T_{max} , MRT and AUC), one of two common mathematical approaches can be applied: a compartmental or a non-compartmental approach (Bonate, 2011). The compartmental approach assumes that the body consists of multiple compartments and proposes a hypothetical model to estimate PK

parameters and indices (Bonate, 2011). The non-compartmental analysis approach uses a numeric method and does not rely on compartmental segmentation of the body (Yamaoka et al., 1978); it can be applied to any route of administration to calculate primary PK parameters such as Cl , V_d and $t_{1/2}$, and secondary parameters (or indices) such as F , C_{max} , T_{max} , AUC and MRT . The only assumption in the non-compartmental model is that absorption and disposition of the drug obey first-order kinetics (Baggot and Giguère, 2013).

In order to perform the PK analyses required for this research, commercial assays for posaconazole, cefovecin and amoxicillin were not available. Thus, assays for all three drugs were developed by the author. The next section of the literature review addresses HPLC for the quantification of drug concentrations in plasma, including a description of sample preparation and assay validation.

1.2.3 Determination of drug concentrations in biological matrices

There are many bioanalytical procedures to quantify drug concentrations in biological matrices for a variety of purposes including PK studies. The aim is to develop reliable, accurate, fast and efficient procedures for qualitative and quantitative analyses (Nováková and Vlčková, 2009). Bioanalytical methods can be categorised into three groups: microbiological assays (bioassays) (Bennett et al., 1966, Simon and Yin, 1970), physicochemical assays such as HPLC (Nováková and Vlčková, 2009) and immunological assays such as enzyme-linked immunosorbent assay (ELISA) (Findlay et al., 2000).

1.2.3.1 High-performance liquid chromatography

Any analytical technique that requires separation of different molecules from the sample mixture is termed chromatography (Meyer, 2004). For separation, the sample mixture is introduced into the mobile phase (either gas or liquid) which carries the sample through a stationary phase (e.g. column or plane) (Meyer, 2004, Moldoveanu and David, 2017d). While

passing through the stationary phase, the different molecules are retained from the flowing mobile phase and are later released back into the mobile phase as an aggregation which undergoes detection and is displayed in the output (Moldoveanu and David, 2017d). If liquid or gas is used as the mobile phase, the process is known as liquid chromatography (LC) or gas chromatography (GC), respectively. The stationary phase can be either a solid, surface-active, porous material in small-particle form or a thin film of liquid coated on a solid support or column wall (Meyer, 2004).

Liquid chromatographic separation modes include adsorption chromatography, reversed-phase chromatography, chemically-bonded-phase chromatography, ion-exchange, ion-pair and ion chromatography, size-exclusion chromatography, and affinity chromatography (Meyer, 2004). One of the most important mechanisms of chromatographic separation is based on the polarity of the whole molecule, or that associated with specific regions of the molecule (Moldoveanu and David, 2017c). With adsorption chromatography, a relatively polar material with a high specific surface area (e.g. silica being the most popular, or alumina and magnesium oxide) is used as the stationary phase and the mobile phase is relatively non-polar incorporating an organic solvent ranging from heptane to tetrahydrofuran. The separation effect is provided by different attractions of various molecules in the mixture to the stationary phase. Generally, polar (a.k.a. water-soluble, hydrophilic or lipophobic) compounds are eluted later than non-polar (a.k.a. fat-soluble, hydrophobic or lipophilic) compounds (Meyer, 2004). In reversed-phase chromatography, the rules of the adsorption chromatography are reversed, and the stationary phase is non-polar while the mobile phase is relatively polar, and generally, non-polar compounds are eluted later than polar compounds (Meyer, 2004).

High-performance liquid chromatography can be a very efficient system to separate complicated mixtures of different compounds by molecular weight, polarities and acid–base properties in various biological matrices (Nováková and Vlčková, 2009). As the stationary

phase contains very small particles, high pressure is required to force the mobile phase through the column and this gives rise to the term ‘high-pressure liquid chromatography’ (Meyer, 2004). The detection output is represented as a chromatogram, with the x -axis as the assay run time and the y -axis as the signal intensity (Meyer, 2004). The chromatogram can provide directly both qualitative and quantitative information because, for a standardised set of conditions, each compound in the mixture has its own elution or retention time (the point at which the signal appears on the recorder or screen) which is displayed as a peak of which both the area and height are proportional to the amount of the corresponding substance (Meyer, 2004, Moldoveanu and David, 2017c).

The HPLC system comprises of a solvent supply system (solvent reservoir and degasser), a high-pressure pump, a sample injection device that delivers the samples into the mobile phase, a column or stationary phase (usually preceded by a smaller, more readily disposable pre-column that protects the column from blockages, although separation occurs within the main column) and detector(s) for the eluates (i.e. the components leaving the column) (Meyer, 2004, Moldoveanu and David, 2017c). The whole system is connected to a computer for controlling all components (mobile phase composition and / or pressure, sample injection volume, column temperature, etc.) and data recording and processing (see Figure 1-3). The mobile phase composition can be changed (i.e. the percentage of each solvent during the analysis run time for each sample can be increased or decreased) known as a ‘gradient’ elution or can be kept unchanged which is referred to an ‘isocratic’ elution (Moldoveanu and David, 2017c).

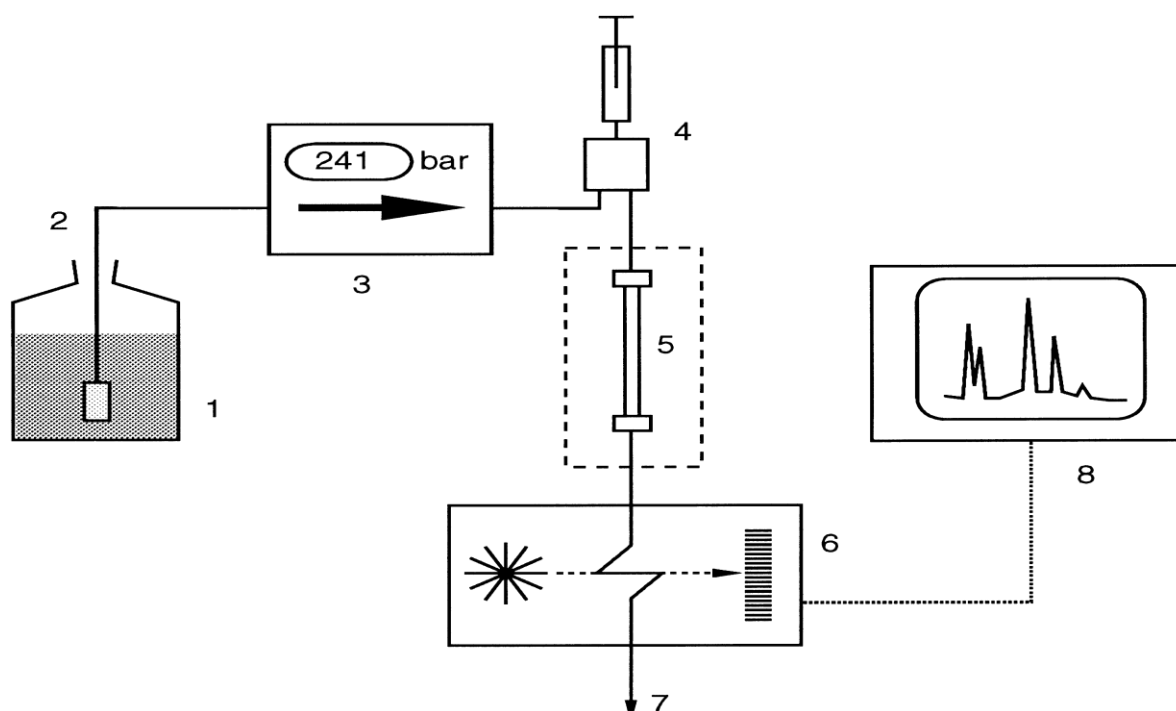


Figure 1-3 Schematic diagram of an HPLC unit, 1: solvent reservoir; 2: transfer line with frit; 3: pump (with manometer); 4: sample injection; 5: column (with oven); 6: detector; 7: waste; 8: data acquisition. The Figure is adapted from ‘Practical High-Performance Liquid Chromatography’ Meyer V. R. 4th edition, Chapter 1: Introduction, p: 9 (Meyer, 2004).

The detection and measurement in the HPLC system is generally based on the physicochemical properties of the separated analytes which are different from that of the mobile phase (Moldoveanu and David, 2017d). Accordingly, the HPLC system can incorporate various detector types including ultraviolet visible (UV-Vis), photo diode array (PDA), electrical conductivity, fluorescence, mass spectrometer (MS) and refractive detector (Choi, 2011). The UV-Vis is only adjustable to a single wavelength of interest while the PDA detector, which is the advanced form of UV-Vis detector, can register multiple wavelengths simultaneously (Poole, 2003). Therefore, the PDA is suitable to monitor the spectrum for identifying the optimal wavelength to detect the target molecule(s).

Different detectors have different sensitivity ranges such as 0.1 – 1 ng for a UV-Vis detector; 1 – 10 pg for a fluorescence detector and 1 pg – 1 ng for a mass spectrometer (Moldoveanu and David, 2017b, Moldoveanu and David, 2017c).

As many compounds absorb UV in the range of 190 – 800 nm, and light absorption is proportional to the concentration of the analyte of interest (Moldoveanu and David, 2017b), the UV-Vis is the most frequently used detector worldwide for HPLC (Poole, 2003, Moldoveanu and David, 2017b). However, due to the lack of specificity, the UV-Vis detector is not routinely used for characterisation of unknown compounds (Moldoveanu and David, 2017b, Moldoveanu and David, 2017c).

Florescence detection is intrinsically more sensitive than absorption detection, but its application is restricted because only a small proportion of organic compounds and drugs that absorb light are naturally florescent (Poole, 2003, Moldoveanu and David, 2017c).

In mass spectrometry molecules are ionised and separated based on their mass-to-charge ratio (m/z). A mass spectrum is a fingerprint of the molecule and therefore, MS detection typically is capable of identifying unknown compounds (Poole, 2003, Moldoveanu and David, 2017a). Liquid chromatography / mass spectrometry (LC/MS) has considerably higher sensitivity (can detect concentrations as low as pg/mL) and selectivity, and is a powerful instrument for identification and determination of the compounds (Moldoveanu and David, 2017a).

The refractive detector has the lowest sensitivity compared to other detectors but is the best option for compounds with minimal or no UV absorption (such as alcohols, carbohydrates, fatty acids and polymers) (Siddiqui et al., 2017).

1.2.3.2 Sample preparation

The sample is the biological material that contains the analyte, which is the molecule of interest, and the matrix which comprises the remaining sample components (Moldoveanu and David, 2017d). Therefore, both parts of the sample must be considered to ensure accurate and reliable drug concentration quantification. Sample preparation (a.k.a. sample extraction, sample clean-up or sample treatment) is usually required prior to HPLC analysis to separate the analyte of

interest from other interfering substances present in the biological matrix and to minimise or possibly eliminate those matrix substances (Kole et al., 2011, Moldoveanu and David, 2017d).

There are three conventional sample preparation techniques: liquid-liquid extraction (LLE), protein precipitating extraction (PPE) and solid phase extraction (SPE) (Kole et al., 2011).

In LLE, non-polar immiscible organic solvents such as ethyl acetate, chloroform, tert-butyl methyl ether (TBME) or hexane are used to partition the analyte of interest into either the aqueous or organic layer. Polar compounds are typically concentrated in the aqueous layer while non-polar drugs are extracted into the organic layer (Li et al., 2006).

The PPE method is a fast sample clean-up in which proteins are converted into insoluble compounds and precipitated using organic solvents, salts, acids or metal ions. Organic solvents such as acetonitrile and methanol reduce the dielectric constant of the plasma protein solution, thereby, facilitating electrostatic protein interactions which precipitate proteins (Polson et al., 2003). However, other endogenous insoluble components in the biological matrix cannot be removed by the PPE which can cause more interferences and increase the pressure in the HPLC system, thereby, blocking the system or damaging the column (Polson et al., 2003).

Solid phase extraction resembles the liquid chromatographic process for separating the drug from the biological matrix. The SPE apparatus usually consists of a cartridge that contains one of many sorbent-packing materials. Intermolecular forces between the sorbent, the sample and different solvents lead to the analyte being initially retained within the sorbent but in the final step is eluted from the sorbent. Different types of SPE phases include reversed-phase (RP-SPE), normal-phase and ion exchange (Li et al., 2006). The latter is more selective than RP-SPE and distinguishes analytes and matrix in terms of their relative hydrophobicity (Majors, 2013). A typical non-polar SPE has four steps: conditioning the cartridge, loading the samples, washing and eluting the analytes, and each of these steps requires optimisation for separating the analyte of interest (Majors, 2013).

1.2.3.3 *Validation of analytical methods*

Analytical method validation is required to demonstrate that the method used to quantitate the analyte(s) in a given biological matrix (e.g. plasma, serum, urine, etc.) is suitable for the intended use. The fundamental requirements for validation of an analytical method include demonstrating selectivity (or specificity), sensitivity, accuracy, precision, linearity of the calibration range, recovery and stability of the analyte in pre-spiked samples (i.e. samples containing known concentrations of the drug) (Food and Drug Administration, 2001, International Conference on Harmonisation, 2005, Tiwari and Tiwari, 2010). When there is a requirement to modify the chromatographic conditions or sample preparation techniques, revalidation of the modified aspects of the assay should be considered (Dadgar et al., 1995).

Selectivity (or specificity) describes the ability of an analytical method to distinguish and quantitate the analyte of interest from other compounds including endogenous matrix elements, metabolite(s), decomposition products, concomitant medication and other exogenous xenobiotics in the sample (Food and Drug Administration, 2001). The assay should be selective (able to differentiate the target substance) at all concentration ranges of the assay, especially at the lower limit of quantification (LLOQ). Blank samples (i.e. matrix sample containing neither the drug of interest nor the internal standard) should be analysed for the presence of endogenous peaks that may interfere with that of the target substance (Food and Drug Administration, 2001).

When small changes in the analyte concentration result in large changes in the analytical response function, the method is considered sensitive (Karnes et al., 1991, Causon, 1997). Sensitivity is expressed as the LLOQ and the upper limit of quantification (ULOQ) which are the limits of calibration or standard curve range. The LLOQ is the lowest concentration of an analyte that can be quantified with acceptable precision and accuracy. Any values that fall outside the calibration range cannot be quantified reliably (Causon, 1997, Tiwari and Tiwari,

2010) and is not acceptable (Peters et al., 2007). Therefore, any values below the LLOQ can only be semi-quantitated or presented as qualitative data. The lowest limit of detection (LLOD) is the lowest concentration of an analyte in the sample that can be detected or differentiated from background noise, but it is not necessarily quantitated as an exact value (International Conference on Harmonisation, 2005, Peters et al., 2007). One approach to determine the LLOQ and LLOD is based on the standard deviation (SD) of the response and the slope of the standard curve (International Conference on Harmonisation, 2005):

$$LLOD = \frac{3.3\sigma}{S}$$

$$LLOQ = \frac{10\sigma}{S}$$

where σ is the SD of the response (e.g. the SD of y-intercepts of the regression lines) and S is the slope of the standard curve of the analyte.

Accuracy (expressed as bias, %) defines the closeness of the mean of the measured values and the true values (concentration) whereas the precision (expressed as the coefficient of variation, CV, %) refers to the closeness of repeated sample values. The bias and CV should be within 15 % of the actual values, except for the LLOQ where it should not be more than 20 % (International Conference on Harmonisation, 2005). Quality control (QC) samples usually comprise of three known concentrations: low, middle and high, each determined with at least three replicates that undergo HPLC analysis and are used to confirm both intra- and inter-day accuracy and precision in order to satisfy the International Conference of Harmonisation (2005) criteria.

$$Bias (\%) = \frac{(\text{measured value} - \text{true value})}{\text{true value}} \times 100$$

$$CV (\%) = \frac{\text{standard deviation (SD)}}{\text{mean}} \times 100$$

Recovery (expressed as %) characterises the extraction efficiency of an analytical method. The recovery is performed by comparing the analytical results of the extracted spiked samples (at three concentrations: low, medium, and high) with unextracted standards at the same initial concentration in a simple matrix such as phosphate buffered saline (PBS) or mobile phase that represents 100 % recovery. The greater recovery of the analyte from the matrix, the more desirable; but in some situations, the recovery might be compromised intentionally for better selectivity. Nevertheless, recovery results are acceptable if they are consistent, precise and reproducible (Dadgar et al., 1995, Food and Drug Administration, 2001).

An internal standard (IS) is widely used in LC to increase the accuracy and precision of the results by minimising the inherent system errors such as volume errors caused by solvent evaporation, sample preparation procedures and injection-to-injection errors. Accordingly, a known volume and concentration of the internal standard is added to the standard, QC and actual study samples prior to the analysis. Therefore, instead of using the absolute peak height or area of the analyte, the ratio of its peak height or area to that of the internal standard is applied to plot the standard curve and to determine the concentration of the unknown samples (Haefelfinger, 1981, Usher et al., 2015).

To ensure a reliable assay, the relationship between the analyte concentration in the sample and the corresponding detector response should be determined by analysing the pre-spiked matrix-based standard samples and plotting the corresponding results vs the respective concentrations. The standard (calibration) curve is further assessed statistically to check the linearity (Tiwari and Tiwari, 2010). The standard curve should consist of a blank sample, a zero sample (i.e. matrix sample containing IS), six to eight non-zero (pre-spiked) samples which cover the expected range, including the LLOQ. The same biological matrix as the samples in the intended study should be used to prepare a standard curve and the QC samples, i.e. spiking the samples with known concentrations of the analyte (Food and Drug Administration, 2001).

Stability tests should be conducted on the stock solution and QC samples (high and low concentrations) to evaluate the drug(s) maintenance during the workup process, laboratory conditions, three freeze/thaw cycles (if the real samples are kept frozen) and long-term storage (under the same condition as real study samples). The data obtained from the analysis of the study samples should be accompanied by information regarding the stability of the drug in a given matrix under specific conditions, to ensure that the drug concentration in the samples has been maintained during storage, prior to analysis (Dadgar et al., 1995).

As this research project is interested in the PK profiles of selected antimicrobial drugs in the koala, the next section describes some characteristics of the koala to appreciate the relevance of the outcomes of the research chapters.

1.2.4 The koala

The koala (*Phascolarctos cinereus*) is an iconic Australian marsupial that epitomises the diversity of Australia's unique fauna. As a significant tourist attraction, the koala's economic contribution to the Australian economy via international and domestic tourism has been estimated to be worth between \$1.1 to \$2.5 billion annually (Hundloe and Hamilton, 1997, NSW Government Information, 2017). The koala is a member of the mammalian infraclass *Marsupialia*, and a common characteristic of marsupial species is that they carry and protect their young in a pouch (Andrew, 2015).

Mature koalas weigh 4 to 15 kg, with the average weight between 5 to 8 kg of those that live in the more northern and warmer regions of their range. Koalas in QLD are smaller than those in southern Australia such as Victoria (Vic.) where mature male koalas average 12 kg (Blanshard and Bodley, 2008). Koalas are nocturnal and generally have a sedentary, arboreal lifestyle which is facilitated by a low metabolic rate to conserve energy and compensate their low energy diet (Dawson and Hulbert, 1970, Blanshard and Bodley, 2008). The body

temperature of mature koalas is 35.5 to 36.5 °C (Blanshard and Bodley, 2008). The metabolic rate of marsupials is approximately 30 % lower than that of eutherian species (species which nourish the foetus via a placenta) (Dawson and Hulbert, 1970).

The koala diet is almost exclusively *Eucalyptus* spp. foliage (Hume, 1999). Approximately 90 % of the koala's daily energy requirements are met by digestion of the cell content fraction of eucalypt leaves. *Eucalyptus* spp. leaves are highly fibrous, low in protein and contain high concentrations of cellulose fibres and a variety of phytochemicals such as phenolics, tannins, cyanogenic glycosides and essential oils (EOs). Many of these phytochemicals are described as plant secondary metabolites (PSMs) (Freeland and Janzen, 1974, Hume, 1999, Stupans et al., 2001, El-Merhibi et al., 2007). Plants use PSMs as a defence to avoid ingestion by herbivores or insects, or attack by microbial disease (Freeland and Janzen, 1974). Mature koalas are estimated to ingest 62 to 324 g DM day⁻¹ of *Eucalyptus* foliage (Marsh et al., 2014). Consumption of a similar quantity of *Eucalyptus* oil by most other species would be toxic and potentially fatal (Stupans et al., 2001).

The koala is a monogastric hindgut fermenter. The digested carbohydrates, lipids, and amino acids are absorbed in the small intestine (Cork et al., 1983); however, the small intestine is relatively short compared to other species, and particulate and soluble materials transit through the small intestine rapidly (approximately 6 and 60 minutes, respectively) (Cork and Warner, 1983). The koala has the largest caecum relative to body size compared to other species (Blanshard and Bodley, 2008). Fermentative digestion of their eucalypt foliage diet, some protein synthesis and denaturing of secondary compounds occurs slowly in the caecum (Snipes et al., 1993, Hume, 1999, Blanshard and Bodley, 2008). Figure 1-4 shows the gastrointestinal tract from stomach to rectum of the mature koala.

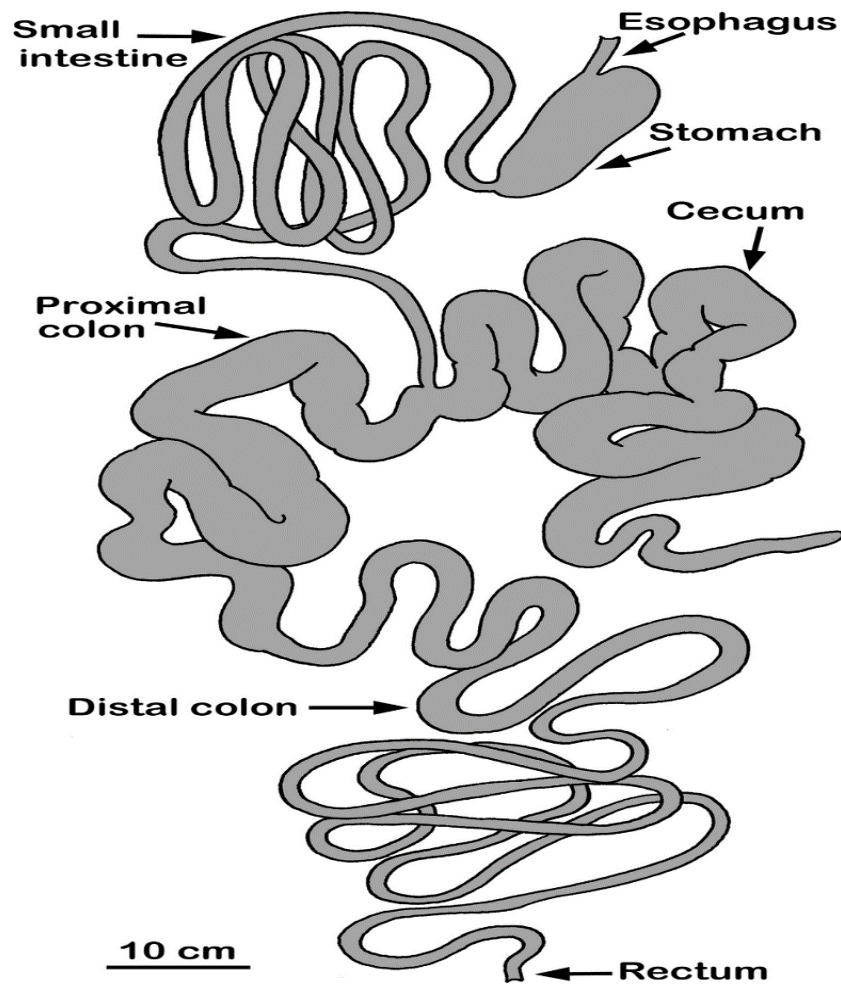


Figure 1-4 Gastrointestinal tract from stomach to rectum of the mature koala, drawn by Sally Pope. From ‘Review of some pharmacokinetic and pharmacodynamic properties of anti-infective medicines administered to the koala (*Phascolarctos cinereus*)’ by Govendir (2017) in *Journal of Veterinary Pharmacology and Therapeutics*, <https://doi.org/10.1111/jvp.12435>.

Plant secondary metabolite absorption in koalas seems to be restricted by some physiological defence systems such as the involvement of gut flora to initiate some breakdown of these substances (Freeland and Janzen, 1974) and the rapid transit time through the small intestine that potentially reduces the degree of absorption (Cork and Warner, 1983). The koala also possesses some extremely efficient hepatic metabolic enzymatic pathways such as those mediated by cytochrome P450 (CYP) 2C that could accelerate the elimination of some absorbed PSMs (McLean and Foley, 1997, Stupans et al., 2001, El-Merhibi et al., 2007, Kimble et al., 2014).

Wild koalas are found in forests and woodlands of eastern Australia from QLD to Vic. Continued clearing of natural koala habitats has fragmented and scattered wild populations in some areas of QLD, NSW, Vic. and South Australia (SA) (Blanshard and Bodley, 2008). The population of wild koalas has substantially diminished in QLD, NSW and the Australian Capital Territory (ACT) due to urban expansion - which necessitated the Australian Federal Government officially classifying wild koala populations in these locations as 'vulnerable to extinction' in April 2012 (Department of Sustainability Environment Water Population and Communities, 2012). Wild koala populations are also threatened by bushfires and droughts, traumatic injuries from vehicle strikes, predation by feral and domestic species, pollutants and disease, particularly the infectious disease chlamydiosis in northern populations (Melzer et al., 2000, Griffith et al., 2013, Narayan and Williams, 2016). Many rehabilitation centres and non-governmental organisations are devoted to the care and conservation of koalas (Griffith, 2010). Consequently, veterinary therapeutic drugs registered for domestic species are frequently administered to treat traumatised and diseased koalas (Blanshard and Bodley, 2008).

The most significant infectious disease of the koala is chlamydiosis caused by either *Chlamydia pecorum* or *Chlamydia pneumoniae* (Jackson et al., 1999, Polkinghorne et al., 2013). Clinical signs of this disease include ocular and urogenital tract disease which can lead to blindness and / or infertility, respectively (Obendorf and Handasyde, 1990, Polkinghorne et al., 2013). Another significant disease in wild and captive koalas is cryptococcosis caused by the fungal yeast *Cryptococcus gattii* that is a part of the *Cryptococcus neoformans* species complex (Krockenberger et al., 2003, Chen et al., 2014). The treatment of both infectious diseases in koalas has been challenging (Govendir, 2017). Koala retrovirus (KoRV) is found in the genome of many koalas and is considered to increase the risk of other diseases such as chlamydiosis by suppressing the immune system (Maher and Higgins, 2016).

Many studies investigated the anatomical and physiological adaptations of the koala to its diet while less attention has been paid to the therapeutic properties of the eucalypt foliage and its effect on the koala's body. Constituents of *Eucalyptus* plants especially their EOs have shown promising applications in medicinal products for human and veterinary use due to their reported biological activities including their antimicrobial efficacy (Turek and Stintzing, 2013). As the last chapter of this thesis is focussed on the antibacterial activity of koala blood matrices, the next section provides a summary of reported bioactivities for *Eucalyptus* spp. constituents.

1.2.5 The constituents and biological activities of *Eucalyptus* spp.

The *Eucalyptus* genus of plants belongs to the *Myrtaceae* family, native to Australia with approximately 900 species and subspecies (Russo et al., 2015). Species grow in a wide range of climatic conditions and are widely distributed throughout Australia except in arid central Australia and dense rainforests (reviewed Ghisalberti, 1996). Different parts of *Eucalyptus* trees (e.g. roots, leaves and tree gum) have traditionally been used by indigenous Aboriginal populations for medicinal purposes such as antiseptics and as a treatment for colds, wounds and eye infections (Clarke, 1987, Wigmore et al., 2016). Likewise, in Brazilian folk medicine, constituents of *Eucalyptus* spp. plants are used for a variety of medicinal purposes such as anti-inflammatory, analgesic and antipyretic remedies and for respiratory infections such as colds, flu and sinus congestion (Silva et al., 2003).

Extracts from *Eucalyptus* spp. plants reportedly have various biological activities such as analgesic, anti-inflammatory (Silva et al., 2003), anti-hyperglycaemic (Gray and Flatt, 1998) and antioxidant (Lee and Shibamoto, 2001) properties as well as being used as insect repellents (Nerio et al., 2010); eucalypt EOs are responsible for most of these properties. The EOs are odorous, volatile, organic compounds produced from the secondary metabolism of aromatic plants. They are complex and variable mixtures composed of hydrocarbons (e.g. monoterpenes

(C₁₀H₁₆) and sesquiterpenes (C₁₅H₂₄) such as 1,8-cineole and α -pinene) and oxygenated compounds (e.g. alcohols, esters, ethers, aldehydes, ketones, lactones, phenols and phenol ethers), and are usually concentrated in the leaves, stems, bark or fruit (Conner, 1993, El-Merhibi et al., 2007, Nerio et al., 2010). Only 10 % of all 3,000 known EOs have commercial importance in the cosmetic, food and pharmaceutical industries (Nerio et al., 2010). The composition of EOs varies considerably between plant species and varieties as well as within the same variety from different geographic areas (Nerio et al., 2010). Plant health, the age of the leaves, the season and soil factors as well as habitat and climate can also affect EO composition (El-Merhibi et al., 2007, Turek and Stintzing, 2013).

Additionally, *Eucalyptus* extracts and EOs have antibacterial and antifungal activities against a wide range of microorganisms (Sartorelli et al., 2007, Ghalem and Mohamed, 2008, Gilles et al., 2010, Tyagi and Malik, 2011, Elaissi et al., 2011, Wigmore et al., 2016). *In-vitro* studies show that EOs of *E. globulus* exhibit antibacterial activity against ATCC strains of some pathogens including *Escherichia coli* (*E. coli*), *Bacillus cereus*, *Klebsiella pneumonia* and *Staphylococcus aureus* (*S. aureus*) (Tohidpour et al., 2010). The *S. aureus* is the most susceptible pathogen to the EOs of *E. globulus* (MIC to inhibit *S. aureus* = 51.36 μ g/mL). Some isolates of methicillin-resistant *S. aureus* (MRSA) tested in this study also displayed susceptibility to the EOs of *E. globulus* (MIC to inhibit MRSA \leq 85.6 μ g/mL). Similarly, the EO of *E. globulus* MIC was lower to inhibit gram-positive pathogens (*B. subtilis* and *S. aureus*) (MIC to inhibit both pathogens = 2.25 mg/mL) than gram-negative pathogens (the MIC to inhibit *E. coli* = 4.5 mg/mL; MIC to inhibit *Pseudomonas aeruginosa* and *P. fluorescens* = 9 mg/mL) (Tyagi and Malik, 2011). An alcoholic extract (70 % ethanol) of *E. globulus* leaves was prepared and tested for antibacterial activity against a range of gram-positive and gram-negative pathogens (Dezsi et al., 2015). *Eucalyptus globulus* exhibited higher antibacterial activity against *S. aureus* (MIC = 50 μ g/mL) and *Listeria monocytogenes* (MIC = 30 μ g/mL)

than *E. coli*, *Bacillus subtilis* and *Salmonella typhimurium* (the extract MIC to inhibit all three isolates > 100 µg/mL) (Dezsi et al., 2015).

Essential oils of 20 *Eucalyptus* species were characterised based on their composition and their antibacterial activity inhibiting *E. coli*, *P. aeruginosa*, *S. aureus* and *Enterococcus faecalis* was reported by Elaissi et al. (2011). Antibacterial activity varied considerably with the EOs from each plant species and the bacterial strains (Elaissi et al., 2011). Generally, the most susceptible and resistant strains were *S. aureus* and *P. aeruginosa*, respectively (Elaissi et al., 2011).

In an *in-vivo* study, dried leaves of *E. globulus* were incorporated in the diet (62.5 g/kg) and drinking water (2.5 g/L) of male albino rats with or without diabetes. *E. globulus* inhibited candidiasis in both normal and diabetic rats and improved the condition of both groups compared to controls (Bokaeian et al., 2010). The EOs extracted from *E. globulus* also stimulated the innate cell-mediated immune response by stimulation of phagocytosis both *in vivo* and *in vitro* (Serafino et al., 2008).

Prior to 2010, there were no published studies on PK profiles of any first-line therapeutic drugs in koalas. Up till then, the dosage regimens of therapeutic drugs for koalas were similar to that administered to dogs and cats (Blanshard and Bodley, 2008). Several studies have been published since 2010 on the PK of therapeutic drugs administered to koalas (Griffith et al., 2010, Govendir et al., 2012, Black et al., 2013a, Black et al., 2013b, Kimble et al., 2013a, Black et al., 2014, Govendir et al., 2015, Budd et al., 2017). Most of these demonstrated differences in the PK profile such as absorption and metabolism of the investigated drugs in koalas compared to other species. Published PK studies in koalas include some antimicrobials (fluoroquinolones, fluconazole and phenicols) and the NSAID, meloxicam that are summarised below.

1.2.6 Pharmacokinetic studies of some therapeutic drugs in the koala

1.2.6.1 Fluoroquinolones

The first published study on any therapeutic drug in koalas investigated the absorption of two fluoroquinolones, enrofloxacin and marbofloxacin, administered p.o. and s.c. to diseased koalas (Griffith et al., 2010). Prior to this study, the suggested dosage of enrofloxacin was 5 mg/kg p.o. twice daily (Blanshard and Bodley, 2008) for the prophylaxis of some bacterial infections and as a potential systemic treatment for chlamydiosis. Both drugs demonstrated relative poor oral absorption compared to s.c. administration; however, the extent of s.c. absorption was lower than that reported in some other species such as the dog (Griffith et al., 2010). The suggested dosage of enrofloxacin at 5 mg/kg seemed inefficacious and unlikely to fulfil the PK/PD target (C_{\max}/MIC ratio = 8 – 10) which required the C_{\max} to be at least 2.5 $\mu\text{g/mL}$. Although the clinical signs of chlamydiosis improved with enrofloxacin and the bacterial load reduced during the treatment, the bacterial load increased following treatment withdrawal (Griffith, 2010). Subsequently, an additional study investigated the disposition of enrofloxacin after i.v. injection (10 mg/kg) in clinically normal koalas (Black et al., 2013a). The outcome was consistent with the earlier conclusions that enrofloxacin plasma concentrations at 10 mg/kg appeared inadequate against chlamydial pathogens but might be efficacious to treat pathogenic bacterial infections susceptible to drugs with MICs ≤ 0.03 $\mu\text{g/mL}$ (Black et al., 2013a).

1.2.6.2 Phenicols

As chloramphenicol was the other drug used for the systemic treatment of chlamydiosis in koalas in 2012, the PK profile of chloramphenicol base (administered at 60 mg/kg s.c. daily) and its effect on chlamydial shedding when administered to koalas infected with chlamydiosis was described (Govendir et al., 2012). Despite preventing chlamydial shedding for 2 – 63 days after treatment cessation, the dosage failed to control severe urogenital disease (Govendir et

al., 2012). A subsequent study reported the PK profile of chloramphenicol sodium succinate administered at 25 mg/kg i.v. to clinically normal koalas and compared the PK profile of this formulation to the 'base' formulation, both administered as single boluses at 60 mg/kg s.c. (Black et al., 2013b). The PK profile of the sodium succinate after i.v. injection was similar to those reported in other species. However, as expected, there were significant differences between the PK of two formulations when administered s.c. The sodium succinate demonstrated much higher plasma concentrations and was eliminated much faster than the oily base which exhibited an absorption rate limited profile (Black et al., 2013b). When both formulations were administered at 60 mg/kg s.c., the median elimination half-life of the sodium succinate was 1.45 h and the base was 13.14 h (Black et al., 2013b).

In 2013, due to the withdrawal of chloramphenicol base formulation from the Australian veterinary market, a research project was undertaken to investigate the PK of florfenicol following i.v. and s.c. administrations as an alternative to chloramphenicol to treat chlamydiosis in koalas (Budd et al., 2017). The results were not promising and florfenicol appeared unlikely to be efficacious against chlamydiosis at doses tolerable to koalas. It was recommended not to administer florfenicol s.c. as repeated administration increases the risk of potentially fatal dysbiosis.

1.2.6.3 Fluconazole

The PK profile of the antifungal fluconazole (administered at 10 mg/kg p.o. and i.v.) in clinically normal koalas revealed absorption rate limited disposition after oral administration (Black et al., 2014). Oral bioavailability was low and variable in contrast with almost complete oral bioavailability (1.00) in most other species. The plasma protein binding of fluconazole was approximately four times higher than that in other species. Although the Vd exhibited an allometric relationship with other species, Cl and $t_{1/2}$ did not. The Cl of fluconazole was about seven times faster in the koala than the value predicted by bodyweight scaling. The suggested

dose rate 10 mg/kg every 12 h was predicted to be inadequate in the koala to meet the proposed PK/PD target for *Cryptococcus gattii* (proposed AUC/MIC = 192 vs estimated AUC/MIC = median: 6.92, range: 2.57 – 12.66).

1.2.6.4 Other antimicrobials with severe side effects when administered to koalas

Although macrolides and tetracyclines are used to treat chlamydiosis in humans (Senn et al., 2005, Kohlhoff and Hammerschlag, 2015), erythromycin (Brown et al., 1984) and oxytetracycline (Osawa and Carrick, 1990) are reported to cause emaciation and death in koalas. Some antimicrobials, particularly those active against anaerobic pathogens, can cause life-threatening dysbiosis in koalas (Osawa et al., 1993). Doxycycline administration p.o. (either 0.25 mg/kg twice daily for 8 days or 2.5-5 mg/kg once daily) and i.m. (2.5 mg/kg once weekly for 28 days) caused dysbiosis in koalas (Griffith, 2010).

1.2.6.5 Non-steroidal anti-inflammatory drugs - meloxicam

Meloxicam was the most frequently administered NSAID to koalas (de Kauwe et al., 2014). The PK profile was investigated following i.v. (0.4 mg/kg), s.c. and p.o. (0.2 mg/kg) administration to clinically normal koalas (Kimble et al., 2013a). Meloxicam had extremely poor oral bioavailability and rapid Cl in the koala compared to other mammalian species and the previously suggested dosage seemed inappropriate in the koala. For example, the half-life of meloxicam in koalas is 1.19 h (range: 0.71 – 1.62 h) (Kimble et al., 2013a), 24 h in dogs (Busch et al., 1998) and approximately 13 h in humans (Turck et al., 1996). A comparative *in-vitro* study using a hepatic microsomal assay revealed that many weakly acidic NSAIDs, including flurbiprofen, diclofenac and meloxicam except for indomethacin, are unlikely to have PK applicability because of the *in-vitro* rapid intrinsic hepatic clearance (Cl_{int}), indicating a faster Cl and shorter $t_{1/2}$ in the koala (Kimble, 2015).

1.2.6.6 What has been learnt from PK studies in koalas?

The lessons from these aforementioned studies indicate that when drugs are administered orally to koalas they are poorly absorbed (such as enrofloxacin, fluconazole and meloxicam); however, first pass metabolism could also be a contributing factor; there appears to be some differences in drug binding (such as enrofloxacin and fluconazole) to plasma proteins between koalas and other species; some drugs such as meloxicam are rapidly metabolised in this species; some antimicrobial agents such as erythromycin and oxytetracycline cause emaciation and death in koalas so *in-vivo* antimicrobial studies in the koala must be undertaken carefully.

This research examined the PK profiles of the antifungal drug posaconazole and the antibacterials cefovecin and amoxicillin. The next section summarises the relevant information about these three agents in human medicine and veterinary practice.

1.2.7 Drugs studied in this thesis

1.2.7.1 Posaconazole

Posaconazole is a triazole antifungal drug with a chemical formula of $C_{37}H_{42}F_2N_8O_4$ (illustrated in Figure 1-5). It is a third-generation, lipophilic azole with one of the broadest spectrums of activity of all azoles (Hof, 2006). Posaconazole is efficacious against dimorphic fungi (such as *Blastomyces* spp., *Coccidioides* spp., *Candida* spp. and *Histoplasma* spp., both in their yeast and filamentous forms), *Aspergillus* spp., dematiaceous fungi (i.e. dark fungi that contain melanin in their cell wall) and zygomycetes (i.e. fungi which reproduce sexually by producing resistant spores) (Hof, 2006). Posaconazole is also indicated for salvage therapy against other drug-resistant, fungal infections (Segal et al., 2005, Ashbee et al., 2013).

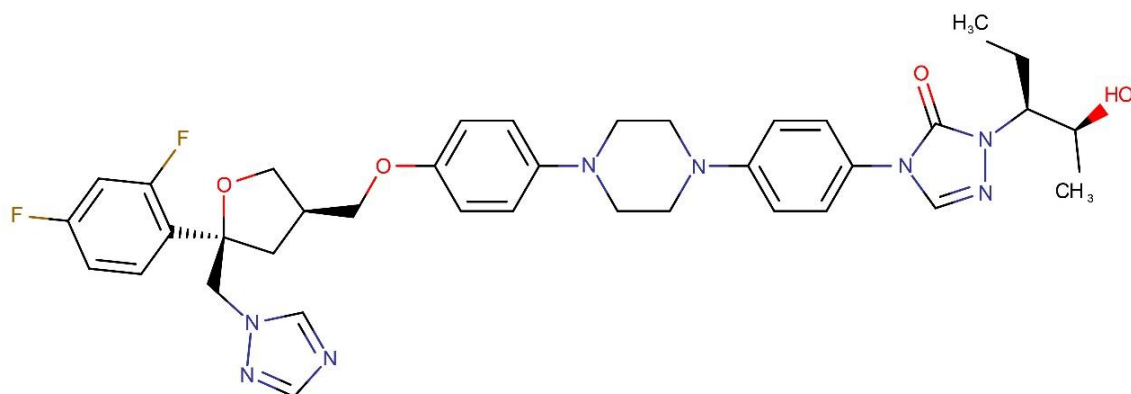


Figure 1-5 Posaconazole structure; the Figure is modified from the DrugBank database (2017), <https://www.drugbank.ca/drugs/DB01263> [accessed 29 Aug 2017].

1.2.7.1.1 Administration and dosage

Posaconazole is registered for use in humans and is available as a 40 mg/mL oral suspension, delayed release tablets and as an intravenous infusion (Noxafil, MSD, Macquarie Park, NSW) and has been used to treat fungal infections in cats (Krockenberger et al., 2010, Evans et al., 2011), dogs (Kendall and Papich, 2015, Corrigan et al., 2015, Cook et al., 2016), rabbits (Perfect et al., 1996) and rats (Ullmann et al., 2007) with minimal adverse effects.

Oral itraconazole is the current systemic, first-line, broad-spectrum, antifungal drug for most subcutaneous and systemic disseminated fungal infections in companion animals. However, itraconazole can reduce appetite and cause signs of hepatotoxicity in some cats (Malik et al., 2005). Fluconazole has been the drug of choice to treat feline cryptococcosis, the most common fungal infection in cats in Australia. However, feline cryptococcal meningitis requires more aggressive therapy with fluconazole combined with the potentially nephrotoxic drug amphotericin B, or amphotericin B with flucytosine (a.k.a. 5-fluorocytosine). Posaconazole is used occasionally in Australian veterinary practice to treat fungal infections in cats (McLellan et al., 2006, Wray et al., 2008, Krockenberger et al., 2010, Evans et al., 2011) and *Aspergillus* spp. infections in dogs (Corrigan et al., 2015). Posaconazole appears palatable and has a low

incidence of adverse effects in humans (Courtney et al., 2003), dogs (Kendall and Papich, 2015) and cats (Mawby et al., 2016). However, an additional challenge when treating fungal infections is the long duration of treatment. Posaconazole's major disadvantage is cost, which can be substantial for months of therapy. One bottle of Noxafil (40 mg/mL; volume of 105 mL) costs AU \$651.56 (in 2017) which equates approximately to AU \$3.25 – 4.00 per day to treat a fungal infection in a cat at the current suggested oral dosage (5 – 7.5 mg/kg/day) (Barrs and Talbot, 2014).

1.2.7.1.2 Mechanism of action

Like other azoles, posaconazole inhibits ergosterol production in the fungal cell membrane by binding to and inhibiting CYP (lanosterol-14 α -demethylase) which is present in most fungi except *Pneumocystis* and *Pythium* spp. (Hof, 2006). Posaconazole has a high affinity to bind to its target (Hof, 2006). As posaconazole's chemical structure differs from that of fluconazole and voriconazole: it can interact with an additional domain of the target so that it might inhibit mutated strains resistant to fluconazole and voriconazole (Hof, 2006). Posaconazole inhibits efflux pumps in fungi, so it can remain active when other azoles are already inactive (Hof, 2006).

1.2.7.1.3 Pharmacokinetics of posaconazole in some species

Pharmacokinetic studies have recently been published in the dog (Kendall and Papich, 2015) and cat (Mawby et al., 2016).

Oral absorption is improved by the presence of Fatty food (Krishna et al., 2009a) and when the total daily dose is divided into two to four doses (Li et al., 2010). Oral bioavailability was 0.48 when a 10 mg/kg suspension was administered to recently fed dogs (Nomeir et al., 2000). This value was reported as 0.26 (0.08 – 0.79) and 1.59 (0.85 – 5.00) when posaconazole was administered to dogs as an oral suspension (6 mg/kg, $n = 6$) or as delayed-release tablets (mean

dose: 6.9 mg/kg, $n = 5$), respectively (Kendall and Papich, 2015). Depending on the dosage and food, oral bioavailability of posaconazole is variable in humans and dogs (Li et al., 2010, Kendall and Papich, 2015). Following daily administration of 40 mg/kg for eight consecutive days via an oral suspension to fed dogs, serum concentrations were higher than that following a single dose, but the $AUC_{0-\infty}$ following a single dose was similar to the AUC_{0-24} following multiple doses indicating no untoward accumulation (Nomeir et al., 2000).

Posaconazole is highly protein-bound, predominately to albumin, in humans and dogs ($> 98\%$ and $> 99\%$, respectively) (Li et al., 2010, Kendall and Papich, 2015). Posaconazole is widely distributed to tissues (Li et al., 2010). The geometric mean of the V_{dss} in dogs is 3.28 L/kg (range: 2.25 – 6.45) (Kendall and Papich, 2015). In humans, it ranges from approximately 5 to 25 L/kg which may suggest extensive extravascular distribution and penetration into intracellular spaces (Li et al., 2010). There is some Phase II metabolism which is responsible for biotransformation of posaconazole into the glucuronide conjugate. Cytochrome p450 (CYP) enzymes do not play a significant role in its metabolism, but posaconazole inhibits CYP3A4 and p-glycoprotein and is therefore implicated in some drug interactions (Greer, 2007, Ashbee et al., 2013).

Less than 1 % of posaconazole is excreted unchanged in urine and 66 % is excreted unchanged in faeces, indicating renal elimination is a minor excretion pathway (Li et al., 2010).

There is some suggestion that posaconazole should be used cautiously in cats because of their inherent decreased rate of glucuronide conjugation. However, case reports of posaconazole 5 mg/kg p.o. administered once daily for many months to cats demonstrated efficacy with minimal adverse effects (McLellan et al., 2006, Wray et al., 2008, Krockenberger et al., 2010, Evans et al., 2011). The only adverse effect reported in cats has been erythema and pruritus of the pinnae and superficial excoriations of the skin in the affected region from self-trauma associated with rubbing (McLellan et al., 2006).

Posaconazole is slowly eliminated (Li et al., 2010). The $t_{1/2}$ of posaconazole in dogs is approximately 29, 24 and 42 h when given i.v. (3 mg/kg) and p.o. either by oral suspension or delayed-release tablet (6 mg/kg), respectively (Kendall and Papich, 2015). The clearance in dogs is 0.08 L/h/kg (Kendall and Papich, 2015). Some PK parameters and indices in dogs after i.v. or p.o. administration are summarised in Table 1-1.

Table 1-1 Pharmacokinetic parameters and indices in dogs after administration of posaconazole p.o. or i.v (Kendall and Papich, 2015)

| Parameters/indices | Mean Value (Range) | | |
|--|-----------------------------|--|---|
| | i.v. 3 mg/kg ($n = 6$) | oral suspension 6 mg/kg ($n = 6$) | delayed-release tablet 6.9 mg/kg ($n = 5$) |
| C_{\max} ($\mu\text{g/mL}$) | 1.29 (0.79 – 2.23) | 0.42 (0.19 – 0.81) | 1.80 (1.15 – 3.55) |
| T_{\max} (h) | - | 7.69 (4.18 – 34.28) | 9.48 (3.36 – 25.41) |
| $AUC_{(0-\infty)}$ ($\mu\text{g/mL}\cdot\text{h}$) | 38.40 (13.35 – 63.76) | 19.73 (6.43 – 75.43) | 130.39 (106.81 – 229.70) |
| K_a^* | - | 0.35 (0.03 – 0.90) | 0.44 (0.07 – 2.36) |
| α^* (h^{-1}) | 1.72 (0.32 – 11.05) | - | - |
| V_{dss} (L/kg) | 3.28 (2.25 – 6.45) | - | - |
| K_{el}^* (h^{-1}) | 0.02 (0.02 – 0.03) | 0.03 (0.01 – 0.05) | 0.02 (0.01 – 0.02) |
| Cl_b (mL/h/kg) | 78.13 (47.05 – 224.75) | - | - |
| $t_{1/2}$ (h) | 29.29 (19.93 – 35.71) | 23.92 (14.18 – 48.85) | 41.71 (31.59 – 60.54) |
| MRT (h) | 41.95 (28.68 – 51.36) | - | - |
| F (%) | - | 26 (7.8 – 79.0) | 159 (85 – 500) |

* K_a : absorption rate constant, α : distribution rate constant, K_{el} : elimination rate constant

1.2.7.1.4 Pharmacodynamic information

In a model of aspergillosis in neutropaenic rabbits, the minimal highly effective plasma concentration was 1 $\mu\text{g/mL}$ (Petraitiene et al., 2001). A trough posaconazole plasma concentration of 1 $\mu\text{g/mL}$ increases the probability of treatment efficacy in human patients with invasive aspergillosis (Ashbee et al., 2013). Both the posaconazole MIC and the genotype of

the invading pathogen are important determinants of exposure-response (PK/PD) relationship for efficacious posaconazole therapy (Ashbee et al., 2013).

The AUC_{24}/MIC is the best predictive PK/PD parameter for triazole efficacy in preclinical animal models of disseminated candidiasis (Lewis, 2011, Lepak and Andes, 2014). Preclinical PD studies of fluconazole, voriconazole, posaconazole and ravuconazole revealed that their antifungal PD target was similar (defined as 50 % of the maximal effect) across the drug class as long as the unbound drug concentrations are taken into account (Lewis, 2011, Lepak and Andes, 2014). An AUC_{24}/MIC of approximately 25 (range: 12 – 25) is required to achieve 50 % of the maximal effect of triazole family to treat *Candida* spp. (Lewis, 2011). Therefore, an AUC_{24}/MIC of 25 – 50 would mean that drug concentrations remaining near the MIC over 24 h period would be expected to achieve this target ($1 \times MIC \times 24 \text{ h} = AUC_{24}/MIC$) (Lewis, 2011, Lepak and Andes, 2014).

Considering the high plasma protein binding of posaconazole (Li et al., 2010, Kendall and Papich, 2015), the free concentration of posaconazole (the pharmacologically active part of a drug) would be expected to be appreciably less than the MICs for many fungal pathogens and therefore, theoretically results in inadequate antifungal efficacy. However, clinical trials in humans demonstrate a high degree of clinical efficacy (Lignell et al., 2011, Dolton et al., 2012). It was reported that unbound posaconazole concentrations of only 10 % of the known fungal MIC in serum have significant antifungal effects against *Candida* spp. isolates while this effect was not seen in protein-free media (Lignell et al., 2011). It was suggested that a flux of protein-bound posaconazole to its fungal binding target is likely attributable to this observed PD effect (Lignell et al., 2011). Furthermore, the high efficacy of posaconazole despite its lower plasma / serum concentration than MIC in clinical trials may also be attributable to its lipophilicity which results in high intracellular concentrations. For instance, posaconazole concentrations within alveolar cells can be 40 times greater than that in serum. In another study in humans,

there were increased intracellular concentrations of posaconazole in peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) compared to the plasma concentration after oral posaconazole administration (Conte et al., 2009, Farowski et al., 2010).

1.2.7.2 Cefovecin

Cefovecin (INN) is a semi-synthetic third-generation cephalosporin of the beta-lactam antibacterial group with a chemical formula of $C_{17}H_{18}N_5NaO_6S_2$ (illustrated in Figure 1-6). It was first registered in Australia in 2007 (APVMA, 2007) as an aqueous solution for a bolus s.c. administration with a long duration of action to treat specific bacterial infections in dogs and cats (Stegemann et al., 2006b, Stegemann et al., 2006c).

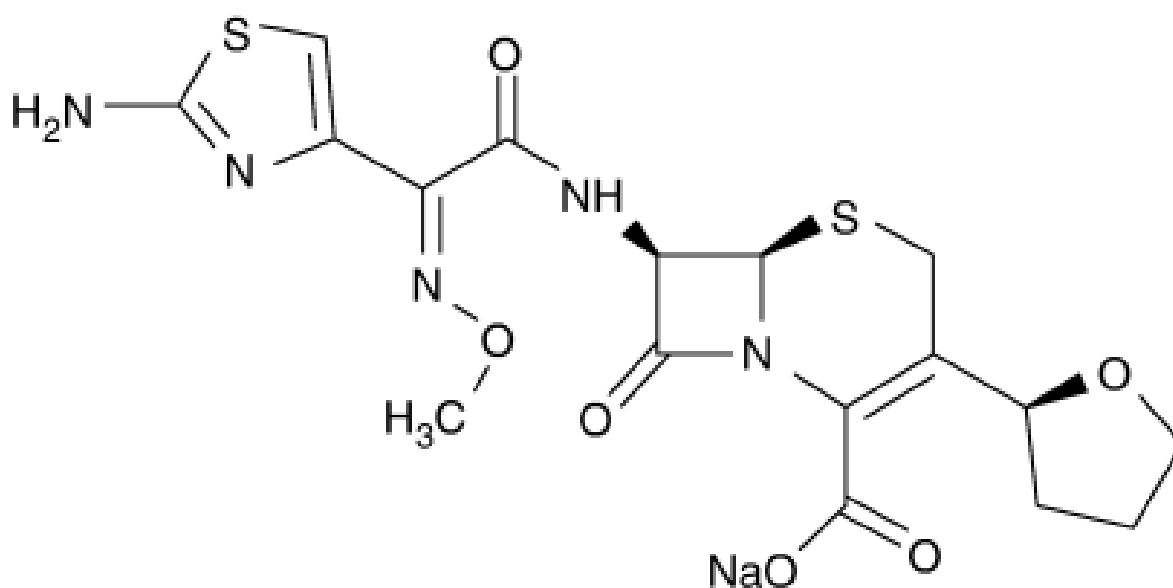


Figure 1-6 Cefovecin structure from ‘Pharmacokinetics and pharmacodynamics of cefovecin in dogs’ by Stegemann et al. (2006) in *Journal of Veterinary Pharmacology and Therapeutics*, 29, 6, p: 509.

1.2.7.2.1 Administration and dosage

Cefovecin has a broad-spectrum of activity against gram-positive and gram-negative pathogens (both aerobic and anaerobic) and is approved in many countries to treat bacterial infections including those commonly involved in skin infections, infected wounds, abscesses and urinary

tract infections in dogs and cats (Stegemann et al., 2007a, Stegemann et al., 2007b, Six et al., 2009). Cefovecin has a long elimination half-life in dogs and cats (approximately 5 and 7 days, respectively) and long antibacterial activity in transudates for at least 14 days (Stegemann et al., 2006c, Stegemann et al., 2006b). A single s.c. injection of 8 mg/kg b.w. dosage of cefovecin is recommended to be efficacious for at least 10 to 14 days in these species (Stegemann et al., 2006c, Stegemann et al., 2006b). For infections that require antibacterial action for longer than 14 days, the injection can be repeated at 14-day intervals on two to four occasions in dogs and cats (Prescott, 2013a).

1.2.7.2.2 Mechanism of action

Like other beta-lactam antibacterials (such as penicillins, cephalosporins, carbapenems and monobactams), cefovecin prevents formation of the bacterial cell wall during cell division by interfering with the final stage of peptidoglycan synthesis. Peptidoglycan synthesis in the cell wall relies on penicillin binding proteins such as transpeptidase and other peptidoglycan-active enzymes. Beta-lactams bind to and inhibit those enzymes that catalyse cross-linkage of peptidoglycan polymer units forming the bacterial cell wall (Prescott, 2013a).

1.2.7.2.3 Pharmacokinetics of cefovecin in some species

The PK profile of cefovecin has been studied in dogs (Stegemann et al., 2006b), cats (Stegemann et al., 2006c), some nonhuman primates (Papp et al., 2010, Raabe et al., 2011, Bakker et al., 2011), hens (Thuesen et al., 2009), alpacas (*Vicugna pacos*) (Cox et al., 2014b) as well as some aquatic species (García-Párraga et al., 2010, Steeil et al., 2014, García-Párraga et al., 2015, Lee et al., 2016) and non-domesticated animals (Thuesen et al., 2009, Nardini et al., 2014, Lee et al., 2016, Sypniewski et al., 2017). Some PK parameters and indices of cefovecin in some species have been summarised in Tables 1-2 and 1-3.

Cefovecin is primarily eliminated via the kidneys (approximately 70 – 80 %), and liver metabolism is minimal (Stegemann et al., 2006b, Papp et al., 2010).

After s.c. administration of cefovecin, almost the same C_{\max} was achieved within 6 h in dogs and 2 h in cats (121 and 141 $\mu\text{g/mL}$ in dogs and cats, respectively) with almost full bioavailability (0.99) for both species (Stegemann et al., 2006b, Stegemann et al., 2006c).

The PK profile of cefovecin between nonhuman primates and domestic carnivores is not consistent (Raabe et al., 2011). The terminal half-lives of cefovecin after s.c. administration have been reported in the following species: olive baboons (*Papio anubis*) [9.17 ± 1.84 h, (Raabe et al., 2011)], rhesus macaques (*Macaca mulatta*) [about 8 h, (Papp et al., 2010, Bakker et al., 2011, Raabe et al., 2011)], cynomolgus macaques (*Macaca fascicularis*) [6.3 ± 1.8 h and 4.95 ± 1.47 h, (Papp et al., 2010, Raabe et al., 2011)] and squirrel monkeys (*Saimiri sciureus*) [2.6 ± 0.1 h, (Papp et al., 2010)]. These are much shorter than those reported in dogs [133 ± 16 h, (Stegemann et al., 2006b)] and cats [166 ± 18 h, (Stegemann et al., 2006c)]. The $\text{AUC}_{0-96\text{h}}$ was much lower in nonhuman primates than that in dogs and cats (see Tables 1-2 and 1-3). The C_{\max} of cefovecin in these species is reported as 2 to 3-fold lower than that in dog and cat plasma (approximately 40 – 90 $\mu\text{g/mL}$ achieved within ≤ 2 h in nonhuman primates (Papp et al., 2010, Bakker et al., 2011, Raabe et al., 2011) compared to 121 $\mu\text{g/mL}$ in dogs (Stegemann et al., 2006b) and 141 $\mu\text{g/mL}$ in cats (Stegemann et al., 2006c). However, similarly to dogs and cats, a relatively small $V_{d_{ss}}$ was reported in cynomolgus macaques [0.16 ± 0.02 L/kg and 0.105 ± 0.018 L/kg, (Papp et al., 2010, Raabe et al., 2011)] and olive baboons [0.097 ± 0.024 L/kg, (Raabe et al., 2011)], likely indicating a high percentage of cefovecin binding to plasma proteins in these species (Raabe et al., 2011). High clearance rates of cefovecin were reported in olive baboons [9.36 ± 1.38 mL/h/kg, (Raabe et al., 2011)] and cynomolgus macaques [15 ± 5 and 17.58 ± 2.76 mL/h/kg, (Papp et al., 2010, Raabe et al., 2011)] which were attributed to decreased plasma concentrations in these species (Papp et al., 2010).

Although *in-vitro* PPB of cefovecin in nonhuman primates was relatively high at four different concentrations ranging from 0.5 to 50 $\mu\text{g/mL}$ [cynomolgus macaques (96.2 to 97.7 %), rhesus macaques (93.8 to 97.8 %) and squirrel monkeys (83.6 to 92.1 %) (Papp et al., 2010)], these values were less than those reported in dogs [96 to 98.7 %, (Stegemann et al., 2006b)] and cats [99.5 to 99.8 %, (Stegemann et al., 2006c)]. The difference in the elimination half-life in nonhuman primates, dogs and cats could partly be related to the different percentage of protein binding of cefovecin as a greater fraction of free drug is available for kidney filtration in the primates (Raabe et al., 2011) and that active renal reabsorption of cefovecin appears to occur in dogs and cats but not in nonhuman primates (Raabe et al., 2011).

The PK profile of cefovecin in hens also differs from that in dogs and cats (Thuesen et al., 2009). Following s.c. injection in hens, the plasma C_{max} ($6 \pm 2 \mu\text{g/mL}$) was attained at approximately $17 \pm 3 \text{ min}$ (Thuesen et al., 2009). The exposure ($\text{AUC}_{0-\infty}$) was smaller than that in dogs and cats [$8 \mu\text{g.h/mL}$ in hens (Thuesen et al., 2009) vs $10400 \mu\text{g.h/mL}$ in dogs (Stegemann et al., 2006b) and $22700 \mu\text{g.h/mL}$ in cats (Stegemann et al., 2006c)]. The elimination half-life was reported as $0.9 \pm 0.3 \text{ h}$ (Thuesen et al., 2009) in hens which is much shorter than that reported in dogs [$133 \pm 16 \text{ h}$, (Stegemann et al., 2006b)] and cats [$166 \pm 18 \text{ h}$, (Stegemann et al., 2006c)]. In hens, the mean Cl values were $1252 \pm 185 \text{ mL.h/kg}$ and the mean Vd was $1.6 \pm 0.5 \text{ L/kg}$, reflecting lower protein binding of cefovecin as high protein binding will result in a longer half-life and a decreased Vd (Thuesen et al., 2009).

In the alpaca, the Vd_{ss} is similar to that of carnivores and nonhuman primates ($0.086 \pm 0.029 \text{ L/kg}$ in alpacas) (Cox et al., 2014b) (see Table 1-2). However, other PK parameters and indices were very different to those described in dogs, cats and nonhuman primates (Cox et al., 2014b). The clearance reported as $7.1 \pm 1.8 \text{ mL/h/kg}$ (Cox et al., 2014b) is much faster than that in dogs [0.76 mL/h/kg , (Stegemann et al., 2006b)] and cats [0.35 mL/h/kg , (Stegemann et al., 2006c)] but slower than that in nonhuman primates [mean: 14 mL/h/kg , (Papp et al., 2010, Raabe et al.,

2011)]. The shorter terminal half-life [16.9 h, (Cox et al., 2014b)] compared with cats [166 h, (Stegemann et al., 2006c)] and dogs [133 h, (Stegemann et al., 2006b)] could be explained with the faster Cl in alpacas (Cox et al., 2014b). The mean PPB of cefovecin in alpacas was 80 % (Cox et al., 2014b).

Table 1-2 Pharmacokinetic parameters and indices in various species following i.v. administration of cefovecin (mean \pm SD)

| Species | dose (mg/kg) | AUC _{0-∞} (μ g.h/mL) | Vd _{ss} (L/kg) | Cl _b (mL/h/kg) | MRT (h) | t _{1/2} (h) |
|--|-----------------|---------------------------------------|-------------------------------------|--------------------------------|---------------------|------------------------------------|
| Dogs (Stegemann et al., 2006b) | 8 | 10500 \pm 1700 | 0.12 \pm 0.01 | 0.76 \pm 0.13 | 165 | 136 \pm 12 |
| Cats (Stegemann et al., 2006c) | | 22900 \pm 2970 | 0.09 \pm 0.01 | 0.35 \pm 0.04 | 256 | 184 \pm 12 |
| Cynomolgus acaques (Papp et al., 2010, Raabe et al., 2011) | 2 8 | 461 \pm 75.30 | 0.16 \pm 0.02 0.11 \pm 0.02* | 15 \pm 5 17.58 \pm 2.76 | 6.12 \pm 0.89 | 8.50 \pm 1.20 4.79 \pm 0.73 |
| Olive baboons (Raabe et al., 2011) | 8 | 863 \pm 139 | 0.10 \pm 0.02 | 9.36 \pm 1.38 | 10.50 \pm 1.30 | 8.70 \pm 0.78 |
| Alpacas (Cox et al., 2014b) | 8 | 1187 \pm 269 | 0.09 \pm 0.03 | 7.10 \pm 1.80 | 12 \pm 2 | 10.30 \pm 1.60 |

*significant difference between males and females

Table 1-3 Pharmacokinetic parameters and indices in various species following s.c. administration of cefovecin (mean \pm SD)

| Species | dose (mg/kg) | C _{max} (μ g/mL) | T _{max} (h) | AUC _{0-∞} (μ g.h/mL) | t _{1/2} (h) |
|---|-----------------|---|---|---|---|
| Dogs (Stegemann et al., 2006b) | 8 | 121 \pm 51 | 6.20 \pm 3 | 10400 \pm 1900 | 133 \pm 16 |
| Cats (Stegemann et al., 2006c) | 8 | 141 \pm 12 | 2 \pm 2 | 22700 \pm 3450 | 166 \pm 18 |
| Hens (Thuesen et al., 2009) | 10 | 6 \pm 2 | 0.28 \pm 0.05 | 8 \pm 1 | 0.87 \pm 0.27 |
| Squirrel monkeys (Papp et al., 2010) | 8 | 42 \pm 9 | 0.50 (0.50) | 128 \pm 38* | 2.60 \pm 0.10 |
| Rhesus macaques (Papp et al., 2010, Bakker et al., 2011, Raabe et al., 2011) | 8 8 8 | 47 \pm 8 79 \pm 5.70 93.50 \pm 6.61 | 2 (0.2 – 4) 0.95 \pm 0.14 0.87 \pm 0.31 | 520 \pm 70* 656 \pm 79 965 \pm 204 | 8 \pm 0.60 6.60 \pm 1 8.40 \pm 2.53 |
| Cynomolgus macaques (Papp et al., 2010, Raabe et al., 2011) | 8 8 | 46 \pm 6 62.80 \pm 13.80 | 1 (1) 0.75 \pm 0.28 | 468 \pm 97* 546 \pm 298 | 6.30 \pm 1.80 4.95 \pm 1.47 |
| Olive baboons (Raabe et al., 2011) | 8 | 75.90 \pm 8.40 | 2.83 \pm 1.83 | 1130 \pm 288 | 9.17 \pm 1.84 |
| Alpacas (Cox et al., 2014b) | 8 | 108 \pm 28 | 2.80 \pm 1.30 | 1673 \pm 579 | 16.90 \pm 8.30 |
| Bamboo sharks (Steeil et al., 2014) | 8 | 52.08 \pm 16.03 | 0.37 \pm 0.14 | - | 2.02 \pm 4.62 |
| Juvenile green iguanas (Thuesen et al., 2009) | 10 | 35 \pm 12 | 0.33 \pm 0 | 190 \pm 0 | 3.90 \pm 0 |
| Atlantic horseshoe crabs (Steeil et al., 2014) | 8 | 26.01 \pm 3.84 | 0.62 \pm 0.31 | - | 37.70 \pm 9.04 |
| Hermann's tortoises (Nardini et al., 2014) | 8 | 27.37 \pm 4.43 | 1.22 \pm 1.14 | 220.35 \pm 36.18** | 20.78 \pm 12.62 |
| Sea otters (Lee et al., 2016) | 8 | 70.60 \pm 14.60 | 2.90 \pm 1.50 | 3447.8 \pm 439 | 41.60 \pm 4.70 |
| Red-eared slider (Sypniewski et al., 2017) | 10 | 32.30 \pm 23.80 | 2.40 \pm 1.20 | 190 \pm 54 | 6.80 \pm 1.20 |

*AUC_{0-96h}**AUC_{0-last}

Table 1-4 Percentage of *in-vitro* PPB of cefovecin in some species

| Species | Concentrations (µg/mL) | Plasma protein binding (%) |
|--|---------------------------|-------------------------------|
| Dogs (Stegemann et al., 2006b) | 10, 25, 50, 100 | 96 – 98.7 |
| | 200 | 72.6 |
| | 300 | 56.4 |
| Cats (Stegemann et al., 2006c) | 10, 25, 50, 100 | 99.5 – 99.8 |
| | 200 | 82.7 |
| | 300 | 66.6 |
| Squirrel monkeys (Papp et al., 2010) | 0.05, 0.5, 5 | 92.1 – 89.1 |
| | 50 | 83.6 |
| Cynomolgus macaques (Papp et al., 2010, Raabe et al., 2011) | 0.05, 0.5, 5, 50 | 97.7 – 96.2 |
| | 0.1, 1, 10, 100 | 82.5 – 88.5 |
| Rhesus macaques (Papp et al., 2010) | 0.05, 0.5, 5 | 97.8 – 97.4 |
| | 50 | 93.8 |
| Alpacas (Cox et al., 2014b) | 1.5, 3.5, 35, 150 | 69 – 91 (79 ± 10) |
| Hermann's tortoises (Nardini et al., 2014) | 1, 5, 10 | 37.9 – 50.2 |
| Sea otters (Lee et al., 2016) | 0.5, 5, 50 | 78 |

1.2.7.2.4 Antibacterial activity of cefovecin

The antibacterial susceptibilities of a wide range of pathogens (1,660 canine and 981 feline isolates) to cefovecin were tested (Stegemann et al., 2006a) (summarised in Table 1-5). The MIC₉₀ of cefovecin to inhibit growth of susceptible canine and feline pathogens like *Staphylococcus pseudintermedius*, *E. coli* and *S. aureus* were 0.25, 1 and 2 µg/mL, respectively (Stegemann et al., 2006a).

Table 1-5 Susceptibility of canine and feline pathogens collected in the European Union and the United States to cefovecin (Stegemann et al., 2006a)

| Pathogen group (no. of isolates tested; origin) | MIC (µg/ml) | | | |
|--|-------------|--------|--------|-----------------|
| | Mode ** | 50 % | 90 % | Range |
| <i>S. intermedius</i> (270; European Union) | 0.12 | 0.12 | 0.25 | ≤ 0.06 – 8 |
| <i>S. intermedius</i> (231; United States) | 0.12 | 0.12 | 0.25 | ≤ 0.06 – > 32 |
| <i>S. aureus</i> (36; European Union) | 1 | 1 | 2 | 0.50 – > 32 |
| Coagulase-negative <i>Staphylococcus</i> spp. (21; European Union) | 0.12 | 0.25 | 4 | 0.12 – 32 |
| Coagulase-negative <i>Staphylococcus</i> spp. (89; United States) | 0.12 | 0.12 | 2 | ≤ 0.06 – 8 |
| Coagulase-positive <i>Staphylococcus</i> spp. (24; European Union) | 0.25 | 0.25 | 0.50 | 0.12 – > 32 |
| β-Hemolytic <i>Streptococcus</i> spp. (86; European Union) | ≤ 0.06 | ≤ 0.06 | 0.12 | ≤ 0.06 – 16 |
| β-Hemolytic <i>Streptococcus</i> spp. (22; United States) | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 – 8 |
| <i>S. canis</i> (66; United States) | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 – ≤ 0.06 |
| <i>Streptococcus</i> spp. (27; United States) | ≤ 0.06 | ≤ 0.06 | 0.50 | ≤ 0.06 – 0.50 |
| <i>Enterococcus</i> spp. (31; European Union) | > 32 | > 32 | > 32 | ≤ 0.06 – > 32 |
| <i>Enterococcus</i> spp. (45; United States) | > 32 | > 32 | > 32 | ≤ 0.06 – > 32 |
| <i>P. multocida</i> (193; European Union) | ≤ 0.06 | ≤ 0.06 | 0.12 | ≤ 0.06 – 2 |
| <i>P. multocida</i> (188; United States) | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 – 0.12 |
| <i>E. coli</i> (260; European Union) | 0.50 | 0.50 | 1 | 0.12 – > 32 |
| <i>E. coli</i> (223; United States) | 0.50 | 0.50 | 1 | 0.12 – > 32 |
| <i>Proteus</i> spp. (71; European Union) | 0.25 | 0.25 | 0.25 | 0.12 – 8 |
| <i>P. mirabilis</i> (110; United States) | 0.25 | 0.25 | 0.50 | 0.12 – 0.50 |
| <i>Klebsiella</i> spp. (11; European Union) | 0.50 | 0.50 | 1 | 0.25 – 1 |
| <i>K. pneumoniae</i> (16; United States) | 0.50 | 0.50 | 1 | 0.25 – 2 |
| <i>Enterobacter</i> spp. (39; European Union) | 1 | 1 | 32 | 0.12 – > 32 |
| <i>Enterobacter cloacae</i> (20; United States) | 2 | 1 | 2 | 0.50 – 8 |
| <i>Pantoea agglomerans</i> (23; United States) | 0.25 | 0.25 | 1 | ≤ 0.06 – 2 |
| <i>Acinetobacter baumannii</i> (16; United States) | 16 | 16 | 32 | 8 – 32 |
| <i>Prevotella</i> spp. (75; European Union) | 0.25 | 0.25 | 1 | ≤ 0.06 – 8 |
| <i>Prevotella</i> spp. (11; United States) | ≤ 0.06 | 0.25 | 4 | ≤ 0.06 – 8 |
| <i>Fusobacterium</i> spp. (26; European Union) | ≤ 0.06 | 0.12 | 1 | ≤ 0.06 – 2 |
| <i>Fusobacterium</i> spp. (66; United States) | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 – 1 |
| <i>Bacteroides</i> spp. (32; European Union) | 0.25 | 0.25 | 2 | ≤ 0.06 – 8 |
| <i>Clostridium</i> spp. (15; European Union) | 0.25 | 0.50 | 16 | ≤ 0.06 – > 32 |
| <i>Peptostreptococcus</i> spp. (21; European Union) | 0.50 | 0.50 | 1 | 0.12 – 2 |
| <i>Porphyromonas</i> spp. (29; United States) | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 | ≤ 0.06 |
| <i>Corynebacterium</i> spp. (11; United States) | 1 | 1 | 4 | 0.25 – > 32 |

*The information in the Table was extracted from a table in ‘Antimicrobial activity and spectrum of cefovecin, a new extended-spectrum cephalosporin, against pathogens collected from dogs and cats in Europe and North America’ by Stegemann et al. (2006) in *Antimicrobial Agents and Chemotherapy*, 50, 7, p: 2288-2290.

** Mode: most often occurring MIC value

1.2.7.3 Amoxicillin

Amoxicillin (INN) or amoxycillin (ANN) is a broad-spectrum, acid-stable, semi-synthetic aminopenicillin antibacterial of the beta-lactam group with a chemical formula of $C_{16}H_{19}N_3O_5S$ (illustrated in Figure 1-7). It is active against gram-positive and some gram-negative pathogens (both aerobic and anaerobic) (Papich and Riviere, 2009). Amoxicillin is sensitive to destruction by beta-lactamases produced by some bacteria such as *Staphylococcus* spp. and *Enterobacteriaceae*. These enzymes rupture the beta-lactam ring and could be partly or exclusively responsible for the resistance of many bacteria to beta-lactam antibacterials (Papich and Riviere, 2009). Therefore, amoxicillin is commonly administered with beta-lactamase inhibitors such as clavulanic acid or sulbactam which are potent, irreversible inhibitors of many beta-lactamases (Neu and Fu, 1978, Papich and Riviere, 2009, Prescott, 2013a). Clavulanic acid, isolated from the culture fluid of gram-positive bacteria *Streptomyces clavuligerus*, also has a beta-lactam structure but weak antibacterial activity (Reading and Cole, 1977, Neu and Fu, 1978). Potassium clavulanate is commonly combined with penicillins such as amoxicillin (Papich and Riviere, 2009).

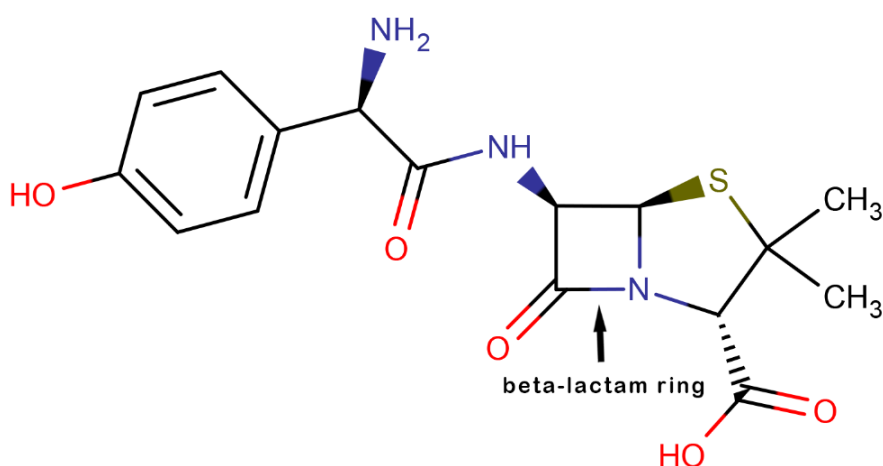


Figure 1-7 Amoxicillin structure, the Figure is modified from the DrugBank database (2017), <https://www.drugbank.ca/drugs/DB01060> [accessed 29 Aug 2017]

Amoxicillin/clavulanic acid (INN) or co-amoxiclav (BAN) is registered for use in humans and animals. This combination is available in oral and injectable formulations such as palatable tablets (50, 250 and 500 mg), palatable drops (amoxicillin trihydrate 50 mg/mL and potassium clavulanate 12.5 mg/mL), injectable suspension (amoxicillin trihydrate 140 mg/mL and clavulanate potassium 35 mg/mL in a fractionated coconut oil base) and as intra-mammary antibacterial for lactating cows in Australia with many generic formulations available. Amoxicillin is indicated in humans and in many animal species for treating respiratory, gastroenteric, urinary tract, soft tissue and other infections (Palmer et al., 1976, Keefe, 1977, Prescott, 2013a).

1.2.7.3.1 Administration and dosage

The usual dosage of amoxicillin in animals is 10 to 20 mg/kg p.o. (twice or three times daily) or i.m. and s.c. (twice daily) (Prescott, 2013a). Amoxicillin is administered to koalas at a dose-rate 10 – 12.5 mg/kg (alone, or in combination with clavulanic acid) to treat some bacterial infections either p.o., i.m. or s.c., although some clinicians avoid oral administration of this drug to koalas p.o. (Blanshard and Bodley, 2008) as it reportedly alters the normal gastrointestinal flora resulting in dysbiosis and death (personal communication with Dr Amber Gillett Veterinarian, Australia Zoo Wildlife Hospital (AZWH), Beerwah, QLD).

1.2.7.3.2 Mechanism of action

Amoxicillin like other beta-lactam antibacterials binds to penicillin-binding proteins (PBPs) and interferes with cell wall synthesis, killing susceptible bacteria (Papich and Riviere, 2009).

With negligible intrinsic antibacterial activity, clavulanic acid irreversibly binds to a wide variety of beta-lactamases (Papich and Riviere, 2009), thereby, protecting beta-lactamase sensitive penicillins such as amoxicillin and increasing amoxicillin's efficacy against many

species of bacteria. The combination of these two drugs is usually bactericidal at one or two dilutions below the MIC compared to when amoxicillin is used alone (Prescott, 2013b).

1.2.7.3.3 Pharmacokinetics of amoxicillin in some species

The PK profile of amoxicillin, alone or in combination with clavulanic acid, has been studied in humans (Arancibia et al., 1980) and many other species including dogs (Ten Voorde et al., 1990, Kung and Wanner, 1994), sheep (Craigmill et al., 1992, Carceles et al., 1995a, Elsheikh et al., 1999, Fernandez et al., 2007), goats (Carceles et al., 1995a, Carceles et al., 1995b, Escudero et al., 1996, Elsheikh et al., 1999), turkeys (Carceles et al., 1995c, Jerzsele et al., 2011), horses (Wilson et al., 1988, Baggot et al., 1988, Ensink et al., 1992), cows (Rutgers et al., 1980, Nouws et al., 1986), chickens (Carceles et al., 1995c, Jerzsele et al., 2009), pigeons (Escudero et al., 1998), pigs (AgersØ and Friis, 1998, Anfossi et al., 2002, Reyns et al., 2007), the northern elephant seal (*Mirounga angustirostris*), the Pacific harbor seal (*Phoca vitulina*) (Gulland et al., 2000) and tammar wallabies (*Macropus eugenii*) (McLelland et al., 2009). Co-administration of amoxicillin with clavulanic acid does not appear to alter the PK profile of amoxicillin in any species (Reyns et al., 2007, Baggot and Giguère, 2013).

Two chemical formulations of amoxicillin are available: sodium and trihydrate. The former is rapidly absorbed, reaches a maximum blood concentration within an hour and is suitable for i.v., i.m. and s.c. injection while the latter is more stable and has a slower absorption profile to prolong the action and may be injected i.m. or s.c. (Papich and Riviere, 2009). After i.v. injection of sodium amoxicillin, the plasma concentration falls to its half concentration in less than 2 h in all species (see Table 1-6).

Generally, penicillins distribute within the intravascular spaces and extracellular fluid and, in most studies, the $V_{d_{ss}}$ of amoxicillin in horses (Wilson et al., 1988, Ensink et al., 1992), foals (Baggot et al., 1988), cows (Rutgers et al., 1980), goats (Craigmill et al., 1992, Carceles et al.,

1995a, Carceles et al., 1995b, Escudero et al., 1996), sheep (Craigmill et al., 1992, Craig, 1995, Fernandez et al., 2007) and dogs (Kung and Wanner, 1994) ranges between 0.1 to 0.3 L/kg which is consistent among penicillins. The $V_{d_{ss}}$ appears higher in birds (Carceles et al., 1995c, Escudero et al., 1998, Abo El-Sooud et al., 2004, Jerzsele et al., 2011) and pigs (AgersØ and Friis, 1998) with the exception of one study that reported lower $V_{d_{ss}}$ of amoxicillin in pigs [0.34 L/kg, (Reyns et al., 2007)]. This V_d indicates that amoxicillin could reach intracellular concentrations in these species (AgersØ and Friis, 1998, Papich and Riviere, 2009). Plasma protein binding of amoxicillin has been studied in chickens [1.63 – 15.5 %, (Abo El-Sooud et al., 2004)], dogs [13 %, (Abo El-Sooud et al., 2004)] and humans [17 – 18 %, (Sutherland et al., 1972, Abo El-Sooud et al., 2004)].

Aminopenicillins are metabolised somewhat by hydrolysis of the beta-lactam ring, producing microbiologically-inactive metabolites. Facilitated by their relatively low protein binding, aminopenicillins and their metabolites are excreted by glomerular filtration and active tubular secretion (Papich and Riviere, 2009). A summary of the PK parameters and indices of amoxicillin after i.v. administration is provided in Table 1-6.

Table 1-6 Pharmacokinetic parameters and indices of amoxicillin sodium after i.v. administration in various species (mean \pm SD)

| Species | dose (mg/kg) | AUC _{0-∞} (mg.h/L) | Vd _{ss} (L/kg) | Cl _b (L/h/kg) | MRT (h) | t _{1/2} (h) |
|--|-----------------|--------------------------------|----------------------------|-----------------------------|-----------------|-------------------------|
| Horse | 10 | 37.0 \pm 3.82 | 0.19 \pm 0.01 | 0.27 \pm 0.03 | - | 1.43 \pm 0.28 |
| (Wilson et al., 1988, Ensink et al., 1992, Errecalde et al., 2001) | 10 | - | 0.32 \pm 0.07 | 0.34 \pm 0.05 | - | 0.66 \pm 0.06 |
| | 40 | 42.61 \pm 7.43 | 0.43 \pm 0.16 | 0.44 \pm 0.047 | - | 0.84 \pm 0.40 |
| Foal | 20 | - | 0.26 \pm 0.80 | 0.34 \pm 0.46 | - | 0.74 \pm 0.06 |
| (Baggot et al., 1988) | | | | | | |
| Cow | 4 | 11.09 \pm 0.98 | 0.27 \pm 0.01 | 0.36 \pm 0.01 | - | 1.34 \pm 0.26 |
| (Rutgers et al., 1980) | | | | | | |
| Pig | 9 | 23.50 \pm 3.70 | 0.55 \pm 0.05 | 0.37 \pm 0.06 | 1.50 \pm 0.20 | - |
| (AgersØ and Friis, 1998, | 9 | 17 \pm 3.40 | 0.63 \pm 0.17 | 0.52 \pm 0.10 | 1.20 \pm 0.20 | - |
| Reyns et al., 2007) | 20 | 35.00 \pm 5.64 | 0.34 \pm 0.09 | 0.58 \pm 0.09 | 0.60 \pm 0.17 | 0.37 \pm 0.26 |
| Goat | 20 | 186.17 \pm 21.33 | 0.16 \pm 0.02 | 0.11 \pm 0.01 | 1.52 \pm 0.20 | |
| (Craigmill et al., 1992, | 20 | 185.51 \pm 9.81 | 0.17 \pm 0.02 | 0.11 \pm 0.01 | 1.57 \pm 0.09 | 1.20 \pm 0.17 |
| Carceles et al., 1995a, | 10 | 14.91 \pm 2.15 | 0.47 \pm 0.259 | 0.68 \pm 0.096 | - | 1.11 |
| Carceles et al., 1995b, | | | | | | |
| Escudero et al., 1996) | | | | | | |
| Sheep | 20 | 231.36 \pm 23.15 | 0.16 \pm 0.00 | 0.09 \pm 0.01 | 1.82 \pm 0.19 | 1.43 \pm 0.16 |
| (Carceles et al., 1995a, | 10 | 21.83 \pm 8.00 | - | - | 0.48 \pm 0.15 | 0.38 \pm 0.09 |
| Fernandez et al., 2007), 11, | 10 | 16.73 \pm 1.85 | 0.22 \pm 0.02 | 0.61 \pm 0.07 | - | 0.77 |
| Chicken | 10 | 12.81 \pm 0.55 | 0.98 \pm 0.07 | 0.78 \pm 0.03 | 0.88 \pm .030 | 1.28 \pm 0.05 |
| (Carceles et al., 1995c, Abo | 20 | 16.75 \pm 2.31 | 1.59 \pm 0.13 | 1.19 \pm 0.08 | 1.17 \pm 0.14 | 1.03 \pm 0.11 |
| El-Sooud et al., 2004, | 40 | 13.85 \pm 0.14 | 1.00 \pm 0.02 | 0.80 \pm 0.006 | - | 1.07 \pm 0.02 |
| Jerzsele et al., 2009) | | | | | | |
| Turkey | 10 | 12.72 \pm 0.28 | 0.70 \pm 0.03 | 0.78 \pm 0.02 | 0.89 \pm 0.05 | 1.28 \pm 0.03 |
| (Carceles et al., 1995c, | 20 | 19.97 \pm 2.00 | 1.52 \pm 0.09 | 1.00 \pm 0.09 | 1.28 \pm 0.16 | 1.12 \pm 0.09 |
| Jerzsele et al., 2011), | | | | | | |
| Dog | 20 | - | 0.31 \pm 0.102 | 0.20 \pm 0.07 | 1.60 \pm 0.40 | - |
| (Kung and Wanner, 1994) | | | | | | |
| Beagle | 15 | 56.45 \pm 7.21 | - | 0.27 \pm 0.03 | - | 1.18 \pm 0.13 |
| (Ten Voorde et al., 1990) | | | | | | |
| Pigeon | 20 | 19.82 \pm 1.98 | 0.99 \pm 0.06 | - | 1.47 \pm 0.15 | 1.22 \pm 0.09 |
| (Escudero et al., 1998) | | | | | | |

Amoxicillin trihydrate when injected i.m. to horses and pigs has a half-life of 20.65 h (Wilson et al., 1988) and 15.5 h (AgersØ and Friis, 1998), respectively and for dogs and cows, 6.8 and 8.8 h, respectively (Nouws et al., 1986, Ten Voorde et al., 1990). The shorter terminal half-life of amoxicillin trihydrate administered i.m. to chickens, goats, sheep and tammar wallabies (1 – 2 h) demonstrates rapid elimination in these species (Elsheikh et al., 1999, Abo El-Sooud et al., 2004, McLelland et al., 2009). Following i.m. administration of amoxicillin trihydrate, C_{\max} was reached at 15 min in chickens (Abo El-Sooud et al., 2004), 1 h in sheep and goats (Elsheikh et al., 1999, Fernandez et al., 2007) and 2 h in tammar wallabies (McLelland et al., 2009). Some PK parameters and indices of i.m. amoxicillin administration are provided in Table 1-7.

Table 1-7 Pharmacokinetic parameters and indices following i.m. administration of amoxicillin sodium and amoxicillin trihydrate in various species (mean \pm SD)

| Species | dose (mg/kg) | C _{max} (μ g/mL) | T _{max} (h) | AUC _{0-∞} (mg.h/L) | MRT (h) | t _{1/2} (h) |
|---|-----------------|-----------------------------------|-------------------------|--|-----------------|-------------------------|
| Amoxicillin sodium | | | | | | |
| Sheep (Fernandez et al., 2007) | 10 | 13.42 \pm 5.36 | 0.36 \pm 0.21 | 15.05 \pm 1.82 | 1.07 \pm 0.30 | 0.55 \pm 0.15 |
| Turkeys (Carceles et al., 1995c) | 20 | 8.03 \pm 1.23 | 0.38 \pm 0.04 | 15.27 \pm 1.54 | 1.86 \pm 0.17 | 1.44 \pm 0.10 |
| Chickens (Carceles et al., 1995c) | 20 | 7.99 \pm 1.01 | 0.38 \pm 0.05 | 14.32 \pm 1.22 | 1.51 \pm 0.13 | 1.31 \pm 0.09 |
| Pigeon (Escudero et al., 1998)* | 5 | 5.81 \pm 0.85 | 0.38 \pm 0.05 | 15.06 \pm 1.67 | 1.77 \pm 0.19 | 1.52 \pm 0.09 |
| Amoxicillin trihydrate | | | | | | |
| Horse (Wilson et al., 1988) | 10 | - | - | 33.43 \pm 6.07 | - | 20.65 \pm 9.98 |
| Pig (AgersØ and Friis, 1998) | 14.7 | 5.10 \pm 0.80 | 2.00 \pm 0.70 | 33.10 \pm 3.90 | 8.80 \pm 2.60 | 15.5 \pm 8.30 |
| Dog (Ten Voorde et al., 1990) | 15 | 7.64 \pm 3.91 | 1.61 \pm 0.50 | 57.40 \pm 12.59 | - | 6.98 \pm 3.85 |
| Cows (Nouws et al., 1986) | 10 | 2.60 \pm 1.10 | 2.60 \pm 0.60 | 28.50 \pm 7.20* | - | 8.80 \pm 5.00 |
| Pre-ruminant calves (Nouws et al., 1986) | 10 | 6.00 \pm 2.50 | 2.40 \pm 1.10 | 53.20 \pm 21.50* | - | 5.80 \pm 2.20 |
| Ruminant calves (Nouws et al., 1986) | 10 | 3.80 \pm 0.97 | 1.90 \pm 0.90 | 24.8 \pm 1.46* | - | 2.90 \pm 0.60 |
| Sheep (Fernandez et al., 2007) | 10 | 2.48 \pm 0.54 | 0.98 \pm 0.15 | 15.40 \pm 1.05 | 8.57 \pm 2.78 | - |
| Tammar wallaby (McLelland et al., 2009) | 10 | 4.51 \pm 0.74 | 2.00 \pm 0.00 | 14.82 \pm 4.10 | 4.03 \pm 0.85 | 1.88 \pm 0.60 |
| Nubian goat (Elsheikh et al., 1999) | 10 | 11.03 \pm 0.97 | 0.85 \pm 0.11 | 28.10 \pm 3.03 | 2.03 \pm 0.25 | 1.05 \pm 0.14 |
| Desert sheep (Elsheikh et al., 1999) | 10 | 9.47 \pm 1.33 | 0.98 \pm 0.15 | 25.21 \pm 2.15 | 2.15 \pm 0.16 | 1.19 \pm 0.16 |
| Chickens (Abo El-Sooud et al., 2004) | 10 | 5.20 \pm 0.23 | 0.26 \pm 0.04 | 10.67 \pm 0.43 | - | 1.09 \pm 0.034 |

*Data is AUC_{0-last time}

Bioavailability of amoxicillin varies among species from 0.50 to 0.70 orally and 0.70 to 1.20 intramuscularly (documented in Table 1-8). This could be explained by interspecies biological variation and the use of different sites of injection (Abo El-Sooud et al., 2004). One study

demonstrated that the injection site affected amoxicillin absorption in dairy cows which could be attributed to the administration to areas with different blood flow (Rutgers et al., 1980). It has also been reported that injection into the neck muscle is absorbed more rapidly and completely than an injection into the gluteal muscles or rear legs in both cattle and horses (Papich and Riviere, 2009). Another study reported that the age of ruminants had an effect on the relative systemic availability; there was a higher AUC of the amoxicillin trihydrate in pre-ruminant calves (3 – 4 weeks) than in 5-month-old ruminant calves and dairy cows (shown in Table 1-7).

Table 1-8 Bioavailability of amoxicillin (p.o. and i.m.) in different species (mean \pm SD)

| Species | Route | |
|---------------------|--|---|
| | p.o. | i.m. |
| Horse | 0.10 (Wilson et al., 1988) 0.50 \pm 0.01 (Ensink et al., 1992) | - |
| Pig | 0.23 \pm 0.06 (Reyns et al., 2007) 0.31 \pm 0.15 (AgersØ and Friis, 1998) 0.38 \pm 0.16 (Reyns et al., 2009) | 0.82 \pm 0.08 (AgersØ and Friis, 1998) |
| Goat | 0.27 \pm 0.04 (Carceles et al., 1995b) | - |
| Nubian goat | - | 0.91 \pm 0.09 (Elsheikh et al., 1999) |
| Sheep | - | 0.73 (Fernandez et al., 2007) |
| Desert sheep | - | 0.95 \pm 0.06 (Elsheikh et al., 1999) |
| Foal | 0.36 \pm 0.13 (Baggot et al., 1988) | - |
| Pre-ruminant calves | 0.37 \pm 0.07 (Soback et al., 1987) | - |
| Chicken | 0.64 \pm 0.03 (Jerzsele et al., 2009) 0.61 \pm 0.03 (Abo El-Sooud et al., 2004) | 0.85 \pm 0.05 (Carceles et al., 1995c) 0.77 \pm 0.03 (Abo El-Sooud et al., 2004) |
| Turkey | 0.60 \pm 0.03 (Jerzsele et al., 2011) | 0.78 \pm 0.05 (Carceles et al., 1995c) |
| Dog | 0.64 \pm 0.18 (Kung and Wanner, 1994) | 1.03 \pm 0.23 (Ten Voorde et al., 1990) |
| Pigeon | - | 0.76 \pm 0.07 (Escudero et al., 1998) |
| Cows | - | 1.19 \pm 0.15 (Rutgers et al., 1980) |

1.2.7.3.4 Antibacterial activity of amoxicillin

At AZWH, amoxicillin (with or without clavulanic acid) is used to treat pneumonia in koala joeys but is not administered to mature koalas due to a perceived risk of inducing dysbiosis. There is a great variation in bacterial species isolated from clinical cases with pneumonia in captive or free-ranging koalas. Potential primary or secondary pathogens isolated from koalas with pneumonia and / or other respiratory tract pathology include *Bordetella bronchiseptica*, *Pseudomonas* spp., *Streptobacillus moniliformis*, *Staphylococcus epidermidis*, *Nocardia asteroides*, *Cryptococcus neoformans* complex, *Corynebacterium* spp., *Enterobacter agglomerans*, *Proteus* spp., α -haemolytic *Streptococcus* spp., *diphtheroids*, *Pasteurella* spp., *S. aureus*, *E. coli* and *Acinetobacter lwoffii* (Blanshard and Bodley, 2008).

The MIC of ampicillin (used to test the susceptibility of pathogens to amoxicillin) has been reported for susceptible strains of *E. coli*, *Staphylococcus* spp., *Streptococci* (β -haemolytic group and viridans group) as (≤ 0.25 $\mu\text{g/ml}$), *Listeria* spp. (≤ 2 $\mu\text{g/ml}$), and *Enterobacteriaceae* and *Enterococci* (≤ 8 $\mu\text{g/ml}$) (CLSI, 2013b).

The MIC₉₀ concentrations of amoxicillin/clavulanic acid for many susceptible gram-positive and gram-negative pathogens are less than 0.50 $\mu\text{g/mL}$ (Prescott, 2013b) (see Table 1-9). The spectrum of activity of amoxicillin/clavulanic acid is similar to that of a first- or second-generation cephalosporins (Prescott, 2013b).

Table 1-9 MIC₉₀ (µg/mL) of amoxicillin/clavulanic acid against selected veterinary pathogens (Prescott, 2013b)

| Organism | MIC ₉₀ | Organism | MIC ₉₀ |
|------------------------------|-------------------|---------------------------|-------------------|
| Gram-positive cocci | | | |
| <i>S. aureus</i> | 0.50 | <i>S. disgalactiae</i> | ≤ 0.13 |
| <i>S. intermedius</i> * | 0.25 | <i>S. suis</i> | ≤ 0.13 |
| <i>S. agalactiae</i> | ≤ 0.13 | | |
| Gram-positive rods | | | |
| <i>A. pyrogens</i> | 0.25 | <i>L. monocytogenes</i> | 0.25 |
| Gram-negative aerobes | | | |
| <i>A. pleuropneumoniae</i> | 0.50 | <i>P. multocida</i> | 0.25 |
| <i>B. bronchiseptica</i> | 2 | <i>Pseudomonas</i> spp. | ≥ 32 |
| <i>E. coli</i> | 8 | <i>P. mirabilis</i> | 0.50 |
| <i>H. somni</i> | 0.06 | <i>Salmonella</i> | 2 |
| <i>M. bovis</i> | 0.06 | | |
| <i>M. haemolytica</i> | 0.13 | | |
| Anaerobic bacteria | | | |
| <i>B. fragilis</i> | 0.50 | <i>P. asaccharolytica</i> | 1 |
| <i>C. perfringens</i> | 0.50 | <i>Fusobacterium</i> spp. | ≥ 32 |

The Table is from ‘Other Beta-lactam Antibiotics: Beta-lactamase inhibitors, Carbapenems, and Monobactams’ in *Antimicrobial Therapy in Veterinary Medicine* by Giguère, S. et al. (2013), 5th edition, p: 179.

* *S. intermedius* is now named *S. pseudintermedius* (Devriese et al., 2005)

1.3 Aims of the thesis

A significant challenge in veterinary medicine is to avoid blindly extrapolating a drug dosage between species which might have significant differences in their anatomy, biochemistry, physiology, dietary or behavioural characteristics (Toutain et al., 2010). Biological differences can cause numerous interspecies differences in the PK profile of drugs (Toutain et al., 2010). The koala is a good example where dosages that were extrapolated from those for dogs and cats resulted in surprising and less than ideal PK profiles. Recent published studies confirm that extrapolation of dosage regimens from other species to the koala seems inappropriate and

currently suggested dose regimens of some drugs such as enrofloxacin, marbofloxacin, fluconazole, florfenicol and meloxicam are insufficient to provide therapeutic plasma concentrations in the koala (Griffith et al., 2010, Govendir et al., 2012, Black et al., 2013b, Kimble et al., 2013a, Black et al., 2014, Budd et al., 2017).

Antimicrobials are frequently administered to koalas to treat infectious diseases and the dosage regimen used for koalas has traditionally been extrapolated from domesticated animal species such as the dog and cat or based on ‘trial and error’ with minimal evaluation of drug efficacy (Blanshard and Bodley, 2008, Griffith, 2010). Thus, this research project was undertaken to investigate the PK of three antimicrobials in koalas. Two drugs cefovecin and amoxicillin were selected due to specific requests by veterinarians – Dr Larry Vogelneust, Senior Veterinarian at Taronga Zoo, Mosman, NSW, and Dr Amber Gillette, Senior Veterinarian at AZWH, Beerwah, QLD, to better understand these drugs’ specific PK profiles in the koala and to determine the applicability of a long-acting cephalosporin cefovecin in selected marsupials by screening the *in-vitro* plasma protein binding. The *in-vivo* PK of the third drug posaconazole in clinically normal koalas was investigated to evaluate it as an alternative for fluconazole to treat cryptococcosis.

As explained in the relevant research chapters, the HPLC with UV detection method was problematic for cefovecin and amoxicillin and resulted in low sensitivity of both assays which was not ideal for PK studies. Therefore, microbiological methods were also trialled to estimate plasma concentrations of these drugs.

The antibacterial activities of koala plasma and serum were assessed in the last chapter. Although the studies in this chapter were not part of the original objectives of this thesis, after conducting the microbiological assays in Chapter 6, they were undertaken to assist in understanding the variation in the results of broth microdilution inhibition assay over different time points of individual koalas and to provide some information of antibacterial activities of

koala plasma and serum. This information could be fundamentals for future research to assist koalas overcome infectious disease.

Thus, the specific objectives of this research were:

- 1) To develop and validate HPLC-PDA methods to quantify plasma concentrations of the antifungal posaconazole and the antibacterials cefovecin and amoxicillin in koala plasma
- 2) To investigate the PK of posaconazole, cefovecin and amoxicillin in koalas and to use the PK profiles of each drug in combination of PD principles to estimate the efficacy of current dosage regimens
- 3) To determine the *in-vitro* plasma protein binding of cefovecin in selected Australian marsupials in order to evaluate whether cefovecin has therapeutic application in these marsupials
- 4) To develop microbiological assays to determine cefovecin and amoxicillin concentrations in koala plasma in order to confirm the HPLC results of both drugs
- 5) To screen the antibacterial activity of koala plasma and serum against gram-positive and gram-negative pathogens and to compare this activity with those from other selected species

Chapter 2

**Pharmacokinetics of posaconazole in koalas
after intravenous and oral administrations**

2.1 Abstract

Posaconazole is a third-generation antifungal drug with a broad-spectrum anti-fungal activity. It is registered for use in humans and reportedly has excellent activity to inhibit all *Cryptococcus* spp. serotypes tested *in vitro*, including isolates with reduced fluconazole susceptibility. Posaconazole has been used to treat a variety of fungal infections in dogs and cats with minimal adverse effects.

The PK profile of posaconazole in clinically normal koalas after a single oral dose of 6 mg/kg ($n = 6$) and a single intravenous bolus of 3 mg/kg ($n = 2$) was investigated. Serial plasma samples were collected over 24 h (i.v.) and 36 h (p.o.), and plasma concentrations of posaconazole were quantified by validated HPLC assays. A non-compartmental PK analysis of the data was performed. Following i.v. administration, estimates of the median (range) of plasma Cl and V_{dss} were 0.15 (0.13 – 0.18) L/h/kg and 1.23 (0.93 – 1.53) L/kg, respectively. The median (range) elimination half-life after i.v. and p.o. administration was 7.90 (7.62 – 8.18) and 12.58 (10.06 – 15.99) h, respectively. Oral bioavailability varied from 0.43 to 1.06 (median: 0.64). Following oral administration, C_{max} (median: 0.73, range: 0.55 – 0.93 $\mu\text{g/mL}$) was achieved in 8 h (range: 6 – 12 h). The *in-vitro* plasma protein binding of posaconazole was 99.25 ± 0.29 %. Based on posaconazole PK/PD targets for some yeasts such as disseminated candidiasis, posaconazole is predicted to be an efficacious treatment for cryptococcosis in koalas.

2.2 Introduction

The systemic fungal disease cryptococcosis affects many species including koalas (Krockenberger et al., 2002). *Cryptococcus gattii* is considered the principle pathogen responsible for cryptococcosis in koalas and is part of the *Cryptococcus neoformans* species complex comprised of *C. gattii* and *C. neoformans* (Krockenberger et al., 2003, Chen et al.,

2014). The spectrum of presentation of this infection in koalas varies from asymptomatic carriage of cryptococcal organisms in the nasal mucosa, subclinical disease, localised disease of the nasal cavity to severe systemic disease often with nervous involvement (Krockenberger et al., 2002). Colonisation of this fungal yeast among respiratory tract cells with no clinical signs is attributed to the close association between koalas and *Eucalyptus* spp. trees which are natural hosts for *C. gattii* (Ellis and Pfeiffer, 1990, Krockenberger et al., 2002). The interactions between host, pathogen and environment is complex and occasionally lead to clinical disease affecting approximately 2.5 % of koalas necropsied at The University of Sydney between 1980 and 2003 (Stalder, 2003).

Drugs used for the systemic treatment of clinical cryptococcosis in koalas include fluconazole (Woods and Blyde, 1997, Wynne et al., 2012, Kido et al., 2012, Govendir et al., 2015), itraconazole (Kido et al., 2012, Woods and Blyde, 1997, Wynne et al., 2012) and ketoconazole (Woods and Blyde, 1997). A treatment protocol of daily fluconazole administration with additional subcutaneous administration of amphotericin is also reported (Woods and Blyde, 1997, Wynne et al., 2012, Kido et al., 2012, Govendir et al., 2015). Black et al. (2014) demonstrated that oral fluconazole administration alone at 10 mg/kg twice daily is unlikely to be effective against *Cryptococcus gattii*. Moreover, therapeutic plasma concentrations (MIC_{90} to inhibit *C. gattii* = 16 µg/ml) were not attained in some infected koalas treated with fluconazole orally 10 – 15 mg/kg twice a day with or without amphotericin (Govendir et al., 2015) and some degree of nephrotoxicity is expected when amphotericin B is administered to people and animals (Burgess and Birchall, 1972, Davis et al., 2009).

Posaconazole has excellent activity to inhibit all *Cryptococcus* spp. serotypes tested *in vitro*, including isolates with reduced fluconazole susceptibility (Thompson et al., 2009) and has been used to treat a variety of fungal infections in dogs and cats with minimal adverse effects (McLellan et al., 2006, Wray et al., 2008, Krockenberger et al., 2010, Evans et al., 2011,

Corrigan et al., 2015, Cook et al., 2016). Therefore, the aim of this study was to document changes in plasma concentrations of clinically normal koalas after a single oral bolus of 6 mg/kg. Pharmacokinetic data were also obtained from samples collected from two koalas administered 3 mg/kg, i.v. On the basis of the PK profiles, it was hypothesised whether the oral dosage was likely to be efficacious against *C. gattii*.

2.3 Materials and methods

2.3.1 Intravenous administration of posaconazole

This part of the experiment was conducted by Associate Professor Merran Govendir in association with Drs Cindy Stadler and Julie Barnes at Los Angeles Zoo, California, USA as an intravenous posaconazole formulation was only available in the USA at the time of study. The PK analysis was undertaken by the author of the thesis.

The Animal Management Committee at the Los Angeles Zoo approved posaconazole administration to two long-term captive female koalas (age: both 13 years; weights: 6.4 kg and 5.6 kg). These animals were considered clinically normal on the basis of regular physical examinations that included blood collection to determine their haematology and biochemical analyte values. The koalas were anaesthetised with isoflurane in 100 % oxygen for placement of a 20-gauge, 3.75 cm intravenous catheter into the cephalic vein. A cap was attached to the catheter and flushed with heparinised saline. The cap and catheter were secured with tape and bandage, for drug administration and serial blood collection. Blood (5 mL) was collected into lithium heparin tubes to determine baseline haematological and biochemical analyte values ($t = 0$ h). Whilst under general anaesthesia, a single i.v. bolus of Noxafil (Merck, Kenilworth, NJ, USA) was administered at 3 mg/kg. After blood samples were collected at 0.25 and 0.5 h, the animals were recovered from anaesthesia. Further blood samples were then collected at 0.75, 1, 2, 4, 8, 12 and 24 h. Samples were centrifuged within 1 h of collection, and plasma transferred into plain tubes. Plasma was stored at -20°C and protected from light. After

recovery, the koalas were housed individually in pens, and supplied with food (various *Eucalyptus* spp.) and water *ad-libitum*.

2.3.1.1 Drug analysis

Samples were couriered on dry ice to the Clinical Pharmacology Laboratory at the College of Veterinary Medicine, North Carolina State University, Raleigh, NC, to determine posaconazole concentrations as documented elsewhere (Kendall and Papich, 2015) and was performed within weeks of sample collection.

2.3.2 Oral administration of posaconazole

An oral suspension of posaconazole (Noxafil, MSD, Macquarie Park, NSW) was administered to six koalas at Taronga Zoo, Mosman, NSW. This study was approved by Taronga Conservation Society Australia; Animal Ethics Committee (AEC) (protocol: 3c/06/15).

2.3.2.1 Animals

Six long-term captive koalas, three of each gender, aged 3.3 to 6.9 years (median: 4.3 years) and weight 6.7 to 8.1 kg (median: 7.5 kg), were recruited opportunistically from the Taronga Zoo, Mosman, NSW, Australia. These animals were considered clinically normal based on physical examinations and on the basis of haematological and biochemical analyte values within normal reference ranges. During the study, koalas were housed individually in pens and supplied with food (various *Eucalyptus* spp.) and water *ad-libitum*.

2.3.2.2 Drug administration and blood collection

Koalas were anaesthetised with alphaxalone (Alfaxan, Jurox Pty Ltd, Rutherford, NSW) at 3 mg/kg i.m. and maintained under anaesthesia with isoflurane in 100 % oxygen via a face mask for catheter placement as described above. A 20-gauge, 3.75 cm catheter was placed into the cephalic vein and bandaged in place for the duration of blood collection. The animals were

then recovered from anaesthesia. Blood (5 mL) was collected 2 h after recovery in lithium heparin tubes to determine baseline haematology and biochemistry analyte values ($t = 0$ h). The following morning, posaconazole suspension was administered 6 mg/kg p.o. Then, serial blood samples (up to 3 mL) were collected into lithium heparin tubes at the following time points: 1, 2, 4, 6, 8, 12, 24 and 36 h. The cap and catheter were flushed with heparinised saline after each collection. Samples were centrifuged within 1 h of collection, and plasma was transferred into plain tubes. Plasma was stored at -80°C and protected from light until analysis.

2.3.2.3 Drug Analysis

Posaconazole concentrations in plasma samples were quantified within weeks of collection at the Veterinary Pharmacology Laboratory at The University of Sydney. The HPLC system (Shimadzu, Rydalmere, NSW) consisted of a CBM-20A module, a Shimadzu LC-20AT delivery unit, DGU-20A degassing solvent delivery unit, SIL-20A auto injector, CTO-20AC column oven and SPD-20A diode array detector. A Shimadzu class VP data system (ver. 7.4; Shimadzu, Rydalmere, NSW) was used for chromatographic control, data collection, and data processing. Chromatographic separation was performed with a Synergy MAX-RP-80 Å [$4\text{ }\mu\text{m}$, $150\text{ mm} \times 4.6\text{ mm}$, (i.e. column packing particle size, column length \times internal diameter)] (Phenomenex, Lane Cove, NSW) with a 1 mm Opti-guard C-18 pre-column (Choice Analytical, Thornleigh, NSW) at ambient temperature. The isocratic mobile phase used was the mixture of 50 mM phosphate buffer (pH: 6.2) and acetonitrile (40:60 v/v) at a flow rate of 1 mL/min and the UV wavelength used for detection was 262 nm. The retention times of posaconazole and the IS (itraconazole) were approximately 4.2 and 10.9 min, respectively.

2.3.2.4 Sample preparation

A stock solution of posaconazole (1 mg/mL) and itraconazole as the IS (0.5 mg/mL) were prepared in 100 % methanol and acetonitrile, respectively. Posaconazole was further diluted with 50 % of methanol to give a series of working solutions of 0.25, 0.5, 1, 2.5, 5, 10, 25, 50 and 100 µg/mL. Prepared stock solution was stored at – 20 °C and working solutions were freshly prepared at each analysis. A working solution of the IS was prepared at the concentration of 1 µg/mL in acetonitrile. Blank pooled koala plasma ($n = 6$) was used for validating the chromatographic condition. For the preparation of plasma calibration standards (0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL) and QC samples (0.05, 0.5 and 5 µg/mL), the appropriate amount of working standard solutions of posaconazole were spiked into blank pooled koala plasma and stored at – 20 °C.

2.3.2.5 Plasma sample extraction

The proteins within the plasma samples (100 µL) were precipitated by adding 1:1 volume of acetonitrile containing 1 µg/mL of the IS and then, sonicated for 5 min and centrifuged twice at 14,000 g for 5 min. Following this, 30 µL of the supernatant was directly injected into the HPLC system.

2.3.2.6 HPLC Method validation

The selectivity was established by analysing blank koala plasma to identify endogenous interferences around the retention times of posaconazole and the IS. Posaconazole concentrations in plasma samples were quantified via calibration curves (0.0125, 0.025, 0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 µg/mL); whereby weighted least square regression ($1/x$) was used to normalise the SD of the residuals. The LLOQ was determined based on calibration curves using the formula $LLOQ = 10 \times \sigma/S$ in which σ is the SD of the y-intercepts for the regression lines and S is the mean slope of the weighted regression lines ($n = 3$) (International

Conference on Harmonisation, 2005). An acceptance criterion for LLOQ was defined as precision (CV) and accuracy within $\pm 20\%$ of the nominal concentration with repeated analyses (International Conference on Harmonisation, 2005). Intra- and inter-day precision, expressed as CV (%), were analysed from triplicates of QC samples (0.05, 0.5 and 5 $\mu\text{g/mL}$), both within a day, and on three different days, respectively. Intra- and interday accuracy, expressed as bias was determined by a percentage difference between estimated value and the nominal value of posaconazole $[100 - (\text{estimated value} / \text{nominal value} \times 100)]$. Absolute recovery of posaconazole was determined by comparing the peak area of pre-spiked plasma samples at concentrations of 0.05, 0.5 and 5 $\mu\text{g/mL}$ ($n = 3$) and 1 $\mu\text{g/mL}$ of the IS with corresponding concentrations of cefovecin and the IS in deionised water. Long-term stability (up to 5 months) of posaconazole (0.5 and 5 $\mu\text{g/mL}$; $n = 3$) in pre-spiked koala plasma samples at $-20\text{ }^{\circ}\text{C}$ was determined. Each assay was conducted in triplicate and statistical data analysis was achieved by ANOVA and unpaired student t-tests using GraphPad Prism 7.02 (GraphPad Software, Inc. CA, USA), where the minimum significance level for all statistical tests was set at $P < 0.05$.

2.3.3 Plasma protein binding assay

The proportion of posaconazole bound to koala plasma proteins was determined using frozen-thawed pooled plasma of six clinically normal koalas. In brief, the pH of pooled plasma (approximately 8) was adjusted to 7.4 and then analytical grade posaconazole was added to plasma aliquots to yield 0.5, 1 and 2.5 $\mu\text{g/mL}$ concentrations. Each concentration (as 5 replicates) were incubated in a rapid equilibrium chamber (Thermo Fisher Scientific, Scoresby, Vic.) at $37\text{ }^{\circ}\text{C}$ for 4 h at 250 rpm during which the unbound drug diffused into a chamber containing PBS (according to the manufacturer's instructions). At the end of incubation, equal volumes from each chamber were removed, and after sample preparation, as described above, injected to the HPLC system. The percentage of drug bound to the plasma proteins was determined using the following equation:

$$PPB (\%) = 100 \% - \left(\left[\frac{\text{drug concentration at buffer chamber}}{\text{drug concentration at plasma chamber}} \right] \times 100 \% \right)$$

2.3.4 Pharmacokinetic analysis

Pharmacokinetic parameters and indices for i.v. and p.o. administration were determined as a non-compartmental model using PKSolver (Zhang et al., 2010). The C_{\max} and T_{\max} were determined by visual inspection of the plasma concentration vs. time curve. The elimination rate constant (K_{el}) was estimated by semi-log linear regression of the terminal slope, and elimination half-life ($t_{1/2}$) was estimated by $\ln 2 / K_{el}$. The AUC and AUMC from 0 to last observed concentration (AUC_{0-t} and $AUMC_{0-t}$, respectively) were determined by the linear trapezoidal method. The AUC and AUMC from the last observed concentration to infinity were determined by:

$$AUC_{t-\infty} = \frac{C_{last}}{K_{el}}$$

$$AUMC_{t-\infty} = \left(\frac{C_{last} \times t_{last}}{K_{el}} \right) + \left(\frac{C_{last}}{K_{el}^2} \right)$$

The mean residence time (MRT), clearance (Cl), volume of distribution at steady state (Vd_{ss}), volume of distribution during pseudoequilibrium (V_{area}) and oral bioavailability (F) were determined by the following equations:

$$MRT = \frac{AUC_{t-\infty}}{AUMC_{t-\infty}}$$

$$Cl = \frac{dose_{i.v.}}{AUC_{(i.v.) 0-\infty}}$$

$$Vd_{ss} = Cl \times MRT$$

$$V_{area} = \frac{Cl}{K_{el}}$$

$$F = \frac{(dose_{i.v.} \times AUC_{p.o.})}{(dose_{p.o.} \times AUC_{i.v.})}$$

All PK parameters and indices were expressed as median (range).

2.4 Results

2.4.1 Method development and optimisation

The retention times of posaconazole and the IS were approximately 4.2 and 10.2 min, respectively. No endogenous interference was observed at the retention times of both posaconazole and the IS.

The Synergy MAX-RP-80 Å (4 µ, 150 × 4.6 mm) provided the best sensitivity and peak resolution of the analytes. A mixture of 60 % acetonitrile and 40 % phosphate buffer (50 mM, pH: 6.2) was the best mobile phase to ensure the sharpest (narrow) peaks with minimal interference. The chromatograms of QC samples and blank koala plasma are shown in Figure 2-1.

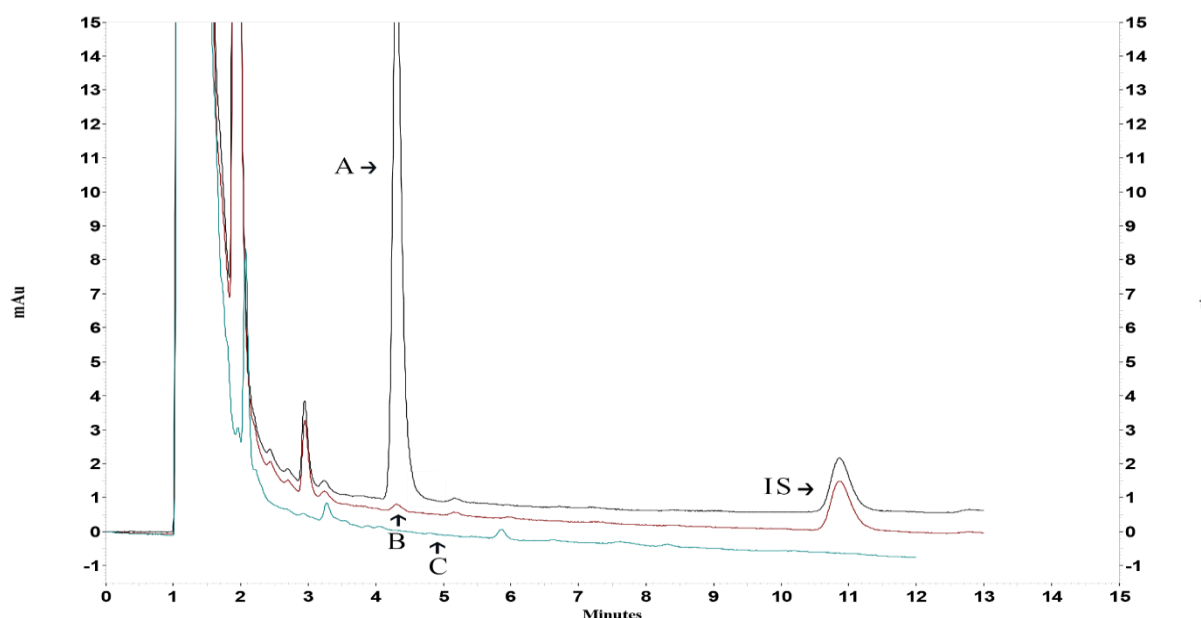


Figure 2-1 Chromatograms of high QC (5 µg/mL of posaconazole) (**A, black trace**) and low QC (0.05 µg/mL) (**B, red trace**) samples (retention time of 4.2 min); IS (itraconazole) with a retention time of 10.9 min; and (**C, green trace**) pooled blank koala plasma at the UV wavelength of 262 nm.

2.4.2 HPLC method validation

2.4.2.1 Selectivity

Pooled blank koala plasma and extracted koala samples, pre-spiked with posaconazole (0.05 and 5 µg) and IS (1 µg/mL) were used to check the selectivity of this method. No endogenous components from koala plasma interfered with elution of the analytes.

2.4.2.2 Linearity, LLOQ, accuracy and precision

The plasma peak ratio (area of posaconazole peak divided by the area of the IS peak) vs the concentration was plotted and was linear for the concentration range used (0.0125–5 µg/mL). The mean regression calibration curves ($n = 3$) were expressed as $y = 0.866 (\pm 0.015, \text{SD}) x + 0.01 (\pm 0.002, \text{SD})$, with a weighting factor of $1/x$ and the correlation coefficient value (R^2) for each curve ≥ 0.9996 . Based on the SD of the y-intercepts and the slope of the curves, the LLOQ of posaconazole was estimated to be 0.02 µg/mL. Intra- and inter-day precision expressed as CVs ranged from 0.83 to 2.35 % and 1.88 to 3.35 %, respectively. Intra- and interday accuracy expressed as a percentage of the bias ranged from – 9.54 to 0.84 % and –5.83 to – 0.89 %, respectively as illustrated in Table 2-1. These values satisfied the guidelines regarding assay reliability (International Conference on Harmonisation, 2005).

Table 2-1 Precision and accuracy for the QC samples (0.05, 0.5, and 5 µg/mL of posaconazole) (triplicates per day for 3 days)

| Nominal concentration (µg/mL) | Measured concentrations (µg/mL) | | Precision (CV, %) | | Accuracy (Bias, %) | |
|----------------------------------|------------------------------------|----------|----------------------|----------|-----------------------|----------|
| | Intraday | Interday | Intraday | Interday | Intraday | Interday |
| 0.05 | 0.05 | 0.05 | 4.12 | 4.52 | 0.47 | – 6.61 |
| 0.5 | 0.47 | 0.51 | 2.92 | 2.02 | 5.24 | – 1.49 |
| 5 | 5.20 | 5.26 | 2.71 | 1.88 | – 3.92 | – 5.22 |

2.4.2.3 Recovery

The mean absolute recovery rates \pm SD of posaconazole from the 0.05, 0.5, and 5 $\mu\text{g/mL}$ QC samples ($n = 3$) were $99.78 \pm 3.76 \%$; $95.62 \pm 1.76 \%$; and $93.98 \pm 1.82 \%$, respectively. The mean absolute recovery rate of the IS was $104.95 \pm 4.19 \%$ ($n = 9$).

2.4.2.4 Stability

Quality control plasma samples (0.5 and 5 $\mu\text{g/mL}$ posaconazole) were stable at a minimum of 5 months when stored at -20°C . The results are illustrated in Table 2-2.

Table 2-2 Stability of posaconazole (0.5 and 5 $\mu\text{g/mL}$) in the QC samples during long-term storage at -20°C

| Concentration ($\mu\text{g/mL}$) | Peak area of cefovecin / IS (Mean \pm SD) | |
|---------------------------------------|---|-----------------|
| | First day | After 5 months |
| 0.5 | 0.06 ± 0.01 | 0.06 ± 0.00 |
| 5 | 4.74 ± 0.18 | 4.71 ± 0.10 |

2.4.3 Pharmacokinetic results

The PK parameters and indices estimated for posaconazole are summarised in Table 2-3. The plasma concentration vs time curves administered p.o. ($n = 6$) and i.v. ($n = 2$) are presented in Figure 2-2 A and B, respectively. The $\text{AUC}_{\text{extrapolated}}$ of the oral curves from three koalas were greater than 20 % (range: 20.20 – 27.51 %) of their $\text{AUC}_{0-\infty}$ while this value for the other koalas were less than 20 % (range: 11.27 – 18.71 %). The *in-vitro* PPB of posaconazole in pooled koala plasma (pooled protein concentration: 64 g/L) was $99.25 \pm 0.29 \%$.

Table 2-3 Pharmacokinetic parameters and indices (median and range) estimated following administration of posaconazole p.o. (6 mg/kg, $n = 6$) and i.v. (3 mg/kg, $n = 2$) to normal koalas

| Parameters and indices | Oral administration (6 mg/kg) | Intravenous administration (3 mg/kg) |
|---|----------------------------------|---|
| C_{\max} ($\mu\text{g/mL}$) | 0.73 (0.55 – 0.93) | 6.98 (5.17 – 8.78) |
| T_{\max} (h) | 8 (6 – 12) | - |
| $t_{1/2}$ (h) | 12.58 (10.06 – 15.99) | 7.90 (7.62 – 8.18) |
| $V_{d_{ss}}$ (L/kg) | - | 1.23 (0.93 – 1.53) |
| V_{area} (L/kg) | - | 1.76 (1.40 – 2.14) |
| K_{el} (h^{-1}) | 0.06 (0.04 – 0.07) | 0.09 (0.08 – 0.09) |
| Cl (L/h/kg) | - | 0.15 (0.13 – 0.18) |
| AUC (0– ∞) ($\mu\text{g/mL}\cdot\text{h}$) | 15.77 (9.47 – 23.32) | 20.03 (16.51 – 23.54) |
| AUMC (0– ∞) ($\mu\text{g/mL}\cdot\text{h}^2$) | 388.80 (165.08 – 686.02) | 155.60 (139.23 – 171.97) |
| MRT (h) | 23.17 (17.43 – 29.42) | 7.87 (7.30 – 8.43) |
| F_{oral} | 0.64 (0.43 – 1.06) | - |
| AUC ₂₄ /MIC ₉₀ * | 38.13 (28.51 – 53.16) | 5 (59.55 – 86.17) |

*Posaconazole MIC₉₀ of *Cryptococcus gattii* ≤ 0.25 $\mu\text{g/mL}$ (Thompson et al., 2009), AUC/MIC₉₀ target of 25 has been recommended for disseminated candidiasis (Lewis, 2011).

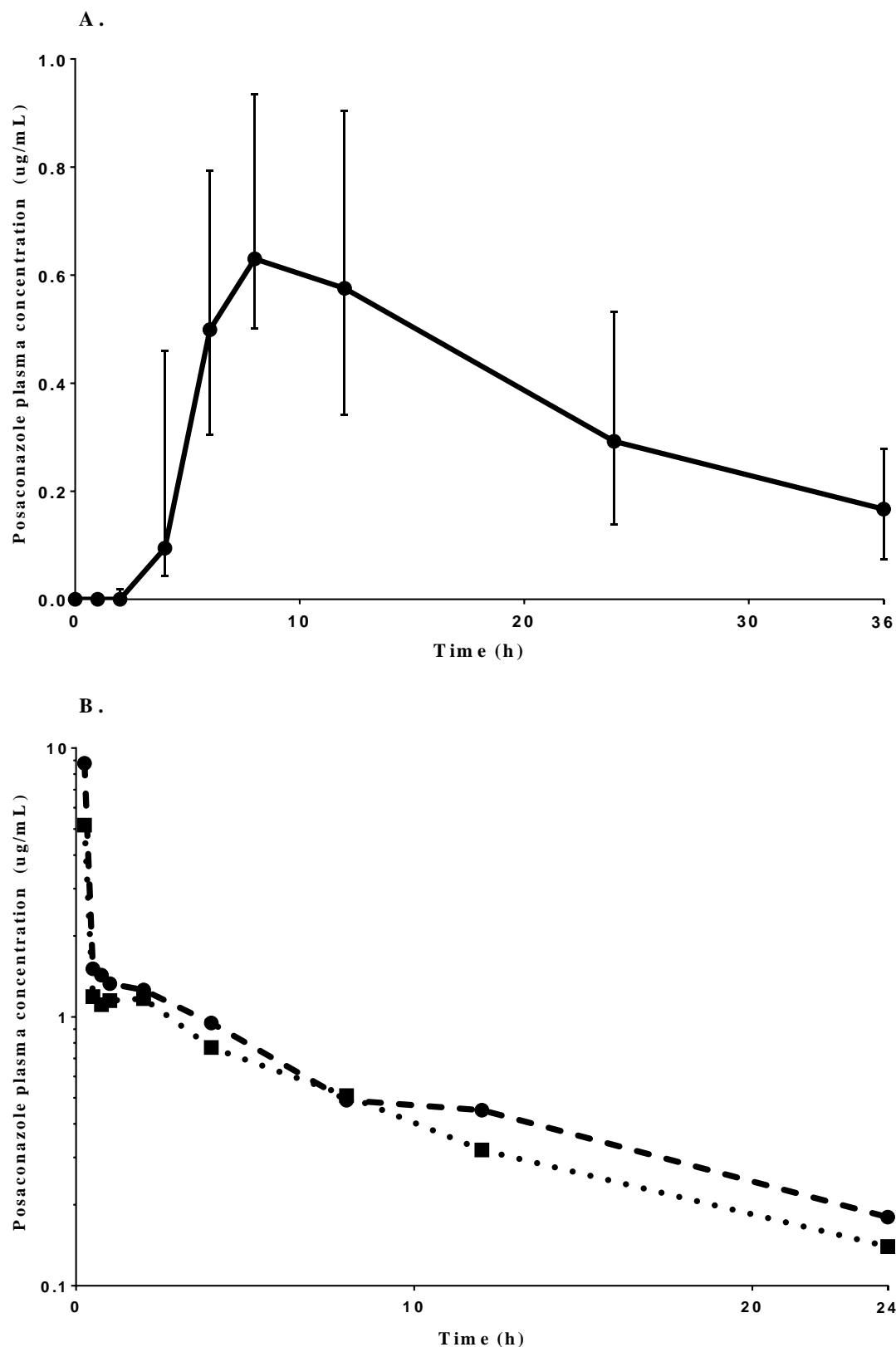


Figure 2-2 Posaconazole plasma concentration vs time curve. **A.** 6 mg/kg p.o. (median \pm IQR) ($n = 6$), LLOQ = 0.02 μ g/mL, **B.** 3 mg/kg i.v. ($n = 2$), LLOQ = 0.04 μ g/mL (Kendall & Papich, 2015).

Moreover, in two koalas at the time 0, one extra chromatogram peak was observed around the retention time of posaconazole. This peak was identified as alphaxalone by running a working solution of alphaxalone through the column and using the diode array detector.

2.5 Discussion

This is the first study to report the PK profile of posaconazole in clinically normal koalas and indicates that posaconazole could be a useful drug for treating systemic fungal diseases in this species. No adverse effects were observed for either the p.o. or i.v. routes in any animal.

As the intravenous formulation was only available in the US at the time of study, the intravenous and oral administration occurred at different zoos, and the plasma drug concentrations were quantified by different laboratories, both with a validated posaconazole assay. A procedural difference was that the two koalas administered the i.v. formulation remained anaesthetised until the 30-minute sample was collected and were then recovered. This was a condition stipulated by Los Angeles zoo in order to minimise stress to the animals during blood collection at 15 and 30 min after drug administration. As posaconazole is reported to undergo some hepatic metabolism and the parent molecule is primarily excreted via the faeces in people (Courtney et al., 2003, Krieter et al., 2004), it was not expected that general anaesthesia would have significantly delayed drug elimination although the elimination pathway/s of these drugs in koalas is/are unknown. The animals that underwent general anaesthesia and i.v. administration at Los Angeles Zoo were considerably older (13 years) than those that underwent oral administration at Taronga Zoo (median: 4.29 years). However, the haematology and biochemical analytes of these animals were within normal ranges.

The $t_{1/2}$ of posaconazole in koalas [median (range), i.v.: 7.90 (7.62 – 8.18); p.o.: 12.58 (10.06 – 15.99) h] was shorter than that reported in dogs [i.v.: 19.93 – 35.71 h; p.o.: 14.18–48.85 h (Kendall and Papich, 2015)], cats [i.v.: 57.7 ± 28.4 ; p.o.: 38.7 ± 15.02 h (Mawby et al., 2016)]

and humans [median terminal $t_{1/2}$: 15 – 35 h; p.o.: 20 – 66 h (Li et al., 2010)]. The shorter $t_{1/2}$ in koalas might be explained by higher median Cl (0.15 L/h/kg) and lower V_{dss} (1.23, range: 0.93 – 1.53 L/kg) compared to those in dogs [Cl: 0.08 L/h/kg; V_{dss} : 3.28, range: 2.25 – 6.45 L/kg, (Kendall and Papich, 2015)] and cats [Cl: 0.028 L/h/kg; V_{dss} : 1.86 ± 0.299 L/kg, (Mawby et al., 2016)]. The $t_{1/2}$ of the oral posaconazole formulation was longer than that when oral fluconazole is administered to koalas [4.69, range: 2.47 – 8.01 h, (Black et al., 2014)] which indicates that the oral posaconazole may have a convenient once-daily dosing interval in koalas.

The T_{max} (average: 8 h, range: 6 – 12 h) was approximately similar to that recorded in dogs at a dosage of 6 mg/kg [average: 7.69 h, range: 4.18 – 34.28 h, (Kendall and Papich, 2015)]. The median C_{max} of posaconazole (oral suspension 6 mg/kg) in koalas was greater (0.73 μ g/mL) than that reported for dogs [0.42 μ g/mL (Kendall and Papich, 2015)].

Posaconazole has a reasonable oral bioavailability range in koalas (median: 0.64, range: 0.43 – 1.06) which is interesting as bioavailability of oral formulations in koalas is generally lower than in other species such as carnivores. For example, the oral bioavailability of meloxicam is negligible in koalas (Kimble et al., 2013a) vs 1.00 in dogs (Busch et al., 1998) and that of fluconazole in koalas is reported to be variable with a median 0.53 (range: 0.20 – 0.97) (Black et al., 2014) while in humans is > 0.90 (Brammer et al., 1990). The oral bioavailability of posaconazole was variable across the koalas and such variability has been noted in dogs [average: 0.26, range: 0.08–0.79, (Kendall and Papich, 2015)] and humans [variable depending on dosage regimen and food, (Li et al., 2010)]. Posaconazole is extremely insoluble which may be responsible for such variable oral absorption (Kendall and Papich, 2015). Furthermore, the oral absorption of posaconazole can be increased by the presence of food in people (Courtney et al., 2004), increased acidity of the gastric and / or small intestinal environment (Krishna et al., 2009b) and co-administration with fatty foods or a nutritional supplement (Courtney et al.,

2004). The pH of the koala forestomach is 2.7 ± 0.1 and hind-stomach is 1.9 ± 0.2 (Cork et al., 1983) and may have had a role in enhancing posaconazole absorption.

No metabolites were detected in the plasma of orally administered koalas (via searching for any additional chromatogram peaks). This is not surprising as posaconazole is primarily excreted in faeces unchanged and to some extent metabolised by glucuronidation with reported negligible involvement of oxidative mechanisms (Li et al., 2010, Ashbee et al., 2013). Alternatively, the LC conditions for measuring the parent drug may not have been optimal for the detection of additional metabolic peaks.

In this study, p.o. posaconazole was administered with no additional supplement as the palatability was acceptable to the koalas. Further investigation of oral absorption when administered with a fatty supplement is warranted.

Posaconazole has been shown to exhibit potent *in-vitro* activity against many cryptococcal species (Sabatelli et al., 2006, Espinel-Ingroff et al., 2012, Pfaller et al., 2013) but posaconazole is not considered as primary therapy for cryptococcosis in people owing to the lack of data (Perfect et al., 2010). There is also some ambiguity about the required posaconazole plasma concentrations that are efficacious for fungal diseases in people (Dolton et al., 2012). It has been reported that maintenance of a trough plasma concentration (C_{min}) of 1 $\mu\text{g/mL}$ with invasive fungal infections increases the probability of therapeutic response (Ashbee et al., 2013). For example, an average plasma concentration of 1.25 $\mu\text{g/mL}$ is associated with the highest clinical response for invasive aspergillosis (Walsh et al., 2007), however, the genotype of the pathogen is important when making such recommendations (Ashbee et al., 2013). A posaconazole dosage used in this study (6 mg/kg p.o. once daily) failed to maintain plasma concentrations above 1 $\mu\text{g/mL}$ over 24 h. However, the $\text{AUC}_{24}/\text{MIC}$ ratio has also been advocated as the most predictive PK/PD index of posaconazole efficacy for yeasts. This target is reported to be 25 (range: 12 – 25) for disseminated candidiasis (Lewis, 2011); specifically,

17 for disseminated *Candida albicans* infections (Li et al., 2010). To the best of this author's knowledge, this index has not been established for *C. gattii*. Given the MIC₉₀ value of posaconazole for *Cryptococcus gattii* ($\leq 0.25 \mu\text{g/mL}$) (Thompson et al., 2009), the median (range) of AUC₀₋₂₄/MIC₉₀ for p.o. suspension at 6 mg/kg and i.v. solution at 3 mg/kg were determined as 38.13 (28.51 – 53.16) and 72.86 (59.55 – 86.17), respectively. Thus, the dose of 6 mg/kg p.o. and 3 mg/kg i.v. exceeded the AUC/MIC target of 25 in all koalas which may indicate efficacious antifungal activity of posaconazole. Posaconazole exhibited *in-vitro* plasma protein binding of $99.25 \pm 0.29 \%$ in koala plasma which is consistent with that reported in dogs (99 %) (Kendall and Papich, 2015) and humans (98 %) (Li et al., 2010). Bound drug in the blood serves as reservoir for the physiologically active free drug concentration, and therefore, may prolong the drug action (Kratochwil et al., 2002). Taking this into consideration, the high degree of binding of plasma proteins to posaconazole might facilitate its efficacy. It is generally accepted that only the free fraction of a drug in plasma is pharmacologically active (Lignell et al., 2011); given the degree of posaconazole plasma protein binding, the unbound posaconazole in the plasma would be far below the MIC for many fungal pathogens but the unbound posaconazole concentration of only 10 % of the known fungal MIC in serum have demonstrated a significant antifungal effect against *Candida* spp. while such effect has not been observed in protein-free media (Lignell et al., 2011). Therefore, it is suggested that a flux of protein-bound posaconazole to its fungal binding target is likely to be responsible for the efficacy of posaconazole (Lignell et al., 2011). Other factors that contribute to posaconazole's efficacy may include its lipophilicity, resulting in the intracellular concentration exceeding that in serum or plasma (Conte et al., 2009, Farowski et al., 2010, Campoli et al., 2011, Campoli et al., 2013).

A major disadvantage of posaconazole for veterinary medicine is the cost, as a single 105 mL bottle of the 40 mg/mL oral formulation (Noxafil, MSD, Macquarie Park, NSW) is

approximately AU \$651.56 at the time of writing (2017). The present study, however, suggests that the oral formulation may well be efficacious for the treatment of systemic fungal diseases in koalas. Further studies are required to determine optimal dosage regimens in koalas to achieve therapeutic concentrations. Clinical trials are also required to investigate the efficacy of posaconazole in infected koalas (either with sub-clinical or clinical infections) as some aspects of the fluconazole PK profile varied between clinically normal, sub-clinical (antigen positive with no obvious clinical signs of disease) and clinically affected animals (Govendir et al., 2015). Further, a repeated dose study is required in koalas with fungal infections. Pharmacokinetic study of delayed-release posaconazole tablets may further reduce the frequency of drug administration although administration to koalas without crushing the tablet is difficult and would likely interfere with the delayed-release properties. Moreover, therapeutic drug monitoring of posaconazole during treatment is recommended because of interpatient variability in the posaconazole PK profile in humans and dogs (Dolton et al., 2012, Kendall and Papich, 2015).

Chapter 3

***In-vitro* binding of cefovecin to plasma proteins in selected Australian marsupials and the horse**

3.1 Abstract

Cefovecin is a semi-synthetic, third-generation cephalosporin antibacterial that was first registered in Australia in 2007 as an aqueous solution for subcutaneous administration to dogs and cats. It is highly bound to plasma proteins (more than 90 %) in dogs, cats and monkeys. Binding of drugs to plasma proteins influences drug disposition. Slow release of cefovecin from plasma proteins results in a long elimination half-life in dogs and cats. Consequently, cefovecin seems to have attracted the interest of veterinarians for the treatment of bacterial infections in exotic species. Determination of the degree of cefovecin binding to plasma proteins, especially in non-domesticated species, provides valuable information as to whether a single bolus of cefovecin may have a prolonged therapeutic action. The aim of this study was to determine the *in-vitro* plasma protein binding of cefovecin in selected Australian marsupials and in the horse. An HPLC method to determine cefovecin concentrations in plasma was developed and validated. The plasma protein binding of cefovecin was determined by the UF method. The percentage of bound cefovecin to plasma protein in the selected marsupial species ranged from approximately 13 % (koala) to 36 % (red kangaroo) compared to 93 % in the horse. The low proportion of cefovecin binding to marsupial plasma proteins suggests that cefovecin will have a shorter duration of action in koalas and other marsupial species than in dogs and cats.

3.2 Introduction

Stress is a frequent cause of morbidity and mortality in hospitalised, non-domesticated wildlife animals (Myers, 2006). Administration of a long-acting antimicrobial is of interest to veterinarians, as such a formulation may reduce the amount of handling stress for these animals (Papp et al., 2010, Myers, 2006). Binding of drugs to plasma proteins is an important factor that influences drug disposition, and particularly for a time-dependent antimicrobial such as cefovecin, will also affect efficacy (Olson and Christ, 1996, Kratochwil et al., 2002). Bound

drug in the blood stream serves as reservoir for the physiologically active free drug concentration, and therefore, prolongs the duration of drug action (Kratochwil et al., 2002). However, a high proportion of protein binding may not be the only factor contributing to the long half-life of cefovecin in some species. Other factors such as tubular reabsorption in the kidneys in dogs and cats may also occur (Stegemann et al., 2006b, Stegemann et al., 2006c). Consequently, the study of protein binding across species, especially in wildlife, provides valuable information as to whether cefovecin may have applications for the treatment of other species. The aim of this study was to determine the *in-vitro* PPB of cefovecin in some selected Australian marsupials including the koala, eastern ring-tailed possum (*Pseudocheirus peregrinus*), common brush-tailed possum (*Trichosurus vulpecula*), eastern grey kangaroo (*Macropus giganteus*), red kangaroo (*Macropus rufus*), and Tasmanian devil (*Sarcophilus harrisii*). These species were selected on the request of Dr Larry Vogelnest, senior veterinarian at Taronga Zoo (Mosman, NSW). Determination of cefovecin binding to horse plasma proteins was also undertaken as a relatively large amount of plasma could be collected with minimal adverse effects on the horse (*Equus caballus*) and cefovecin plasma binding to equine plasma proteins has been reported previously as approximately 92 – 93 % (Valitutto et al., 2011). Therefore, horse plasma was used for the early development of the cefovecin assay and as a positive control.

3.3 Materials and Methods

3.3.1 Drug analysis

Cefovecin concentrations in plasma samples were quantified by HPLC. The HPLC system was described previously in Chapter 2. The chromatographic separation was performed with a Luna C18 (2) 100 Å New Column, 5 µm, 250 x 4.6 mm (Phenomenex, Lane Cove, NSW) attached to a 1-mm Opti-guard C-18 pre-column (Choice Analytical, Thornleigh, NSW). The mobile phase was an isocratic mixture of 17 % acetonitrile and 0.1 % formic acid in water (modified

from a method provided by Professor Mark Papich [College of Veterinary Medicine, North Carolina State University, NC, USA]). The column temperature was maintained at 40 °C. The flow rate of mobile phase was set at 1.5 mL/min. The diode array detector was set at the wavelength of 261 nm. The total run time for each sample was 17 min. The retention times of cefovecin and sulfamethoxazole (IS) were 7.5 and 15.5 min, respectively.

3.3.2 Sample preparation

A primary stock standard solution of cefovecin (2 mg/mL) was prepared in water and further diluted to obtain a series of working solutions of 20, 40, 100, 200, 500, 5000 and 2000 µg/mL. A stock solution of sulfamethoxazole (2 mg/mL) was prepared in acetonitrile. Prepared stock solution was stored at – 20 °C and working solutions were freshly prepared at each analysis. Blank pooled koala plasma ($n = 6$) was used for validating the chromatographic condition. For the preparation of plasma calibration standards (1, 2, 5, 10, 25, 50 and 100 µg/mL) and QC samples (4, 40, and 80 µg/mL), the respective cefovecin working standard solutions were spiked into blank pooled koala plasma and stored at – 20 °C.

3.3.3 Plasma sample extraction

The proteins within the plasma samples (100 µL) were precipitated by adding a 1:1 volume of acetonitrile containing 25 µg/mL of the IS and then centrifuged at 14,000 g for 5 minutes. The supernatant was removed and then dried in a Speed Vac concentrator (SPD 121P, Thermo Fisher Scientific, Scoresby, Vic.). After reconstituting the samples in 100 µL of mobile phase, centrifugation was repeated at the same speed and duration. Following this, 10 µL of supernatant was injected into the HPLC system.

3.3.4 HPLC method validation

Assay selectivity was established by analysing blank plasma of the selected marsupial species (koala, common brush-tailed possum, eastern ring-tailed possum, red kangaroo, eastern grey

kangaroo and Tasmanian devil) and the horse to identify endogenous interference around the retention times of cefovecin and the IS. Cefovecin concentrations in plasma samples were quantified via calibration curves (1, 2, 5, 10, 25, 50 and 100 µg/mL); whereby weighted least square regression ($1/x$) was used. The LLOQ was determined based on calibration curves using the formula $LLOQ = 10 \times \sigma/S$ in which σ is the SD of the y-intercepts for the regression lines and S is the mean slope of the weighted regression lines ($n = 5$). An acceptance criterion for LLOQ was defined as precision (CV) and accuracy within $\pm 20\%$ of the nominal concentration with repeated analyses (International Conference on Harmonisation, 2005). Intra- and interday precision, expressed as CV (%), were analysed from triplicates of QC samples (4, 40 and 80 µg/mL), both within a day, and on three different days, respectively. Intra- and interday accuracy, expressed as bias was determined by a percentage difference between estimated value and the nominal value of cefovecin [$100 - (\text{estimated value} / \text{nominal value} \times 100)$]. Absolute recovery of cefovecin was determined by comparing the peak area of pre-spiked plasma samples at concentrations of 5, 25 and 50 µg/mL ($n = 3$) and 25 µg/mL of the IS with the peak area of corresponding concentrations of cefovecin and the IS in deionised water. The stability of pre-spiked plasma (5, 25 and 50 µg/mL) was tested at room temperature for 8 h and in 4 °C for 4 days. Three freeze/thaw cycles (over a 5-day period) were assessed with pre-spiked plasma samples (5, 25 and 50 µg/mL; $n = 3$). Long-term stability (up to 18 months) of pre-spiked plasma samples (5, 25 and 50 µg/mL; $n = 3$) at $-20\text{ }^{\circ}\text{C}$ was determined. Each assay was conducted in triplicate and statistical data analysis was achieved by ANOVA and unpaired student t-tests using GraphPad Prism 7.02, where the minimum significance level for all statistical tests was set at $P < 0.05$.

3.3.5 Blood collection

Blood collection from koalas was approved by The University of Sydney AEC (protocol: 2015/877). The animals were considered clinically normal based on regular physical

examination and blood collection to check their haematology and biochemical analyte levels. Horse blood was obtained from the Horse Unit of SSVS following The University of Sydney animal ethics approval (protocol: 2015/736). Blood samples from the other Australian marsupials (at least 3 animals per species) were opportunistically collected by the veterinarians at Taronga Zoo and after centrifugation, plasma samples were stored at – 20 °C until analysis.

3.3.6 Plasma protein binding assay

The *in-vitro* plasma protein binding of cefovecin was determined by the modified UF assay (Dow, 2006). Initially, the non-specific binding of cefovecin to the ultrafiltrate device membrane (Centrifree Centrifugal Filters-30K, Merck Millipore, Bayswater, Vic.) was determined using phosphate buffer (pH: 7.4) instead of plasma, and was between 4 to 6 %. Then, total plasma protein (TPP) concentrations as well as the pH of frozen-thawed, pooled, drug-free plasma samples from different species were measured prior to the assay. The pH of plasma (approximately 8) was adjusted to 7.4. Fresh horse plasma was also used to compare its results with frozen horse plasma. Analytical grade cefovecin was added to 700 µL plasma aliquots to yield 10, 50 and 100 µg/mL concentrations. These concentrations were selected as it was used previously to determine the *in-vitro* PPB of cefovecin in dogs and cats (Stegemann et al., 2006b, Stegemann et al., 2006c). Samples were incubated for 30 minutes in a water bath at 37 °C for horse samples and 36 °C for marsupial samples to mimic their respective body temperatures (Dawson and Hulbert, 1970, Degabriele and Dawson, 1979, Tyndale-Biscoe, 2005). To determine the total drug concentration, 100 µL of plasma from each tube (A) was removed, and after sample preparation, injected to the HPLC system. The remaining plasma was then transferred to the reservoir of the ultrafiltrate device, which had a membrane of a molecular weight cut-off 30 kDa. The ultrafiltrate device was centrifuged with a fixed 45-degree angle rotor and spun at 5,800 g for 18 minutes at room temperature. After centrifugation, the filtrate was used for determining the free drug concentration (C). All samples were prepared

and analysed in triplicate and analysed by ANOVA test using GraphPad Prism 7.02 if required.

The PPB of cefovecin was determined using the following equation:

$$\text{PPB (\%)} = 100 - \left(\frac{C}{A} \right) \times 100$$

3.4 Results

3.4.1 Method development and optimisation

Based on the UV spectra, the greatest area under the cefovecin peak was at the wavelength of 261 nm. The retention times of cefovecin and the IS were approximately 7.5 and 15.5 min, respectively. No endogenous interference was observed at the retention time of cefovecin nor the IS. The chromatograms of pre-spiked (5 and 50 µg/mL of cefovecin) and blank pooled koala plasma are shown in Figure 3-1.

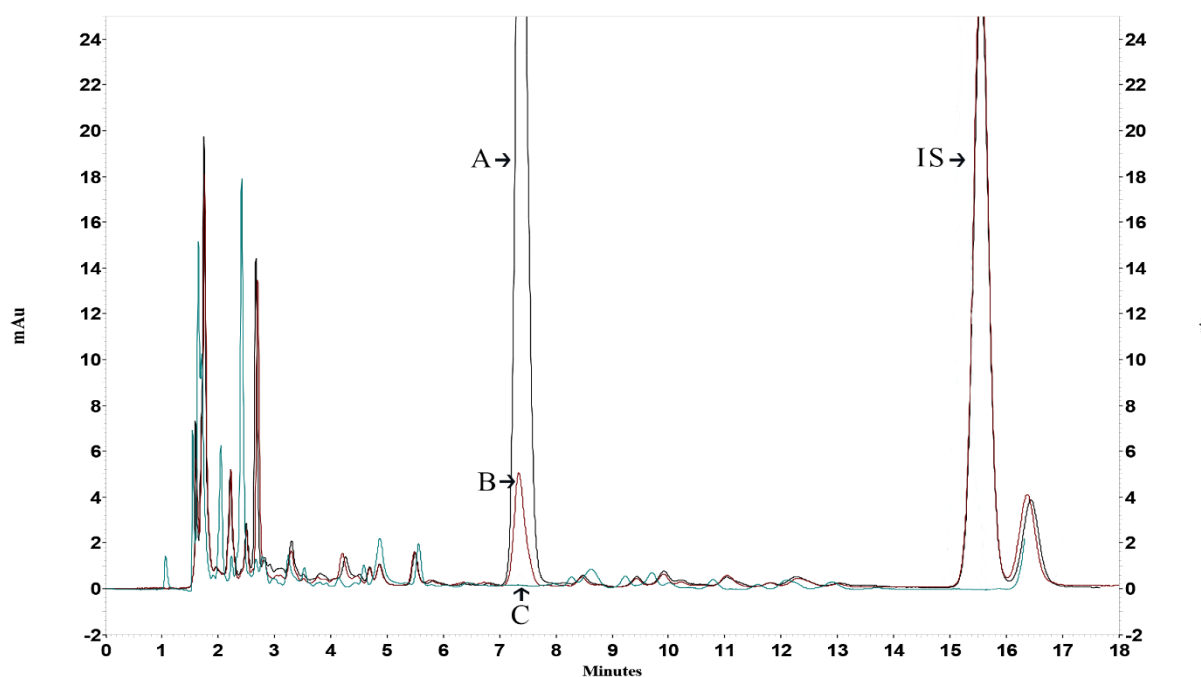


Figure 3-1 Chromatograms of extracted pre-spiked koala plasma containing 50 µg/mL (**A, black trace**) and 5 µg/mL (**B, red trace**) of cefovecin (retention time of 7.5 min) and 25 µg/mL of the IS (sulfamethoxazole, retention of 15.5 min); pooled blank koala (**C, green trace**) plasma at the UV wavelength of 261 nm.

3.4.2 HPLC method validation

3.4.2.1 Selectivity

Pooled blank koala plasma and extracted koala plasma pre-spiked with cefovecin (5 and 50 µg) and IS (25 µg/mL) were used to check the selectivity of this method (Figure 3-1). Moreover, the selectivity of the method was also checked using the plasma from the other marsupial species and the horse. Because of the limited volume of plasma available, only blank plasma of these species were used. No endogenous constituents from plasma interfered with the elution of the cefovecin nor the IS.

3.4.2.2 Linearity, LLOQ, accuracy and precision

The plasma peak ratio (the area of cefovecin peak divided by the area of IS peak) vs the concentration was plotted and found to be linear for the concentration range used (1 – 100 µg/mL). The mean regression calibration curves ($n = 5$) were expressed as $y = 0.027 (\pm 0.001, \text{SD}) x - 0.001 (\pm 0.003, \text{SD})$, with a weighting factor of $1/x$, the correlation coefficient value (R^2) for each curve ≥ 0.999 . Based on the SD of the y -intercepts and the slope of the curves, the LLOD and LLOQ of cefovecin were estimated to be 0.34 and 1.02 µg/mL, respectively. Intra- and inter-day precision expressed as CVs ranged from 0.97 to 4.29 % and 0.73 to 4.52 %, respectively. Intra- and interday accuracy expressed as a percentage of the bias ranged from -7.31 to 3.31 % and -7.31 to 14.37 %, respectively. These values satisfied the guidelines regarding assay reliability (International Conference on Harmonisation, 2005). The average of precision and accuracy of QC samples (4, 40, and 80 µg/mL) are summarised in Table 3-1.

Table 3-1 Precision and accuracy for the QC samples (4, 40, and 80 µg/mL of cefovecin) (triplicates per day for 3 days)

| Nominal concentration (µg/mL) | Measured concentrations (µg/mL) | | Precision (CV, %) | | Accuracy (Bias, %) | |
|-------------------------------|---------------------------------|--------------|-------------------|----------|--------------------|----------|
| | Intraday | Interday | Intraday | Interday | Intraday | Interday |
| 4 | 3.98 ± 0.11 | 3.82 ± 0.11 | 3.04 | 2.76 | 0.42 | 4.51 |
| 40 | 40.60 ± 1.74 | 39.50 ± 1.27 | 4.29 | 3.19 | – 1.50 | 1.24 |
| 80 | 80.70 ± 1.05 | 77.66 ± 1.49 | 0.97 | 1.82 | – 0.87 | 2.93 |

3.4.2.3 Recovery

The mean absolute recovery rates ± SD as a percentage of cefovecin from the 5, 25, and 50 µg/mL QC samples ($n = 3$) were 82.56 ± 3.88 ; 92.01 ± 4.09 ; and 89.01 ± 0.34 , respectively.

The mean absolute recovery rate of the IS was 86.93 ± 2.27 ($n = 9$).

3.4.2.4 Stability

Assessment of the long-term stability of cefovecin in koala plasma demonstrated that QC plasma samples (5, 25 and 50 µg/mL cefovecin) were stable when tested after 8 h at room temperature, 4 days at 4 °C and 18 months when stored at – 20 °C as demonstrated in Table 3-2. No significant changes of cefovecin concentration in the QC samples were observed after three freeze/thaw cycles over a 5-day period.

Table 3-2 Stability of cefovecin in koala plasma containing 5, 25, 50 µg/mL of cefovecin during freeze/thaw cycles and long-term storage at – 20 °C

| Concentration (µg/mL) | Peak area of cefovecin / IS (Mean ± SD) | | | |
|-----------------------|---|-------------|---------------------|-----------------|
| | Freeze/thaw cycles | | Long-term stability | |
| | First day | Last day | First day | After 18 months |
| 5 | 0.12 ± 0.01 | 0.13 ± 0.01 | 0.12 ± 0.01 | 0.12 ± 0.01 |
| 25 | 0.67 ± 0.03 | 0.63 ± 0.00 | 0.68 ± 0.03 | 0.62 ± 0.02 |
| 50 | 1.30 ± 0.03 | 1.26 ± 0.02 | 1.34 ± 0.02 | 1.18 ± 0.06 |

3.4.3 *In-vitro* plasma protein of cefovecin in selected marsupial species

The reported range of TPP concentrations for the respective species and the measured TPP of the pooled samples are provided in Table 3-3.

Table 3-3 Reference range of TPP and measured TPP of the Australian marsupial species and the horse

| Species | Normal range of TPP (g/L) | Measured TPP (g/L) |
|--|----------------------------------|-----------------------|
| Koala (Blanshard and Bodley, 2008) | 60 – 75 | 66 |
| Tasmanian devil (Holz, 2008) | 60 ± 6 | 64 |
| Eastern grey kangaroo (Vogelnest and Portas, 2008) | 60 ± 8 | 70 |
| Common brush-tailed possum (Johnson and Hemsely, 2008) | Male: 59 – 79 Female: 51 – 89 | 70 |
| Eastern ring-tailed possum (Johnson and Hemsely, 2008) | 43 – 67 | 56 |
| Red kangaroo (Vogelnest and Portas, 2008) | 64 ± 10 | 62 |
| Horse (Riond et al., 2009) | 51 – 72 | 70 |

The results of *in-vitro* plasma protein binding of cefovecin in selected Australian marsupial species as well as those in the horse are summarised in Table 3-4 and Figure 3-2.

Table 3-4 *In-vitro* plasma protein binding (%) of cefovecin (10, 50 and 100 µg/mL) in Australian marsupial species and the horse (determined by ultrafiltration)

| Concentration (µg/mL) | Koala | Tasmanian devil | Eastern grey kangaroo | Common brush-tailed possum | Eastern ring-tailed possum | Red kangaroo | Horse |
|--------------------------|--------------|--------------------|-----------------------------|----------------------------------|----------------------------------|-----------------|--------------|
| 10 | 11.9 | 11.89 | 24.05 | 21.24 | 35.55 | 36.46 | 95.47 |
| 50 | 11.29 | 15.99 | 21.41 | 27.25 | 34.17 | 39.52 | 92.94 |
| 100 | 15.46 | 20.03 | 19.55 | 31.03 | 35.25 | 32.27 | 90.18 |
| Mean ± SD | 12.88 ± 2.25 | 15.97 ± 4.07 | 21.67 ± 2.26 | 26.69 ± 4.94 | 34.99 ± 0.72 | 36.08 ± 3.64 | 92.86 ± 2.65 |

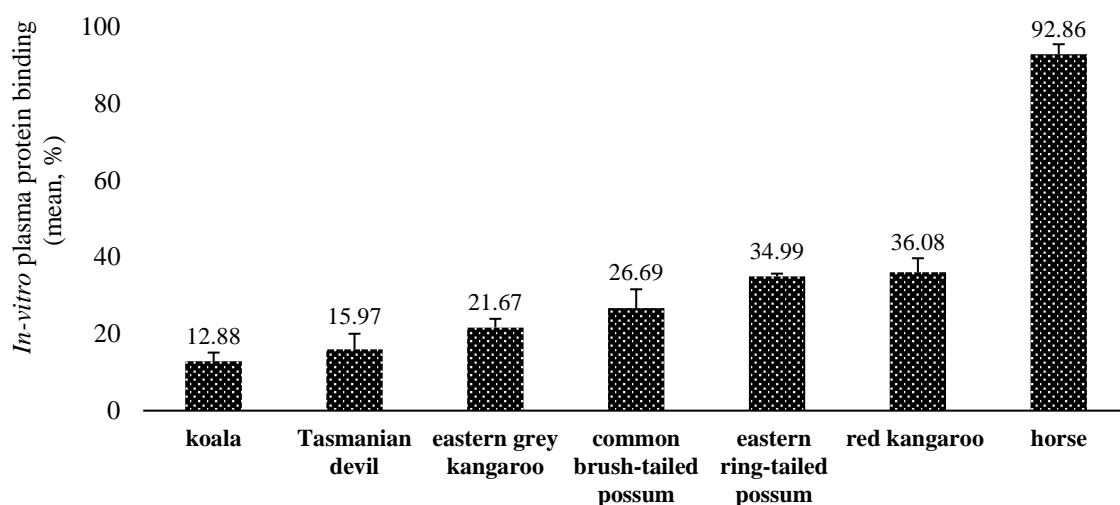


Figure 3-2 Mean plasma protein binding values (%) of cefovecin (10, 50 and 100 $\mu\text{g/mL}$) in selected Australian marsupial species and horse (determined by ultrafiltration). The error bars show the SD.

The mean protein binding values of koala and horse plasma pre-spiked with cefovecin at concentration range between 10 to 300 $\mu\text{g/mL}$ were obtained through a different set of experiments and are depicted in Figure 3-3. While there were no significant differences between bound cefovecin percentages at different concentrations in koala plasma, the PPB values of cefovecin at the concentration of 200 and 300 $\mu\text{g/mL}$ decreased considerably in horse plasma ($P < 0.0001$).

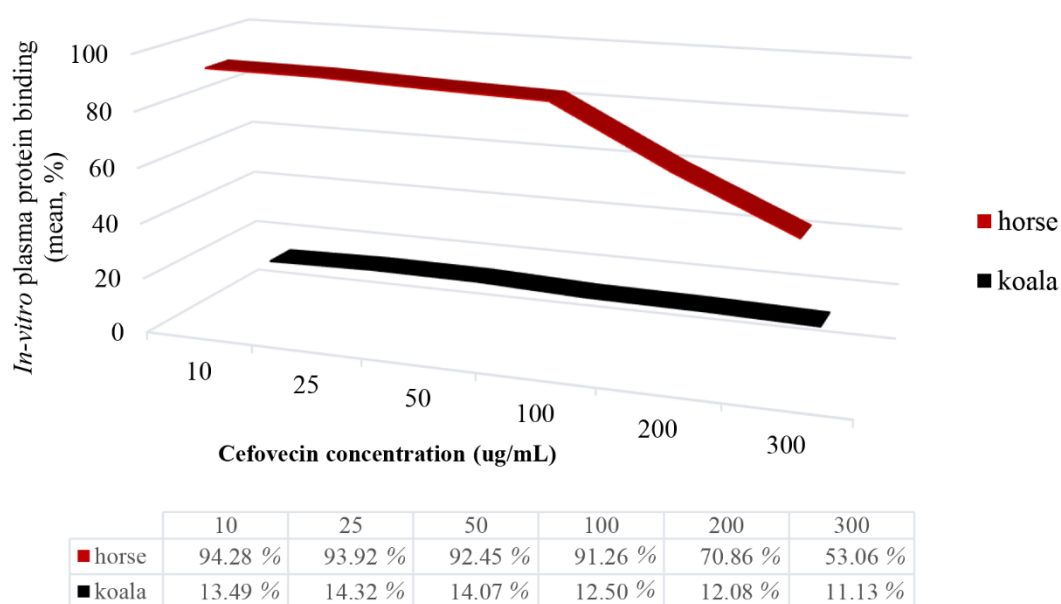


Figure 3-3 Mean percentage of bound cefovecin (10 to 300 µg/mL) in pooled koala and horse plasma determined by ultrafiltration. The table under the graph shows the mean percentage of cefovecin bound to plasma proteins of the horse and the koala at different concentrations; the mean \pm SD of all concentrations for the koala and horse was 12.93 ± 1.13 and 82.58 ± 16.95 , respectively. The reduction of bound cefovecin at higher concentrations in the horse shows the saturation of plasma proteins ($P < 0.0001$).

3.5 Discussion

An HPLC method with a water-based mobile phase and PDA detector was developed and validated for the detection of cefovecin in plasma. A simple sample extraction procedure was undertaken by protein precipitation with 1:1 volume of acetonitrile. This method, with linearity range of 1 – 100 µg/mL and LLOQ of 1.02 µg/mL, was suitable for this *in-vitro* study.

Frozen plasma of the Australian marsupial species was used to batch the results. The pH of frozen plasma is reported to vary from that of fresh plasma which could affect drug binding to plasma proteins (Riviere and Buur, 2011, Riedel, 2013). Therefore, the pH of the samples was adjusted to 7.4 prior to the experiments. Moreover, no significant differences were observed in the PPB values of cefovecin (10, 50 and 100 µg/mL) when tested with the fresh (mean: 92.66

± 1.52 %) vs thawed frozen horse plasma (mean: 92.86 ± 2.65 %). Due to the small amounts of plasma obtained from the marsupials, the plasma was frozen prior to assay and it was not possible to conclude whether this affected the results.

The PPB in the selected marsupial species ranged from 12.88 ± 2.25 % (koala) to 36.08 ± 3.64 % (red kangaroo) which were large differences compared to those in the horse (92.86 ± 2.65 %) and other studied mammals (Stegemann et al., 2006c, Stegemann et al., 2006b, Bakker et al., 2011, Papp et al., 2010, Cox et al., 2014b, Raabe et al., 2011, Valitutto et al., 2011). The PPB of cefovecin varies among species (Colclough et al., 2014). It is assumed many drugs bind to plasma albumin and interspecies differences in PPB is mostly attributable to differences in the amino acid sequences of plasma albumin. Thus, species differences in the conformation of plasma albumin could affect its binding capacity (Baggot, 2001, Colclough et al., 2014). Acidic drugs such as β -lactam antibiotics largely bind to albumin and basic drugs, e.g. macrolides, often bind to other proteins, particularly α_1 -acid glycoprotein (AAG) (Baggot, 2001, Colclough et al., 2014). Cefovecin is a basic drug but there is no published information to which plasma protein(s), cefovecin binds, and this was not established in this study.

Cefovecin shows high protein binding in mammals such as dogs (Stegemann et al., 2006b), cats (Stegemann et al., 2006c), lions (Valitutto et al., 2011), squirrel monkeys (Papp et al., 2010), cynomolgus macaques (Papp et al., 2010, Raabe et al., 2011), rhesus macaques (Papp et al., 2010), alpacas (Cox et al., 2014b), tigers, bears and horses (Valitutto et al., 2011) (illustrated in Table 1-4). Our results in the horse were consistent with those reported previously (92.70 ± 0.26 and 93.99 ± 0.32 for spiked horse plasma containing 1 and 10 $\mu\text{g/mL}$ cefovecin, respectively; Valitutto et al. 2011). To this author's knowledge, the information has not been published in a peer-reviewed journal.

Cefovecin was highly bound to proteins in horse plasma; over a concentration range of 10 – 100 $\mu\text{g/mL}$, the percentage of protein binding was 94.27 % to 90.69 %. At higher

concentrations, 200 and 300 µg/mL, the protein binding reduced to 70.97 % and 53.08 %, respectively. This trend is consistent with those reported for dogs (Stegemann et al., 2006b) and cats (Stegemann et al., 2006c), indicating saturation of plasma proteins for cefovecin binding at the concentration of 200 and 300 µg/mL in these species (Stegemann et al., 2006b). This trend was not observed in koala plasma.

Some birds possess a low PPB capacity for cefovecin (Valitutto et al., 2011). The plasma half-life in hens is reported to be 0.90 h following s.c. administration. This study demonstrated that cefovecin with a 14-day dosing interval is not suitable to treat bacterial infections in hens and most likely in several other birds (Thuesen et al., 2009). Similarly, selected Australian marsupials showed low PPB of cefovecin. However, the *in-vitro* PPB could deviate from that in the live animal as *in-vivo* protein binding can be affected by the concentration, structure and function of plasma proteins which can vary with age and disease, as well as the presence of competing endogenous and exogenous compounds such as dietary constituents or other therapeutic drugs (Lindup and Orme, 1981, Sansom and Evans, 1995).

The dissociation rates of the cefovecin from plasma proteins of these marsupials were not determined. Nevertheless, this value can be of great importance when there is a high PPB of a drug as high PPB and slow dissociation rate can affect ADME of the drug significantly (see section 1.2.2.2 of chapter 1).

The low proportion of cefovecin binding to plasma proteins in marsupials might indicate that cefovecin has a much shorter duration of action in these species and that it might have a different duration of efficacy in these species. This should be confirmed by further *in-vivo* studies. An *in-vivo* study of cefovecin administration to koalas is reported in the next chapter.

Chapter 4

**Plasma concentrations of cefovecin
administered subcutaneously to koalas**

4.1 Abstract

Cefovecin was administered subcutaneously to six koalas at a single dose of 8 mg/kg. Blood was collected at 0 h and then serial blood samples were collected 3, 6, 24, 48, 72 and 96 h after drug administration. Plasma concentrations of cefovecin were determined using an HPLC method. An HPLC method developed to detect cefovecin concentrations $< 1 \mu\text{g/mL}$ did not have acceptable accuracy. Therefore, the method validated in Chapter 3 with an LLOQ of $1.02 \mu\text{g/mL}$ was used to assay the plasma samples from koalas. Cefovecin plasma concentrations were not quantifiable in four koalas and in two other koalas a mean plasma concentration of $1.04 \pm 0.01 \mu\text{g/mL}$ was quantitated after 3 h. It was concluded that, unlike in dogs and cats, cefovecin is likely to have a short duration of action (hours rather than days) in koalas.

4.2 Introduction

Cefovecin is administered to koalas prophylactically or to treat bacterial infections by some veterinarians, who might assume the same duration of action in koalas as in dogs and cats. The result of the *in-vitro* PPB of cefovecin in the koala ($12.88 \pm 2.25 \%$) suggests that a single s.c. bolus injection of cefovecin could have a short duration of action in koala plasma. Therefore, the aim of this chapter was to investigate cefovecin plasma concentration administered to live koalas to confirm whether its duration of therapeutic action in plasma is shorter than that in dogs and cats, and to estimate the correct administration frequency in koalas.

4.3 Materials and Methods

4.3.1 Animals

Six captive koalas (3 males and 3 females), aged 3.3 to 6.9 (median: 4.3) years and weight 6.7 to 8.1 kg (median: 7.5 kg), were recruited opportunistically from Taronga Zoo display collection (Mosman, NSW). They were considered clinically normal based on regular physical examinations and on the basis of haematological and biochemical analyte values within normal

reference ranges. During the study koalas were housed in pens either singly or in groups, and supplied with various *Eucalyptus* spp. and water *ad-libitum*. This study was approved by Taronga Conservation Society Australia with the AEC protocol number of 3c/06/15.

4.3.2 Drug administration and blood collection

Koalas were anaesthetised with alphaxalone (Alfaxan, Jurox Pty Ltd, Rutherford, NSW) at 3 mg/kg i.m. and maintained under anaesthesia with isoflurane in 100 % oxygen via a face mask to place a 20-gauge catheter into the cephalic vein. Blood (5 mL) was collected in lithium heparin tubes 2 h after recovery to determine baseline haematology and biochemistry analyte values, and to confirm the absence of medications ($t = 0$ h). The next morning cefovecin (Convenia, Zoetis Animal Health, West Ryde, NSW) was administered to koalas at 8 mg/kg s.c., the same dosage recommended for dogs and cats (Stegemann et al., 2006b, Stegemann et al., 2006c). To determine the cefovecin plasma concentration, serial blood samples (up to 3 mL) were collected into lithium heparin tubes at the following time points: 3, 6, 24, 48, 72 and 96 h. The cap and catheter were flushed with heparinised saline after each collection. Samples were centrifuged within 1 h of collection and plasma was transferred into plain tubes. Plasma was stored at -80°C , transported promptly on ice to The University of Sydney where the samples were stored at -20°C and protected from light until analysis.

4.3.3 New HPLC method optimisation

The HPLC-PDA method described in the previous chapter had a LLOQ of $1.02\text{ }\mu\text{g/mL}$ suitable for an *in-vitro* assay where concentrations of cefovecin $> 1\text{ }\mu\text{g/mL}$ were required to be detected. However, to determine cefovecin concentrations in plasma samples collected from koalas after s.c. administration of cefovecin, many modifications of the HPLC assay were trialled to achieve a lower LLOQ with acceptable accuracy and precision. Such modifications included revision of the organic solvent, pH of the mobile phase, sample preparation technique and a

change of column. The chromatographic separation with the best results was with a Luna 5u C18(2) 100 Å New Column 250 x 4.6 mm (Phenomenex, Lane Cove, NSW) attached to a 1-mm Opti-guard C-18 pre-column (Choice Analytical, Thornleigh, NSW). The mobile phase was an isocratic mixture of 19 % acetonitrile and 81 % potassium phosphate buffer (20 mM) adjusted with phosphoric acid to pH 2.15. The column temperature was maintained at 40 °C. The flow rate of the mobile phase was set at 1 mL/min and the diode array detector was set at the wavelength of 261 nm. Fluconazole was used as the IS. The total run for each sample took 13 min. The retention times of cefovecin and the IS were 7.7 and 10.5 min, respectively.

4.3.4 Sample preparation

To extract cefovecin from plasma samples, LLE and PPE including methods reported in other species (Stegemann et al., 2006b, Thuesen et al., 2009, Nardini et al., 2014) were trialled. As these techniques resulted in poor recovery and / or selectivity of cefovecin from koala plasma, SPE was undertaken to minimise interference by endogenous matrix compounds.

4.3.4.1 Solid phase extraction using Oasis HLB cartridges

A SPE method using Oasis HLB cartridges (Hydrophilic-Lipophilic Balance cartridges, Waters, Rydalmere, NSW) (Cox et al., 2014a) was used. The cartridges were connected to a Vac Elut vacuum manifold (Waters, Rydalmere, NSW) with a maximum vacuum of 5 psi. Each plasma sample (100 µL) was mixed with 400 µL of methanol (15 % in water), 25 µL of the IS (100 µg/mL) and 25 µL of phosphoric acid (10 % in water). The mixture was vortexed at high speed for 5 s, allowed to stand for 5 min, then centrifuged at 10,000 g for 10 min. All SPE cartridges were conditioned with 1 mL of methanol then washed with 1 mL water. After loading the samples, the cartridges were washed with 1 mL methanol (25 % in water). Cefovecin was then eluted with 1 mL methanol and 1 mL methanol with 3 % ammonium hydroxide. The eluate was dried in a Speed Vac concentrator (SPD 121P, Thermo Fisher Scientific, Scoresby, Vic.).

After reconstituting the samples in 100 μ L of mobile phase, the samples were re-centrifuged at 10,000 g for 5 min and 20 μ L of the supernatant was injected into the HPLC system.

4.3.4.2 Solid phase extraction using Oasis MCX cartridges

SPE was also performed using a protocol recommended by Waters Corporation (Rydalmere, NSW) optimised for cephalexin extraction (a difficult amphoteric analyte with two acid dissociation constants (pK_a) of 2.6 & 7.3) using Oasis MCX (mixed-mode reversed-phase/strong cation-exchange) cartridges (Waters Sample Preparation, 2014). Briefly, each plasma sample was diluted and acidified with phosphoric acid (4 % in water). After conditioning and washing the cartridges (with methanol followed by water), respective aliquots were loaded, and the cartridges were washed with formic acid (2 % in water). The cefovecin was then eluted with either methanol or 5 % ammonium hydroxide in methanol-acetonitrile (60:40 v/v).

4.3.4.3 Micro-disc solid-phase extraction

Due to the lack of selectivity of the abovementioned SPE methods and on consultation with Agilent Technologies Australia (Mulgrave, Vic.), a more specific cartridge, the ‘SPEC-DAU micro-disc’ (Agilent Technologies, Mulgrave, Vic.) was trialled to extract cefovecin. This cartridge is a mixed-mode, reversed-phase/strong cation-exchanger (RP/SCX). Cefovecin is a polar molecule charged at almost any pH. It has three pK_a : the carboxyl has pK_a of 2.6, the amine has pK_a of 3.2 and the amino group on the ring has pK_a of 11.1 (information provided by Professor Mark Papich, College of Veterinary Medicine, North Carolina State University, NC, USA). If the samples containing cefovecin are diluted in an acidic buffer, then conditioned with low pH after loading samples into the cartridge; the cefovecin amine moiety will be charged (NH_4^+) and the acidic group on the cefovecin will be protonated ($-COOH$) and uncharged. If the final elution involves a high pH eluent (such as ammonium hydroxide), the

amine moiety becomes neutral and the cefovecin should elute (Majors, 2013). Therefore, the method of Li et al. (2004) was modified to extract cefovecin from the plasma samples using SPEC-DAU micro-disc SPE cartridges. This method was modified via several preliminary experiments to optimise the sample pH, and washing and elution conditions for maximum recovery and selectivity. Ultimately, plasma samples (100 μ L) were diluted with 800 μ L of 0.1 M potassium phosphate buffer (pH: 6.0) and then spiked with 10 μ L of the working IS solution (fluconazole 200 μ g/mL) to give a final plasma concentration of 20 μ g/mL of the IS. The SPEC-DAU micro-disc SPE cartridges were connected to a Vac Elut vacuum manifold and conditioned with 0.5 mL of methanol followed by washing with 0.5 mL of 0.1 M potassium phosphate buffer (pH: 6.0). The samples were then allowed to run through the disc at a flow rate of less than 1 mL/min. Subsequently, the cartridges were rinsed with 0.5 mL of 1 M acetic acid and dried under vacuum for 2 to 3 min. Then, the tips of the Vac Elut delivery needles were wiped. Analytes were eluted twice with 0.5 mL of 5 % ammonium hydroxide in acetonitrile-methanol (60:40 v/v). The eluents were dried under vacuum in a Speed Vac concentrator and the dried residue was reconstituted in 100 μ L of mobile phase. The mixture was then vortexed and centrifuged at 14,000 g for 5 min and 20 μ L of supernatant was directly injected into the HPLC system.

4.3.5 New HPLC method validation

The validation was conducted with the HPLC condition and micro-disc SPE as described above. The selectivity was established by analysing blank koala plasma to identify endogenous interference around the retention time of both cefovecin and the IS. Absolute recovery of cefovecin was determined by comparing the peak area of pre-spiked plasma samples at concentrations of 0.125, 1 and 4 μ g/mL ($n = 3$) and 20 μ g/mL of the IS with corresponding concentrations of cefovecin and the IS in the mobile phase. The recovery test was performed in duplicate. Cefovecin concentrations in plasma samples were quantified via calibration

curves (0.05, 0.125, 0.25, 0.5, 1.25, 2.5 and 5 $\mu\text{g/mL}$); whereby weighted least square regression ($1/x$) was used. The LLOD and LLOQ were determined based on the SD of the y-intercepts and the average of the slope of the calibration curves. An acceptance criterion for LLOQ was defined as precision (CV) and accuracy within $\pm 20\%$ of the nominal concentration with repeated analyses (International Conference on Harmonisation, 2005). Nonlinear regression by weighting factor $1/x$ was used to test the linearity of the curve using GraphPad Prism 7.02, with a minimum significance level for all statistical tests set at $P < 0.05$. Intra-day precision, expressed as CV (%), was analysed from triplicates of QC samples (0.05, 0.5 and 5 $\mu\text{g/mL}$) within a day. Intra-day accuracy, expressed as bias, was determined by a percentage difference between estimated value and the nominal value of cefovecin [$100 - (\text{estimated value} / \text{nominal value} \times 100)$].

4.3.6 Drug analysis

As the validation of the HPLC method with the micro-disc SPE sample preparation did not satisfy the published guidelines regarding assay reliability (International Conference on Harmonisation, 2005) (as explained in Sections 4.4.1 and 4.4.2), although not ideal, the HPLC method with LLOD: 0.34 $\mu\text{g/mL}$ and LLOQ: 1.02 $\mu\text{g/mL}$ described in the previous chapter was used to analyse cefovecin concentrations in the plasma samples.

4.4 Results

4.4.1 New HPLC method optimisation

The sensitivity (i.e. a reduction in the LLOQ) of the new assay was improved with the following chromatographic conditions: a Luna 5 μ C18 (2) 100 Å New Column 250 x 4.6 mm and an isocratic mixture (pH: 2.15) of 19 % acetonitrile and 81 % potassium phosphate buffer (20 mM) as the mobile phase. The use of SPEC-DAU micro-disc SPE cartridges enabled high selectivity and superior recovery compared to the other sample preparation methods. Therefore,

the sample preparation was performed with micro-disc SPE. The chromatograms of QC samples and blank koala plasma are shown in Figure 4-1.

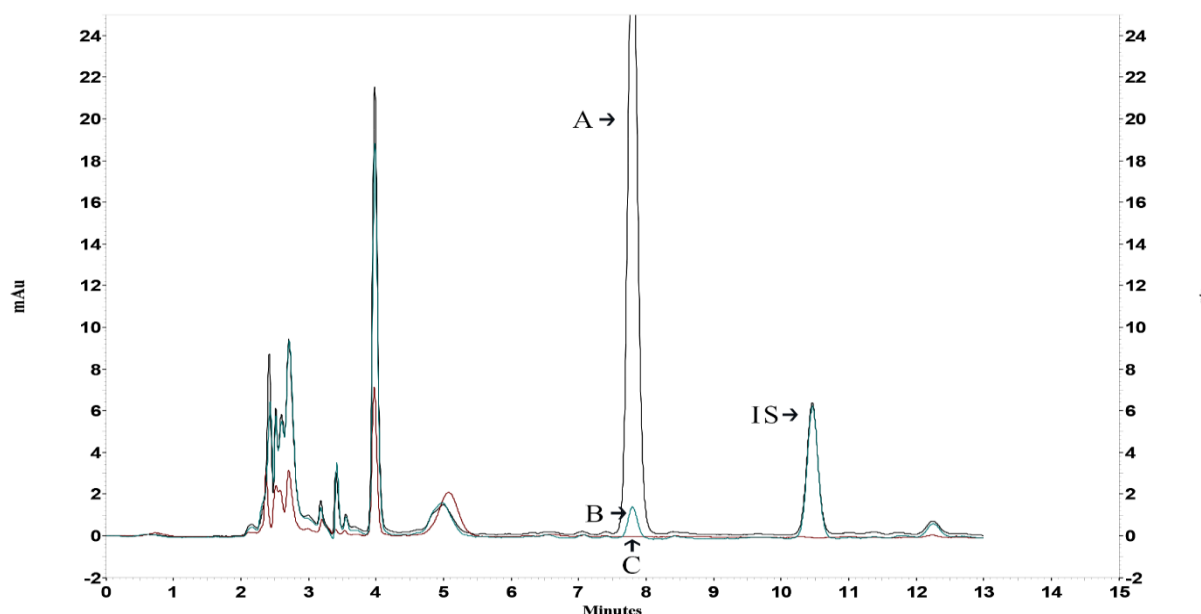


Figure 4-1 Chromatograms of high QC (5 $\mu\text{g/mL}$ of cefovecin) (A, **black trace**) and low QC (0.25 $\mu\text{g/mL}$) (B, **green trace**) samples (retention time of 7.7 min); IS (fluconazole) with a retention time of 10.5 min; and (C, **red trace**) pooled blank koala plasma at the UV wavelength of 261 nm.

4.4.2 New HPLC method validation

For 0.05, 0.5 and 5 $\mu\text{g/mL}$ of cefovecin, the recovery (%) during the method development was 69.29 %, 84.63 % and 86.89 %, respectively. However, during validation, the recovery of cefovecin across concentrations of interest were inconsistent and varied from 50 to 80 % while fluconazole recovery was stable and ≥ 90 % (as documented in Table 4-1).

Table 4-1 The recovery (%) of cefovecin and the IS (20 $\mu\text{g/mL}$) from the QC samples (0.125, 1 and 4 $\mu\text{g/mL}$, triplicate in each run) using micro-disc SPE

| Repetition | Cefovecin concentration ($\mu\text{g/mL}$) | | | IS concentration (fluconazole, 20 $\mu\text{g/mL}$) |
|---------------|--|------------------|------------------|---|
| | 0.125 | 1 | 4 | |
| 1 | 53.35 | 70.91 | 72.78 | 92.56 |
| 2 | 62.01 | 64.99 | 80.44 | 91.19 |
| Mean \pm SD | 57.68 \pm 6.12 | 67.95 \pm 4.19 | 76.61 \pm 5.42 | 91.88 \pm 0.97 |

Using these conditions, the LLOQ was determined to be **0.23 µg/mL**. However, the accuracy values of the standard curves exceeded $\pm 20\%$ of the nominal concentration (range: – 51.93 to 55.39). The values of intraday accuracy for the QC samples did not satisfy the published guidelines regarding assay reliability (International Conference on Harmonisation, 2005). The average precision and accuracy for the QC samples (0.05, 0.5, and 5 µg/mL) are summarised in Table 4-2.

Table 4-2 Precision and accuracy for the QC samples (0.05, 0.5, and 5 µg/mL of cefovecin) (triplicates per day for 3 days)

| Nominal concentration (µg/mL) | Intraday measured concentrations (µg/mL) | Intraday accuracy (Bias, %) | Intraday precision (CV, %) |
|----------------------------------|--|--------------------------------|-------------------------------|
| 0.05 | 0.06 \pm 0.01 | – 20.93 \pm 19.13 | 0.17 |
| 0.5 | 0.63 \pm 0.01 | – 25.85 \pm 2.30 | 0.02 |
| 5 | 5.89 \pm 0.83 | – 17.75 \pm 16.59 | 0.14 |

4.4.3 Pharmacokinetic results

Cefovecin plasma concentrations were determined using the HPLC method described in Chapter 3 with the PPE method (LLOD: 0.34 µg/mL and LLOQ: 1.02 µg/mL). Cefovecin concentrations were only detectable at time 3 h in two koalas (0.72 \pm 0.05 and 0.73 \pm 0.07 µg/mL) and quantifiable at time 3 h in two other koalas (1.03 \pm 0.03 and 1.05 \pm 0.01 µg/mL). No drug was detectable in the plasma of the remaining two koalas nor in the plasma at the other time points of the other four koalas (0 h, 6 h, 24 h, 48 h, 72 h and 96 h). Based on these results, no further PK analyses were possible.

4.5 Discussion

Cefovecin plasma concentrations for the *in-vitro* study were assayed by HPLC-PDA with LLOQ of 1.02 µg/mL (see the previous chapter). Moreover, the HPLC with UV detection has been used to detect cefovecin concentration in plasma samples of white bamboo sharks and

Atlantic horseshoe with an LLOQ of 0.1 µg/mL (Cox et al., 2014a), and of hen, iguana and monkey plasma with an LLOQ of 0.5 µg/mL (Thuesen et al., 2009, Bakker et al., 2011). However, several difficulties were encountered to develop a reliable HPLC method with greater sensitivity to detect cefovecin concentrations in koala plasma *in-vivo*.

First, during method development, endogenous compounds in the koala plasma matrix interfered with the cefovecin peak on the chromatogram. Highly heterogeneous biological matrices such as plasma can contain many endogenous substances such as proteins and lipids which can obscure the drug peaks (including that of the IS) on the chromatogram; this matrix can compromise assay selectivity and sensitivity (Hall et al., 2012, Hooshfar and Bartlett, 2016). The heterogeneity of endogenous substances in koala plasma seems complex, presumably due to the koala's eucalypt foliage diet, causing greater interferences and challenges in the HPLC method optimisation for drug analysis (Stupans et al., 2001, Kimble et al., 2013b). Moreover, in our laboratory, variable peaks and variations in the endogenous peak height and AUC on chromatograms are frequently observed in plasma matrices between koalas from different regions of Australia and even within the same region (unpublished observations). Therefore, the HPLC method was modified and revalidated with pooled blank plasma from the koalas involved in the study.

Second, as most biological matrices are polar and water soluble, both biological matrices and polar compounds such as cefovecin and amoxicillin are eluted early during reversed-phase chromatography. Thus, polar components are subject to more interferences by biological matrices than non-polar compounds that are eluted later during the separation by reversed-phase chromatography (Meyer, 2004, Hall et al., 2012).

Third, the complexity of chemical composition of cefovecin (possessing three pK_a) caused many obstacles when optimising the new HPLC method. As PPE and LLE did not remove the

organic interference in the plasma samples effectively, a more rigorous sample clean-up (SPE) was undertaken to eliminate the interferences and increase the method's sensitivity. Despite promising results during the preliminary method development for cefovecin, the accuracy for the standard curves and QC samples of cefovecin did not meet the accepted criteria regarding assay reliability (International Conference on Harmonisation, 2005). Cefovecin recovery varied during validation for as yet unknown reasons. As cefovecin did not exist in either the load or wash fractions (fractions collected after loading the samples and washing the cartridges, respectively), it might have been retained strongly on the column but was not eluted completely even with higher percentages of ammonium hydroxide (up to 8 %). Other possibilities are secondary interactions between the sorbent and the analyte, or a highly retentive sorbent that could cause irreversible adsorption of some analytes on the cartridges (Majors, 2013, CHROMacademy, 2016).

Initially, two options were considered as the IS: fluconazole and sulfamethoxazole. The latter was used in Chapter 3. Both drugs eluted later than cefovecin; however, the fluconazole retention time was sooner with the conditions described in Chapter 4, reducing the total run time.

Although bioanalytical methods with better sensitivities as low as 0.05 µg/mL (Stegemann et al., 2006c) and 0.005 µg/mL (Papp et al., 2010) are reported using LC-tandem mass spectrometry, the equipment was not available to replicate these methods.

Analysing samples from koalas after cefovecin administration at 8 mg/kg s.c. yielded limited pharmacological information but no adverse effects were observed in any koala following administration. The paucity of cefovecin concentrations above the LLOQ (1.02 µg/mL) meant that the PK indices such as T_{max} , C_{max} and $t_{1/2}$ could not be determined and, therefore, one of the aims of this chapter, i.e. to estimate the correct administration frequency (see Section 4.2), was not met.

The low cefovecin concentrations were not thought to be due to poor stability of cefovecin in plasma, as cefovecin in koala plasma was stable when tested after 18 months storage at – 20 °C.

When planning this study, cefovecin plasma concentrations were expected to be detectable for at least four days, therefore, samples were collected up to 96 h. An assay with lower LLOQ and collection of blood at more time points prior to 6 h could have facilitated better characterisation of some PK indices but, despite many attempts, developing a validated HPLC method with greater sensitivity in this species was difficult.

Administering cefovecin at 8 mg/kg s.c. to dogs gave a mean C_{max} of 121 µg/mL (Stegemann et al., 2006b). However, the same dose rate administered to koalas achieved a plasma concentration of ≤ 1 µg/mL at time 3 h and 6 h. This might be due to the poor bioavailability of s.c. cefovecin and / or a fast elimination rate. Similarly, a low C_{max} (0.83: 0.66 – 1.52) was previously observed following s.c. administration of enrofloxacin (5 mg/kg) which was attributed to limited absorption due to poor vascularity around the injection site or fast elimination in koalas (Griffith et al., 2010). The previous observations of the low percentage of cefovecin PPB (12.88 ± 2.25 %) might suggest a very short half-life of cefovecin in koalas as the PPB of cefovecin was reported to be less than 10 % in birds such as brown pelican (*Pelecanus occidentalis*) and American flamingo (*Phoenicopterus ruber*) (Valitutto et al., 2011) and negligible in the red-eared slider (a semiaquatic turtle, *Trachemys scripta elegans*) (Sypniewski et al., 2017) and, consequently, the plasma half-life of s.c. cefovecin was 0.87 ± 0.27 h in Lohmann hens (*Gallus gallus domesticus*) (Thuesen et al., 2009) and 6.8 ± 1.2 h in the red-eared slider (Sypniewski et al., 2017) compared to 5.5 and 6.9 d in the dog and cat, respectively (Stegemann et al., 2006b, Stegemann et al., 2006c) (see Table 1-3). The difference in half-lives of cefovecin in these species was partly attributed to differences in the binding of cefovecin to plasma proteins (Thuesen et al., 2009, Sypniewski et al., 2017).

The LC conditions for measuring cefovecin concentrations were not optimal for detecting any possible metabolites. Metabolites were not expected as cefovecin hepatic metabolism is insignificant in its elimination in dogs and nonhuman primates (Stegemann et al., 2006b, Papp et al., 2010). As cefovecin is primarily eliminated via kidneys (Stegemann et al., 2006b, Papp et al., 2010), it might be worth investigating its accumulation in urine. This was not attempted because low plasma concentrations were not expected, and the additional resources were not available to catch urine over four days. Moreover, cystocentesis in conscious koalas is challenging due to the koala's urinary bladder lying within the pelvic cavity, frequently surrounded by a large caecocolon (Blanshard and Bodley, 2008).

Cefovecin, a cephalosporin antibacterial, is described as having time-dependent activity (Craig, 1995, Jacobs, 2001, Turnidge and Paterson, 2007). Successful therapy is predicted if the effective concentration of cefovecin during the dosage interval is maintained above 50 to 70 % of the antimicrobial MIC for gram-negative pathogens and less than 40 to 50 % of MIC concentration for gram-positive pathogens (Craig, 1995, MacVane et al., 2014, Lee et al., 2016). To this author's knowledge, no studies have investigated cefovecin concentrations required to inhibit the growth of bacteria isolated from any Australian marsupial species. However, the concentration required usually depends on the antimicrobial agent and pathogen rather than the host. The MIC₉₀ of cefovecin to inhibit bacterial species like *S. intermedius* (now known as *S. pseudintermedius*) and *E. coli* is $\leq 1 \mu\text{g/mL}$ in dogs and cats (Stegemann et al., 2006a) and sea otters (Lee et al., 2016). Thus, the MIC₉₀ of $1 \mu\text{g/mL}$ was used as the therapeutic target of cefovecin to draw some conclusions.

Therapeutic dosing intervals of cefovecin for dogs and cats were determined using the free concentration of cefovecin in plasma and transudate (Food and Drug Administration, 2008). Transudate concentrations were not collected for this study. Free plasma concentration of cefovecin for two koalas was estimated by multiplying total plasma concentration of cefovecin

(mean: 1.04 $\mu\text{g/mL}$) by 0.87, where 0.87 is free percentage of cefovecin in koala plasma. Thus, the mean free cefovecin plasma concentration was considered $\leq 0.9 \mu\text{g/mL}$ in all koalas at $t = 3 \text{ h}$ only.

A PK/PD integration using the total concentration of cefovecin ($T > \text{MIC}_{90}$) demonstrates that a dosing interval of 7.5 h for gram-positive and 4 h for gram-negative pathogens might be required for maintaining the target concentration of 1 $\mu\text{g/mL}$ for 40 and 70 % of dosing intervals for effective therapy. However, this estimation is only based on the results of two of the six koalas and the cefovecin concentration was $< 1 \mu\text{g/mL}$ at $t = 3 \text{ h}$ in four other koalas. Based on the estimated free cefovecin plasma concentration ($\leq 0.9 \mu\text{g/mL}$), this target concentration is unlikely to be maintained after three hours using the current dose of 8 mg/kg s.c. for koalas. In contrast to the koala, total cefovecin plasma concentration remained above 1.0 $\mu\text{g/mL}$ for 21 days when 8 mg/kg was administered s.c. to dogs and cats and the unbound cefovecin concentration in transudate was maintained above 1 $\mu\text{g/mL}$ for 8 days in dogs and 3 days in cats (Stegemann et al., 2006b, Stegemann et al., 2006c). The cefovecin duration in plasma above the MIC for pathogens with lower susceptibility to cefovecin (e.g. $\text{MIC} \leq 0.25$) was not established due to the concentrations below the LLOQ.

On the evidence presented here, albeit the small sample size (the cefovecin plasma concentrations of six koalas), this study suggests a single s.c. bolus of cefovecin 8 mg/kg is unlikely to be efficacious to treat bacterial diseases for more than 3 h in koalas and does not offer the long duration of efficacy that it does in dogs and cats. Cefovecin could be an antibacterial option for pathogens with $\text{MIC} < 1 \mu\text{g/mL}$ in koalas but to maintain the therapeutic concentration of cefovecin above the MIC, a much shorter dosage interval than 14 days such as repeating dosage every 6 to 12 h in the koala, might be required.

Chapter 5

**Plasma concentrations of amoxicillin
administered subcutaneously to koalas**

5.1 Abstract

An HPLC method was developed and validated to detect amoxicillin concentrations in koala plasma. Six koalas were injected subcutaneously with 10 mg/kg amoxicillin trihydrate. Serial blood samples were collected over 24 h and plasma concentrations of amoxicillin were measured using HPLC. Although plasma concentrations were not quantifiable in four koalas, the plasma concentrations in one koala at 1, 2 and 4 h were 0.65 ± 0.18 , 0.57 ± 0.22 and 0.43 ± 0.21 $\mu\text{g/mL}$ respectively, and in the other koala the concentrations were 0.41 ± 0.0 and 0.41 ± 0.02 $\mu\text{g/mL}$ at 0.5 h and 1 h, respectively. However, the HPLC stability test demonstrated that amoxicillin content in plasma reduced by approximately 60 % from the time the samples were collected to when the results were finalised (i.e. when stored at -20°C for five months). Therefore, the results require cautious interpretation.

5.2 Introduction

The chapter ‘Koalas’ in ‘Medicine of Australian Mammals’ (Blanshard and Bodley, 2008), suggests that amoxicillin at a dose of 10 – 12.5 mg/kg (alone, or in combination with clavulanic acid) can be administered p.o., i.m. or s.c. to koalas to treat some bacterial infections; although the authors note that some clinicians avoid oral administration to koalas (Blanshard and Bodley, 2008). As no information about the PK profile of amoxicillin in koalas exists, upon the request of Dr Amber Gillett from the AZWH, this research was conducted to develop an HPLC method for quantitating amoxicillin concentrations in koala plasma and to investigate plasma concentrations of amoxicillin trihydrate following a single, s.c. injection of 10 mg/kg to koalas.

5.3 Materials and methods

5.3.1 Animals

Six clinically normal, captive koalas (3 males and 3 females), aged 3.64 to 10.44 (median: 7.17) years and weight 6.8 to 9.2 (median: 8.35) kg, were recruited from the Sydney Taronga Zoo display collection. These koalas were considered clinically normal based on regular physical examinations and haematological and biochemical analyte values within normal reference ranges. The koalas were housed singly or in groups in pens and supplied with various *Eucalyptus* spp. and water *ad-libitum*. This study was approved by the Taronga Conservation Society Australia, AEC protocol number 3d/06/16.

5.3.2 Drug administration and blood collection

Koalas were anaesthetised with alphaxalone (Alfaxan, Jurox Pty Ltd, Rutherford, NSW) at 3 mg/kg i.m. and maintained under anaesthesia with isoflurane in 100 % oxygen via a face mask for catheter placement. Blood (5 mL) was collected in lithium heparin tubes to determine baseline haematology, biochemistry values and to confirm the absence of medications (t = 0 h). The animals were then recovered from anaesthesia. The next morning amoxicillin trihydrate (Betamox 150 mg/mL, Norbrook Laboratories Australia Pty Ltd, Tullamarine, Vic.) was administered at 10 mg/kg s.c., the dose rate currently suggested for koalas (Blanshard and Bodley, 2008). To determine amoxicillin plasma concentration, serial blood samples (up to 3 mL) were collected into lithium heparin tubes at 0.5, 1, 2, 4, 6, 8, 12 and 24 h. The cap and catheter were flushed with heparinised saline after each collection. Samples were centrifuged within 1 h of collection and plasma was transferred into plain tubes. Plasma was stored at – 80 °C and transported on ice within days of collection to The University of Sydney, where they were stored at – 20 °C and protected from light until analysis. While the amoxicillin assay was undergoing validation for plasma samples of Taronga Zoo koalas, an average of 165 days (or 5.5 months) elapsed between the koalas being medicated and the confirmation of the amoxicillin concentrations in the koala plasma.

5.3.3 Drug Analysis

Amoxicillin concentrations in plasma samples were quantified by the HPLC system described in Chapter 2. Chromatographic separation initially involved comparing the sensitivity of the following columns: AtlantisTMdC18, 3 μ m, 150 \times 2.1 mm (Waters, Rydalmere, NSW), Hypersill ODS, 5 μ m, 150 \times 4.6 mm (Supelco, Bellefonte, Pennsylvania, USA), Nova-Pak C18, 4 μ m, 300 \times 3.9 mm (Waters, Rydalmere, NSW), Synergi MAX-RP 80Å, 4 μ m, 150 \times 4.6 mm (Phenomenex, Lane Cove, NSW), Synergi Hydro-RP 80 Å, 4 μ m, 250 \times 4.6 mm (Phenomenex, Lane Cove, NSW) and Polaris C18-A, 5 μ m, 150 \times 4.6 mm (Agilent Technologies, Mulgrave, Vic.). Different concentrations of methanol (4 to 20 %) and 20 mM sodium phosphate buffer at a range of pH (2 to 7) were trialled to modify the mobile phase and to optimise peak resolution and retention times of amoxicillin and the IS. Cefadroxil was selected as the IS (Abreu et al., 2003).

Optimal chromatographic separation was performed with the Polaris C18-A column (5 μ m, 150 \times 4.6 mm) attached to a 1 mm Opti-guard C-18 pre-column (Choice Analytical, Thornleigh, NSW) at 35 °C. The isocratic mobile phase was 20 mM sodium phosphate buffer (pH: 3.15) and methanol (95:5 v/v) at a flow rate of 1 mL/min. The UV wavelength for the target analytes was 228 nm. The total run time for each sample was 15 min. The retention times of amoxicillin and the IS (cefadroxil) were 7.2 and 9.1 min, respectively.

5.3.4 Sample preparation

A stock solution of amoxicillin (0.5 mg/mL) and the IS (125 μ g/mL) were prepared in water. Amoxicillin was further diluted with water to give a series of working solutions of 5, 10, 20, 25, 50, 100, 200, 250 and 300 μ g/mL. Prepared stock solution was stored at – 20 °C and working solutions were freshly prepared for each analysis. Blank pooled koala plasma ($n = 6$) was used for validating the chromatographic condition. For the preparation of plasma

calibration standards (0.2, 0.4, 0.8, 1, 2, 4, 8, 10 and 12 µg/mL) and QC samples (0.4, 1 and 8 µg/mL), the appropriate amount of working standard solutions of amoxicillin were spiked into blank pooled koala plasma and stored at – 20 °C.

5.3.5 Plasma sample extraction

After trialling numerous procedures (as discussed in Section 5.4), a new LLE method was developed to prepare samples for amoxicillin analysis in plasma. Each plasma sample (240 µL) was mixed with 10 µL of the IS (125 µg/mL) and 15 µL of phosphoric acid. After mixing for 15 s, 1700 µL of TBME was added and vortexed for 15 s and then allowed to stand for 5 min. After removing the supernatant, to extract the amoxicillin, 700 µL of dichloromethane was added to the mixture and vortexed for 15 s. The supernatant was then collected and centrifuged for 15 min at 14,000 g. The volume of 40 µL of the supernatant was diluted with 60 µL of the mobile phase and after centrifugation at 14,000 g for 15 min, 20 µL was injected into the HPLC system.

5.3.6 HPLC method validation

Assay selectivity was established by analysing blank koala plasma to identify endogenous interference around the retention times of both amoxicillin and the IS. Amoxicillin concentrations in plasma samples were quantified via standard curves based on the following concentrations: 0.2, 0.4, 0.8, 1, 2, 4, 8, 10 and 12 µg/mL using a weighted least square regression ($1/x$). The LLOQ was determined based on the standard curves using the formula $LLOQ = 10 \times \sigma/S$ in which σ is the standard deviation (SD) of the y-intercepts for the regression lines and S is the mean slope of the weighted regression lines ($n = 7$). An acceptance criterion for LLOQ was defined as precision (CV) and accuracy within $\pm 20\%$ of the nominal concentration with repeated analyses (International Conference on Harmonisation, 2005). Intra- and inter-day precision, expressed as CV (%), were analysed from triplicates of QC

samples (0.4, 1 and 8 µg/mL), within a day and on three days, respectively. Intra- and inter-day accuracy, expressed as bias, was determined by a percentage difference between estimated value and the nominal value of amoxicillin [$100 - (\text{estimated value} / \text{nominal value} \times 100)$]. Absolute recovery of amoxicillin and the IS was determined by comparing the peak area of pre-spiked plasma samples at concentrations of 0.4, 1 and 8 µg/mL ($n = 3$) and 5 µg/mL of the IS with corresponding concentrations of amoxicillin and the IS in the mobile phase. Three freeze/thaw cycles (over 6 days) were assessed with pre-spiked plasma samples at three concentrations (0.4, 1 and 8 µg/mL; $n = 3$). Long-term stability of amoxicillin was determined with pre-spiked samples at two concentrations (1 and 8 µg/mL; $n = 3$) when stored for 5 months at $-20\text{ }^{\circ}\text{C}$ and protected from light. Each assay was conducted in triplicate and analysed by ANOVA and unpaired student t-tests using GraphPad Prism 7.02, with the minimum significance level at $P < 0.05$.

5.4 Results

5.4.1 Method development and optimisation

The Polaris C18-A column provided the best sensitivity and peak resolution of the analytes. A mixture of 5 % methanol and 95 % sodium phosphate buffer (20 mM, pH: 3.15) was the best mobile phase to ensure the sharpest (narrowest) peaks with minimal interference. Figure 5-1 illustrates the chromatograms of pre-spiked (0.5 and 2.5 µg/mL of amoxicillin) and blank pooled koala plasma.

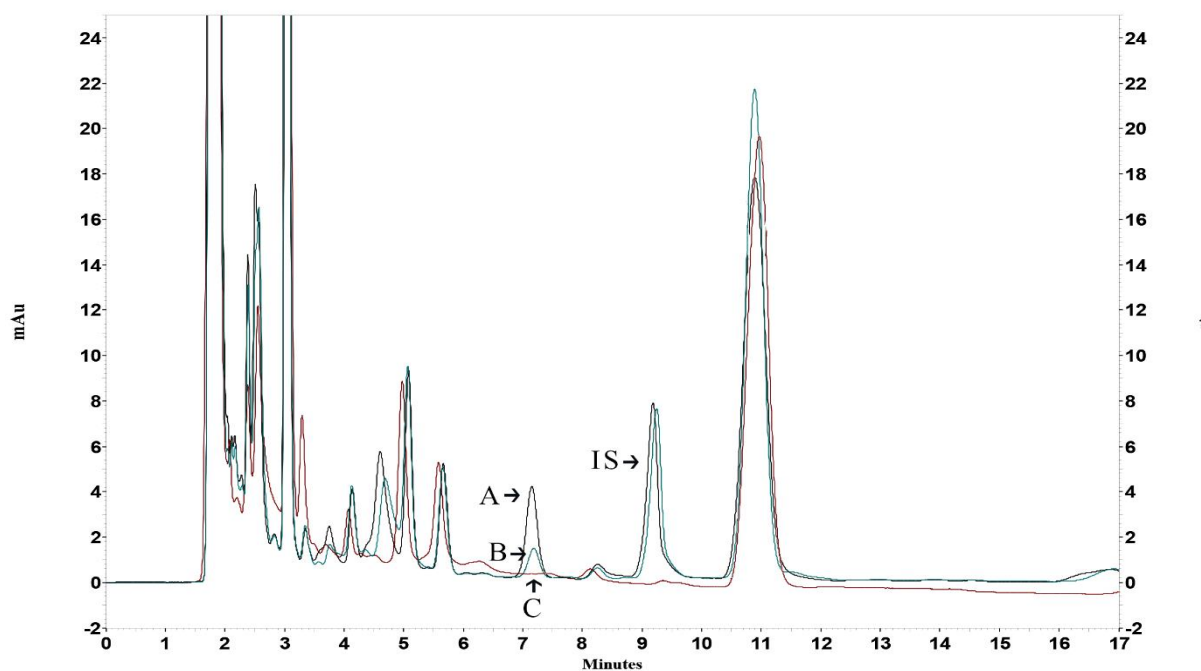


Figure 5-1 Chromatograms of extracted pre-spiked koala plasma containing 2.5 µg/mL (A, **black trace**) and 0.5 µg/mL (B, **green trace**) of amoxicillin (retention time of 7.2 min) and 5 µg/mL of the IS (cefadroxil, retention time of 9.1 min); and pooled blank koala plasma (C, **red trace**) at a UV wavelength of 228. Note the endogenous plasma peaks (in all three traces) prior to 7 min and at 11.5 min.

5.4.2 HPLC method validation

5.4.2.1 Selectivity

Pooled blank koala plasma and extracted koala plasma pre-spiked with amoxicillin (0.5 and 2.5 µg) and the IS (5 µg/mL) were used to check the selectivity of this method. No endogenous components from koala plasma interfered with elution of the amoxicillin but due to minimal interference with the IS peak, the ratio of peak height of the analytes was used instead of the ratio of area under the peak.

5.4.2.2 Linearity, LLOQ, accuracy and precision

The plasma peak ratio (the height of the amoxicillin peak divided by the height of the IS peak) vs the concentration were plotted and found to be linear for the concentration range used (0.2 – 12 µg/mL). The mean regression calibration curves ($n = 7$) were expressed as $y = 0.1206 (\pm 0.0077, \text{SD}) x - 0.0214 (\pm 0.0097, \text{SD})$, with a weighting factor of $1/x$ and the correlation

coefficient value (R^2) for each curve was ≥ 0.99 . Based on the SD of the y-intercepts and the slope of the curves, the LLOD and LLOQ of amoxicillin were estimated to be 0.13 $\mu\text{g/mL}$ and 0.39 $\mu\text{g/mL}$, respectively. Precision (CV) and accuracy of the calibration curves were both $\leq 20\%$. The average of precision and accuracy for the QC samples (0.4, 1, and 8 $\mu\text{g/mL}$) are summarised in Table 5-1. Intra- and inter-day precision, expressed as CVs, ranged from 0.03 to 1.50 % and 0.031 to 0.20 %, respectively. Intra- and inter-day accuracy expressed as a percentage of the bias, ranged from -6.67 to 10.92% and -14.80 to 11.27% , respectively. These values satisfied the ICH guidelines regarding assay reliability (International Conference on Harmonisation, 2005).

Table 5-1 Precision and accuracy for the QC samples (0.4, 1, and 8 $\mu\text{g/mL}$ of amoxicillin) (triplicates per day for 3 days)

| Nominal concentration ($\mu\text{g/mL}$) | Measured concentrations ($\mu\text{g/mL}$) | | Precision (CV, %) | | Accuracy (Bias, %) | |
|---|---|-----------------|----------------------|----------|-----------------------|----------|
| | Intraday | Interday | Intraday | Interday | Intraday | Interday |
| 0.4 | 0.40 ± 0.02 | 0.41 ± 0.02 | 0.05 | 0.07 | -0.72 | -2.00 |
| 1 | 1.00 ± 0.04 | 0.97 ± 0.07 | 0.04 | 0.08 | -0.18 | 2.54 |
| 8 | 7.78 ± 0.24 | 7.97 ± 0.33 | 0.03 | 0.04 | 2.70 | 0.35 |

5.4.2.3 Recovery

The mean absolute recovery rates \pm SD of amoxicillin from the 0.4, 1, and 8 $\mu\text{g/mL}$ QC samples ($n = 3$) were $72.26 \pm 5.23\%$; $70.44 \pm 2.02\%$; and $73.64 \pm 3.24\%$, respectively. The mean absolute recovery rate of the IS was $95.29 \pm 7.15\%$ ($n = 9$).

5.4.2.4 Stability

There was no loss of amoxicillin concentration after three freeze/thaw cycles over 6 days but the QC samples (1 and 8 $\mu\text{g/mL}$ amoxicillin in koala plasma) were not stable after 5 months as amoxicillin degraded significantly after 5 months at -20°C (determined by a reduced AUC of the amoxicillin peak compared to time 0). The results are presented in Table 5-2.

Table 5-2 Stability of amoxicillin in the QC samples (1 and 8 µg/mL) during long-term storage (5 months) at – 20 °C

| Concentrations (µg/mL) | The AUC reduction after 5 months compared to the time 0 (%) |
|------------------------|---|
| 1 | 54.34 |
| 8 | 63.32 |

5.4.3 Pharmacokinetic results

Given the LLOQ (0.39 µg/mL), plasma concentrations of amoxicillin in four koalas were not quantifiable at all time points (see Table 5-3).

Table 5-3 Amoxicillin concentration in koala plasma ($n = 6$) following s.c. administration at 10 mg/kg, LLOQ of the HPLC method: 0.39 $\mu\text{g/mL}$. The koalas are ordered chronologically by date of amoxicillin administration.

| Animals' information | | Amoxicillin concentration ($\mu\text{g/mL}$) | | | | |
|--|----------------------|--|----------------------|-----------------------------------|-----------------------------------|----------------------|
| Sample collection date | | | | | | |
| Sample analyses date | | | | | | |
| Koala number | Koala 1 | Koala 2 | Koala 3 | Koala 4 | Koala 5 | Koala 6 |
| Sex | M | M | F | F | M | F |
| Weight | 8.8 kg | 8.4 kg | 6.8 kg | 7.9 kg | 9.2 kg | 7.8 kg |
| Drug injection date | 25 Aug 16 | 30 Aug 16 | 13 Sep 16 | 13 Sep 16 | 15 Sep 16 | 21 Sep 16 |
| Final results date (days after drug injection) | 17 Feb 17 (161 d) | 27 Feb 17 (180 d) | 24 Feb 17 (164 d) | 23 Feb 17 (163 d) | 23 Feb 17 (161 d) | 27 Feb 17 (159 d) |
| Time after drug injection | | | | | | |
| 0 h | - | - | - | 0.18 \pm 0.00 | 0.15 \pm 0.00 | - |
| 0.5 h | - | - | - | 0.41 \pm 0.00 | 0.38 \pm 0.05 | - |
| 1 h | - | - | - | 0.41 \pm 0.02 | 0.65 \pm 0.18 | - |
| 2 h | - | - | - | 0.38 \pm 0.01 | 0.57 \pm 0.22 | - |
| 4 h | - | - | - | 0.31 \pm 0.04 | 0.43 \pm 0.21 | - |
| 6 h | - | - | - | 0.31 \pm 0.02 | 0.30 \pm 0.08 | - |
| 8 h | - | - | - | 0.31 \pm 0.02 | 0.27 \pm 0.05 | - |
| 12 h | - | - | - | 0.32 \pm 0.01 | 0.24 \pm 0.01 | - |
| 24 h | - | - | - | 0.17 \pm 0.01 | 0.23 \pm 0.04 | - |

* All values for koalas 1 to 3 and 6 were not detectable

** The values that are between LLOD (0.13 $\mu\text{g/mL}$) and LLOQ (0.39 $\mu\text{g/mL}$) have also been reported in this table, however, these values are not necessarily the real values as they are below the LLOQ of the HPLC method.

5.5 Discussion

An HPLC method with a PDA detector and an isocratic mobile phase was developed to detect amoxicillin in koala plasma. As explained in Chapter 4, similar to cefovecin, developing an HPLC method to determine amoxicillin concentrations in koala plasma was challenging and

required numerous modifications to overcome selectivity issues. Various HPLC methods are reported for amoxicillin determination in biological fluids using UV detection (Muth et al., 1996, Gulland et al., 2000, Hoizey et al., 2002, Abreu et al., 2003, Foroutan et al., 2007, Jerzsele et al., 2009) and fluorescence detection (Mascher and Kikuta, 1998, Wibawa et al., 2002). Several sample extraction techniques have been reported, such as PPE (Mascher and Kikuta, 1990, Gulland et al., 2000, Hoizey et al., 2002, Abreu et al., 2003, Jerzsele et al., 2009), LLE (Foroutan et al., 2007, Ajitha et al., 2010), SPE (Pingale et al., 2012, Gaikwad et al., 2013) and ultrafiltration (Muth et al., 1996, Matar, 2006). Detecting amoxicillin in koala plasma was not expected to be so challenging as numerous HPLC methods using UV detection in other species are published (summarised in Table 5-4). The intrusion of multiple endogenous peaks within the koala plasma matrix eliminated many chromatographic conditions and sample extraction techniques that had been used to detect amoxicillin in the biological tissues and fluids of other species. The optimal isocratic mobile phase of 5 % methanol and 95 % sodium phosphate buffer (20 mM, pH: 3.15) was similar to other published HPLC conditions (Foroutan et al., 2007, Ahmed et al., 2011). During method optimisation, as amoxicillin retention time was altered by small pH changes, mobile phase solutions with pH values from 2 to 7 were trialled. A pH of 3.15 produced the best mobile phase condition.

Based on the UV spectrum consistent with the previous report of Tippa and Singh (2010), the maximum response of amoxicillin peak was at the wavelength of 228 nm. However, detection wavelengths less than 230 nm reportedly results in greater interference by low-molecular-mass endogenous substances, necessitating more rigorous drug extraction (Mascher and Kikuta, 1998).

Table 5-4 A summary of HPLC conditions with UV detection to determine amoxicillin concentrations

| Sample matrix | Sample preparation * | Mobile phase | Column; Thermostat temperature*; UV Wavelength | Linearity range; LLOQ ($\mu\text{g/mL}$) |
|---|--|---|--|--|
| Human plasma (Muth et al., 1996) | Ultrafiltration IS: cefadroxil | Gradient Solvent A and B (10 mM heptane sulfonate and 30 mM sodium dihydrogen phosphate in a solution of purified water-methanol (75:25 and 92:8 v/v, respectively) (pH: 2.5) | Spherisorb ODS II (5 μm , 250 x 4.6 mm); 40 °C; 230 nm | 0.05 – 25.4; 0.05 |
| Seal plasma (Gulland et al., 2000) | PPE: perchloric: citrate/phosphate buffer | Isocratic 5 % acetonitrile 95 % phosphate buffer (pH: 5) | Zorbax SB-C8 (4.6 mm \times 15 cm) 40 °C; 229 nm | 0.156 – 10; 0.656 |
| Human plasma (Hoizey et al., 2002) | PPE: methanol Recovery: 95 – 99 % | Gradient: Solvent A (potassium dihydrogen phosphate & tetramethylammonium chloride (pH: 2.5) Solvent B (acetonitrile) | Lichrospher column 100 RP8 (5- μm , 250 \times 4 mm); Ambient temp; 220 nm | 0.625 – 20; 0.625 |
| Human plasma (Abreu et al., 2003) | PPE: methanol IS: cefadroxil Recovery: >90 % | Isocratic 5 % acetonitrile 95 % sodium phosphate buffer (pH: 4.8) | Lichrosorb® 10 μm RP18; Ambient temp; 229 nm | 1 – 50; 1 |
| Human plasma (Foroutan et al., 2007) | PPE: acetonitrile then LLE: dichloromethane Recovery: 93.8 % IS: allopurinol | Isocratic 4 % methanol 96 % sodium phosphate buffer (pH: 3) | Chromolith Performance column (RP-18e, 100 mm \times 4.6mm); - 228 nm | 0.2 – 12; 0.015 |
| Broiler chicken plasma (Jerzsele et al., 2009) | PPE: methanol Recovery: 95 % | Isocratic: 10 % methanol 90 % potassium phosphate buffer (pH: 7) | C18 analytical column (10- μm , 250 \times 4 mm); - 220 nm | 0.2 – 20; 0.16 |
| Bulk drug & pharmaceutical dosage forms (Ahmed et al., 2011) | Dissolving in mobile phase and then sonication Recovery: 99 % | Isocratic 5 % methanol 95 % potassium phosphate buffer | Hypersil C18 (250 mm \times 4.6 mm); Ambient temp; 254 nm | 20 – 100; 1.254 |

* Some articles do not include the IS used and / or thermostat temperature.

Numerous sample extraction techniques such as PPE, LLE, SPE and ultrafiltration were trialled to clean up the koala plasma samples. Ultimately, sample preparation was undertaken by LLE as only this method enabled elimination of interfering substances around the retention time of amoxicillin. In LLE, by adding non-polar organic solvent, the sample is partitioned into two portions: an aqueous layer which contains the polar compounds and an organic layer which concentrates the non-polar compounds (Li et al., 2006).

Some publications describe two steps for plasma sample preparation (Foroutan et al., 2007, Ajitha et al., 2010). First, plasma proteins are precipitated and then the amoxicillin is extracted from the supernatant using dichloromethane. Replicating the published methods did not improve assay selectivity. Therefore, LLE was trialled using different organic solvents including chloroform, ethyl acetate, heptane, hexane and TBME. A mixture of the different volumes of organic solvents (e.g. TBME and ethyl acetate 1:1 volume, etc.) was also tested. Then, the aqueous layer of each solution was mixed with dichloromethane to extract amoxicillin. Those organic solvents that resulted in cleaner chromatograms underwent the next optimisation step to determine whether acidifying the plasma samples improves extraction. Ultimately, the procedure with the best selectivity and recovery for amoxicillin involved plasma samples initially being acidified with phosphoric acid and then amoxicillin extraction with TBME and dichloromethane.

The assay linearity range selected was 0.2 – 12 µg/mL as the C_{\max} of amoxicillin trihydrate administered via extravascular routes did not exceed 12 µg/mL in most species (see Table 1-7).

Initially, the HPLC method was developed to determine the plasma concentration of amoxicillin and clavulanic acid (Appendix I); however, clavulanic acid was found to be very unstable. There was an approximate 46 % loss of the clavulanic acid in all QC samples (1, 5 and 10 µg/mL) after one-month storage at – 20 °C (Appendix II). As clavulanic acid acts as a potent bacterial beta-lactamase inhibitor, co-administration of amoxicillin with clavulanic acid does not appear to alter PK parameters of amoxicillin in any species (Reyns et al., 2007, Prescott, 2013a). Therefore, amoxicillin alone was selected for investigation.

The plasma concentration of amoxicillin was below the LLOQ in most of the koalas and only above the LLOQ in two of the six koalas at some time points. However, the stability test results do not support these results. Unfortunately, amoxicillin degraded during storage at –

20 °C between collection and analysis time, resulting in low amoxicillin concentrations in the plasma samples. The amoxicillin HPLC assay was initially developed and validated for koalas at AZWH, QLD. This assay had been validated with AZWH koala plasma prior to sample collection. However, staffing issues when amoxicillin administration and plasma collection were requested prevented AZWH from assisting with the study. Fortunately, Dr Larry Vogelneust and the Animal Ethics Committee of the Taronga Zoo approved recruiting collection koalas for the study. The Taronga Zoo koala plasma samples showed additional interference (different to that of the AZWH koala plasma), which required further assay modification and additional validation which delayed quantifying the amoxicillin plasma concentrations.

There are differing reports on amoxicillin stability in human plasma or serum: amoxicillin was stable for 50 days in human serum at – 70 °C while amoxicillin concentration degraded 25 – 35 % when stored at – 20 °C (Mascher and Kikuta, 1998). Amoxicillin was stable for at least 1 month in human plasma stored at – 20 °C (Foroutan et al., 2007) and – 70 °C (Matar, 2006), and for 61 days after storage at – 70 °C (Yaxin et al., 2016). A stock solution of amoxicillin was stable at – 70 °C for 4 months (Yaxin et al., 2016). To the best of this author's knowledge, there are no reports of amoxicillin's stability for more than two months in plasma or serum. Our stability test showed about a 60 % reduction in the amoxicillin concentration in QC samples stored for 5 months at – 20 °C.

Amoxicillin (with or without clavulanic acid) is administered to treat bacterial infections, especially pneumonia, in koalas. Bacterial species such as *B. bronchiseptica* and *E. coli* have been isolated from captive and free-ranging koalas with pneumonia (Blanshard and Bodley, 2008). There is no published information on the plasma breakpoints to inhibit these pathogens in koalas. Theoretically such breakpoints are primarily determined by the pathogen and drug rather than the host species. The following published information was extrapolated for use in

the koala: MIC₉₀ of amoxicillin/clavulanic acid to inhibit the growth of *B. bronchiseptica* (in pigs) = 2 µg/ml (Prescott, 2013b), the MIC₉₀ of amoxicillin to inhibit *susceptible* strains of *E. coli* (non-urinary) from dogs and cats ≤ 0.25 µg/mL (CLSI, 2013b), and the susceptibility breakpoint to inhibit *Staphylococci* spp. and *Streptococci* spp. ≤ 0.25 µg/mL (CLSI, 2013b). As there was approximately 60 % loss of the amoxicillin, the real plasma concentrations of the samples above LLOQ can be estimated to be 1.625, 1.425 and 1.05 µg/mL at time 1, 2 and 4 h, respectively in koala 5, and 1.025 µg/mL at time 0.5 and 1 h in koala 4. These estimates suggest that the plasma concentration of amoxicillin at 10 mg/kg s.c. in koalas might reach the MIC breakpoints for pathogens inhibited with amoxicillin MIC ≤ 1 µg/ml.

The PK/PD relationship that determines therapeutic efficacy for time-dependant antibacterials such as amoxicillin is the time interval that the drug concentration exceeds the MIC in serum or plasma. Although the data are limited, if the amoxicillin concentration exceeded 1 µg/mL for at least 4 hours, the frequency of administration would need to be a minimum of every 8 hours [as the dosage interval should be maintained above 40 to 50 % of the MIC for both susceptible gram-positive and gram-negative pathogens (Jacobs, 2001, MacVane et al., 2014)]. This might be impractical to treat non-domesticated species (injection of a drug every 8 h) if there are other efficacious antibacterials such as enrofloxacin and chloramphenicol. To effectively treat pneumonia, antimicrobials must penetrate the blood-bronchus barrier and attain sufficient concentrations at the sites of infection to reach or exceed the antibacterial MIC (Valcke et al., 1990, Honeybourne, 1994, Agersø et al., 1998). Lipophilic drugs such as enrofloxacin are more likely to reach this site than hydrophilic drugs such as amoxicillin (Valcke et al., 1990, Honeybourne, 1994, Papich and Taboada, 2005). As amoxicillin concentration in bronchial mucosa is approximately 40 % of its concurrent serum concentration (Honeybourne, 1994, Cook et al., 1994), higher dose rates might be required to achieve the therapeutic concentration at the site of infection to treat pneumonia in adult koalas.

Enrofloxacin could be a better choice to treat pneumonia in koalas, as its PK profile is known (Griffith et al., 2010, Black et al., 2013a). Enrofloxacin can be a better choice to treat feral animal bites infected by aerobic pathogens, but it has poor efficacy against anaerobic pathogens (Giguère and Dowling, 2013). Amoxicillin with or without clavulanic acid is active against anaerobic pathogens (Prescott, 2013a) but susceptibility testing against these pathogens has not been standardised to predict the amoxicillin concentrations required to inhibit anaerobic pathogens injected via bites. Blanshard and Bodley (2008) proposed a dose rate of metronidazole, another lipophilic drug, with efficacy against anaerobic pathogens, but its PK profile in koalas is not established.

It might be worth repeating this study to confirm the amoxicillin PK profile in koalas. Minimum storage duration and temperatures appear critical to assay amoxicillin. As the stability test at – 20 °C was finished in June 2017, repeating the study which required reapplication of animal ethics approval and time intensive processes of *in-vivo* drug administration, blood collection and sample analysis were not possible at the late stage of this candidature. However, this chapter indicates it is doubtful that repeating this study will do anything more than confirm that amoxicillin's efficacy is too short to provide practical therapy in koalas.

Chapter 6

Microbiological assays to estimate cefovecin and amoxicillin concentrations in koala plasma

6.1 Abstract

Cefovecin and amoxicillin concentrations in koala plasma (using the medicated plasma from studies described in Chapters 4 and 5) were determined in agar disc diffusion assays to investigate their ability to inhibit the growth of *E. coli* ATCC 25922 and *Bacillus* sp. VPB 498. The assay sensitivity for both cefovecin and amoxicillin was too low as the lowest detectable concentrations were 8 µg/mL for cefovecin and 4 µg/mL for amoxicillin. Consequently, broth microdilution inhibition assays were used to test the remaining plasma samples' abilities to inhibit *E. coli* ATCC 25922. The results indicated the highest cefovecin concentration was at $t = 3$ h, which supports the HPLC results of the cefovecin study. With the amoxicillin samples, no significant growth reduction after amoxicillin administration (i.e., samples collected at time 0.5 h onwards), indicating that amoxicillin concentrations did not exceed 4 µg/mL, the amoxicillin MIC to inhibit *E. coli* ATCC 25922. As reported in Chapter 5, amoxicillin instability was likely to have affected this assay.

6.2 Introduction

Antimicrobial concentrations in biological fluids have been determined using bioassays and chromatographic assays. Bioassays are simpler, faster and cheaper than HPLC methods which require costly equipment, and laborious method development and validation. A major disadvantage of bioassays is their lower specificity to differentiate the analyte of interest from other active metabolites and antimicrobials present in the sample (Perea et al., 2000, Stead, 2000, Böttcher et al., 2001). Furthermore, microbial inhibition assays tend to be less sensitive, accurate and precise than HPLC (Perea et al., 2000, Böttcher et al., 2001). However, the bioassay is sufficiently accurate and precise to be used for some drug analyses in biological fluids (Foulstone and Reading, 1982, Perea et al., 2000, Driscoll et al., 2012). For instance, an agar disc diffusion test to determine plasma concentration of amoxicillin trihydrate in sheep

plasma was reported to have a LLOQ of 0.06 µg/mL and accuracy and precision were both within $\pm 5\%$ (Fernandez et al., 2007).

The HPLC methods described in Chapters 3, 4 and 5 did not have ideal sensitivity and could not quantitate concentrations lower than 1.02 µg/mL and 0.39 µg/mL for cefovecin and amoxicillin in koala plasma, respectively. Therefore, this investigation used additional assays to confirm cefovecin and amoxicillin concentrations in the same samples determined by HPLC.

6.3 Materials and methods

All microbial procedures used aseptic techniques to avoid extraneous microbial contamination.

6.3.1 Bacterial strains and culture conditions

E. coli ATCC 25922 and *Bacillus* sp. VPB 498 strains were used. The pathogens were sub-cultured onto tryptose agar plates (Invitrogen, San Diego, CA, USA) and incubated at 35 °C for 18 – 24 h.

6.3.2 Inoculum preparation

Bacterial colonies selected from the sub-cultured plates were emulsified in Phoenix ID broth (Becton, Dickinson & Co., North Ryde, NSW) to produce 0.5 McFarland solution (equivalent to 1.5×10^8 CFU/mL, i.e., colony-forming units per mL; a unit to estimate the number of viable bacteria in a sample) as determined by a PhoenixSpec™ nephelometer (Becton, Dickinson & Co., North Ryde, NSW).

6.3.3 Agar disc diffusion assays

The agar disc diffusion assay was performed according to the Clinical and Laboratory Standards Institute (CLSI) standards (CLSI, 2013a) to estimate cefovecin and amoxicillin concentrations in samples collected for the *in-vivo* studies described in Chapters 4 and 5.

Primary stock solutions of cefovecin (1 mg/mL) and amoxicillin trihydrate (1 mg/mL) were freshly prepared in sterile MQ water and phosphate buffer (pH: 6) (CLSI, 2013b), respectively, for each assay. Aliquots of the stock solutions were added to pooled koala plasma to obtain concentrations of 0.25, 0.5, 1, 2, 4, 8 and 16 µg/mL of cefovecin, and 0.5, 1, 2, 4, 8, 16 and 32 µg/mL of amoxicillin as a standard curve. The inoculum suspensions of both pathogens were spread as a lawn across Mueller-Hinton agar (MHA) plates with a cotton swab (Becton, Dickinson & Co., North Ryde, NSW). Then, the standard dilutions and the remaining plasma samples of cefovecin and amoxicillin *in-vivo* studies were applied to 6 mm antimicrobial ‘blank’ discs (CT998B; Oxoid Australia Pty Ltd, West Heidelberg, Vic.) in one of the following two ways: firstly, discs were placed on the agar plates and 20 µL of each sample was pipetted directly onto a disc. Each sample was repeated in triplicate. Secondly, for the assay with *E. coli* only, three discs soaked for 3 h in either each sample or standard dilutions were placed at different locations on the agar plates. Blank koala plasma and commercial discs containing 30 µg of cefovecin and 2, 10 and 25 µg of amoxicillin (Oxoid Australia Pty Ltd, West Heidelberg, Vic.) served as controls. The plates were then incubated at 35 °C for 18 – 24 h after which zone diameters of growth inhibition, where possible, were measured to the nearest millimetre.

6.3.4 Broth microdilution inhibition assays

Due to the apparent low sensitivity of the agar disc diffusion assay to determine cefovecin and amoxicillin concentrations in koala plasma, broth microdilution inhibition assays using reference strain *E. coli* ATCC 25922 and sterile 96-well culture plates (Nunclon™ Delta

Surface, Thermo Fisher Scientific, Scoresby, Vic.) were performed to determine whether the antibacterial agents (apart from endogenous antimicrobial components of plasma) present in the koala plasma after drug administration could further reduce the growth of *E. coli* compared to $t = 0$ h. The inoculum and sample volumes were the same as those prescribed by the CLSI for the broth microdilution susceptibility assay (CLSI, 2013a). To prepare the inoculum, a solution with 0.5 McFarland turbidity was diluted with Mueller-Hinton broth (MHB) to produce a concentration of 1.5×10^6 CFU/mL (CLSI, 2013a). A volume of 100 μ L of each sample was added to each well of a plate. Into each well, 10 μ L of the inoculum was added to give a bacterial concentration of 1.5×10^5 CFU/mL and a final volume of 110 μ L. Each sample was tested at least in duplicate and for each sample a proper blank (as an OD_{zero}) was also prepared simultaneously. A well containing MHB only and a positive control (MHB and inoculum) were included in each plate to establish that the MHB was uncontaminated and that the assay supported bacterial growth, respectively. Wells of six and seven two-fold serial standard dilutions of the cefovecin (8 – 0.25 μ g/mL) and amoxicillin (32 – 0.5 μ g/mL), respectively, in MHB were also prepared in triplicate to determine the MIC of cefovecin and amoxicillin to inhibit *E. coli*. Contents of each well were mixed with a pipette, covered and incubated for 18 – 24 h at 35 °C. After incubation, the wells were visually examined for \pm bacterial growth and semi-quantitated by measuring the turbidity at 620 nm (Widdel, 2007) relative to the respective blank well as OD_{zero} (an automated microplate reader, Halo LED 96, Dynamica Pty Ltd, Clayton, Vic.). The OD_{zero} is the reference optical density (OD) caused by the medium which is subtracted from the observed OD to determine the OD caused by the cells (Widdel, 2007). The MIC was considered as the lowest concentration of the cefovecin and amoxicillin that inhibited visible growth of the bacteria.

6.3.5 Statistical analysis

The mean bacterial growth data between groups were analysed statistically using ANOVA and Tukey's multiple comparisons test using GraphPad Prism 7.02, where the minimum significance level for all statistical tests was $P < 0.05$.

6.4 Results

6.4.1 Agar disc diffusion assays

6.4.1.1 Cefovecin

Neither bacterial strains' growth was inhibited by any disc impregnated with or soaked in cefovecin *in-vivo* samples (at different time points). A small zone of inhibition of *E. coli* was only observed at the standard dilutions of 8 and 16 $\mu\text{g/mL}$ of cefovecin for soaked discs (see Figure 6-1 A). The growth inhibition zones of commercial cefovecin discs 30 μg on *E. coli* and *Bacillus* sp. lawns were 27 and 15 mm, respectively (see Figure 6-1 A, B and C).

6.4.1.2 Amoxicillin

Similar to the cefovecin, there was no growth inhibition of both bacterial strain by any discs either impregnated with or soaked in amoxicillin *in-vivo* samples (at different time points). The lowest concentrations of amoxicillin that inhibited *E. coli* and *Bacillus* sp. growth were 50 and 4 $\mu\text{g/mL}$ respectively (Figure 6-1 C and D). For the latter strain, however, the inhibition zone margins for all the discs were not distinct. The growth inhibition zones of the commercial amoxicillin discs (2, 10 and 25 μg) on the *E. coli* lawn were 8, 18 and 22 mm, respectively (Figure 6-1.C). The corresponding values on the *Bacillus* sp. lawn were approximately 10, 14 mm and 16 mm (Figure 6-1 D).

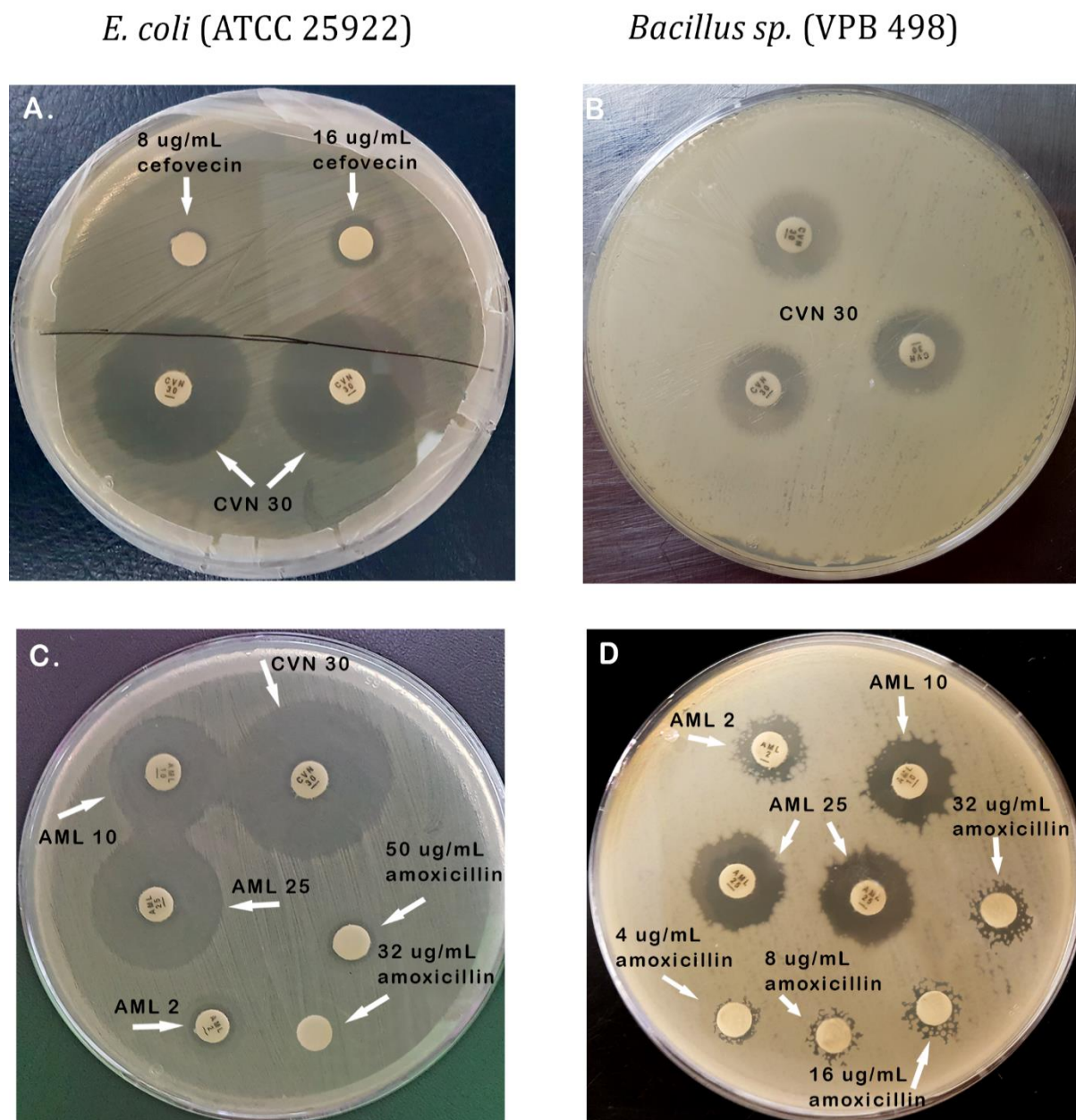


Figure 6-1 Agar disc diffusion test of cefovecin and amoxicillin using two isolates (*E. coli* ATCC 25922 and *Bacillus* sp. VPB 498) on MHA. **A.** Commercial cefovecin discs (CVN: 30 μ g) and the discs soaked at cefovecin standard dilutions (8 and 16 μ g/mL) on the *E. coli* lawn; **B.** Commercial cefovecin discs (30 μ g) on the *Bacillus* sp. Lawn; **C.** Commercial amoxicillin discs (AML: 2, 10 and 25 μ g) and cefovecin (CVN: 30 μ g) and the discs impregnated with amoxicillin standard dilutions 32 and 50 μ g/mL on the *E. coli* lawn; **D.** Commercial amoxicillin discs (AML: 2, 10 and 25 μ g) and the discs impregnated with amoxicillin standard dilutions (4, 8, 16 and 32 μ g/mL) on the *Bacillus* sp. lawn.

6.4.2 Broth microdilution inhibition assays

Unlike the positive controls (MHB and inoculum), there was no bacterial growth in the negative controls (MHB only) on each plate. The MIC of cefovecin and amoxicillin to inhibit *E. coli* ATCC 25922 were 0.5 µg/mL [within the range of 0.5 – 2 µg/mL specified by CLSI VET01-S2 (CLSI, 2013b)] and 4 µg/mL [within the range of 2 – 8 µg/mL specified by CLSI VET01-S2 for ampicillin (CLSI, 2013b)], respectively (Figure 6-2 and 6-3).

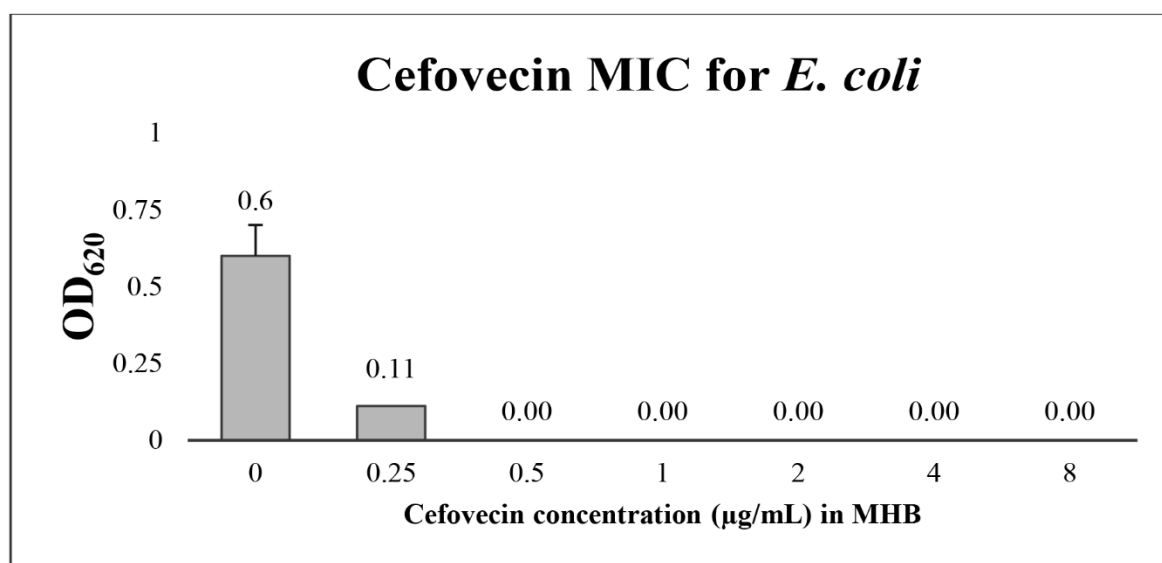


Figure 6-2 MIC of cefovecin to inhibit *E. coli* ATCC 25922 growth, serial dilutions of cefovecin were prepared in MHB and incubated with *E. coli* inoculum at 35 °C for 18 – 24 h. Bacterial growth was estimated by measuring the OD₆₂₀. The lowest concentration of cefovecin that inhibited *E. coli* growth was 0.5 µg/mL.

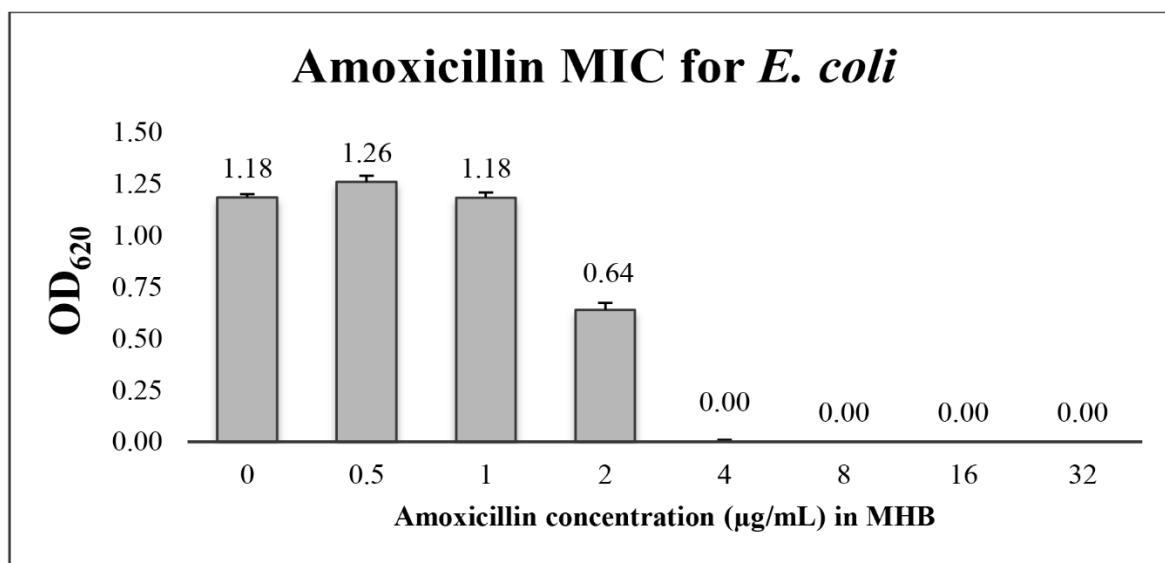


Figure 6-3 MIC of amoxicillin to inhibit *E. coli* ATCC 25922 growth, serial dilutions of amoxicillin were prepared in MHB and incubated with *E. coli* inoculum at 35 °C for 18 – 24 h. Bacterial growth was estimated by measuring the OD₆₂₀. The lowest concentration of amoxicillin that inhibited *E. coli* growth was 4 µg/mL.

6.4.2.1 Cefovecin

The *E. coli* growth when incubated with koala plasma samples of cefovecin *in-vivo* study is shown in Figures 6-4 and 6-5. Bacterial growth with plasma samples was less than that in the positive controls. The *E. coli* growth was significantly less at time 3 h compared to time 0 h ($P < 0.0011$) and 24 h ($P < 0.0156$) (see Figure 6-4).

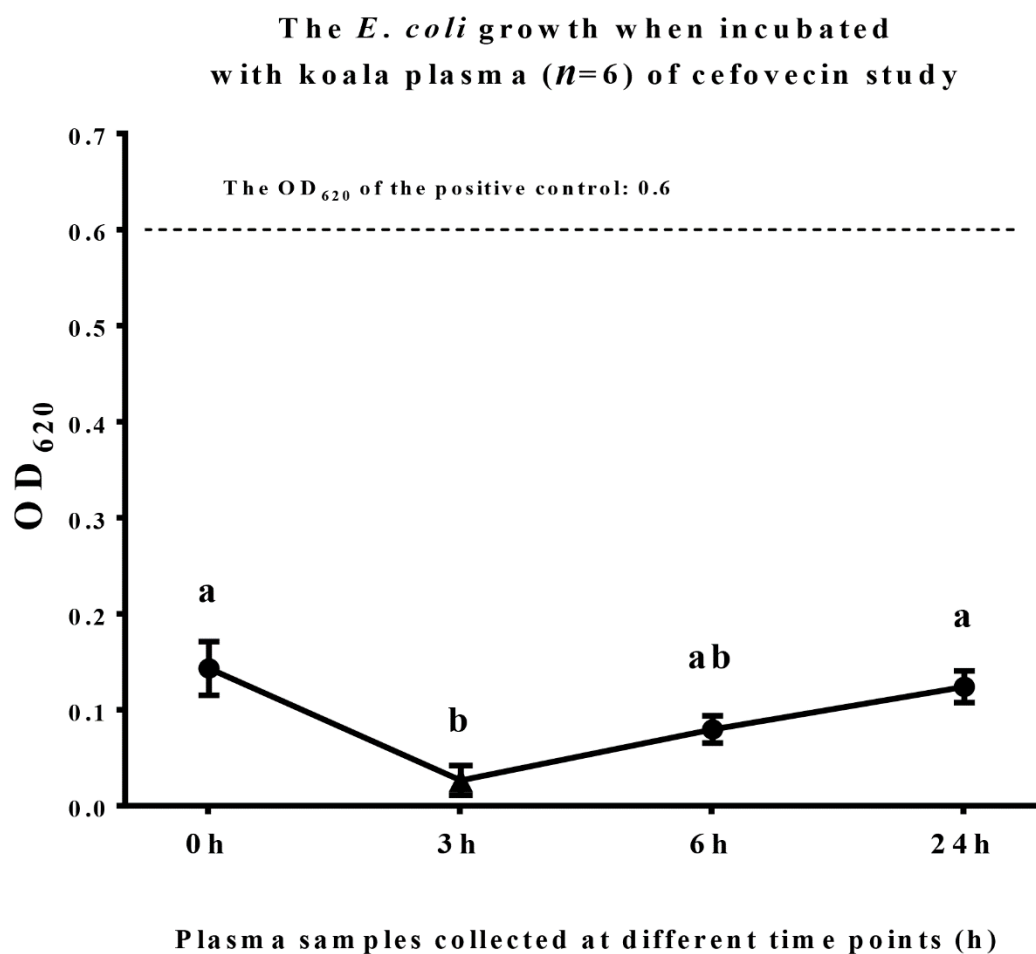


Figure 6-4 *E. coli* growth incubated with plasma samples from six koalas at different time points following cefovecin administration at 8 mg/kg s.c. Bacterial growth was estimated by measuring the OD₆₂₀. The symbols represent the mean growth of *E. coli* at each time point, and the error bars show the SE of the means. Different letters indicate a significant difference between means of each group ($P < 0.05$). The P value for the comparison between the mean of time 3 h vs 0 and 24 h was 0.0011 and 0.0156, respectively.

Given the individual koala samples (Figure 6-5), lowest *E. coli* growth was at time 3 h of five koalas. Moreover, *E. coli* had almost no growth with plasma samples at time 6 h of koala 3 and time 0 h of koala 5.

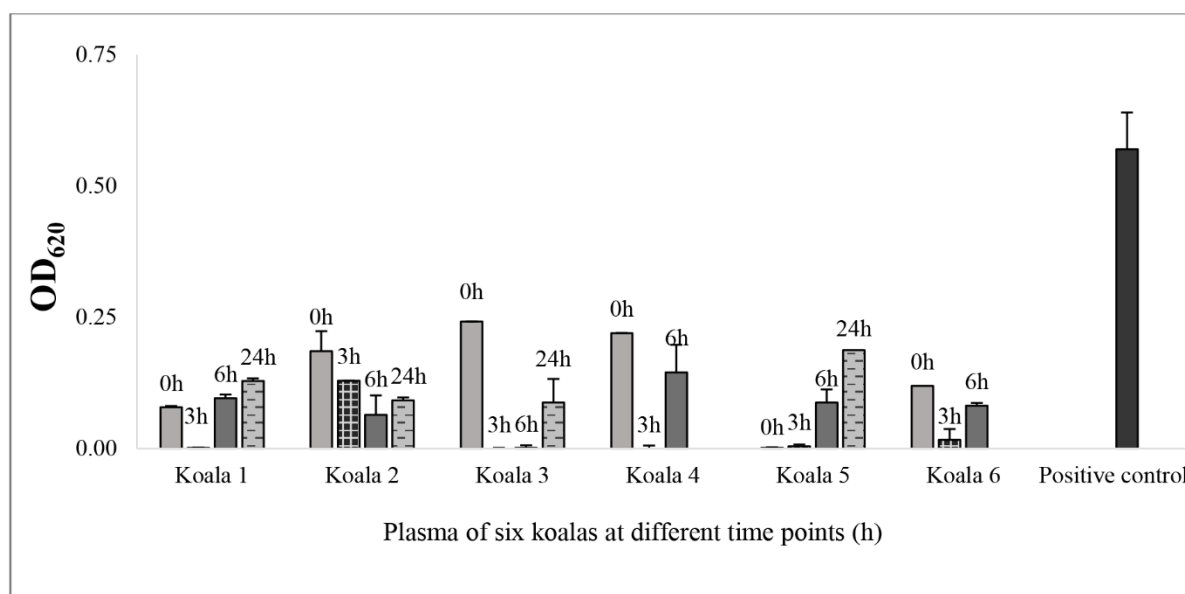


Figure 6-5 *E. coli* growth incubated with plasma samples from six koalas at different time points following cefovecin administration at 8 mg/kg s.c. Bacterial growth was estimated by measuring the OD₆₂₀. Each column represents the mean growth of *E. coli*, and the error bars show the SD of the means. The cefovecin concentration with the HPLC method (LLOD: 0.34 and LLOQ: 1.02 µg/mL) was only detected at time 3 h of koala 1 (1.03 µg/mL), koala 4 (1.05 µg/mL), koala 5 (0.73 µg/mL) and koala 6 (0.72 µg/mL).

6.4.2.2 Amoxicillin

The *E. coli* growth incubated with koala plasma samples of amoxicillin *in-vivo* study is shown in Figures 6-6 and 6-7. Similar to the cefovecin, amoxicillin samples inhibited *E. coli* growth compared to the positive controls. Bacterial growth, however, fluctuated between different time points and the lowest *E. coli* growth was at time 0 h and 24 h (see Figure 6-6). *E. coli* growth in individual koala plasma samples collected over 24 h also fluctuated at sequential time points.

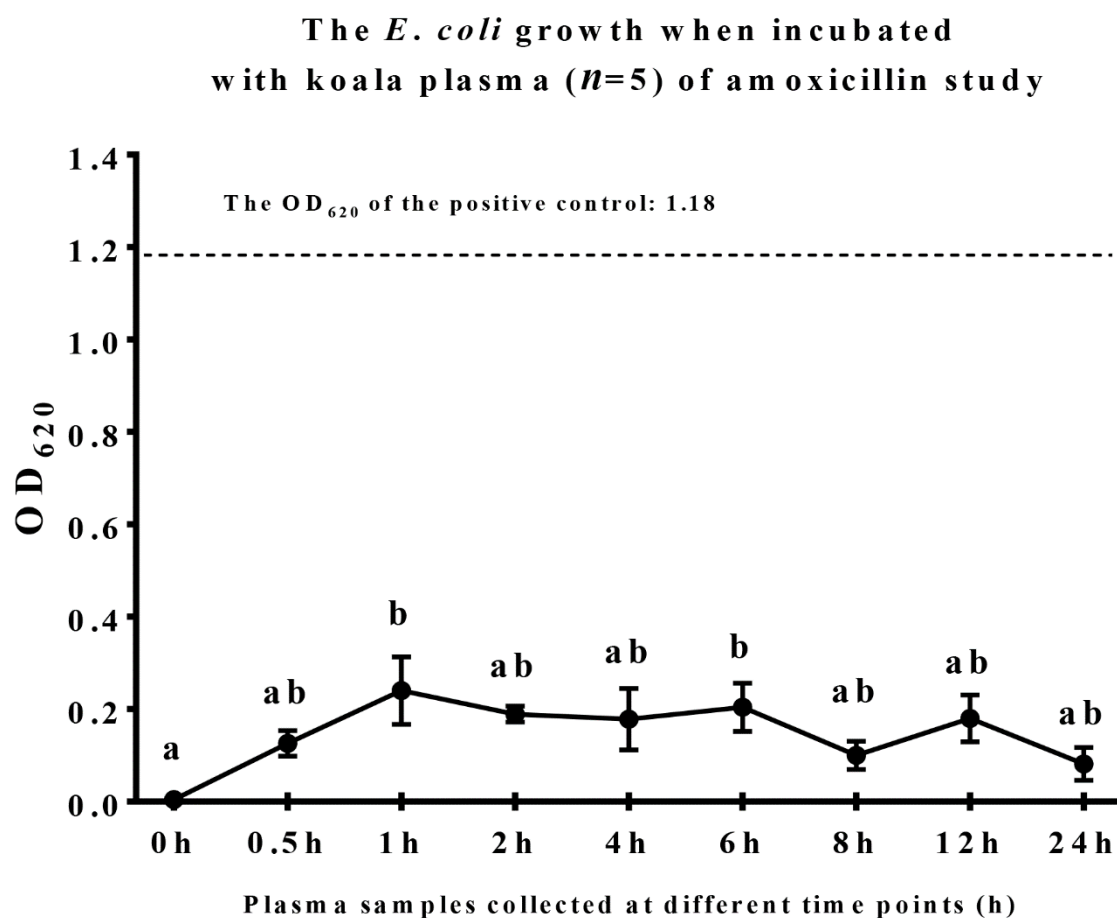


Figure 6-6 *E. coli* growth when incubated with plasma samples from five koalas at different time points following amoxicillin administration at 10 mg/kg s.c. Bacterial growth was estimated by measuring the OD₆₂₀. The symbols represent the mean growth of *E. coli* at each time point, and the error bars show the SE of the means. Different letters indicate a significant difference between means of each group ($P < 0.05$). The P values for the comparison between the mean of time 0 h vs 1 h and 6 h were 0.0068 and 0.0316, respectively

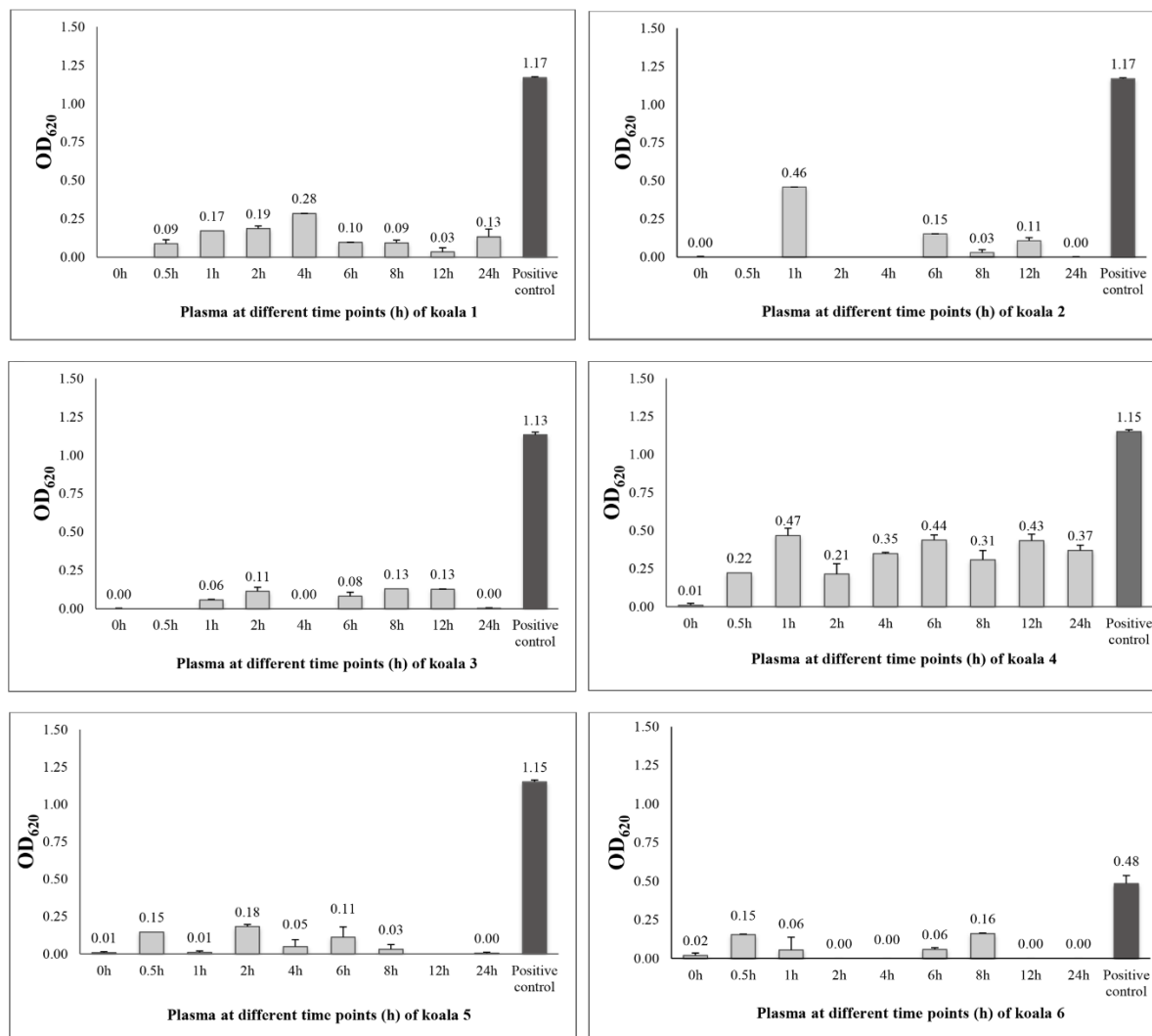


Figure 6-7 *E. coli* growth incubated with plasma samples from six koalas at different time points following amoxicillin administration at 10 mg/kg s.c. The koalas are ordered by date of amoxicillin administration as per Table 5.3. Bacterial growth was estimated by measuring the OD₆₂₀. Each column represents the mean growth of *E. coli* and the error bars the SD of the means. The amoxicillin concentration with HPLC method (LLOD: 0.13 and LLOQ: 0.39 µg/mL) was only quantitated in koala 4 at time 0.5 h (0.41 µg/mL) and 1 h (0.41 µg/mL), and koala 5 at time 1 h (0.65 µg/mL), 2 h (0.57 µg/mL) and 4 h (0.43 µg/mL).

The assay for koala 6 was conducted separately to other koalas and this koala's amoxicillin concentrations were not incorporated in Figure 6-7. Also, due to the paucity of samples, 75 µL of each sample was used instead of 100 µL to run the assay for this koala's samples.

6.5 Discussion

Although low concentrations of antimicrobials have been detected by some bioassays, the agar disc diffusion assay performed here was not sensitive enough to detect concentrations below 8 µg/mL using *E. coli* for cefovecin and 4 µg/mL using *Bacillus* sp. for amoxicillin. The agar disc diffusion procedure described by the CLSI was followed (CLSI, 2013a) and commercial cefovecin and amoxicillin discs (30, and 2, 10 and 25 µg, respectively) were used as the positive controls. The inhibition zones of commercial discs on the *E. coli* agar plate (cefovecin 30 µg: 26 – 28 mm and amoxicillin 10 µg: 18 mm) were within the range prescribed by Table 4 of CLSI standard VET01-S2 (25 – 30 mm for cefovecin and 16 – 22 mm for ampicillin) (CLSI, 2013b).

Initially, the samples were manually applied directly to the top surface of the discs placed on the bacterial lawns as this method was successful in previous studies (Wilson et al., 2006, Griffith, 2010). However, this method showed low sensitivity. According to the instructions for commercial disc preparation (Oxoid Australia, Pty Ltd, West Heidelberg, Vic.), 10 µL of an antibacterial stock solution is added to each disc. For example, to have a final concentration of 30 µg cefovecin in each disc, 10 µL of cefovecin stock solution 3,000 µg/mL is placed on each disc. This commercial disc with 30 µg produced an inhibition zone of 27 mm. In this study, 20 µL of the plasma standard solutions containing 0.5, 1, 2, 4, 8 and 16 µg/mL of cefovecin were added to each disc, so the concentrations of the respective discs were 0.01, 0.02, 0.04, 0.08, 0.16 and 0.32 µg, too low to inhibit growth around the discs placed on the *E. coli* lawn. The discs were then soaked in the samples and standard dilutions for 3 h before being placed on the bacterial lawns, followed by incubation at 35 °C for 18 – 24 h, but this method did not significantly improve sensitivity.

As a result of low sensitivity with *E. coli*, the *Bacillus* sp. was used because it had been recommended by Bennett et al. (1966) when testing ampicillin and this in-house strain was

used successfully in another study by Wilson et al. (2006). When comparing growth inhibition zones of the cefovecin commercial discs on both *E. coli* and *Bacillus* sp. lawns, the *E. coli* inhibition zones were larger, indicating *E. coli* is a more sensitive indicator than *Bacillus* sp. to cefovecin. The presence of colonies within the inhibition zones on the *Bacillus* sp. lawn (seen in Figure 6-1 D) suggests heteroresistance to amoxicillin in this species (El-Halfawy and Valvano, 2015). However, due to this assay's insensitivity, improving the sensitivity and investigating the heteroresistance were considered unproductive and abandoned.

The lack of assay sensitivity of the agar disc diffusion inhibition assay could be attributed to interactions between one or more of the following factors: the koala plasma, use of insensitive bacterial strains and / or issues with the agar that inhibited drug diffusion (Driscoll et al., 2012).

Broth microdilution susceptibility assays were more sensitive to inhibit *E. coli* growth with lower concentrations of antibacterial agents. The cefovecin MIC in MHB was determined to be 0.5 µg/mL; therefore, cefovecin concentrations greater or equal to 0.5 µg/mL of cefovecin present in plasma could inhibit *E. coli* growth but the actual inhibitory concentration could not be quantified. Despite the variation in *E. coli* growth with plasma samples within the cefovecin study, the growth was inhibited more at 3 h than time 0 or 24 h. These findings are consistent with the HPLC results, where cefovecin concentrations were 0.7 – 1 µg/mL in four koalas at time 3 h. Although broth microdilution assays cannot be relied on alone because of the endogenous antimicrobial activity of plasma components (Hirsch, 1960, Taylor, 1983, Yeaman, 1997, Tang et al., 2002), they confirmed the cefovecin HPLC results by demonstrating maximum *E. coli* inhibition at 3 h.

The amoxicillin samples did not reduce *E. coli* growth significantly in samples collected at time 0.5 h onwards, indicating low concentrations of amoxicillin in plasma samples (i.e. lower than the amoxicillin MIC to inhibit *E. coli*). Broth microdilution assays of amoxicillin samples were undertaken almost 5 to 6 months after sample collection (shortly after HPLC analysis).

At this time, the long-term stability of amoxicillin in koala plasma at – 20 °C had not been established. However, stability test results indicate that amoxicillin present in koala plasma samples had been largely degraded. Degradation of amoxicillin is mainly due to the hydrolysis of the beta-lactam ring (Hou and Poole, 1971) which produces microbiologically-inactive metabolites (Papich and Riviere, 2009) unlikely to inhibit *E. coli* growth.

The bioassays were only employed as an adjunctive assay to confirm the HPLC results. The cefovecin broth microdilution assays further support the idea that a single bolus injection does not confer sustained antibacterial activity in koalas. The amoxicillin broth microdilution assays demonstrated no significant changes in *E. coli* growth after drug administration, which also implies amoxicillin concentrations lower than the MIC to inhibit *E. coli* (4 µg/mL). These results are consistent with the HPLC results, indicating low concentrations of amoxicillin.

The intriguing, unique finding from the cefovecin broth microdilution assay was the variability of antibacterial activity between clinically normal koalas even before they were medicated, and between sequential time points of an individual koala. The reason for this variation in *E. coli* growth is explored further in the next chapter.

Chapter 7

Investigation into the antibacterial activities of plasma, serum, delayed-coagulation serum and heated serum of koalas and other selected species

7.1 Abstract

The comparative antibacterial activities of plasma, serum, delayed-coagulation serum and heated serum of koalas and selected species were investigated by broth microdilution inhibition assay using two reference strain pathogens (*E. coli* ATCC 25922 and *S. aureus* ATCC 29213). Compared to the positive controls, both pathogens grew less when incubated with the blood matrices of all species studied. Koala samples varied significantly in inhibiting growth of both pathogens compared to those of the other species.

7.2 Introduction

Whole blood and its components such as serum and plasma, with or without platelets, are recognised to possess antimicrobial activity (Hirsch, 1960, Taylor, 1983, Yeaman, 1997, Tang et al., 2002, López-Medrano et al., 2016). Plasma is the supernatant produced after centrifuging blood mixed with an anticoagulant such as sodium or lithium heparin, citrate or oxalate (Uges, 1988). Serum, in contrast to plasma, excludes fibrinogens (clotting proteins), and is the resulting supernatant when whole blood is allowed to clot and then centrifuged (Uges, 1988). Constituents of serum and plasma that exhibit antimicrobial activity include complement, lysozyme enzymes, antibodies and small antimicrobial peptides (AMPs) (Taylor, 1983). Additionally, an activated coagulation system contributes to innate immunity by entrapping bacteria inside the clot and producing AMPs (Taylor, 1983, Berends et al., 2014).

The *in-vitro* antimicrobial activity of eucalypt extracts and EOs (Sartorelli et al., 2007, Ghalem and Mohamed, 2008, Gilles et al., 2010, Tyagi and Malik, 2011, Elaissi et al., 2011) suggests a hypothesis that the almost exclusive eucalypt diet of koalas could have a synergistic effect on the antimicrobial activity of koala plasma and / or serum. Although their diet is based on the ingestion of one specific plant genus, wild and captive koalas prefer a variety of eucalypt species foliage in their diet (Blanshard and Bodley, 2008). The diet of wild koalas can include

a diverse range of *Eucalyptus* leaves based on tree abundance and species variety within their habitat (Callaghan et al., 2011, Marsh et al., 2014). For example, eight wild koalas monitored for two weeks at Philip Island, Vic., mainly preferred *Eucalyptus viminalis*, *Eucalyptus globulus* and *Eucalyptus ovata* foliage from 139 trees they visited (Marsh et al., 2014). At Taronga Zoo, koalas are fed a variety of *Eucalyptus* species simultaneously, including *Eucalyptus amplifolia*, *Eucalyptus microcorys*, *Eucalyptus molucanna*, *Eucalyptus punctate*, *Eucalyptus robusta*, *Eucalyptus scoparia* and *Eucalyptus tereticornis* (personal communication with Taronga Zoo staff).

The chemical composition and biological activities of many *Eucalyptus* species have been studied (discussed in Chapter 1, Section 1.2.5). Eucalypt EOs are estimated to account for 16 % of the total digestible energy intake by koalas (El-Merhibi et al., 2007). The susceptibility of pathogens to a range of *Eucalyptus* species EOs varies significantly (Elaissi et al., 2011). For example, EOs extracted from *E. viminalis* (Elaissi et al., 2011) and *E. globulus* (Tyagi and Malik, 2011) inhibited *S. aureus* more than *E. coli* while *E. ovata* (Elaissi et al., 2011) and *E. robusta* (Sartorelli et al., 2007) inhibited *E. coli* more than *S. aureus*.

Whilst research has focused on the koala's physiological and anatomical adaptations to deal with its almost exclusive eucalypt foliage diet, little has focussed on the therapeutic properties and effects of a eucalypt diet on the koalas' susceptibility or resistance to infectious diseases. To the best of this author's knowledge, there is no published information about the baseline antibacterial activity of koala blood matrices. Thus, this study aimed to evaluate the antibacterial activity of koala plasma and to compare it with that of other species. Additionally, the antibacterial activities of serum, delayed-coagulation serum (DCS) and heated serum (HS) of koalas and sheep (*Ovis aries*) to inhibit *E. coli* and *S. aureus* were also compared. Delayed-coagulation serum collected after slow coagulation of the blood for experiment 2 is platelet-free and is the blood matrix with the lowest antibacterial activity in humans and rabbits (Hirsch

1960). Hirsch (1960) used the term *plasma serum* to differentiate the serum collected after delayed coagulation of the blood from normal serum (Hirsch, 1960). A third experiment undertaken compared the antibacterial activities of plasma and serum in inhibiting *E. coli* and *S. aureus*.

7.3 Materials and methods

All the following microbial procedures were undertaken using aseptic technique to avoid extraneous microbial contamination of the assays.

7.3.1 Bacterial strains, culture conditions and inoculum preparation

Reference strains *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were used and sub-cultured onto tryptose agar plates (Invitrogen, San Diego, CA, USA) and incubated at 35 °C for 18 – 24 h. Individual bacterial colonies were selected and emulsified in Phoenix ID broth (Becton, Dickinson & Co., North Ryde, NSW). A PhoenixSpec TM nephelometer (Becton, Dickinson & Co., North Ryde, NSW) was used to produce a solution of 0.5 McFarland turbidity (1.5×10^8 CFU/mL). Solutions were further diluted with MHB to produce an inoculum of 1.5×10^6 CFU/mL.

7.3.2 Broth microdilution inhibition assays

Plasma, serum, HS and DCS samples were tested for antibacterial activity by broth microdilution assay to inhibit both reference strains using 96-well culture plates (Nunc™ Delta Surface, Thermo Fisher Scientific, Scoresby, Vic.). The inoculum and sample volumes were the same as prescribed for the broth microdilution susceptibility assay (CLSI, 2013a): a volume of 100 µL of each sample was added to each well. To each well, 10 µL of inoculum was added to give a bacterial concentration of 1.5×10^5 CFU/mL and a final volume of 110 µL. Samples were tested in triplicate and for each sample, proper blanks were prepared concurrently. For every assay run, a well containing only MHB and a positive control (MHB

and inoculum only) were included in each plate to establish that the MHB was uncontaminated and that the assay supported bacterial growth, respectively. Contents of each well were mixed with a pipette and the plates were covered and incubated for 18 – 24 h at 35 °C. After incubation, the wells were visually examined for \pm bacterial growth and semi-quantitated by measuring the turbidity at 620 nm (Widdel, 2007) relative to the respective blank well as OD_{zero} (an automated microplate reader, Halo LED 96, Dynamica Pty Ltd, Clayton, Vic.).

7.3.3 Experimental design

7.3.3.1 Experiment 1: Antibacterial activities of koala, cow (Bos taurus) and horse plasma to inhibit E. coli and S. aureus

Plasma samples underwent broth microdilution inhibition assay against each reference strain. Blood samples were obtained opportunistically from clinically normal, unmedicated koalas at Taronga Zoo ($n = 5$), Sydney, Wildlife World, Darling Harbour ($n = 4$) (both located in central Sydney, NSW), Port Macquarie Koala Hospital in regional/semiurban NSW ($n = 4$), and Currumbin Wildlife Hospital in semi-urban QLD ($n = 2$). Blood samples collected opportunistically from clinically normal horses ($n = 2$) and cows ($n = 2$) at the SSVS farms were used for comparison. Sodium heparin was used as the anticoagulant for all samples. Blood samples were centrifuged for 15 min at 4,000 g and the plasma supernatant was collected promptly and stored at $-20\text{ }^{\circ}\text{C}$ away from light until analysis.

To assess the antibacterial activity of sodium heparin, 1 mL MHB was added to nine commercial blood collection (green-top) tubes containing 102 IU sodium heparin (BD Vacutainer™ Plastic Blood Collection Tubes with sodium Heparin 367874, North Ryde, NSW) and vortexed thoroughly. Then, 200 μL from each tube was added to the first well of three rows to perform eight two-fold dilutions (from 102 to 0.8 IU/mL). Each row of the serial

dilutions was inoculated either with 10 μ L of *E. coli* or *S. aureus* at a density of 10^5 CFU/mL. One row of the serial dilutions without any bacteria served as negative controls.

7.3.3.2 Experiment 2: Comparative activities of serum, DCS and HS of the koala vs sheep to inhibit *E. coli* and *S. aureus*

The abilities of serum, DCS and HS to inhibit *E. coli* and *S. aureus* were also determined by broth microdilution inhibition assay.

Blood samples were obtained opportunistically from five clinically normal, unmedicated koalas at Taronga Zoo, Mosman, NSW and five sheep from the SSVS farm. The blood was divided for serum and DCS preparation. To prepare the serum, the blood was drawn into plain clotting tubes (without anticoagulant) and allowed to clot at room temperature. The samples were then centrifuged for 15 min at 4,000 g and the serum supernatant was collected. The samples were stored at $-20\text{ }^{\circ}\text{C}$ away from light until analysis. Before the assay, some sera were also incubated at $56\text{ }^{\circ}\text{C}$ for 15 – 30 min to inactivate complement (Hein-Kristensen et al., 2013) and were then rapidly cooled on ice.

Platelet-free delayed-coagulation serum (DCS) samples were prepared according to Hirsch (1960). The 15 mL centrifuge tubes (Labtek Pty Ltd, Brendale, QLD) were placed into 50 mL centrifuge tubes (Labtek Pty Ltd, Brendale, QLD) filled with water and then frozen. Blood was collected directly into the cold 15 mL tubes followed by centrifugation for 20 min at 4,000 g at $0\text{ }^{\circ}\text{C}$. Subsequently, the supernatant was collected and incubated at $37\text{ }^{\circ}\text{C}$ for 1.5 h. After centrifugation, the clear serum was removed and stored at $-20\text{ }^{\circ}\text{C}$ and kept away from light until analysis.

7.3.3.3 Experiment 3: Comparative antibacterial activities of koala plasma and serum to inhibit *E. coli* and *S. aureus*

Due to the limited blood volume that can be collected from koalas, there was no possibility to compare the ability of koala plasma vs serum to inhibit bacterial growth in experiment 2. Therefore, this experiment tested for differences between the antibacterial activities of plasma vs serum against both pathogens.

Blood samples were obtained opportunistically from three clinically normal, unmedicated koalas from Taronga Zoo and one koala from the Port Macquarie Koala Hospital. Approximately 7 – 8 mL of blood from each animal provided plasma and serum as described in experiments 1 and 2. The samples were stored at – 20 °C and kept away from light until analysis.

7.3.4 Statistical analysis

The mean bacterial growth data between groups were analysed by ANOVA and Tukey's multiple comparisons test using GraphPad Prism 7.02, with a minimum significance level of $P < 0.05$.

7.4 Results

7.4.1 Experiment 1: Antibacterial activities of koala, cow and horse plasma to inhibit *E. coli* and *S. aureus*

Koala plasma exhibited a wider range of relative growth inhibition of both *E. coli* and *S. aureus* compared to that of cow and horse plasmas (see Figure 7-1 A and B). The growth of *E. coli* and *S. aureus* incubated with plasma from all species were less than the positive controls ($P < 0.0001$) and cow and horse plasma inhibited the growth of *S. aureus* significantly more than koala plasma ($P < 0.0001$).

None of the sodium heparin concentrations (0.80 – 102 IU/mL) inhibited the growth of either *E. coli* or *S. aureus*.

Figure 7-2 A and B demonstrates the antibacterial activity of koala plasma based on animal geographic location. All plasma samples inhibited *E. coli* and *S. aureus* growth to various degrees, and the bacterial growth was significantly less than the respective positive controls ($P < 0.0001$). The only significant difference between locations was between the samples from Darling Harbour, NSW and from Currumbin, QLD ($P < 0.0001$). This observation should be interpreted with caution as the Currumbin data only incorporate two koalas, of which the plasma of one was very inhibitory and the plasma from the other was far less inhibitory.

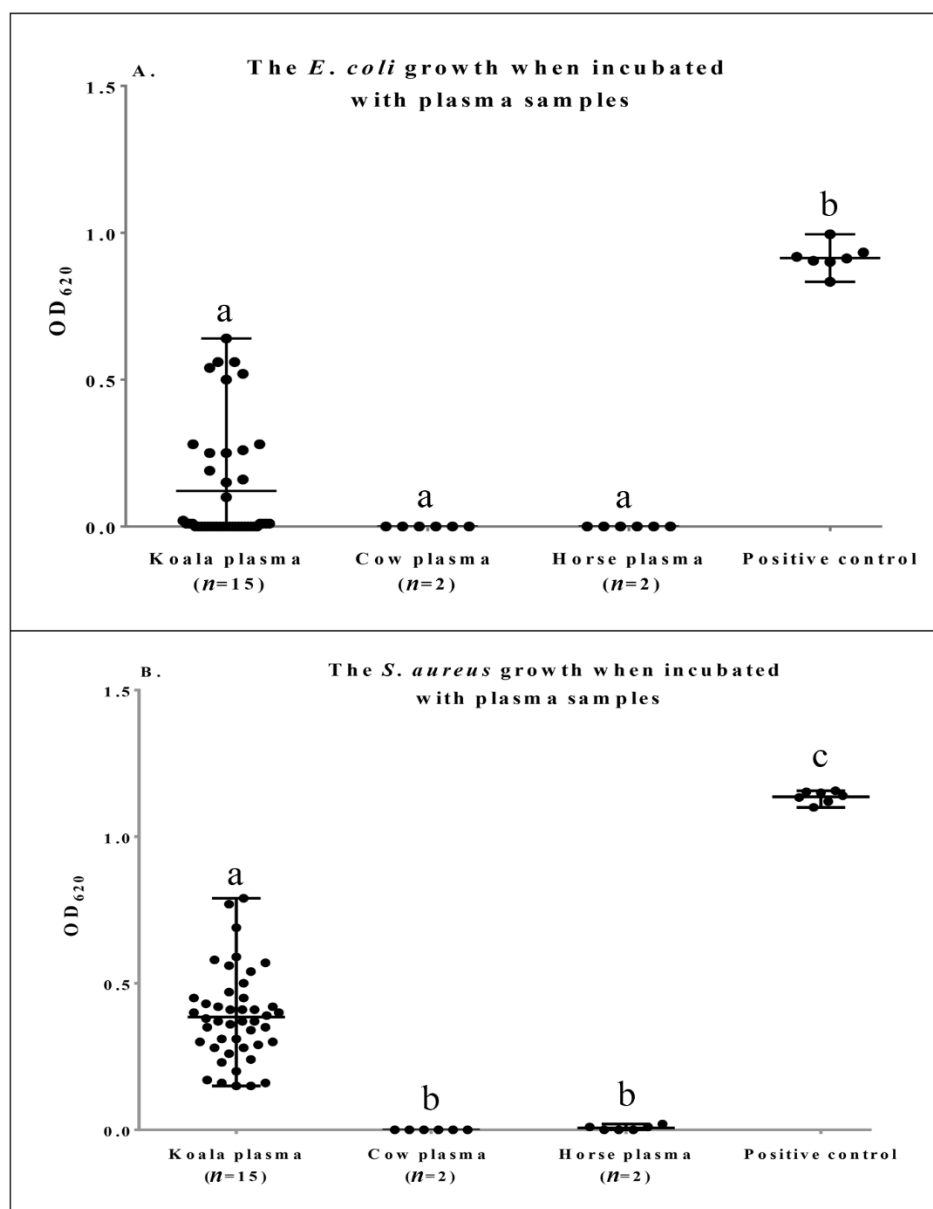


Figure 7-1 *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 29213 (B) growth incubated with different plasma samples at 35 °C for 18 – 24 h. Koala plasma samples were from 15 adult koalas. Cow and horse plasma samples were from two animals each. All animals were clinically normal and un-medicated. The growth of the pathogens was estimated by measuring the OD₆₂₀. Each datum is a replicate. The middle line is the mean and whiskers are the minimum and maximum values (range). Different letters indicate a statistical difference between means of each group in each graph ($P < 0.0001$).

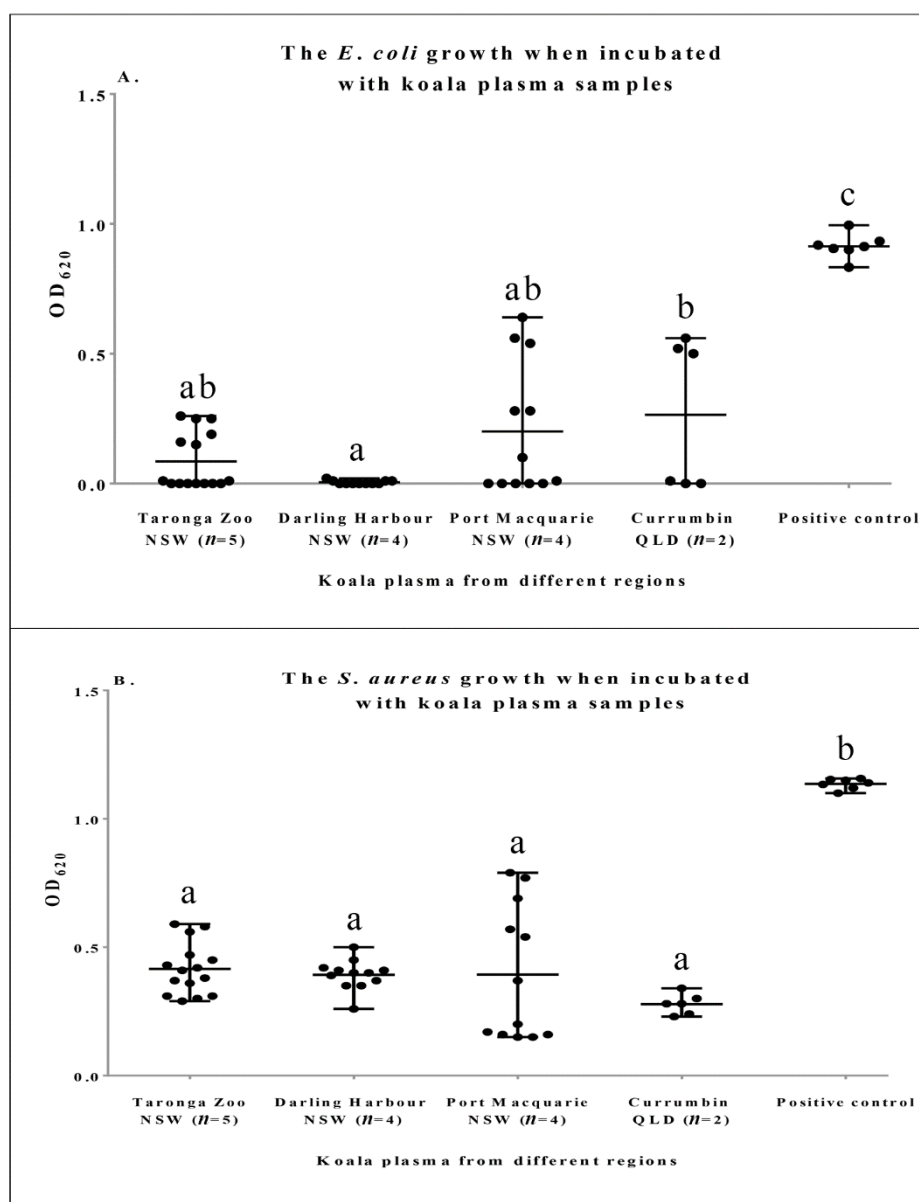


Figure 7-2 *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 29213 (B) growth incubated with koala plasma samples from four different locations at 35 °C for 18 – 24 h. The growth of the pathogens was estimated by measuring the OD₆₂₀. Each datum is a replicate. The middle line is the mean and whiskers are the minimum and maximum values (range). Different letters indicate a significant difference between means of each group in each graph ($P < 0.0001$).

7.4.2 Experiment 2: Comparative activities of serum, DCS and HS of koala vs sheep to inhibit E. coli and S. aureus

Inhibition of *E. coli* and *S. aureus* by koala and sheep DCS, serum and HS are illustrated in Figure 7-3 A and B, respectively. Bacterial growth in all groups was less than the respective positive controls ($P < 0.0001$). Inhibition of *E. coli* and *S. aureus* by sheep DCS, serum and HS had similar means and ranges. Likewise, koala DCS and serum exhibited similar means of activity against both pathogens. Koala DCS and serum resulted in lower mean of *E. coli* growth (i.e. greater inhibition) than HS ($P < 0.0001$). There was a significant difference between mean growth inhibition of *S. aureus* by koala serum and HS ($p = 0.0227$). Sheep samples inhibited *E. coli* more than the koala samples ($P < 0.0001$).

Koala samples for both experiments exhibited more variability (as represented by the OD₆₂₀ range) to inhibit pathogens' growth compared to cow, horse and sheep samples.

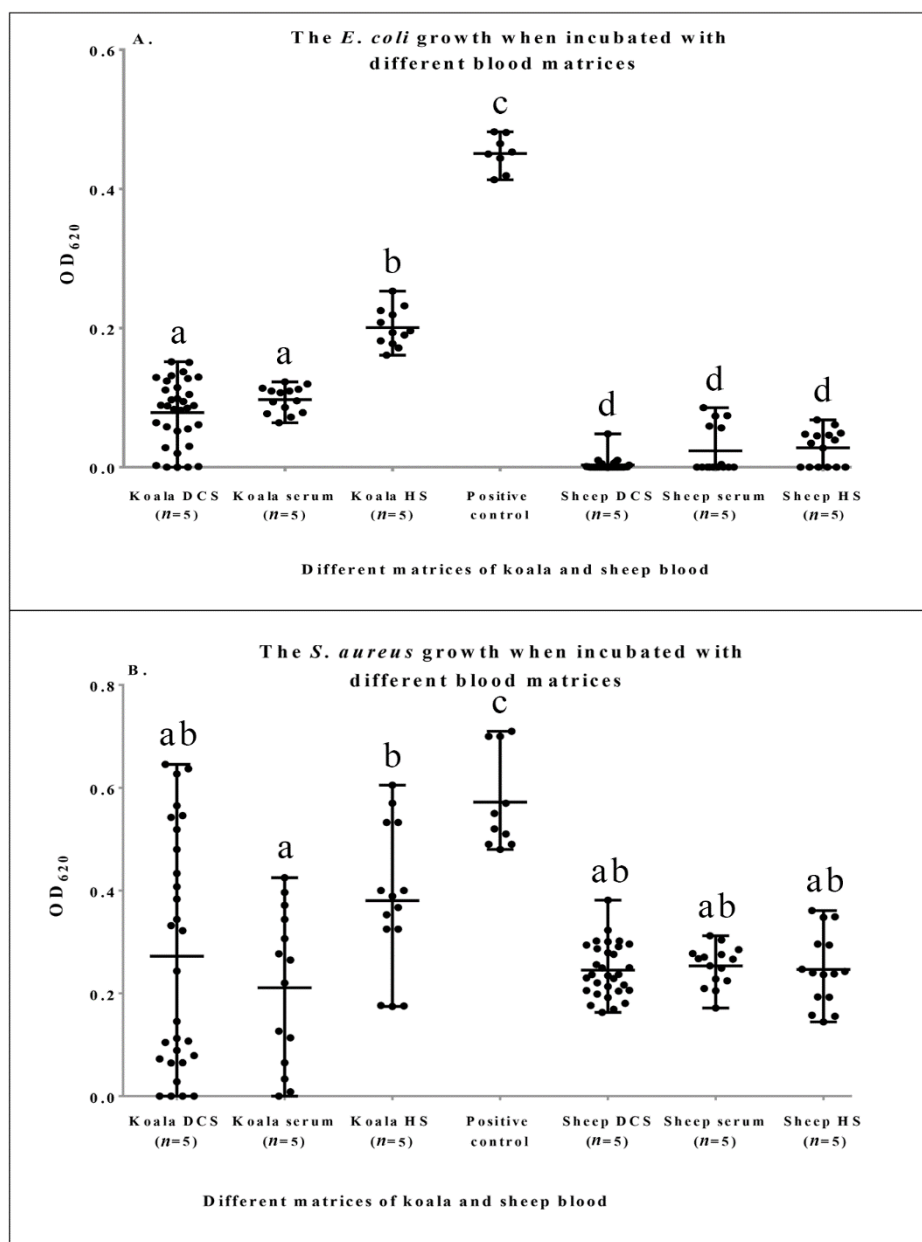


Figure 7-3 *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 29213 (B) growth incubated with different matrices of koalas ($n = 5$) and sheep ($n = 5$) blood at 35 °C for 18 – 24 h. DCS = delayed-coagulation serum; HS = heated serum. The matrices were prepared from the same blood collection from each animal. The growth of the pathogens was estimated by measuring the OD₆₂₀. Each datum is a replicate. The middle line is the mean and whiskers are the minimum and maximum values (range). Different letters indicate a significant difference between means of each group in each graph ($P < 0.05$).

7.4.3 Experiment 3: Comparative antibacterial activities of koala plasma and serum to inhibit *E. coli* and *S. aureus*

The last experiment demonstrated the same mean antibacterial activity of koala plasma and serum against *E. coli*; however, koala serum inhibited *S. aureus* more than plasma ($P < 0.0001$). Koala plasma inhibited *E. coli* more than *S. aureus*. Koala serum had similar antibacterial activities against both pathogens. Similar to the other experiments, koala samples had variable antibacterial activity against both pathogens.

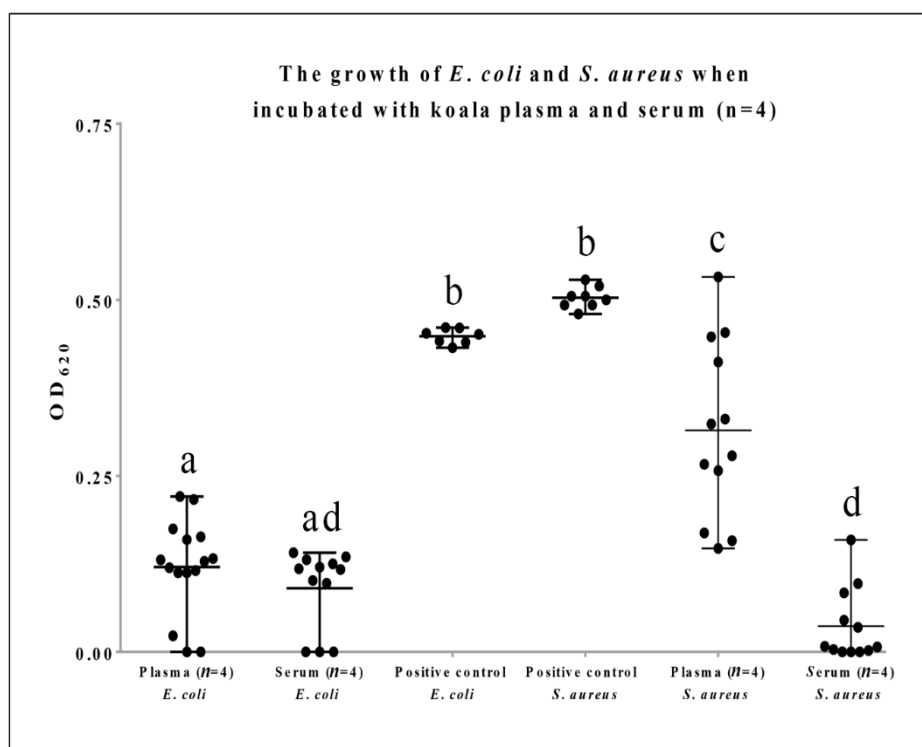


Figure 7-4 *E. coli* ATCC 25922 (A) and *S. aureus* ATCC 29213 (B) growth incubated with koala plasma ($n = 4$) or serum ($n = 4$) at 35 °C for 18 – 24 h. The plasma and serum were prepared from the same blood collection from the same animal. The growth of the pathogens was estimated by measuring the OD₆₂₀. Each datum is a replicate. The middle line is the mean and the whiskers the minimum and maximum values (range). Different letters indicate a significant difference between means of each group ($P < 0.05$).

7.5 Discussion

Comparative, objective, bacterial growth inhibition assays with plasma, DCS, serum and HS established baseline antibacterial activities of koala plasma and serum. Blood-derived fluids of all species studied significantly inhibited bacterial growth compared to positive controls, attributed to innate antimicrobial constituents of serum and plasma.

In this study, *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 represented gram-negative and gram-positive pathogens respectively. These bacterial species have been isolated from koalas with infectious diseases such as pneumonia and other respiratory tract infections and erosive dermatitis due to bite wounds (Blanshard and Bodley, 2008).

The antibacterial activity of sodium heparin (≤ 102 IU/mL) in the 5 mL plasma collection tubes was evaluated to confirm that it did not contribute to the antibacterial activity of the plasma samples. Heparin concentrations ≥ 125 IU/mL and ≥ 250 IU/mL inhibited the growth of *S. aureus* and *E. coli* respectively (Rosett and Hodges, 1980); however, 102 IU did not inhibit growth of *E. coli* or *S. aureus* in this study.

In experiment 1, whilst both pathogens appeared completely inhibited by cow and horse plasma (two animals of each species) *in-vitro*, they displayed much variation in susceptibility to koala plasma. This experiment could be improved by including samples from more cows and horses. The cow and horse (and sheep in experiment 2) were chosen as are large animals and blood was collected from these species opportunistically during veterinary students' practical sessions.

The possible effects of age, sex, degree of relatedness of koalas from different locations and environmental factors such as diet require further investigation with more individuals. These factors were not investigated as koala familial relationships were not available from three locations other than Taronga Zoo. Although two Taronga Zoo koalas in experiment 1 and three

koalas in experiment 3 were related, their samples did not display similar antibacterial activities.

The DCS was described by Hirsch (1960) to investigate the antibacterial activity of rabbit and human blood matrices. This study reported antibacterial activity as a reciprocal of the bactericidal titre, and the lowest antibacterial activity (< 10) was observed with rabbit DCS against *S. aureus*, *Bacillus megaterium* and *B. subtilis* compared to serum (320 – 3200) and HS (160 – 3200). Rabbit serum and DCS both had the same, low antibacterial activity (32) against *E. coli*. In contrast, human serum and DCS displayed the same higher antibacterial activity (256) against *E. coli*. Heating rabbit and human serum (< 2) largely inactivated the antibacterial activity against *E. coli*. In experiment 2, there were no significant differences in the antibacterial activities between DCS and serum samples of koalas and sheep against both pathogens; however, heating reduced the ability of koala serum to inhibit bacterial growth. The increased bacterial growth associated with koala HS could be due to inactivation of labile antimicrobial properties (such as complement) (Taylor, 1983) but this could also be attributed to the inactivation of volatile and thermolabile eucalypt components such as terpenoids derived from the diet in koala blood matrices (Turek and Stintzing, 2013). Further evaluations of EOs in serum and HS might be required to confirm the factors involved in this reduction. The negligible effect of heating on sheep serum antibacterial activity indicates that some heat-stable properties other than complement could be responsible for the antibacterial activity (Taylor, 1983).

In experiment 2, unfortunately no koala plasma was processed from the animals' whole blood to be compared with the serum. Koalas are not large animals (on average 6 to 10 kg) and it was challenging to obtain sufficient blood to obtain DCS, unheated and heated serum samples assayed in triplicate. Therefore, experiment 3 was undertaken to compare koala plasma and serum antibacterial activity processed from the same whole blood collection from each animal.

The results revealed that koala plasma and serum are likely to result in the same relative inhibition of *E. coli*. Nevertheless, the antibacterial activity of koala serum against *S. aureus* was superior to that of plasma, possibly due to different protein concentrations between plasma and serum. Human plasma has greater concentrations of fibrinogen and osteopontin (OPN) than human serum (Ayache et al., 2006) but human serum has higher concentrations of 11 chemokine factors and some platelet-related factors (Ayache et al., 2006) that contribute to the antimicrobial activity of blood matrices (Tang et al., 2002, Yang et al., 2003). Differences between the antibacterial activity of plasma and serum is reported (López-Medrano et al., 2016). Human serum has better antibacterial action than plasma against *E. coli* while human plasma exhibited greater antibacterial activity against *Legionella pneumophila* (López-Medrano et al., 2016).

Experiments 1 and 3 showed that koala plasma inhibited *E. coli* more than *S. aureus*. Gram-negative pathogens such as *E. coli* are more susceptible to serum antibacterial activity such as complement cascade than gram-positive pathogens such as *S. aureus* (Rowley and Wardlaw, 1958, Taylor, 1983). Nevertheless, in experiment 3 koala serum exhibited almost the same antibacterial activity against *E. coli* and *S. aureus*. The same antibacterial activity of plasma and serum against *E. coli* but superior activity of serum against *S. aureus*, could indicate that antimicrobial properties of plasma and serum that inhibit *E. coli* are the same but serum possesses extra components with superior activity against *S. aureus* than the plasma. These components could be related to antimicrobial peptides released during coagulation process which only happens as a precursor to serum removal. For example, when fibrinogen is converted to fibrin via thrombin activation, an antimicrobial peptide (GHR28) is released from the fibrinogen β -chain (Påhlman et al., 2013) which has significant antibacterial activity against *S. aureus* but not *E. coli* (Påhlman et al., 2013). However, the activity of koala serum to inhibit *S. aureus* has been equivocal with broad variation.

The variability in the inhibition of bacterial growth by koala plasma and / or serum was the most significant observation from all three experiments as individual variability in the antimicrobial activity of serum within a species is supposed to be low (Taylor, 1983). It was hypothesised (although it cannot be concluded) that the variable antibacterial inhibition of koala plasma and serum could be due to genetic factors that affect immune system efficacy in inhibiting microbial growth. Additionally, undulations in dietary constituents' concentrations in plasma/serum due to intermittent feeding and nutrient absorption could also have a role in this variation. Moreover, volatile plant compounds are prone to fluctuate naturally in composition due to plant health, leaf age, season, soil factors and the microenvironment and climate (El-Merhibi et al., 2007, Turek and Stintzing, 2013). Consequently, the different bioactivities of *Eucalyptus* spp. might vary the content of nutrients and compounds present in plasma or serum.

Establishing the factor(s) responsible for variation in plasma/serum antimicrobial activities may not be easy; however, investigating changes in serum or plasma antimicrobial activity using serial blood collection with respect to ingestion of a specific eucalypt species and monitoring the feeding time of koalas might be the next step. As the variation in antibacterial activity is a significant finding, these experiments could be expanded to include comparing the plasma or serum activities of other eucalypt folivores such as the common brush-tailed possum and eastern ring-tailed possum. Similar antimicrobial variations in the serum or plasma of these marsupials would provide stronger evidence that ingestion of eucalypt constituents is a significant factor.

Further investigations into the effects of a eucalypt diet on the koala's immune system might help establish new treatments, especially for chlamydiosis, exploiting therapeutic aspects of different *Eucalyptus* species to control koalas' infectious diseases. The findings of this chapter open a further line of inquiry into the role of *Eucalyptus* extracts and EOs in inhibiting

Chlamydia spp. classified as obligate intracellular gram-negative bacteria. Investigations into whether blood matrices' transport antibacterial activity to the cells and contribute to controlling this pathogen, which causes significant morbidity in koalas, are warranted. Furthermore, this research suggests studies to evaluate possible synergistic effects of a eucalypt foliage diet with immune system components including WBC, platelets, RBC and blood matrices (serum and plasma) in controlling cryptococcal infections or even viraemias such as active koala retrovirus (KoRV) infections.

Chapter 8

Final discussion

8.1 General discussion and limitations of this research project

Many observations from this research contribute significantly to expanding knowledge concerning antimicrobials for koalas and other Australian wildlife species. The previously undocumented findings include:

- Generating the first report of the PK profile of posaconazole in koalas and a consequent prediction that it is likely to be an efficacious antifungal drug in this species. Posaconazole was also the first drug documented to have reasonable oral absorption in koalas (bioavailability (F) = median: 0.64, range: 0.43 – 1.06).
- Demonstrating that cefovecin has a short duration of action in koalas with a single s.c. administration. Hypothetically, there might be some accumulation with multiple administrations, but this was not investigated in this research. The outcomes documented in Chapter 4 will stop wildlife veterinarians from administering a single bolus of cefovecin to koalas, assuming that a single bolus provides koalas with adequate antibacterial protection for days. Cefovecin was also found to have a low percentage of binding to plasma proteins in other selected Australian marsupials and in these species is likely to have a similar short duration of action *in vivo*.
- Very surprisingly, in attempting to confirm the plasma concentrations of cefovecin and amoxicillin in koala plasma using bioassays, variable antibacterial activity of koala blood matrices was observed. This requires investigation to explain such variation and whether it is due to fluctuations of eucalypt constituents' concentrations in the circulation and / or other factors.

An additional strength of this research was that for all three antimicrobial drugs an HPLC method was developed and rigorously validated to ensure endogenous plasma interferences were eliminated and to increase the accuracy and precision of each assay. Difficulties in optimising the methods to detect drugs in koala plasma prompted extensive literature reviews

to appraise the most applicable LC procedures and to help find solutions to assay these drugs in koala plasma. However, many endogenous peaks in koala plasma required customisation of HPLC conditions for this species to maximise sensitivity and selectivity with the available equipment.

The HPLC-PDA method developed and validated to determine posaconazole concentration was highly sensitive and could detect posaconazole concentrations as low as 0.02 µg/mL using 100 µL koala plasma. This sensitivity exceeded the methods validated for dog plasma [LLOQ: 0.04 µg/mL using 500 µL plasma (Kendall and Papich, 2015)], cat plasma [LLOQ: 0.05 µg/mL using 500 µL plasma (Mawby et al., 2016)] and rat plasma [LLOQ: 0.05 µg/mL using 200 µL plasma (Khalil et al., 2015)]. The advantage of this method was the use of small volumes of koala plasma (100 µL) with a convenient protein precipitation extraction that is beneficial with limited sample volumes. The method could also detect itraconazole (the internal standard for the posaconazole assay) without endogenous interference.

This research project provided valuable information about the stability of posaconazole and cefovecin in both stock solutions and koala plasma stored at – 20 °C for 5 and 18 months, respectively, indicating both drugs are stable at – 20 °C for these durations. Although cefovecin samples were analysed by HPLC within two months after collection, there were stability QC samples stored at – 20 °C available after 18 months and those samples could be assayed accurately for up to 18 months, which is advantageous when batching samples.

This research also found amoxicillin is unstable in plasma samples stored at – 20 °C. Amoxicillin is a first-line antibacterial frequently administered to animals (often combined with a beta-lactamase inhibitor) and it is very useful to know it loses 4 % of its detectable mass after 1 month and up to 50 to 60 % after 5 months stored at – 20 °C (documented in Chapter 5).

A major unexpected finding was the difficulty extracting cefovecin and amoxicillin from the plasma samples and then assaying them by HPLC. However, another strength of this research was to utilise an alternative method (bioassays) to confirm the HPLC results. Broth microdilution inhibition in this project was assayed according to the CLSI standard (CLSI, 2013a) which specifies that visual inspection is sufficient to detect bacterial growth inhibition. However, Bacterial growth was also estimated by measuring the turbidity at 620 nm (OD_{620}). The plates were carefully vortexed to remove any cell clots that may influence OD_{620} values prior to measuring the turbidity using an automated microplate reader. Although the wells were also examined visually, the OD_{620} approach provided an objective and quantitative method to compare bacterial growth inhibition between time points (Chapter 6) and treatment groups (Chapter 7) rather than just relying on a visual, binary appraisal of \pm bacterial growth.

The proportions of posaconazole and cefovecin bound to plasma proteins were determined using two methods, ultrafiltration and equilibrium dialysis, which were introduced in Chapter 1, Section 1.2.2.2, the equilibrium dialysis method was described in Chapter 2, Section 2.3.3 and the ultrafiltration method was described in Chapter 3, Section 3.3.6. Initially, the percentage of cefovecin bound to plasma proteins of selected species was determined by UF. Although this assay was relatively simple and practical, each replicate required at least 700 μ L. The percentage of bound posaconazole to koala plasma proteins was determined by equilibrium dialysis assay using the commercial 'Rapid ED' (Thermo Fisher Scientific, Scoresby, NSW). The RED device enabled running the assay with smaller volumes of plasma samples, as each replicate only required a plasma volume of 100 to 500 μ L. Therefore, this method was advantageous when only small sample volumes are available. In this research, pooled plasma samples from the same sources underwent both methods to compare the PPB results obtained for cefovecin and posaconazole (illustrated in Appendix III, Table A-2 and A-3). The PPB results in koala and horse plasma obtained by the ED were slightly greater than those obtained

by the UF method. However, both methods produced accurate results consistent with published reports. The difference between the ED and UF PPB are minimal and not expected to have any clinical implications. The PPB of posaconazole in horse, dog and cat plasmas determined by the ED are presented in Appendix III, Table A-3. The value in the dog ($99 \pm 0.18 \%$) is consistent with literature reports $> 99 \%$ using UF assay (Kendall and Papich, 2015).

One of the limitations encountered was the small numbers of animals in each study. Koalas are very valuable animals. All these studies relied on Taronga Zoo providing some of their display animals, and six mature animals were recruited for each *in-vivo* study (oral posaconazole, cefovecin and amoxicillin). The pool of available mature koalas was further restricted by excluding mature females with pouch young and koalas involved in other studies. Although not statistically ideal, pharmacological studies with treatment groups based on six clinically normal mature individuals are considered publishable for non-domesticated animals (e.g., Gharibi et al. (2017)).

Another limitation was the volume of blood available from each animal during each pharmacological study and comparative antibacterial study as described in Chapter 7. Koalas are not large, so collecting adequate volumes of plasma was challenging. Sample preparation methods were undertaken to extract maximal drug from low plasma volumes. For example, it wasn't possible to use 500 μL plasma that had been used in some assays to perform PPE (Kendall and Papich, 2015) or LLE (Foroutan et al., 2007). Thus, sample extraction was optimised to use 100 μL and 240 μL of plasma for PPE and LLE, respectively.

There were Two significant challenges in the HPLC method optimisation for both cefovecin and amoxicillin: i) there was significant interferences by endogenous compounds in the koala plasma around the peaks of interest on the chromatograms and ii) the drugs themselves were problematic for extraction and assay by HPLC. Koala plasma seems to have a complicated heterogeneous matrix probably due to the animals' eucalypt diet (Kimble et al., 2013b) which

can interfere with the analyte of interest, particularly those with polar chemical properties (such as cefovecin and amoxicillin) because biological matrices are mainly polar and are eluted simultaneously with polar drugs early in reversed phase chromatography (Meyer, 2004, Hall et al., 2012). Blank koala plasma from the same (see Figure 8-1) or different regions of Australia (see Figure 8-2) using the same HPLC conditions and sample preparation techniques resulted in variable endogenous peak heights, variable AUC and variable peaks in the chromatograms.

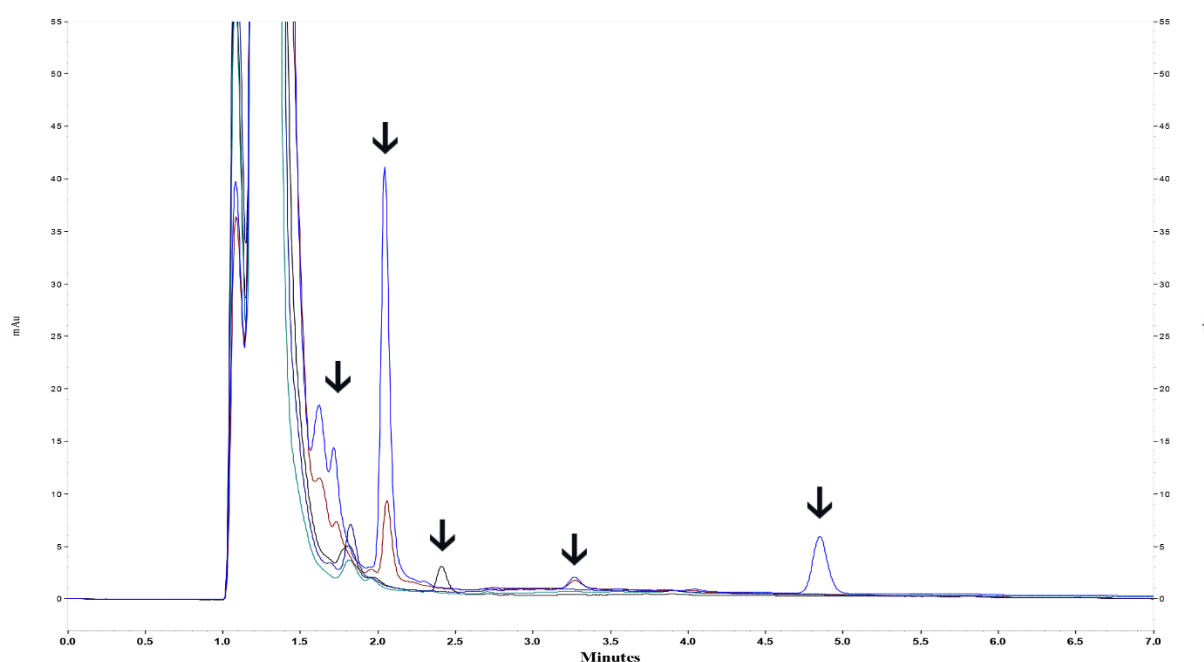


Figure 8-1 Chromatograms of blank plasma from five koalas at Taronga Zoo. Each coloured trace is a chromatogram of an individual koala. Arrows show significant differences between the chromatograms; two koalas have extra peaks at 2.4 and 4.8 min. Also, the AUC and height of the peaks at 1.7 and 2.2 min vary between individuals. Chromatographic separation was performed with a Synergy MAX-RP 80Å at ambient temperature. An isocratic mobile phase consisted of 50 mM phosphate buffer (pH: 6.2) and acetonitrile (40:60 v/v) at a flow rate of 1 mL/min and a UV wavelength of 262 nm. Samples were prepared with protein precipitating extraction using 1 to 1 volume of acetonitrile.

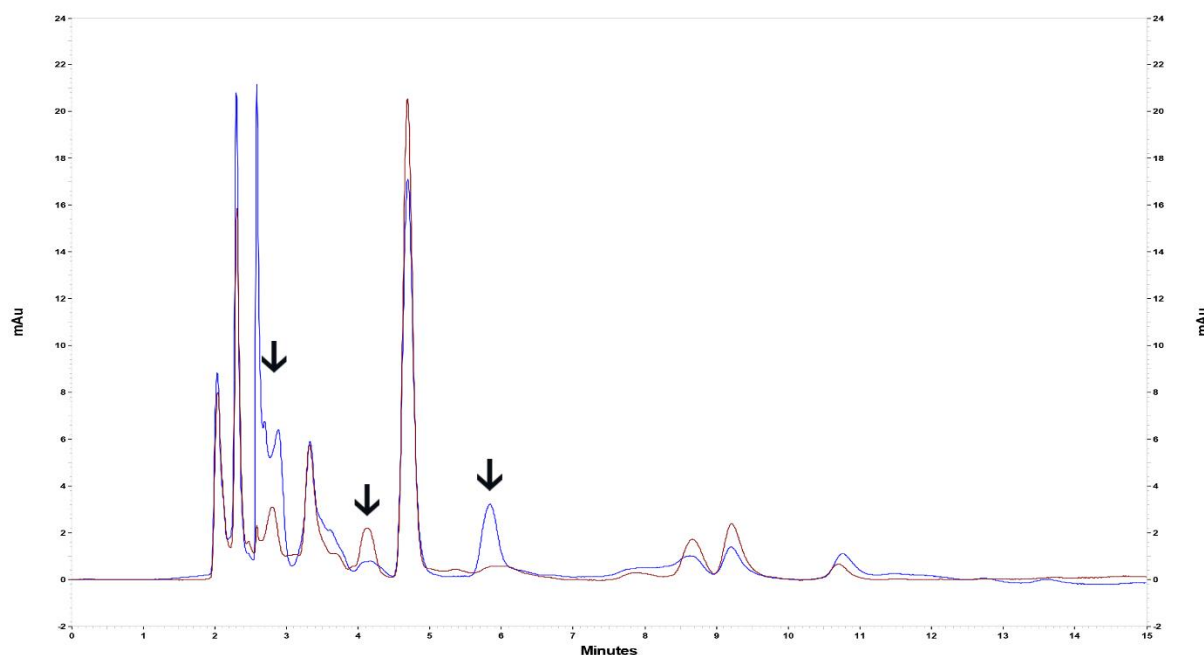


Figure 8-2 Chromatograms of pooled blank koala plasma from Taronga Zoo (**blue**) and Port Macquarie Koala Hospital (**red**). Arrows show significant differences between the chromatograms; an extra peak appears at 5.8 min of the Taronga Zoo sample. Also, the AUC and height of the peaks at 2.8 and 4.2 min vary between the two samples. Chromatographic separation was performed with a Synergi Hydro-RP 80Å at 35 °C. An isocratic mobile phase consisted of sodium phosphate buffer (50 mM, pH: 3.15) and methanol (95:5 v/v) at a flow rate of 1.2 mL/min and a UV wavelength of 228 nm. Samples were prepared with protein precipitating extraction using 1 to 1 volume of methanol.

Higher assay sensitivity for cefovecin might have permitted the detection of lower drug concentrations in the plasma and facilitated generation of a more comprehensive PK profile such as a more accurate half-life and the possibility of PK/PD integration to predict likely efficacy against specific pathogens. Although multiple methods were trialled to increase sensitivity, most of these methods (applicable to extracting the drug from other species) were not successful. The method developed and validated for the cefovecin *in-vivo* assay (described in Chapter 4) did not satisfy the guidelines regarding assay reliability due to variations in cefovecin recovery as the lower concentrations of the calibrators in the standard curve and the QC samples containing 0.05 and 0.5 µg/mL of cefovecin had poor accuracy. The reason for such variation was not understood. It could be strong retention of cefovecin on the SPE

cartridges and / or irreversible adsorption of cefovecin on these cartridges due to secondary interactions between the sorbent and cefovecin (Majors, 2013, CHROMacademy, 2016).

When planning the *in-vivo* cefovecin study, the cefovecin was not expected to be detected so rapidly in the *in-vivo* samples and cefovecin plasma concentration fell to < 1 µg/mL by 6 h. In hind-sight if such a study was to be repeated in koalas, it is recommended that blood collection time points to be concentrated in first 6 h after drug administration as cefovecin plasma concentration fell to < 1 µg/mL by 6 h.

Detection of amoxicillin in koala plasma posed similar problems to those encountered detecting cefovecin in koala plasma. However, an additional problem was amoxicillin instability over time. The amoxicillin assay was initially validated based on the plasma of koalas from AZWH (QLD). When the time came to medicate the koalas, personnel issues prevented the study at Australia Zoo. Dr Larry Vogelnest and Taronga Zoo (NSW) agreed to conduct the study. Endogenous compounds in the samples from these koalas interfered with the amoxicillin peak and the assay required further modification and revalidation; HPLC assay of these samples occurred 5.5 months after arrival. Although the candidate worked consistently between September 2016 and February 2017 to measure amoxicillin concentrations in the plasma samples, the stability test showed that amoxicillin QC samples were unstable after 5 months stored at – 20 °C. Therefore, the results of amoxicillin chapter indicating low plasma concentrations of amoxicillin was likely, in part, to be attributable to amoxicillin instability. The *in-vivo* PK study of amoxicillin in koalas is required to be repeated to confirm the PK profile of amoxicillin in koalas. This component could not be repeated at the late stage of this candidature as repeating the study required reapplication of animal ethics approval and time-intensive processes of *in-vivo* drug administration, blood collection and sample analyses.

More thorough PK profiles were not determined due to the low cefovecin (because of low HPLC method sensitivity) and amoxicillin (because of poor stability) concentrations detected

in most plasma samples at many time points. Although studies of intravenously administered cefovecin and amoxicillin might have provided greater drug plasma concentrations and facilitated calculations of drug V_d and Cl , unlike posaconazole, formulations that can be administered i.v. are not available for these drugs. When planning these studies, it was thought that evaluating these drugs using the route by which they are administered to koalas would provide the most relevant information.

Only clinically normal koalas were recruited for this project and PK studies of these three drugs were not investigated in diseased koalas. Drug PK profiles between sick and clinically healthy animals can differ (Lees et al., 2009). Illness conditions can affect drug absorption, distribution, metabolism and elimination, thereby, influencing the PK profile in diseased individuals (Smith et al., 2012). For example, severe infections such as bacteraemia or pneumonia that stimulate the systemic inflammatory response syndrome (i.e. the presence of at least two of the following conditions: fever, leucocytosis or leukopenia, tachycardia, tachypnoea and hypotension) can increase the V_d , particularly that of hydrophilic antibacterials such as beta-lactams that generally have a low V_d (Blot et al., 2014).

8.2 Recommendations and directions for future research

8.2.1 Use of alternative drug determination methods

Based on the results of HPLC method developments for two polar drugs, other methods could be superior to HPLC-UV for quantitating polar drugs in koala plasma or serum due to the compromised sensitivity and selectivity of this HPLC method. Alternatively, based on the availability of other analytical instruments and time to develop a new method, or to implement methods reported in the literature, other bioanalytical methods with higher sensitivity and selectivity such as HPLC with fluorescence detection, LC/MS or electrochemical detection could be superior for analysing polar drugs in complex biological fluids.

A pilot study prior to the experimental PK studies is suggested to improve the study design, select the appropriate time points for sample collection, select standard curve ranges for HPLC methods and check if endogenous interference will be a problem.

8.2.2 Investigation of itraconazole PK profile and its evaluation as a treatment for fungal disease in koalas

Although posaconazole showed promising efficacy for treating fungal disease in koalas, its cost is a major disadvantage for veterinary practice (a single 105 mL bottle of the 40 mg/mL oral formulation, Noxafil costs approximately AU \$650 in 2017). Itraconazole is a cheaper broad-spectrum antifungal used in koalas, and its PK profile in the koala needs to be described. There are no published studies of itraconazole's PK profile in koalas and as a result of this research a method to detect itraconazole in koala plasma is now available for a future *in-vitro* [hepatic metabolism studies (Kimble et al., 2014)] and / or *in-vivo* studies.

8.2.3 Investigating factors responsible for the variability of antibacterial activity of koala blood matrices

Microbiological assays were initially undertaken to confirm the HPLC findings. However, an intriguing finding from the broth microdilution assay was the variability of antibacterial activity between clinically normal koalas even before they were medicated, and between sequential time points for an individual koala from both the cefovecin and amoxicillin *in-vivo* samples. This variation led to studying the antibacterial activity of koala plasma and serum in Chapter 7. Similar to the observed variability in the chromatograms of koala plasma, antibacterial activities of both koala plasma and serum varied considerably. Several factors can contribute to such variations and the reasons are not clear. As the koalas tested in Chapter 7 were clinically normal and unmedicated, genetic factors could cause this variation. In addition,

as specialist eucalypt folivores, the koala diet could contribute to this variability as digestion of *Eucalyptus* spp. (with variable bioactivities) can vary the content of nutrients and compounds present in plasma or serum. Further investigations are required to discover the reason for such variability in the antibacterial activity of koala blood matrices.

8.2.4 Effects of different *Eucalyptus* spp. leaf extracts and EOs on pathogenic microorganisms such as *Chlamydia* and *Cryptococcus* spp.; diet therapy in koalas and other *Eucalyptus* spp. folivores

Given the biological activities of *Eucalyptus* spp. foliage, some *in-vitro* assays can be conducted to test the antimicrobial activities of different *Eucalyptus* spp. against *Chlamydia* and *Cryptococcus* spp. Effective eucalypt species can be explored for further *in-vivo* studies to control and treat infectious disease in koalas. As immuno-stimulatory (Serafino et al., 2008) and anti-inflammatory (Silva et al., 2003) activities of some *Eucalyptus* EOs are reported, exploring interactions between the diet and the koala immune system is warranted. These experiments can be expanded to other *Eucalyptus* herbivores such as the common brush-tailed possum and eastern ring-tailed possum.

8.3 Conclusions

Results in this thesis indicate promising efficacy of posaconazole to treat systemic fungal diseases in koalas. A single s.c. bolus of cefovecin 8 mg/kg is unlikely to be efficacious to treat bacterial diseases for more than 3 h and does not offer the long duration of efficacy seen in dogs and cats. The *in-vivo* PK study of amoxicillin should be repeated due to the instability of amoxicillin stored at – 20 °C. Variable antibacterial activities of koala plasma and serum could imply synergistic antimicrobial effects of *Eucalyptus* spp. with innate antimicrobial components of koala blood matrices. The reasons for this variability require further investigation.

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Appendix I- HPLC method to determine amoxicillin and clavulanic acid concentrations in koala and horse plasma

Amoxicillin/clavulanic acid concentrations in plasma samples were quantified with HPLC. Chromatographic separation was performed with a Synergi Hydro-RP 80Å, (4µm, 250×4.6 mm) (Phenomenex, Lane Cove, NSW) with a 1 mm Opti-guard C-18 pre-column (Choice Analytical, Thornleigh, NSW) at 35 °C. The isocratic mobile phase consisted of sodium phosphate buffer (50 mM, pH: 3) and methanol (96 : 4 v/v) (Foroutan et al., 2007) at a flow rate of 1.4 mL/min and the UV wavelength used for detection was 228 nm. The retention time of clavulanic acid, the IS (allopurinol) and amoxicillin were approximately 5.5, 6.4 and 11.2 min, respectively. The total run time for each sample was 19 min.

For drug extraction from plasma samples, protein precipitation was performed by adding a 1:1 volume of methanol followed by centrifugation at 14,000 g for 10 min. The supernatant was removed and after further centrifugation at the same speed and duration, 10 µL of supernatant was injected into the HPLC system.

A chromatogram of amoxicillin, clavulanic acid and the IS is provided in Figure A-1

FOROUTAN, S., ZARGHI, A., SHAFATI, A., KHODDAM, A. & MOVAHED, H. 2007. Simultaneous determination of amoxicillin and clavulanic acid in human plasma by isocratic reversed-phase HPLC using UV detection. *Journal of Pharmaceutical and Biomedical Analysis*, 45, 531-534.

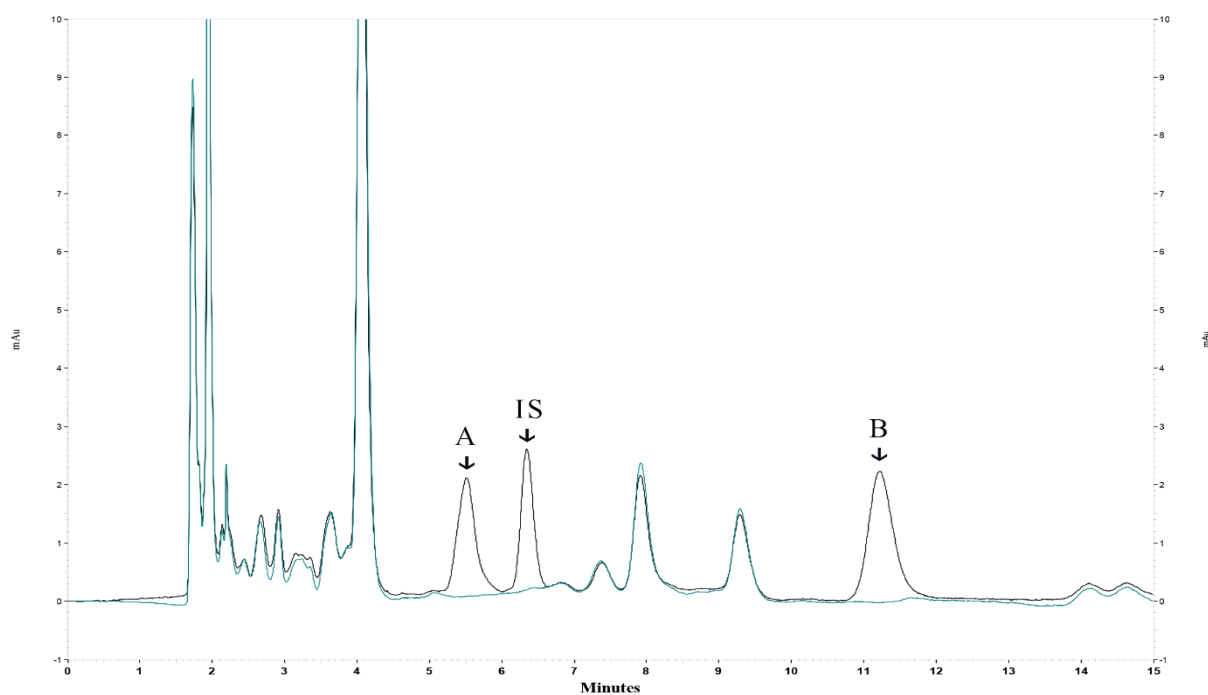


Figure A-1 Chromatograms of extracted pre-spiked koala plasma (**black trace**) containing 10 $\mu\text{g/mL}$ of clavulanic acid (**A**, retention time of 5.5), 5 $\mu\text{g/mL}$ of IS (allopurinol, retention time of 6.4 min) and 5 $\mu\text{g/mL}$ of amoxicillin (**B**, retention time of 11.2 min); and blank koala plasma (**green trace**) at a UV wavelength of 228.

**Appendix II- Summary of stability test results of amoxicillin and clavulanic acid
conducted in October 2014**

Table A-1 Stability of amoxicillin and clavulanic acid in koala plasma pre-spiked with 1, 5 and 10 µg/mL of both drugs when stored at different temperatures

| Concentrations (µg/mL) | Storage temperature (°C) | Storage period (d) | The AUC reduction compared to the time 0 (%) | |
|---------------------------|--------------------------------|-----------------------|---|--------------------|
| | | | Amoxicillin | Clavulanic acid |
| 1 | + 4 | 4 | 17.91 | 47.55 |
| 10 | | | 19.69 | 71.13 |
| 1 | – 20 | 28 | 3.66 | 47.28 |
| 5 | | | 0.97 | 45.14 |
| 10 | | | 0 | 46.15 |
| 1 | – 80 | 34 | 0 | 17.61 |
| 10 | | | 7.25 | 23.84 |

Appendix III- Comparison of equilibrium dialysis and ultrafiltration for the determination of PPB values of cefovecin and posaconazole

Table A-2 *In-vitro* plasma protein binding (%) of cefovecin (10, 50 and 100 µg/mL) in the koala and horse determined by equilibrium dialysis and ultrafiltration.

| Cefovecin concentration (µg/mL) | Koala | | Horse | |
|---------------------------------|----------------------|-----------------|----------------------|-----------------|
| | Equilibrium dialysis | Ultrafiltration | Equilibrium dialysis | Ultrafiltration |
| 10 | 19.37 | 11.9 | 95.60 | 95.47 |
| 50 | 16.85 | 11.29 | 94.79 | 92.94 |
| 100 | 16.21 | 15.46 | 92.56 | 90.18 |
| Mean ± SD | 17.48 ± 1.67 | 12.88 ± 2.25 | 94.32 ± 1.58 | 92.86 ± 2.65 |

The PPB of cefovecin was determined using frozen-thawed pooled plasma of five and three clinically normal koalas and horses. For equilibrium dialysis, the incubation temperature was 37 °C for both animals. Incubation for ultrafiltration was at 36 °C for koalas and 37 °C for horses.

Table A-3 *In-vitro* plasma protein binding (%) of posaconazole (0.5, 1, 2.5 and 5 µg/mL) in the koala, horse, dog and cat determined by equilibrium dialysis and ultrafiltration.

| Posaconazole concentration (µg/mL) | Koala | | Horse | Dog* | Cat* |
|------------------------------------|----------------------|-----------------|----------------------|----------------------|----------------------|
| | Equilibrium dialysis | Ultrafiltration | Equilibrium dialysis | Equilibrium dialysis | Equilibrium dialysis |
| 0.5 | 98.92 | 97.81 | 97.35 | 98.87 | 99.70 |
| 1 | 99.44 | - | - | - | - |
| 2.5 | 99.41 | 96.85 | 98.58 | 99.14 | 95.19 |
| 5 | - | 95.66 | - | - | - |
| Mean ± SD | 99.25 ± 0.43 | 96.77 ± 1.08 | 97.96 ± 0.87 | 99.00 ± 0.18 | 97.45 ± 3.18 |

The frozen-thawed pooled plasma of six koalas, three horses, six dogs and six cats was used to determine the PPB of posaconazole using equilibrium dialysis; the incubation temperature was 37 °C for all species. The same koala samples also underwent ultrafiltration assay at 36 °C. Cat and dog plasma were residual plasma from hospital patients submitted to Veterinary Pathology Diagnostic Services (VPDS), SSVS.