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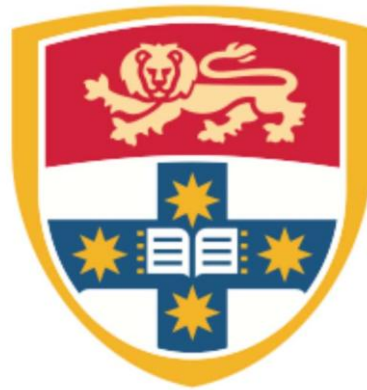
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**Pharmacokinetic aspects of meloxicam in koalas:  
including its hepatic microsomal metabolism  
compared with other selected species**

**By**

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## Abstract

Prior to this research, no disposition studies of meloxicam (nor any other non steroidal anti-inflammatory drugs) had been conducted in koalas (a specialist *Eucalyptus* spp. foliage feeder) despite being readily administered to this species, in the field. Thus, aspects of the *in-vivo* pharmacokinetic profile of meloxicam in the koala and the *in-vitro* metabolism of meloxicam in the koala and selected species were investigated.

In the first stage of the research, a simple, sensitive and improved method using high performance liquid chromatography equipped with photo diode array detection was developed and validated to determine meloxicam concentrations in koala plasma, applicable for *in-vivo* pharmacokinetic study. Following intravenous injection, meloxicam exhibited a rapid plasma clearance of  $0.44 \pm 0.20$  L/h/kg in koalas ( $n = 5$ ). Median plasma terminal elimination  $t_{1/2}$  was 1.19 h (range 0.71 to 1.62 h). In koalas, bioavailability after the subcutaneous injection was approximately 56 to 70 % where oral bioavailability was negligible. Plasma protein binding of meloxicam was about 98%. Three hydroxylated metabolites of meloxicam (M1, M2 and M3) were detected in the koala plasma with one (M1) identified as the 5-hydroxy methyl metabolite. According to the *in-vitro* hepatic microsomal metabolism of meloxicam, it was demonstrated that biotransformation of meloxicam, likely mediated *via* cytochrome P450 enzymes, were much faster in koalas (and also in other *Eucalyptus* spp. foliage feeders: ringtail possums and brushtail possums) compared to rats or dogs. The rank order of apparent *in-vitro* intrinsic clearance was brushtail possums ( $n = 3$ ) (mean:  $394 \mu\text{L}/\text{min}/\text{mg}$  protein) > koalas ( $n = 6$ ) ( $50 \mu\text{L}/\text{min}/\text{mg}$  protein) > ringtail possums ( $n = 2$ ) ( $36 \mu\text{L}/\text{min}/\text{mg}$

protein) (with no significant difference between koalas and ringtail possums) > pooled rats (3.2  $\mu\text{L}/\text{min}/\text{mg}$  protein) > pooled dogs (not determined as the rate of metabolism was too slow). According to the *in-vitro* study, single hydroxylated metabolite (M1) was determined as the major product of meloxicam in brushtail possums and the rat whereas multiple hydroxylated metabolites were observed in the koala (M1, M2, and M3) and the ringtail possum (M1 and M3). Using a well-stirred model, this research showed applicability of predicting *in-vivo* clearance of meloxicam in koalas and the rat from the apparent *in-vitro* intrinsic clearance data (average fold error for prediction was less than 2). While cytochrome P450C9 is the major responsible enzymes for metabolism of meloxicam, the research also found that the stability of other cytochrome P450C9 substrates, particularly non steroidal anti-inflammatory drugs were also generally not stable in hepatic microsomes of koala and other *Eucalyptus.spp* foliage feeders than the rat. Particularly, there was some similarity on the pattern of CYP2C9 substrates stabilities between koala and ringtail possum (*Eucalyptus spp.* foliage specialist feeders).

This research demonstrated that koalas exhibited rapid plasma clearance and extremely poor oral bioavailability of meloxicam compared with other eutherian species. Due to differences in the rate of hepatic metabolism on meloxicam, other eutherians such as rats or dogs are inadequate model for dosage extrapolation of this drug in koalas. Furthermore, as catalytic activity of cytochrome P450C-like enzymes appeared to be different in these *Eucalyptus spp.* foliage feeders, it is highly recommended to consider when extrapolating dosage of therapeutic drugs (cytochrome P450C9 substrates), particularly non steroidal anti-inflammatory drugs, from other eutherians. On the other hand,

as *in-vivo* clearance is one of the pharmacokinetic indexes for determining the dosage of drug, this study demonstrates the utility of *in-vitro* to *in-vivo* scaling as an alternative prediction method of drug clearance in koalas.

# Declaration

The work contained in this thesis, unless where due acknowledgement has been made, is the original research carried out by the author and has not been submitted for any other degree.

**Benjamin Kimble**

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# List of publications

## I. Original publications arising directly from work presented in thesis

- i. **Kimble, B.**, Li, K.M., Govendir, M. (2013). Quantitation of meloxicam in the plasma of koalas (*Phascolarctos cinereus*) by improved high performance liquid chromatography. *Journal of Veterinary Science*, 14(1), 7-14.
- ii. **Kimble, B.**, Black, L. A., Li, K.M., Valtchev, P., Gilchrist, S., Gillett, A., Higgins, D. P., Krockenberger, M. B., Govendir, M. (2013). Pharmacokinetics of meloxicam in koalas (*Phascolarctos cinereus*) after intravenous, subcutaneous and oral administration. *Journal of Veterinary Pharmacology and Therapeutics*, 36(5), 486-493
- iii. **Kimble, B.**, Li, K.M., Valtchev, P., Higgins, D. P., Krockenberger, M. B., Govendir, M. (2014). *In-vitro* hepatic microsomal metabolism of meloxicam in koalas (*Phascolarctos cinereus*), brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*), rats (*Rattus norvegicus*) and dogs (*Canis lupus familiaris*). *Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, 161 (1), 7-14

## II. Conference abstracts

- i. **Kimble B**, Black L, Gilchrist S, A G, Jobbins S, Li K, Govendir M. Plasma pharmacokinetics of meloxicam after oral, subcutaneous and intravenous administration in the koala In: *12th International Congress of the European Association for Veterinary Pharmacology and Toxicology*. Noordwijkerhout, The Netherlands: 2012

- ii. **Kimble, B.**, Li, K., Govendir, M. Hepatic microsomal stability of human CYP2C9 substrates between marsupials (the koala, common ringtail possum and common brushtail possum) and the rat: a preliminary study In: **13th International Congress of the European Association for Veterinary Pharmacology and Toxicology**. Nantes, France: 2015 (*accepted*)

### **III. Publications participated in during *PhD* candidature and indirectly relevant to this thesis**

- i. Govendir, M., Hanger, J., Loader, J., **Kimble, B.**, Griffith, J., Black, L., Krockenberger, M., Higgins, D. (2012) Plasma concentrations of chloramphenicol after subcutaneous administration to koalas (*Phascolarctos cinereus*) with chlamydiosis. ***Journal of Veterinary Pharmacology and Therapeutics***, 35, 147-154.
- ii. Black, L. A., Krockenberger, M. B., **Kimble, B.**, Govendir, M. (2013) Pharmacokinetics of fluconazole following intravenous and oral administration to koalas (*Phascolarctos cinereus*). ***Journal of Veterinary Pharmacology and Therapeutics***, 37, 90-98.
- iii. De Kauwe, T., **Kimble, B.**, and Govendir, M. (2014). Perceived efficacy of analgesic drug regimens used for koalas (*Phascolarctos cinereus*) in Australia. ***Journal of Zoo and Wildlife Medicine***, 45(2): 350-356.
- iv. Govendir, M., Black, L. A., Jobbins, S. E., Kimble, B., Malik, R., Krockenberger, M. B., Some pharmacokinetic indicators of oral fluconazole administration to koalas (*Phascolarctos cinereus*) infected with cryptococcosis (Submitted to ***Journal of Veterinary Pharmacology and Therapeutics***)

## List of abbreviations

ADME	Absorption, distribution, metabolism, and excretion
AUC	Area under the curve
AUC <sub>0-t</sub>	Area under the curve between zero time point to last sampling time point ( <i>t</i> )
AUC <sub>t-∞</sub>	Area under the curve between last sampling time point ( <i>t</i> ) to infinite
AUMC	Area under the first moment curve
<i>Cl</i>	Clearance
C <sub>last</sub>	Last sampling time point
C <sub>max</sub>	Peak drug concentration
COX	Cyclooxygenase
CYP	Cytochrome P450
CV	Coefficient of variation
DME	Drug metabolising enzymes
Drug <sub>(total)</sub>	Total drug concentration
Drug <sub>(free)</sub>	Free drug concentration determined from ultrafiltrate device
F	Bioavailability
F <sub>u</sub>	Unbound fraction of a drug
F <sub>u(mic)</sub>	Unbound fraction of a drug in microsomes
F <sub>u(plasma)</sub>	Unbound fraction of a drug in the plasma
GIT	Gastro-intestinal tract
Hct	Haematocrit
HLB	Hydrophilic-lipophilic balanced
HPLC	High performance liquid chromatography
IVIVE	<i>In-vitro</i> to <i>in-vivo</i> extrapolation
<i>In-vitro Cl<sub>int</sub></i>	<i>In-vitro</i> intrinsic clearance
IM	Intramuscular
IS	Internal standard

IV	Intravenous
$K_a$	Absorption rate constant
$K_{el}$	Elimination rate constant
LC-MS	Liquid chromatography-mass spectrometer
LC-MS/MS	Liquid chromatography tandem mass spectrometer
LLE	Liquid-liquid extraction
LLOD	Lowest limit of detection
LLOQ	Lowest limit of quantification
MeCN	Acetonitrile
MeOH	Methanol
MRT	Mean residual time
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NMR	Nuclear magnetic resonance
NSAID	Non steroidal anti-inflammatory drug
PO	Per os (Oral administration)
POR	P450 oxidoreductase
PD	Pharmacodynamic
PK	Pharmacokinetics
PDA	Photo diode array
PPE	Protein precipitating extraction
PSM	Plant secondary metabolite
QC	Quality control
qd	Every day
$Q_H$	Hepatic blood flow
$R_t$	Retention time
SC	Subcutaneous
SD	Standard deviation
SER	Smooth endoplasmic reticulum
SPE	Solid phase extraction

$t_{1/2}$	Half life
T-CDE	<b>T</b> annin- <b>c</b> omplex <b>d</b> egrading <b>e</b> nterobacteria
$T_{max}$	Time to reach peak drug concentration
UV-Vis	Ultraviolet-Visible
$V_c$	$V_d$ of central compartment
$V_d$	Volume of distribution
$V_{ss}$	$V_d$ at steady state
$V_z$	$V_d$ at pseudo-equilibrium state
vs.	Versus

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# Chapter 1

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**General introduction, literature review and  
aims of the thesis**

## 1. 1. General introduction

This research project focused on aspects of the *in-vivo* pharmacokinetic profile of the non-steroidal anti-inflammatory drug (NSAID) meloxicam in the koala and the *in-vitro* metabolism of meloxicam in the koala and selected species. The hepatic intrinsic clearance rates (*in-vitro*  $Cl_{int}$ ) of meloxicam were applied to predict the *in-vivo* clearance in the koala and rat. As most NSAIDs, including meloxicam, are predominately metabolised by the catalytic activity of cytochrome P4502C9 in humans, this project also investigated cytochrome P4502C-like activity in the koala and the other species.

The literature review, **Chapter 1**, provides a general overview of pharmacokinetics and a comparative species review of cytochrome P450 (CYP) mediated hepatic metabolism on xenobiotics, such as therapeutic drugs. A brief introduction on methods used for the *in-vitro* to *in-vivo* extrapolation to predict drug clearance and an overview of bio-analytical methodology are described. Current information on both the pharmacokinetics of drugs and hepatic metabolism in koalas are reviewed, followed by an overview of the indications of meloxicam in veterinary medicine. This chapter concludes with the specific aims of this research project.

The first stage of this project was concerned with establishing an analytical method for determining meloxicam concentrations for both the *in-vitro* and *in-vivo* studies. Therefore **Chapter 2** describes development and validation of a liquid chromatography method for quantification of meloxicam in koala plasma.

**Chapter 3** describes the plasma pharmacokinetics of meloxicam, as well as its metabolites, when administered orally (0.1-0.2 mg/kg), subcutaneously (0.1-0.2 mg/kg) and intravenously (0.4 mg/kg) to the koala.

**Chapter 4** reports on the hepatic *in-vitro*  $Cl_{int}$  of meloxicam in the koala and in other species, such as the common brushtail possum (*Trichosurus vulpecula*) and common ringtail possum (*Pseudocheirus peregrinus*), whose dietary habits are similar to the koala (*Eucalyptus* spp. foliage feeder). The hepatic *in-vitro*  $Cl_{int}$  of meloxicam was also determined in the rat and dog. Furthermore, *in-vitro*  $Cl_{int}$  data were applied to predict *in-vivo* clearance of meloxicam, an essential pharmacokinetic parameter for estimating drug dosage, for the koala and rat.

As CYP2C is considered as one of the major drug metabolising enzymes and involved in the metabolism of some therapeutically important classes of medicines, including NSAIDs, species differences in CYP2C-like activities between *Eucalyptus* spp. foliage feeders and rat were investigated in **Chapter 5**.

Finally, **Chapter 6** discusses the overall results of the research project and the utility of the *in-vitro* intrinsic metabolism model to not only determine the hepatic clearance of drugs for an individual species but also to predict the *in-vivo* clearance of some drugs, prior to conducting invasive *in-vivo* pharmacokinetic studies. Additionally, limitations of the entire research project are discussed and future directions proposed.

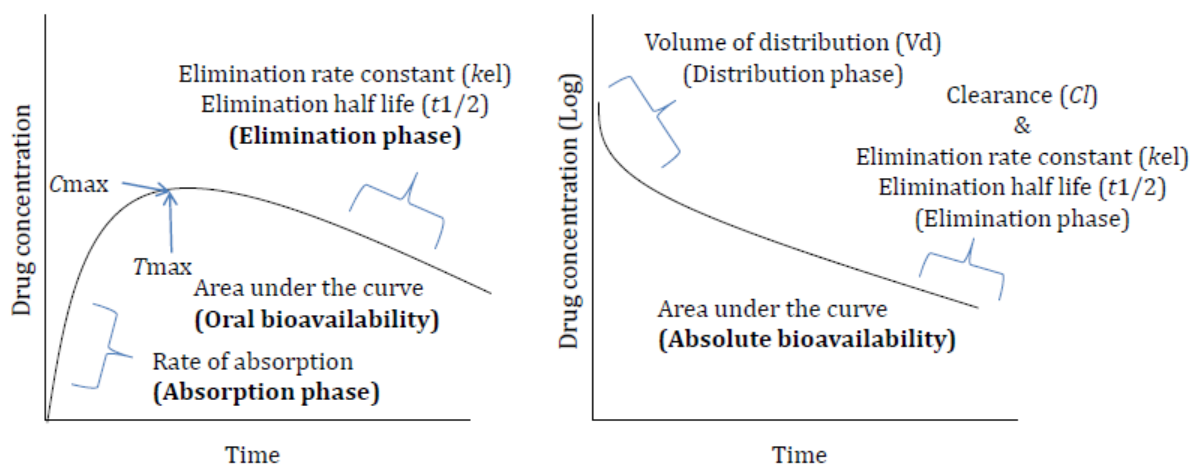
## 1.2. Literature review

### 1.2.1. Pharmacokinetics

Pharmacokinetics (PK) is the study of those factors that affect drugs' disposition or movement in the body and provides quantitative information on how these drugs are processed according to four general processes: **a**bsorption, **d**istribution, **m**etabolism and **e**xcretion: ADME (Wagner, 1981). Accordingly, as ADME is highly dependent on physiological responses, PK is also colloquially referred to as 'what the body does to a drug' (Tozer, 1981). To characterise the ADME of a drug, it is desirable to measure the change in drug concentration in the body, usually graphically represented as a drug concentration vs. time, curve (**Figure 1.2.1**). Any tissue or biological fluid can be sampled to detect drug concentration change over time, however plasma (serum or blood) is commonly used as a sampling medium for drug analysis due to its accessibility (Tozer, 1981). Subsequently, with the assistance of proper mathematical modeling, the PK profile of a drug can be estimated from a drug concentration vs. time course curve.

Drug 'dosage' is defined as the dose rate, as well as the frequency of administration to deliver a sustained and efficacious drug concentration at the site of action (Thompson, 1992). In order to formulate an efficacious dosage, both PK and pharmacodynamic information is required. Pharmacodynamics (PD) is concerned with drug 'effect' or 'response' (Lees et al., 2004a), therefore the integration of both PK and PD data (PK-PD modeling) defines the dose-concentration-response relationship of a drug and is used to predict an efficacious drug dosage (Meibohm and Derendorf, 1997). To formulate an optimal drug dosage, it is ideal to measure the concentrations of a drug at the site of

action which may take place intracellularly (Isoldi et al., 2005), on cell membranes (Yocum et al., 1980) or extracellularly (Mathisen et al., 1981). However, sample collection from some tissue sites can be problematic, therefore plasma (serum or blood) is commonly used as a satisfactory surrogate for PK analyses as it generally correlates with the activity of the drug at the site of action (Baggot, 1978a).



**Figure 1.2.1** Typical plasma (serum or blood) drug concentration vs. time after oral (left) and intravenous administration (right); When the y axis is in log scale and the drug follows first order kinetics, the terminal section of the curve becomes linear as demonstrated by the graph on the right. For some of high clearance drugs, substantial amount of drugs can be eliminated during the distribution phase (Image generated by author).

The area under the drug concentration vs. time curve (known as area under the curve: AUC) is an important PK construct which represents the body's exposure to the drug as well as the change in drug concentration in the sampling compartment over time (Baggot, 1978b). If the rate of change of the drug concentration is *via* a time dependent manner (i.e. first order kinetics: where the amount of drug elimination is proportional

over time) (Rowland and Tozer, 1995), it is general convention for the y axis to be represented as a log scale resulting in the elimination phase becoming linear. While many drugs are eliminated according to the first order kinetics, some drugs such as phenytoin, salicylic acid and ethanol (e.g. in humans) are eliminated *via* zero order kinetics (i.e. the amount of drug eliminated is constant over time) or mixed (zero and first) order kinetics. The trapezoidal rule (either by linear or log linear trapezoidal rule), is popularly utilised for calculation of the AUC between time points (first sampling point after the drug administered and until last sampling point) ( $AUC_{0-t}$ ) (Gabrielsson and Weiner, 2012). The elimination rate constant ( $k_{el}$ ) (the slope of the elimination phase of the concentration vs. time curve) is used to estimate the AUC between the measured drug concentration at the last sampling point ( $C_{last}$ ) to infinite ( $AUC_{t-\infty}$ ).

$$AUC_{t-\infty} = C_{last} / k_{el}$$

#### **1.2.1.1. Absorption**

Absorption is the process whereby a drug enters the circulation from the site of administration, either *via* enteral routes [e.g. oral (per os: PO), sublingual or rectal administration] or parental routes [e.g. subcutaneous (SC), intramuscular (IM), intravenous (IV) administration or by inhalation]. The extent and the rate of drug absorption is generally affected by the physicochemical properties of drugs (e.g. lipophilicity and ionic state), dosage formulation and the physiological environment around the site of administration, e.g. vascularity of absorption site (except for IV administration) (Urso et al., 2002).

Bioavailability (F) is defined as the fraction of the drug which enters the systemic circulation after the administration; consequently IV administered drugs, but not all (e.g. chloramphenicol succinate), have an absolute bioavailability (F = 1) (Urso et al., 2002). The bioavailability for drugs administered *via* other extravascular routes ( $F_{\text{specific route}}$ ) are expressed as a fraction (0 to 1) of the absolute bioavailability **(1)** (Urso et al., 2002).

$$F_{\text{specific route}} = \text{AUC}_{\text{specific route}} / \text{AUC}_{\text{IV}} \dots\dots\dots \mathbf{(1)}$$

With orally administered drugs, the process of absorption can be complicated as several factors may affect its bioavailability. In order to enter the systemic circulation, these drugs need to pass through the gut wall, particularly the small intestine which is the primary absorption site either *via* transcellular, paracellular or transporter-mediated diffusion, and are then transported to the liver *via* portal vein (Caldwell et al., 1995). During this process, depending on the chemical property of drugs, a substantial portion of some drugs are eliminated *via* first-pass metabolism by hepatic enzymes and / or enzymes located along the intestinal wall or by colonised bacteria (Pang, 2003); and may also undergo active extrusion into the intestinal lumen by a variety of cellular transporters, such as the P-glycoprotein efflux pump proteins (Lin et al., 1999). Other factors such as the luminal pH, the presence of digesta which may bind to, or entrap, some drugs and intestinal length and its transit time also may potentially limit absorption through gastro-intestinal tract (GIT) (Caldwell et al., 1995). A drug concentration vs. time course curve provides the opportunity to calculate the absorption rate constant ( $k_a$ ) [e.g. by the method of residuals (Perrier and Gibaldi, 1973)]; and



permits visualization of estimates of the peak plasma concentration ( $C_{\max}$ ) and time to reach  $C_{\max}$  ( $T_{\max}$ ) which are useful indices to estimate the extent and the rate of absorption (e.g.  $C_{\max}/AUC$ ) (Endrenyi et al., 1991).

### 1.2.1.2. Distribution

Once a drug reaches the systemic circulation, depending on its physiochemical properties, many drugs, but not all, may either diffuse or are transported into various body compartments. Volumes of distribution ( $V_d$ ) are hypothetical volumes which indicate how extensively a drug is distributed in the body, and can be expressed as proportionality constants between total amount of drug in the body and plasma concentrations; where  $V_d$  can be expressed as  $V_c$  ( $V_d$  of the central compartment),  $V_{ss}$  ( $V_d$  when the drug is at steady state) or  $V_z$  [ $V_d$  is determined after the drug is equally distributed between tissues and the central compartment (pseudo-distribution equilibrium)] (Kwon, 1996, Toutain and Bousquet-Melou, 2004a). Volume of distribution can be determined by the following relationship **(2)**:

$$\frac{\text{Total amount of drug in the body at time } (t)}{\text{Drug plasma concentration at time } (t) \dots\dots\dots \mathbf{(2)}}$$

Those drugs where the  $V_d$  is  $\sim 0.04$  L/kg [e.g. heparin (Estes, 1980)] are largely confined to plasma; those with a  $V_d \sim 0.6$  L/kg are likely to be fully distributed in all body fluids **(Table 1.2.1)** and those with a  $V_d > 0.6$  L/kg [e.g. amiodarone (Latini et al., 1984)] are likely to be distributed into one or more tissue compartments (Toutain and Bousquet-Melou, 2004a). In general, a drug's lipophilicity, ionic state and chemical properties (e.g.

location of functional group), physiological volumes and perfusion rates for individual tissues are the common factors that affect the Vd (Clausen and Bickel, 1993, Lin and Lu, 1997). Depending on these properties, drugs circulate in the body either ‘unbound’ or complexed to plasma proteins (e.g. albumin, lipoproteins, glycoproteins, or  $\beta$ -globulins) or bind to and accumulate at tissue membranes (Toutain and Bousquet-Melou, 2004a). For example, many of NSAIDs are generally highly protein bound drugs and likely to be confined into the body fluids such as plasma, interstitial or extracellular fluids (Vd ~0.2 L/kg) rather than accumulate at tissue membranes (Verbeeck et al., 1983).

**Table 1.2.1)** Composition of body fluids in human adapted from Armstrong, 2007.

Total body water	0.6 L/kg
Intracellular	0.4 L/kg
Extracellular	0.2 L/kg
Interstitial	0.16 L/kg
Plasma	0.04 L/kg

### 1.2.1.3. Elimination

Elimination, which includes the processes of metabolism and /or excretion, describes the removal of a drug from the body, usually terminating the pharmacological activity of the drug (Tozer, 1981). Metabolism, also referred to as biotransformation, converts a lipophilic drug into less lipophilic, more water soluble compounds (metabolites). As lipophilic drugs, such as NSAIDs (Lees et al., 2004b), are likely to be re-absorbed into systemic circulation when reaching the lumen of the kidney tubules, the major function of metabolism is to increase the efficiency of renal excretion and/or facilitate biliary excretion of the drug (Lin and Lu, 1997). As the AUC of a drug is highly dependent on

elimination processes, clearance ( $Cl$ ) and elimination half life ( $t_{1/2}$ ) are particularly important in order to estimate the dose rate and the frequency required for repeat administration (Toutain and Bousquet-Melou, 2004b, Toutain and Bousquet-Melou, 2004c). Clearance determined from plasma (serum or blood), which refers to the total  $Cl$ , represents the overall rate of elimination of a drug; in which the sum of all the excretory organs contribute to  $Cl$  of that drug in the body (**Table 1.2.2**) (Toutain et al., 2010).

**Table 1.2.2)** Organs that contribute to drug elimination and mechanisms (in human) adapted from Jang et al., 2001, Toutain et al., 2010.

	<b>Hepatic <math>Cl</math></b>	<b>Renal <math>Cl</math></b>	<b>Other organs' <math>Cl</math></b>
<b>Organs</b>	Liver	Kidney	Gut and others
<b>Mechanisms</b>	Metabolism	Excretion	Efflux transporters
	·Phase 1 reaction	·Active secretion	Metabolism
	·Phase 2 reaction	·Glomerular filtration	
	Biliary excretion	Metabolism	

Clearance is the proportionality constant between rate of drug removal (amount per time) and drug concentration (amount per volume), and is expressed as the volume of blood or plasma necessary for eliminating the drug per unit of time **(3)** (Jang et al., 2001).

$$Cl = (\text{Rate of drug removal}) / (\text{drug concentration}) \dots\dots\dots \mathbf{(3)}$$

As the amount of drug administered IV, is equal to the amount of drug eliminated (Jang et al., 2001),  $Cl$  is determined by **(4)**,

$$Cl = \text{Dose}_{IV} / \text{AUC}_{IV} \dots\dots\dots \mathbf{(4)}$$

Subsequently, *Cl* is regarded as one of the most important PK parameters for estimating the dose of drugs **(5)**.

$$\text{Effective dose} = [Cl \times \text{effective (plasma) drug concentration}] / F \dots\dots\dots \mathbf{(5)}$$

Elimination  $t_{1/2}$  is defined as the time required for the drug concentration (generally plasma concentration) to decrease by 50% after  $V_z$  is achieved. Whereas, mean residence time (MRT), the average total time molecules of a given dose spent in the body, indicates the overall persistence of a drug (Toutain and Bousquet-Melou, 2004c). Elimination  $t_{1/2}$  represents the rate constant at the terminal phase of drug elimination which is expressed in time units (Toutain and Bousquet-Melou, 2004c) **(Figure 1.2.1)**. Subsequently, it is used for estimating the dosing interval of the drug as it allows calculation of drug accumulation and time to reach steady state equilibrium in the system (Toutain and Bousquet-Melou, 2004c). When the drug is eliminated *via* first order kinetics, elimination  $t_{1/2}$  can be calculated by **(6)**

$$\begin{aligned} \text{Elimination } t_{1/2} &= 0.693 (\text{natural logarithm of } 2) / k_{el} \\ &\text{or} \\ &= (0.693 \times V_z) / Cl \dots\dots\dots \mathbf{(6)} \end{aligned}$$

#### **1.2.1.4. Pharmacokinetic analysis**

Compartmental and non-compartmental analyses are two common mathematical approaches used for transforming plasma (serum or blood) drug concentration vs. time course curve into PK parameters. Compartmental analysis heavily relies on the

assumption that a body consists of compartment(s) (single or multiple, such as one central, and one or more peripheral compartments) and employs hypothetical models to estimate PK parameters (Bonate, 2011). In contrast, non-compartmental analysis uses statistical moment theory, a numeric method, which assumes no compartments but rather, that the drug is eliminated from a single sampling pool (Yamaoka et al., 1978). Accordingly, the major difference of these two approaches is that compartmental analysis offers a quantitative description of a drug in non-accessible site(s) to predict or simulate drug concentrations (Bonate, 2011). However, because of the complexity, compartmental analysis needs a sophisticated PK software package, such as Phoenix WinNonlin (Cary, North Carolina, USA), where the validity of the selected compartmental model required is based on assumptions on drug movement between compartments (Bonate, 2011). Non-compartmental analysis is a relatively simple approach to estimate basic PK parameters (and indices), such as  $Cl$ ,  $V_d$ , (AUC,  $t_{1/2}$ , MRT,  $C_{max}$  and  $T_{max}$ ), which describe the general disposition of the drug; however its reliability is dependent on the structure of experimental design (e.g. adequate sampling time-points) and has limited capacity to describe the movement of the drug between body compartments (Jaki and Wolfsegger, 2012).

### **1.3. Hepatic metabolism**

The liver is the primary site for metabolism of both endogenous and exogenous compounds (Williams, 1959). Accordingly, for drugs, such NSAIDs, which undergo metabolism in order to be excreted from the body, elimination is generally dependent on hepatic  $Cl$  (Lees et al., 2004b).

In many situations, the metabolism of drugs is mediated *via* the catalytic activity of enzymes, known as drug metabolising enzymes (DME), which are concentrated in the liver. These enzymes are also found at other extra hepatic sites such as GIT (Lin et al., 1999), lung (Zhang et al., 2006) and kidney (Lohr et al., 1998). According to the functionality by which they alter the chemical structure of drugs, DME can be further classified into two major groups; namely those involved in either phase-1 or phase-2 reactions (Williams, 1959, Liska, 1998) **(Table 1.3.1)**.

Drug metabolising enzymes responsible for phase-1 reactions introduce or expose functional groups (e.g. -COOH, -OH, -NH<sub>2</sub> or -SH) on molecules *via* metabolic reactions including oxidation, reduction or hydrolysis (White and Coon, 1980, Nelson and Gordon, 1983). Phase-2 reactions involve a conjugation, in which hydrophilic conjugates such as glucuronide, sulfate, glutathione, acetyl or methyl groups or an amino acid is added to the functional groups established by the phase-1 reaction, to facilitate excretion (Williams, 1959, Liska, 1998). Phase-1 and/or phase-2 reactions can occur either sequentially or separately, depending on the physicochemical properties of the drug. Generally, it is assumed that the pharmacological property of a parent drug is reduced or diminished once it turned into metabolite(s). But in some cases, the metabolite(s) has a greater pharmacological response, such as ciprofloxacin (a metabolite of enrofloxacin) (Rao et al., 2001), or has toxic properties such in case of N-acetyl-p-benzoquinoneimine, a metabolite of paracetamol (also known as acetaminophen) (Mitchell et al., 1973, Dahlin et al., 1984).

**Table 1.3.1)** Drug metabolising enzymes involved in phase-1 and phase-2 reactions adapted from Nebot, 2009.

Phase-1 reactions	Phase-2 reactions
<p><b>Oxidation</b>            Cytochromes P450 monooxygenase system            Flavin-containing monooxygenase system            Alcohol (and aldehyde) dehydrogenase            Monoamine oxidase            Peroxidase</p> <p><b>Reduction</b>            NADPH-cytochromes P450 reductase            Reduced (ferrous) cytochromes P450</p> <p><b>Hydrolysis</b>            Esterases and amidases            Epoxide hydrolase</p>	<p><b>Glucuronidation</b>            UDP-glucuronosyltransferases</p> <p><b>Sulfation</b>            Sulfotransferases</p> <p><b>Glutathione conjugation</b>            Glutathione-S-transferases</p> <p><b>Acetylation</b>            N-acetyltransferases</p> <p><b>Methylation</b>            Methyl Transferase</p>

### 1.3.1. Cytochrome P450

Cytochrome P450 (CYP) is a super family of haeme-thiolate enzymes, and is the most important class of DME for phase-1 reactions. Cytochromes P450 are also known as mixed-function oxidases. They are also essential for the biosynthesis of endogenous steroids (Miller, 1988), and are important for several homeostatic activities such as controlling cholesterol formation and metabolism (Pikuleva, 2006). The name '**P450**' originates from the reduced form of the **p**igment (haeme portion) of the protein; e.g. reduced with dithionite, and subsequent binding to carbon monoxide which results in a unique spectrum of ultraviolet absorption at **450** nm (Omura and Sato, 1964a, Omura and Sato, 1964b). In the liver, CYPs are distributed within the smooth endoplasmic reticulum (SER) of hepatocytes.

The major metabolic pathway catalysed by CYP is oxidation, which includes aromatic and aliphatic oxidations, epoxidation, N (or S)-dealkylations, N (or S)-oxidations and deaminations etc (Leucuta and Vlase, 2006).

As the region of SER is highly non-polar, lipophilic drugs (RH) flow down a concentration gradient to CYP, allowing CYP to catalyse monooxygenation of the drug [as illustrated by reaction (7)] to form a product of oxidation (ROH) and H<sub>2</sub>O; whereas NAD (nicotinamide adenine dinucleotide) or NADPH (nicotinamide adenine dinucleotide phosphate) are the electron donors transported from P450 oxidoreductase (POR) and the cytochrome b<sub>5</sub> (Coleman, 2010). Thus, the CYP system comprises of three enzymes: CYP, POR and cytochrome b<sub>5</sub> (Coleman, 2010).



According to the similarity of amino acid sequences, CYPs are divided into families (>40% similarity), subfamilies (>55% similarity) and eventually into specific isoforms (Zuber et al., 2002). In the example of CYP3A4, CYP stands for cytochrome P450; CYP3 indicates the genetic family; CYP3A indicates the genetic subfamily; CYP3A4 indicates the gene that represents the specific isoform. Until now, 57 CYP isoforms (18 families and 42 subfamilies) have been characterised in humans (**Table 1.3.2**) (Guengerich, 2008). Within CYP families, CYP1, CYP2 and CYP3 are mostly concentrated in the liver and are purportedly responsible for metabolism of approximately 75% of all therapeutic drugs known to be eliminated by hepatic metabolism (Guengerich, 2008). Each subfamily and its isoforms metabolise specific substrates, however single or multiple CYP(s) may be



involved in the drug metabolism at single or multiple sites of the parent drug (Liska, 1998).

**Table 1.3.2)** Classification of human CYP isoforms based on major substrate class adapted from Guengerich, 2008.

<b>Sterols</b>	<b>Xenobiotics</b>	<b>Fatty acids</b>	<b>Eicosanoids</b>	<b>Vitamins</b>	<b>Unknown</b>
1B1	1A1	2J2	4F2	2R1	2A7
7A1	1A2	4A11	4F3	24A1	2S1
7B1	2A6	4B1	4F8	26A1	2U1
8B1	2A13	4F12	5A1	26B1	2W1
11A1	2B6		8A1	26C1	3A43
11B1	2C8			27B1	4A22
11B2	2C9				4F11
17A2	2C18				4F22
19A1	2C19				4V2
21A2	2D6				4 × 1
27A1	2E1				4Z1
39A1	2F1				20A1
46A1	3A4				27C1
51A1	3A5				
	3A7				

Cytochrome P4502C is considered an important CYP subfamily for drug metabolism. In humans, CYP2Cs are subdivided into four isoforms (CYP2C8, CYP2C9, CYP2C18 and CYP2C19) sharing >82% amino-sequence similarities (Miners and Birkett, 1998). It is known to be highly polymorphic and is reportedly responsible for the *Cl* of approximately 15% of those drugs that undergo phase-1 reactions (**Table 1.3.3**) (Rettie and Jones, 2005). In humans, CYP2C9 is considered the major metabolic pathway for NSAIDs and those drugs that are weak organic acids with a *pKa* ranging from 3.8 to 8.1 (Miners and Birkett, 1998). As a result of substrate specificity, tolbutamide, (S)-warfarin, diclofenac and flurbiprofen are most commonly used for investigating the *in-vitro*

activity of human CYP2C9, whereas celecoxib, warfarin or phenytoin are recommended for *in-vivo* studies (FDA, 2012).

**Table 1.3.3)** Examples of common therapeutic drugs metabolised by human CYP2C9 adapted from Rettie and Jones, 2005.

<b>NSAIDs</b>	<b>Oral hypoglycaemics</b>	<b>Oral anticoagulants</b>
Flurbiprofen	Tolbutamide	(S)-Warfarin
Diclofenac	Glyburide	(S)-Acenocoumrol
Naproxen	Glipizide	
Piroxicam	Glimepiride	
Suprofen		
Ibuprofen		
Celecoxib		
Meloxicam		
<b>Diuretics &amp; Uricosurics</b>	<b>Angiotensin-2 blockers</b>	<b>Anticonvulsant</b>
Torsemide	Losartan	Phenytoin
Ticrynafen	Irbesartan	
Sulfinpyrazone-sulfide	Candesartan	
<b>Anti-asthmatic</b>	<b>Anti-cancer agent</b>	<b>Endogenous compound</b>
Zafirlukast	Cyclophosphamide	Arachidonic acid
		5-hydroxytryptamine
		Linoleic acid

#### 1.4. Species differences with reference to PK process

There are substantial interspecies differences in regards to the processes that affect drug disposition, particularly absorption and metabolism (Lin, 1995), which may be attributable to several factors; for example, on a macroscopic level there are anatomical and physiological differences in the GIT (e.g. monogastric species vs. ruminants) or

dietary differences (e.g. herbivores vs. carnivores vs. omnivores) (Kararli, 1995, Toutain et al., 2010). On a molecular level there are differences in the catalytic activity of DME between species or even within a species (e.g. individual-specific multiple gene polymorphism) (Hucker, 1970, Smith, 1991, Nishimuta et al., 2013).

The 'half-life' of gastric emptying time, which influences food or drug absorption rate, varies between the species, for example: mice and rats (10 min), rabbits (30 min), and dogs (90 min) (Clark and Smith, 1984). Compared to humans, dogs have a faster small intestine transit time (e.g. dogs  $111 \pm 17$  min vs. humans 180-300 min) and have a higher and more variable intestinal pH which affects the extent of drug absorption (Clark and Smith, 1984). While intestinal permeability of drugs is influenced by luminal pH, it is suggested that in general, lipophilic drugs have better oral absorption in dogs than in other species (Sharma and McNeill, 2009). For example, oral absorption of nadolol or atenolol (both  $\beta$ -adrenergic receptor antagonists) in dogs is relatively complete (88 to 104%) compared to mice, rats, hamsters, rabbits, monkeys or humans (< 30%) (Clark and Smith, 1984).

Species differences regarding dietary preferences and constituents also affect the degree of drug absorption. Ruminants such as sheep exhibit low oral bioavailability of enrofloxacin compared to non-herbivorous species as a result of drug binding to dietary macromolecules (Lopez-Cadenas et al., 2013). Equine diets, high in cellulose result in binding of several NSAIDs, including phenylbutazone, flunixin, and meclofenamic acid, forming non-absorbable complexes (Toutain and Cester, 2004).

Several species exhibit deficient phase-2 reactions, for example: some isoforms of glucuronide conjugates in cats are lacking than other species (Court and Greenblatt, 1997), and both dogs and pigs lack acetylation and sulphation pathways, respectively (Sharma and McNeill, 2009).

#### 1.4.1. Species differences on CYP mediated metabolism

Although, the basic CYP amino acid sequence is generally conserved across species (**Table 1.4.1**) differences of CYP activity in relation to substrate specificity and rate of metabolic activity have been demonstrated between species (Smith, 1991, , Lin, 1995, Guengerich, 1997, Martignoni et al., 2006).

**Table 1.4.1)** Major drug metabolising CYP in humans, mice, rats, dogs and monkeys adapted from Martignoni et al., 2006.

CYP Family	CYP Subfamily	Human Isoforms	Mouse Isoforms	Rat Isoforms	Dog Isoforms	Monkey Isoforms
CYP1	CYP1A	CYP1A1 & 2	CYP1A1 & 2	CYP1A1 & 2	CYP1A1 & 2	CYP1A1 & 2
	CYP1B	CYP1B1	CYP1B1	CYP1B1	CYP1B1	CYP1B1
CYP2	CYP2A	CYP2A6 & 13	CYP2A4, 5, 12 & 22	CYP2A1, 2 & 3	CYP2A13 & 25	CYP2A23 & 24
	CYP2B	CYP2B6 & 7	CYP2B9 & 10	CYP2B1, 2 & 3	CYP2B11	CYP2B17
	CYP2C	CYP2C8, 9, 18 & 19	CYP2C29, 37, 38, 39, 40, 50, 54 & 55	CYP2C6,7,11,12 13, 22 & 23	CYP2C21 & 41	CYP2C20 & 43
	CYP2D	CYP2D6, 7 & 8	CYP2D9, 10, 11, 12, 13, 22, 26, 34 & 40	CYP2D1, 2, 3, 4, 5 & 18	CYP2D15	CYP2D17, 19, 29 & 30
	CYP2E	CYP2E1	CYP2E1	CYP2E1	CYP2E1	CYP2E1
CYP3	CYP3A	CYP3A4, 5, 7 & 43	CYP3A11, 13, 16, 25, 41 & 44	CYP3A1, 2, 9, 18 & 62	CYP3A12 & 26	CYP3A8

For example, Guengerich investigated catalytic activities of CYP1A2 between humans (using recombinant CYP1A2) and rats (purified from rat liver), in which 75 % of the CYP

amino acid sequences were identical, with several CYP1A2 substrates identified (Guengerich, 1997). The rate of *N*-hydroxylation of 2-amino-3, 8-dimethylimidazo [4, 5-*f*] quinoxaline activity in humans was three fold higher but the rate of *N*-hydroxylation of 1-amino-1-methyl-6-phenylimidazo [4, 5-*b*] pyridine was similar, whereas 7-methoxyresorufin *O*-demethylation activity in rats was five-fold higher than in humans. Moreover, Chauret et al., (1997) compared catalytic activity of CYP from hepatic microsomes (using human CYP1A1/2, CYP2A6, CYP2C8/9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 substrates) between humans, dogs, cats and horses, and inter-species differences were demonstrated. Among several drug metabolising CYP subfamilies (CYP2A, CYP2B, CYP2C, CYP2D, CYP2E and CYP3A), particularly high differences in the catalytic activity of CYP2A, CYP2B and CYP2C subfamilies were demonstrated between species (mice, rats, rabbits, monkeys and humans) (Guengerich, 1997).

As the rate and extent of PK processes for many therapeutic drugs can be variable between species, this has implications when extrapolating drug dosages from one species to another. Therefore it is likely that PK studies (*in-vivo* and where possible *in-vitro*) are required in order to determine dosage modifications for more exotic species, especially when the dosage is expected to deliver bioequivalent plasma concentrations.

### **1.5. Study of *in-vitro* hepatic metabolism**

Although, hepatic metabolism is important for the elimination (or *Cl*) of many drugs, performing *in-vivo* hepatic studies can be practically and ethically problematic in 'non-laboratory' animals especially when the drug is toxic. As an alternative, *in-vitro* hepatic

models are commonly used. Compared to *in-vivo* hepatic studies, performing *in-vitro* hepatic studies provide a snapshot of the hepatic intrinsic capacity of drug metabolism as well as the possibility of identifying metabolic pathway(s) (e.g. phase-1 and / or phase-2 reactions) *via* individual metabolite identification (Zuber et al., 2002).

Several different hepatic sources are available, including whole cellular systems (such as primary hepatocytes, immortalised cell lines, liver slices and liver homogenates) or sub-cellular systems (such as the supernatant fraction of liver homogenates after centrifugation at 9000 g (S9 fraction), microsomes, cytosol and cDNA-expressed individual CYP isoform), which can be used for *in-vitro* hepatic models (Brandon et al., 2003, Plant, 2004, Pelkonen et al., 2005).

The microsomal fraction is a sub-cellular fraction of hepatic tissue where differential centrifugation (centrifugal force of >100,000 g for 60 to 120 min) is commonly used to separate the microsomal fraction from the liver homogenate (Venkatakrisnan et al., 2001). The microsomal fraction consists of fragments of endoplasmic reticular segments which contain most of the phase-1 drug metabolising enzymes such as CYPs and flavin-containing monooxygenases as well as UDP-glucuronyltransferases for phase-2 reactions (Brandon et al., 2003). One of the advantages of utilising the microsomal fraction is that the catalytic activity of CYP can be stable for up to 5 years if stored at - 80 °C (Yamazaki et al., 1997). However, as activity of some cofactors, specifically NADP and NADPH, diminish during the preparation of microsomal fraction, the addition of these cofactors are required to activate the catalytic activity of CYPs (Pelkonen and Turpeinen, 2007). Consequently, with the assistance of tissue homogenisation, centrifugation and

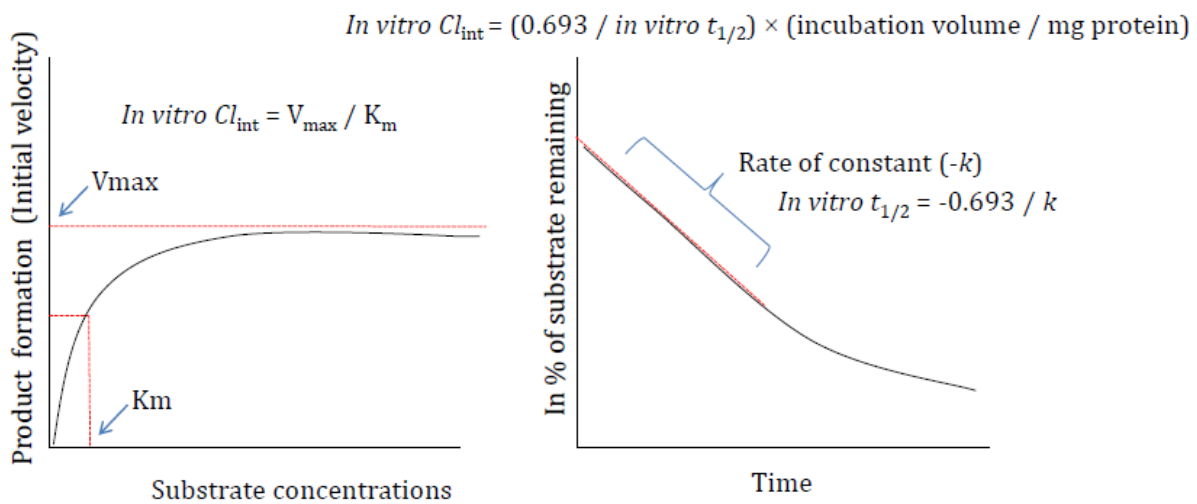
proper analytical techniques, hepatic microsomes, when fortified with proper cofactors are a useful *in-vitro* hepatic model to evaluate stability and metabolic profile of drugs associated with phase-1 reactions, including metabolism mediated *via* CYP (Venkatakrisnan et al., 2001).

Metabolic stability is defined as the fraction of a drug that escapes metabolic attack (Li, 2001). To estimate the stability of a drug, the percentage of the parent drug that disappears when challenged with active microsomes (also expressed as turnover rate) can be determined by the following equation **(8)**:

$$\text{Percentage (\%)} \text{ of drug disappearance} = [1 - (C_0 / C_x)] \times 100 \dots\dots\dots \textbf{(8)}$$

where  $C_0$  is the initial drug concentration and  $C_x$  is the remaining drug concentration after the experimentation.

*In-vitro* intrinsic clearance describes the rate of metabolism based on the catalytic activity of enzymes (e.g. CYP), which in case of microsomes, is expressed as  $\mu\text{L}/\text{min}/\text{mg}$  microsomal protein (Houston, 1994). Broadly, the *in-vitro*  $Cl_{\text{int}}$  is measured from either the initial rate of metabolite (product) formation or the initial depletion rate of the drug (substrate) **(Figure 1.5.1)** (Iwatsubo et al., 1997, Obach, 1999).



**Figure 1.5.1)** Determination of *in-vitro*  $Cl_{int}$  by product formation (left) or substrate depletion (right). (Image generated by the author).

Product formation follows traditional enzyme kinetics, in which its activity is commonly, but not always (Atkins, 2005), explained by Michaelis-Menten kinetics (9) (Michaelis et al., 2011) or alternatively, transformed into linear plots, such as Lineweaver-Burk plot, Hoftstee plot or Edie plot (Dowd and Riggs, 1965).

$$\text{Rate of metabolism} = V_{max} \times C_E / (K_m + C_E) \dots\dots\dots (9)$$

where  $V_{max}$  is maximum velocity or maximal enzyme saturation,  $K_m$  (Michaelis constant), is the substrate concentration required for half of  $V_{max}$ , and  $C_E$  is concentration of drug at the site of enzymatic reaction.

Generally, as the rate of metabolism is assumed to follow first order kinetics, if  $C_E$  is less than  $K_m$  value, then the *in-vitro*  $Cl_{int}$  can be estimated simply by  $V_{max} / K_m$  (Houston, 1994). One of the advantages is it provides *in-vitro*  $Cl_{int}$  as well as the characteristics of enzymatic reaction (Houston, 1994). For example, as the metabolism of a drug catalysed



by enzymes, thus the rate (or velocity) of a product formation progressively increases until all enzymes are saturated by a drug, the  $V_{\max}$  provides an indirect snapshot of amount of enzyme present; as well,  $K_m$  represents the affinity of the drug-enzyme saturation (Houston and Carlile, 1997). However, one of the problems with this approach is the requirement for an authentic standard of the metabolite, which may not be available in many situations.

To minimise the problem of a lack of a metabolite standard, the substrate depletion method uses the initial linear rate of drug disappearance, which assumes first-order kinetics, to estimate *in-vitro*  $Cl_{\text{int}}$  (Obach et al., 1997, Obach, 1999). Thus, it is important to ensure that the substrate concentration is less than the  $K_m$  value, and 1  $\mu\text{M}$  is often used as the arbitrary concentration (Obach, 1999). Although, it does not provide information about the metabolites per se, the substrate depletion is simple and metabolite standards are not required.

## **1.6. Prediction of *in-vivo* $Cl$**

Two different approaches, namely allometric scaling (Riviere et al., 1997, Tang and Mayersohn, 2006) or utilising species specific *in-vitro*  $Cl_{\text{int}}$  (Gillette, 1971, Obach, 1999) are commonly employed for predicting a drug's *in-vivo*  $Cl$ , in which an average fold error of two or less, is regarded as a good estimation of *in-vivo*  $Cl$  (Ito and Houston, 2005).

### 1.6.1. Allometric scaling

Allometric scaling employs an empirical formula established from a power-law relationship between *Cl* and bodyweights **(10)** of selected species to predict *Cl* in the target species based on the assumption that the physiological process (Y) and body weight (W) have a proportional relationship across species (Mahmood, 2007).

$$Y (Cl) = a W^b \dots\dots\dots \mathbf{(10)}$$

where *a* is the allometric coefficient and *b* is the allometric exponent of body weight (W).

Allometric scaling is the most widely used application to predict physiological processes, as it is simple to calculate and successful allometric relationships occur between body weight (or size) and other physiological processes such as basal metabolic rates (Sharma and McNeill, 2009), heart rates, liver weight and hepatic blood flow (Boxenbaum, 1980). Several studies have applied simple allometric scaling to predict drug *Cl* (Riviere et al., 1997, Tang and Mayersohn, 2006) or have used a modified version which includes the Dedrick plot (Dedrick et al., 1970), a two term power function incorporating body weight and brain function (Boxenbaum and Fertig, 1984), with corrections according to maximum life span or brain weight (Mahmood and Balian, 1996). Tang et al., (2006) evaluated simple allometric scaling to predict *in-vivo Cl* of 138 drugs and demonstrated that allometric scaling was more predictable for those drugs eliminated by the kidneys or *via* the bile rather than for those that undergo metabolism. On the other hand, Riviere et al., (1997) demonstrated that lower hepatic *Cl* drugs with high protein binding were not likely to have an allometric relationship with body size or *t*<sub>1/2</sub>. Moreover, Hunter et al., (2008) compared the accuracy of predicting drug *Cl*

(enrofloxacin, salicylic acid, meloxicam, flunixin and gentamicin) in avians using simple allometric scaling established from both mammalian data and avian data. Consequently, the study concluded that mammalian data was not applicable for predicting  $Cl$  in avians, which is probably due to inherent species differences in hepatic metabolism, but prediction of  $Cl$  improved when avian data was used. Therefore when extrapolating dosages between species the selection of appropriate species, which have similar anatomical, physiological, or biochemical properties to the target species, is an important determinant for successful allometric scaling.

#### **1.6.2. *In-vitro* to *in-vivo* extrapolation (IVIVE)**

In order to ameliorate concerns regarding inherent inter-species differences of hepatic  $Cl_{int}$  having potential bias when using allometric scaling, a method that uses species specific hepatic *in-vitro*  $Cl_{int}$  to predict *in-vivo* (or hepatic)  $Cl$ , known as IVIVE, has been proposed as an alternative approach, particularly for drugs that extensively rely on the hepatic metabolism (Rane et al., 1977, Houston, 1994, Obach et al., 1997, Iwatsubo et al., 1997). In order to use *in-vitro*  $Cl_{int}$ , particularly utilising hepatic microsomes, to predict *in-vivo*  $Cl$ , several assumptions are required: 1) the liver is primary  $Cl$  organ; 2) the metabolism by phase-1 reactions greatly exceeds (  $\gg$  ) other metabolism pathways; 3) metabolic  $Cl$  is greater than other  $Cl$  routes (e.g. renal and/or biliary  $Cl$ ) and 4) microsomal metabolism activity  $\gg$  other metabolism pathway activity (Obach et al., 1997).

*In-vitro* to *in-vivo* extrapolation is predicated on the fact that the major factors that govern *in-vivo* *Cl* are hepatic  $Cl_{int}$ , hepatic blood flow ( $Q_H$ ) and the unbound fraction ( $f_u$ ) of drugs, and assumes that the drug delivered to the liver is perfusion-rate limited and only  $f_u$  of drugs are extractable by the liver for metabolic events (Gillette, 1971, Rowland et al., 1973). Thus, with proper mathematical formulae (liver model) that link these factors, it was hypothesised that *in-vivo* hepatic *Cl* could be estimated from *in-vitro*  $Cl_{int}$  under apparent first-order kinetic conditions (Rane et al., 1977). Consequently, *in-vitro*  $Cl_{int}$ , which is based on either hepatic microsomes or hepatic sources, is required to be scaled up to represent whole liver  $Cl_{int}$  (*in-vitro*  $Cl_{int}'$ ) [mg microsomal protein / liver weight (g) per body weight (kg)].

According to several published works, liver models, such as venous equilibrium (well-stirred), undistributed sinusoidal (parallel), distributed sinusoidal and dispersion models, were proposed to explain the anatomical arrangement of hepatic circulation (or  $Q_H$ ) in relation to the delivery of  $f_u$  of drug to the site of metabolic activity in the liver (Gillette, 1971, Rowland et al., 1973, Pang and Rowland, 1977, Wilkinson, 1987). The 'well-stirred' model' **(11)**, the simplest liver model, assumes that concentrations of the  $f_u$  of drug in the liver and plasma are identical and distributed into the liver instantly and homogeneously (Rowland et al., 1973, Pang and Rowland, 1977).

$$\text{Hepatic } Cl = Q_H [(f_u \times Cl_{int}') / (Q_H + f_u \times Cl_{int}')] \dots \text{ (11)}$$

Rane et al., (1977), using the well-stirred model, first demonstrated the correlation between the *in-vivo* hepatic ratio (hepatic  $Cl/Q_H$ ), of several drugs with the *in-vitro*  $Cl_{int}$

(calculated from  $V_{\max}/K_m$ ) sourced from the S9 fraction. Since then, numerous studies have evaluated the *in-vivo* prediction of (hepatic) *Cl* of drugs utilising *in-vitro*  $Cl_{\text{int}}$  data. Ito et al., (2004) compared different liver models (well-stirred, parallel tube, and dispersion) to predict hepatic *Cl* from *in-vitro*  $Cl_{\text{int}}$  data (from hepatocytes and microsomes), demonstrating that the predicted results were comparable, between these models. Further studies indicated that for low *Cl* drugs (< 5mL/min/standard rat weight of 250 g) in particular, results from different liver models were similar (Houston and Carlile, 1997). For simplicity, the well-stirred model is frequently used for the prediction of the *in-vivo* (hepatic) *Cl* of drugs from *in-vitro* data (Ito and Houston, 2004). However, for some high *Cl* drugs, marked differences were demonstrated (Ito et al., 1998).

Baarnhielm et al., (1986) investigated the prediction of hepatic extraction ratio from *in-vitro*  $Cl_{\text{int}}$  data of felodipine, a lipophilic drug that has high hepatic extraction ratio and is highly protein bound, using hepatic microsomes of rat, dog, and man in the well-stirred model. In this study, a more accurate *in-vivo* prediction was demonstrated with *in-vitro*  $Cl_{\text{int}}$  ( $V_{\max}/K_m$ ) when corrected by the non-specific binding to microsomes; that is the unbound drug concentration in the incubation medium ( $f_{u(\text{mic})}$ ) **(12)**.

$$\textit{In-vitro } Cl_{\text{int}} = V_{\max} / (K_m / f_{u(\text{mic})}) \dots \dots \dots \textbf{(12)}$$

Furthermore, Obach (1997) investigated the effect of the non-specific binding of warfarin, imipramine, and propranolol during microsomal incubation in relation to IVIVE. Although disregarding all the binding (e.g.  $f_u$  and  $f_{u(\text{mic})}$ ) provided a better correlation with *in-vivo* *Cl* of imipramine, and propranolol, this study suggested that

generally correcting for the free fraction during the process of *in-vitro* microsomal incubation was required for the calculations (Obach, 1997).

Several studies have evaluated the use of microsomal *in-vitro*  $Cl_{int}$  data, using the substrate depletion method, to predict the *in-vivo* (hepatic)  $Cl$  of drugs (Obach, 1996, Jones and Houston, 2004). Jones and Houston (2004) investigated *in-vivo* prediction of eight benzodiazepines in rat hepatic microsomes to evaluate the effect of microsomal incubation time and microsomal concentration for depletion method. This study suggested that short microsomal incubation times (< 30 min) and the use of low microsomal concentration (< 0.5 mg/mL) provided more accurate *in-vivo* predictions.

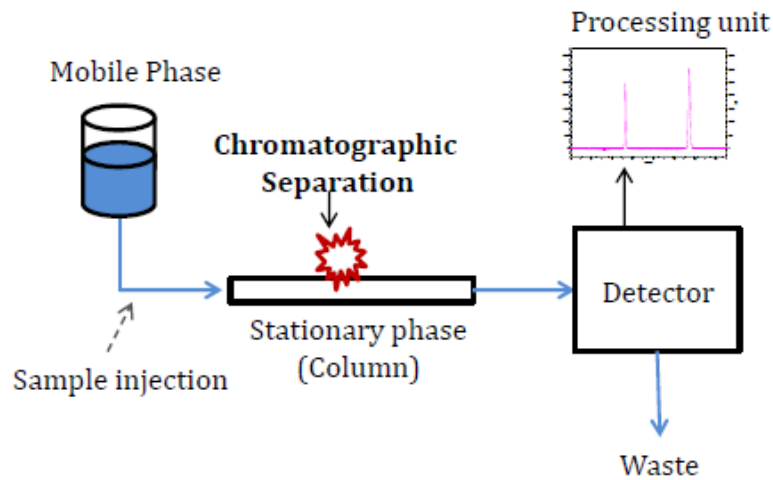
### **1.7. Determination of drug concentration in the biological matrix**

One of the important components of PK studies is the requirement for a robust analytical method to extract the drug from the biological matrix (such as plasma, serum, blood or tissues) and to quantify the drug concentration, and if possible to identify and/or quantify the presence of metabolites. This involves selection of an appropriate analytical technique and development of the assay method or 'conditions', including optimizing sample preparation in order to achieve optimal selectivity (or specificity) of drug detection and to yield maximal extraction for quantification. Assay validation is required to accurately and reliably quantify unknown drug concentrations in the biological matrix and should be performed prior to analysing the samples with unknown drug concentrations (González and Herrador, 2007).

### 1.7.1. High performance liquid chromatography

Immuno-assay and chromatography are the most commonly used analytical techniques for quantification of drug concentrations in a biological matrix. Immuno-assay, which uses an antibody specific for the target drug, is a simple, relatively inexpensive and time efficient technique. But, when a PK application is required, immuno-assay may lack sufficient sensitivity to differentiate the parent drug from its metabolite(s) or from similar homologue(s) within the biological matrix (Hirtz, 1986).

High performance liquid chromatography (HPLC), a form of separation technique, employs the relatively contrasting bonding affinities of two phases, one known as the mobile phase (liquid) and the other is the stationary phase (or column), to separate a mixture of compounds. In contrast to gas chromatography, HPLC offers higher selectivity and sensitivity to analyse thermally liable and non-volatile compounds (Liang et al., 2004). Consequently, HPLC is widely used for drug quantification and is commonly used for PK studies (Bressolle et al., 1996). Separation of mixtures of compounds by HPLC involves the process of mass transfer involving one or more of the following interactions to occur: adsorption, partition, ion exchange, or size exclusion etc (Kupiec, 2004). According to the major mode of interaction used for the separation, liquid chromatography (LC) is further classified into normal, reversed, ion exchange, or gel permeation (size exclusion) chromatography (**Figure 1.7.1**) (Dong, 2006).



**Figure 1.7.1)** Schematic diagram of HPLC; as a mixture of compounds passes through the column, separation occurs due to the intermolecular force created according to the specific mode of interaction. (Image generated by author).

Due to its versatility to separate a wide range of drugs, reversed phase HPLC (RP-HPLC), is most frequently used for PK studies (Shervington et al., 2005, Patel et al., 2013). Reversed phase HPLC employs a column packed with silica, bonded with either a non polar alkyl chain [e.g. octyldecyl (C18), octyl (C8) and butyl (C4)] or further attached with a polar group (e.g. phenyl, amide and -NHs) which provides a lipophilic surface; and utilises relatively hydrophilic solvents, such as a mixture of aqueous solution (water or buffer) and methanol (MeOH), acetonitrile (MeCN), or tetrahydrofuran as the mobile phase. Subsequently, separation is achieved by creating a hydrophobic bonding force and permitting drug retention and slow release from the column depending on the hydrophobic strength of a mobile phase (Bocian and Buszewski, 2012). Thus, retention time is an important marker for identifying a drug. While residual silanols inside the surface of column may create unwanted peak tailing of a drug e.g. interaction with



hydroxyl groups of a drug (Bocian and Buszewski, 2012), often acids (e.g. trifluoroacetic-, glacial acetic-, formic, or phosphoric acid) can protonate the silanols group (Kim et al., 2002) and are included in the mobile phase (~0.1-0.2 %) as a modifier to enhance the chromatogram peak resolution.

Once a drug is separated in the column from its matrix, it then travels through the detector. Several detectors are currently used for HPLC detection such as ultraviolet-visible (UV-vis), photo diode array (PDA), fluorescence, electrical conductivity, refractive detector, or mass spectrometer (MS). While many compounds and drugs, that consist of aromatic groups or  $> C = S$ ;  $> C = O$ ; and  $- N = N -$  groups, have considerable UV absorption ranges and as it is cost effective, HPLC coupled with a UV detector is generally used for PK studies. The PDA detector is an advanced form of UV-vis detector. While UV-vis, either a fixed or variable wavelength type, is only adjustable to a single wavelength of interest, a PDA detector registers multiple wavelengths at once. Within the PDA detector, a multiple spectrum of light is passed through the sample in which the light is further separated into multiple wavelengths and directed to each diode which detects the different wavelengths simultaneously (Choi, 2011). Consequently, one of the advantages of PDA is that it can detect the complete spectrum of the drug thus allowing accurate identification by comparing an unknown compound with a known standard. While the PDA detects the UV-spectrum of a compound, mass spectrometry (MS) allows further measurement of its molecular mass, as the process of MS charges the compound either positively or negatively, *via* electrospray, ion spray, or thermospray, and measures the mass-to-charge ratio ( $m/z$ ) of the compound (Ermer and Vogel, 2000). As

LC-MS (or liquid chromatography-tandem mass spectrometer: LC-MS/MS) can identify general characteristics of some chemical structures, it is commonly applied to elucidate structure of a drug and/or its metabolites (Chen et al., 2007a).

### **1.7.2. Sample preparation**

Due to the complexity of biological matrix, sample preparation is usually a necessary step prior to the HPLC analysis in order to separate the drug from other interfering elements within the matrix. Generally, one of three sample preparation techniques may be used: liquid-liquid extraction (LLE), protein precipitating extraction (PPE) and solid phase extraction (SPE).

Liquid-liquid extraction uses non-polar organic solvents, such as ethyl acetate, chloroform or hexane, that are immiscible to biological fluids, to partition the drug of interest into one of two layers. Lipophilic drugs are generally concentrated in the organic layer or *vice-versa* for hydrophilic drugs (Li et al., 2006). This widely used traditional technique, despite its simplicity and reasonable selectivity, often requires sequential extractions (also referred as back extraction) using different solvents which are laborious and usually yielding low recovery and reproducibility (Li et al., 2006). Liquid-liquid extraction can be inefficient to extract low amounts of hydrophilic drugs or when the volume of the sample is small (Li et al., 2006).

Protein precipitating extraction (PPE) is the simplest technique which uses organic solvents, salts (e.g. ammonium sulphate), acids or metal ions to disrupt protein-drug binding and remove the soluble proteins by converting them into insoluble compounds

using different mode of interactions (Polson et al., 2003). For PK studies, organic solvents [such as methanol (MeOH) and acetonitrile (MeCN)] can efficiently facilitate electrostatic protein interaction by reducing the dielectric constant of the plasma (Polson et al., 2003) For example: about 98% of proteins are precipitated when one volume of plasma is mixed with two volumes of MeCN (Souverain et al., 2004). This process is accomplished by high speed centrifugation to precipitate insoluble proteins so that the supernatant, further concentrated after drying, can be analysed *via* HPLC. However, the major drawback of this technique is the inability to remove other endogenous insoluble components in the biological matrix, thus increasing the risk of increasing pressure in the HPLC system or damaging the column (Polson et al., 2003). Consequently, in the case of mass spectrometry, detection sensitivity may decrease due to ion suppression as the result of presence of phospholipids (Bylda et al., 2014).

Solid phase extraction uses the concept of liquid chromatography to separate the drug from the biological matrix. Intermolecular forces are created between the sorbent, different solvents, and the sample; which initially 'retains' and then 'elutes' the analyte from the biological matrix (Li et al., 2006). Accordingly, different modes of separations are used in SPE sorbents which include reversed phase (e.g. C18, C8 and C4 etc), normal phase (e.g. CN, Si, or NH<sub>2</sub> etc), anion-exchange, cation-exchange or mixed mode (Li et al., 2006).

'Hydrophilic-lipophilic balanced' (HLB) is a sorbent which consists of a copolymer of *m*-divinylbenzene and *N*-vinylpyrrolidone. As in general, it has several advantages over the traditional reversed phase sorbent, which include 1) the ability to extract a wide

spectrum of both polar and non-polar analytes whereas C18 sorbent may require further modification of sample preparation, 2) ease of use (e.g. sorbent drying steps are not required as co-polymeric resins which was first introduced by Waters Oasis HLB are wettable in aqueous solution), 3) good reproducibility (< 5.5 % Relative Standard Deviation: RSD) and 4) reported excellent extraction recovery rates for many drugs (> 85 %) (Li et al., 2006). Subsequently, Baranowska and Kowalski (2011) demonstrated that recovery of Oasis HLB was higher than others (NEXUS and Bond Elut ENV from Varian (Palo Alto, CA) for detecting 15 drugs (including NSAIDs, corticosteroids, beta-blockers and anticonvulsant classes of drugs).

### **1.7.3. Validation of analytical method**

The term validation is to demonstrate that “the analytical procedure is suitable for its intended purpose” [ICH guideline, 2005 (Page 6)]. Thus, it is required to prove that the analytical method is accurate, reliable and suitable to analyse the samples containing unknown drug/analyte concentrations (Bressolle et al., 1996). Generally, validating the analytical method for quantification requires evaluation of following parameters which are sensitivity [e.g. lower limit of quantification (LLOQ)], specificity (or selectivity), precision, accuracy, recovery, and linearity of calibration range (ICH, 2005).

Sensitivity of the analytical method can be expressed as the lowest limit of quantification (LLOQ) and upper limit of quantification which are the lower and upper limit of calibration ranges (Causon, 1997). Thus, any value that falls outside of the assay range cannot be quantified reliably.

Specificity and selectivity are often used interchangeably. Specificity (or selectivity) describes how the analyte of interest is separated and distinguished from other endogenous compounds, including its metabolite(s), from the established analytical condition *via* analysing 'blank' plasma (that is, plasma which does not contain the drug of interest). More specifically, assay specificity demonstrates the absence of endogenous interference when detecting the analyte of interest; however if minimal interference is present 'selectivity' is the term used (Rambla-Alegre et al., 2012). The LLOQ is often used to define the degree of specificity (or selectivity) (Causon, 1997). Accordingly, it is suggested that less than 20% of bias (as defined below) for the LLOQ is recommended (ICH, 2005).

Precision and accuracy indicate errors which may occur during repeated sample preparation (random error) and between true and measured values (systemic error), respectively (Causon, 1997). More specifically, precision refers to closeness of agreement between the repeated samples values (expressed as % of coefficient of variation, CV) **(13)** whereas accuracy (expressed as % of bias) **(14)** refers to the closeness of agreement between the true and measured values (ICH, 2005). Accordingly, less than 15% of both CV and bias, except for LLOQ or upper limit quantification (20%), are recommended with at least nine determinants [three concentrations (low, middle, and high) each determined with no less than three replicates] to satisfy the criteria (ICH, 2005).

$$\text{CV (\%)} = (\text{standard deviation, SD} / \text{mean}) \times 100 \dots\dots\dots \mathbf{(13)}$$

$$\text{Bias (\%)} = [(\text{Measured value} - \text{true value}) / \text{true value}] \times 100 \dots\dots \mathbf{(14)}$$

Recovery (expressed as %) defines the efficiency of extraction or sample pre-treatment procedure as a fraction of the analyte lost during this procedure. Often absolute recovery is used for representing recovery, in which is the amount of analyte remaining in a pre-spiked blank sample after extraction ('extracted') is compared to the same initial concentration in a pure standard that has not undergone extraction ('non-extracted') (15)

$$\% \text{ absolute recovery} = \text{extracted} / \text{non-extracted} \times 100 \dots\dots\dots (15)$$

## **1.8. The koala**

The koala, the last remaining species of the family *Phascolarctidae*, is an iconic Australian marsupial. Although pockets of wild koala populations are distributed along eastern Australia, these populations are fragmented or isolated in woodlands and forests in some areas of the states of Queensland (QLD), New South Wales (NSW), Victoria (VIC) and South Australia (SA) (Vogelnest et al., 2008). The koala not only has cultural significance to the indigenous Australian population but also has great natural significance to all Australians. As a domestic and international tourist attraction, the economic value of koalas to the Australian economy is significant and was estimated at 1.1 billion Australian dollars in 1997 (Hundloe, 1997).

The requirement to protect and conserve the koala is escalating; as the number of wild koalas in QLD, the Australian Capital Territory (ACT) and NSW has significantly declined over recent decades (Department of the Environment, Water, Heritage & the Arts, 2009). Currently, in these states, the status of the koala has been formally recognized as either

'vulnerable' or 'endangered' [the latter classification specifically used for two populations in NSW (Department of the Environment, Water, Heritage & the Arts, 2009)]. The key threats to wild koala populations include habitat loss, climate change, infectious disease (such as the bacterial disease chlamydiosis, fungal disease cryptococcosis and koala retrovirus) and traumatic injuries due to vehicle strikes, predation by feral and domestic dogs and burns from bushfires (Griffith, 2010). A recent historic cohort study (Griffith, 2010), reported that the most common reasons for admission of wild koalas to Koala Preservation Society's koala hospital, Port Macquarie, NSW, during 1975 to 2004, were traumatic incidents (~40% of admissions) followed by localised and/or systemic chlamydiosis (~27.3%).

Wildlife rehabilitation plays an important role in caring for, and treating sick and injured wild koalas to ultimately release them back to their original habitat. Subsequently, medicines, particularly antibiotics, anti-inflammatories and analgesics, are often administered to convalescing koalas. However, although proper dosages are necessary to attain therapeutic plasma drug concentrations, only a few scientific reports on the pharmacokinetics of medicines / therapeutic drugs in koalas are available, and these are limited to antibacterial and antifungal drugs, such as enrofloxacin (Griffith et al., 2010, Black et al., 2013a), chloramphenicol (Govendir et al., 2012, Black et al., 2013b), and fluconazole (Black et al., 2014). Prior to these studies the suggested dosages of therapeutic drugs used in koalas were either extrapolated from other species, such as dogs and humans (Blanshard & Bodley, 2008), or based on anecdotal reports.

The koala, an arboreal folivore, relies almost exclusively on certain *Eucalyptus* spp. foliage as the nutrient source for its survival and accordingly, is classed as a *Eucalyptus* spp. specialist feeder (*E. specialist feeder*) (Stupans et al., 2001). Similar *E. specialist* feeders are the greater glider (*Petauroides volans*) and the common ringtail possum (*Pseudocheirus peregrinus*) whereas the common brushtail possum (*Trichosurus vulpecula*) is classified as *E. generalist feeder* (McLean and Foley, 1997).

*Eucalyptus* spp. foliage contains low nutrient concentrations but high concentrations of cellulose fibers and phyto-chemicals known as 'plant secondary metabolites' (PSM), such as phenols, phloroglucinols, tannins and particularly monoterpenes (the predominant constituent of *Eucalyptus* oil), which have a potential to disrupt digestion or cause toxicity in many other species (Stupans et al., 2001). Koalas are known to have some specialised GIT and detoxification systems to deal with the low levels of nutrients and the high PMS concentrations in *Eucalyptus* spp. foliage (Cork et al., 1983, Osawa et al., 1993, McLean and Foley, 1997).

Similar to other herbivores, koalas use fermentation to support digestion of cellulose fibers; however, unlike foregut fermenters (e.g. ruminants or kangaroos), koalas have a fermentation chamber located in the caecum (a hindgut fermenter) like possums, rabbits and horses (Hume, 1984). Koalas are known to have the largest caecum (200 cm) relative to the body length amongst all hindgut fermenters. Osawa et al., (1993) observed **t**annin-**c**omplex **d**egrading **e**nterobacteria (T-CDE) strategically colonised in the wall of caecum to breakdown tannin-protein complexes to increase digestibility and avoid potential toxic effects. While the role of fermentation is essential for energy



production in most herbivores, it is reported that koalas absorb most of their nutrients (up to 80 %) *via* the small intestine, however ingesta transit time in the small intestine is relatively short (0.1 h for particulate phase and 1 h for solute phase selectively) (Cork et al., 1983). It has been speculated that substantial nutrients for koalas are derived from non-structural carbohydrate and lipid present in the *Eucalyptus* spp. foliage (Cork et al., 1983).

Ingestion of 'Eucalyptus oil', a mixture of dietary monoterpenes such as 1, 8-cineole (>70 % of *Eucalyptus* oil) and *p*-cymene, is reported to be fatal in other species if consumed in large amounts (Stupans et al., 2001). While, the daily intake of *Eucalyptus* spp. foliage by koalas is estimated at about 10 kg, 10% of the dry matter is composed of *Eucalyptus* oil (Pass et al., 2001, Pass et al., 2002) and that amount of oil would be lethal to many other species (Stupans et al., 2001).

### **1.8.1. Hepatic metabolism in koalas**

As koalas are exposed to relatively high concentrations of dietary monoterpenes resulting from their *Eucalyptus* spp. foliage diet; their physiological elimination processes have received considerable attention. Several *in-vivo* studies have noted that koalas (Eberhard et al., 1975, Southwell, 1975) along with other *E.* feeders such as ringtail possums (McLean et al., 1993) and brushtail possums (Southwell et al., 1980) predominately excrete oxidised forms of monoterpenes. Foley et al., (1987) and McLean et al., (1993) observed that greater gliders and brushtail possums completely absorb monoterpenes (*Eucalyptus* oils) in the small intestine, avoiding hindgut fermentation,

suggesting that the primary site for oxidation of terpenes in marsupials is mostly within the liver and less likely by gut microbial activity which was initially suspected as the site of metabolism for herbivores (Freeland and Janzen, 1974).

Southwell et al., (1980) observed novel oxidised metabolites in the excreta of koalas compared to that of brushtail possums, and tried to correlate the greater capability of oxidative metabolism to increased consumption of dietary terpenes. McLean et al., (1993) observed diverse oxidised metabolites of terpenes but a lack of glucuronidation in ringtail possums compared to brushtail possums. Further, Boyle et al., (1999) observed minor and non significant glucuronidation, but multiple hydroxylated terpenes (*p*-cymene) excreted by the *E.* specialist feeders (ringtail possums and greater gliders) in contrast to that of *E.* generalist feeder (brushtail possum) or rats. In subsequent work, Boyle reported that koalas also exclusively excrete diverse oxidised terpenes (*p*-cymene and 1, 8-cineole) and minimal terpenes conjugated with glucuronic acid (Boyle et al., 2000, Boyle et al., 2001).

Pass investigated rates of *in-vitro*  $Cl_{int}$  of terpenes *P*-cymene and 1, 8-cineole on hepatic microsomes of koalas, brushtail possums and rats (Pass et al., 2001, Pass et al., 2002). In these studies, higher rates of *in-vitro*  $Cl_{int}$  of *P*-cymene and 1, 8-cineole were exhibited in both *E.* feeders compared to that of rats at least two fold and seven fold higher, respectively. As well, it was demonstrated that *in-vitro*  $Cl_{int}$  of both terpenes by hepatic microsomes increased when high concentrations of monoterpenes were fed to brushtail possums (Pass et al., 2001, Pass et al., 2002).

A previous study has shown that in *E.* feeders, monoterpenes undergo faster hepatic metabolism compared to rats and humans, and the tendency to utilise phase 1 reactions is greater in *E.* specialist feeders such as koalas and ringtail possums than in the *E.* generalist feeder (brushtail possums), but there is less conjugation (glucuronidation) in the monoterpenes metabolism pathway by *E.* specialist feeders (McLean and Foley, 1997). On the other hand, it was observed that approximately 60 % of urinary glucuronic acid was conjugated with phenolics of *Eucalyptus* spp., and therefore postulated that glucuronidation has a greater role in the metabolism of phenolics (McLean et al., 2003).

Several *in-vitro* studies have investigated phase-1 reactions particularly mediated *via* hepatic CYP in koalas. Stupans et al., (1999) first investigated catalytic activities of CYPs, using hepatic microsomes, in koalas and compared to that of tammar wallabies (*Macropus eugenii*), a grazing herbivore marsupial, and rats. The study reported that between these species, the rates of aminopyrine demethylation (a general indicator for CYP1A, CYP2A, CYP2B, CYP2D and CYP3A activities) and aniline hydroxylation (indicator of CYP2E1 activity) were comparable; whereas relatively low androstenedione 6 $\beta$ - and 16 $\alpha$  hydroxylation activities were demonstrated in koalas (Stupans et al., 1999). Although androstenedione 6 $\beta$ - and 16 $\alpha$  hydroxylation activities are indicators for CYP2C11, a specific isoform of rats, Liapis et al., (2000) observed significantly high tolbutamide hydroxylation (an indicator for CYP2C activity) in hepatic microsomes of koalas compared to that of brushtail possums, rats and humans. Furthermore, it was demonstrated that terpene (1, 8-cineole) induced hydroxylation of

tolbutamide, approximately at a magnitude of two fold, in brushtail possums fed high concentrations of terpenes (higher  $V_{max}$  in terpene treated group) compared to possums fed a non-terpene enriched diet (Liapis et al., 2000). Subsequently, specific isoforms of CYP2C were identified in koalas (CYP2C47 and CYP2C48) (Jones et al., 2008). The activity of erythromycin *N*-demethylation (an indicator for human CYP3A4) was investigated in hepatic microsomes of koalas, tammar wallabies and wombats (El-Merhibi, 2005). The study demonstrated that overall erythromycin *N*-demethylation activity in koalas was two times greater in the female than male, and was notably higher than in the other species. Furthermore, no gender difference was observed in either the tammar wallaby or wombat (El-Merhibi, 2005). Ngo et al., (2000) reported that hydroxylation of lauric acid (an indicator for human CYP4A11 or CYP2E1) in hepatic microsomes of koalas was approximately three to four fold higher than that of rats or wallabies. Subsequently, Ngo et al., (2006) identified and described the CYP isoform (CYP4A15) responsible for hydroxylation of lauric acid in koalas. Thus, these studies have identified that species differences concerning catalytic activities of CYP exist between koalas and other species, particularly higher CYP2C and CYP4A activities.

### **1.8.2. Pharmacokinetic studies in koalas**

Griffith et al., (2010), conducted the first study on the PK of any therapeutic drug in koalas, and reported relatively poor oral absorption of enrofloxacin (20 mg/kg) compared to when administered by SC injection 10 mg/kg SC, to koalas infected with chlamydiosis. Accordingly, a short small intestine transit time, first-pass metabolism, xenobiotics pumped back into the intestine lumen by efflux proteins (such as p-

glycoprotein) and drug entrapment or binding to the gut ingesta (and/or with oral supplementation co-administered with the drug) were postulated as possible factors to account for the lower oral bioavailability of enrofloxacin in the koala (Griffith et al., 2010). Subsequently, Black et al., (2013) investigated disposition of enrofloxacin after IV dosing (10 mg/kg) to clinically normal koalas ( $V_z$ : 2.77 L;  $Cl$ : 2.58 L/h). The estimated SC bioavailability in koalas (41 %) was significantly lower than that of other eutherian species such as rabbits (77 %) (Broome et al., 1991). The additional observation of extremely low peak plasma concentrations of ciprofloxacin, an active metabolite of enrofloxacin in koalas suggested that either the metabolic pathway of enrofloxacin is different to that of many other species, or rapid elimination of ciprofloxacin, known to be metabolised *via* CYP1A2 in humans, occurs in koalas (Black et al., 2013).

Govendir et al., (2012) investigated the PK of chloramphenicol base (active form) in koalas infected with chlamydiosis [(60 mg/kg, SC once a day (s.i.d: semel in die)]. One important speculation arising from this study was that the elimination phase of chloramphenicol, known to conjugate with glucuronic acid for its metabolism in other species (Chen et al., 2007b), was longer in the koala than other species which may have been attributed to one or more following factors: the formulation itself, route of administration, or the disease state of the animals (Govendir et al., 2012). Subsequent to this, Black et al., (2013) further compared different SC injectable formulations of chloramphenicol [base vs. sodium succinate (proactive form), 60 mg/kg, SC] and the disposition of chloramphenicol sodium succinate (25 mg/kg, IV) in clinically normal koalas (Black et al., 2013b). This study demonstrated that  $t_{1/2}$  between two formulations

(base form  $t_{1/2}$ : 13.1 h vs. sodium succinate form  $t_{1/2}$ : 1.4 h) were significantly different, whereas the PK profile of succinate form was comparable to horses ( $1 \pm 0.1$  h) (Pilloud, 1973) and shorter than sheep ( $1.70 \pm 0.02$  h) (Dagorn et al., 1990) and goats ( $1.97 \pm 1.23$  h) (Etuk et al., 2005).

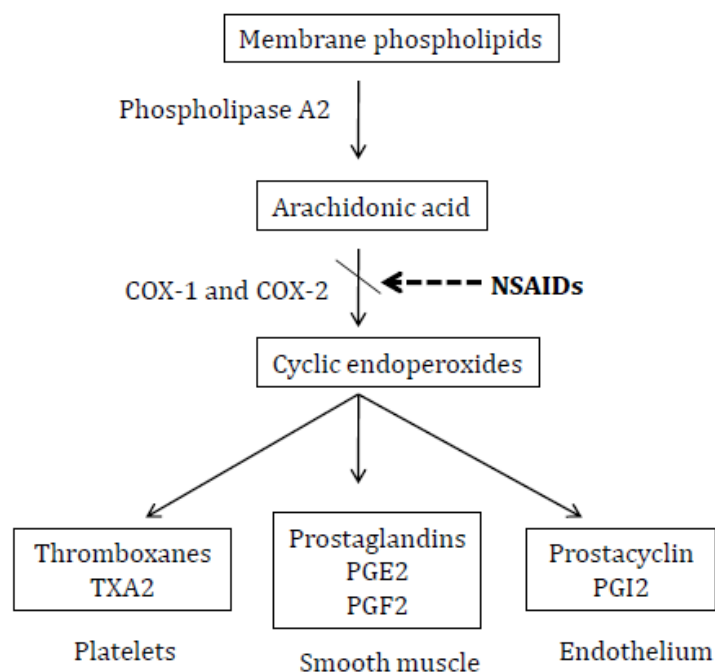
Recently, the PK of fluconazole, currently used for the treatment of the fungal disease cryptococcosis in koalas (Wynne et al., 2012) has been investigated in clinically normal koalas (10 mg/kg, PO and IV) (Black et al., 2014). Although, fluconazole is extensively eliminated unchanged in urine (> 70 %) in other species (Humphrey et al., 1985), thus facilitating allometric scaling across the species to determine the dose rate (Jezequel, 1994), this study revealed a marked difference in  $Cl$  in koalas, which was approximately six times higher than the estimated allometric scaled value. Based on this observation, a few hypotheses were suggested to account for the rapid  $Cl$  in that there may be differences in rates of tubular reabsorption and or active tubular secretion; or differences in metabolic conversion rates in koalas compared to other species (Black et al., 2014). However, this study did not determine the fate of fluconazole (*via* urine or fecal analysis), therefore further studies are warranted to clarify whether fluconazole is excreted unchanged and / or as one or more metabolites. Other important observations from this study were that the fluconazole-plasma protein binding in koalas was ~ four times higher than in other species and that fluconazole had low oral bioavailability (~50 % vs. almost 100% in most other species) (Black et al., 2014).

Accordingly, these studies demonstrate noticeable differences in some PK properties of investigated drugs, including absorption, especially *via* the oral route and/or

metabolism in koalas, and further question the reliability of extrapolating dosage, from another species for administration to koalas.

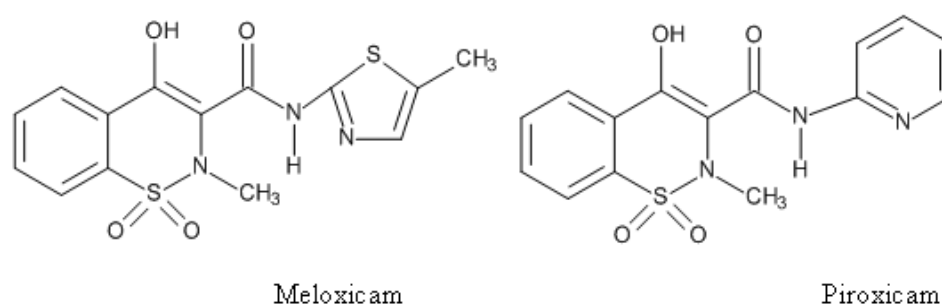
## 1.9. Meloxicam

Meloxicam, [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2*H*-1, 2-benzothiazine-3-carboxamide-1, 1-dioxide], is an oxicam NSAID which is an enolic acidic drug with anti-inflammatory, analgesic and antipyretic activities (Engelhardt et al., 1995). Like many other NSAIDs, meloxicam is known to inhibit activity of inducible cyclooxygenase (COX)-2, thereby preventing formation of prostaglandins (PG), some of which are inflammatory mediators (e.g. PGE<sub>2</sub> and prostacyclin), to reduce inflammation and pain (**Figure 1.9.1**) (Lees et al., 2004b, Hawkey, 2001).



**Figure 1.9.1)** Arachidonic acid - COX inflammatory pathway; NSAIDs such as meloxicam inhibit cyclooxygenase. (Image generated by the author).

Unlike other oxicam NSAIDs or other traditional COX inhibitors, meloxicam, with a chemical structure similar to piroxicam (another oxicam NSAID) (**Figure 1.9.2**) (but with addition of 5-methyl-2-thiazolyl moiety in the pyridine ring) facilitates preferential selectivity on inhibition of COX-2 over COX-1 (**Table 1.9.1**) (Engelhardt, 1996). However, preferential selectivity of meloxicam toward COX-2 inhibition is variable between *in-vitro* and *in-vivo* models (Schattenkirchner, 1997), or it can be species dependent.



**Figure 1.9.2)** Chemical structures of meloxicam and piroxicam (an oxicam NSAID). (Image generated by the author).

**Table 1.9.1)** COX inhibition ratio (COX-2/COX-1) of meloxicam and other NSAIDs tested on the intact cell system of guinea-pig peritoneal macrophages during 6 h of incubation in which a low ratio indicates greater COX-2 selectivity (adapted from Engelhardt, 1996).

NSAIDs	Ratio (COX-2/COX-1)
Meloxicam	0.33
Piroxicam	33
Tenoxicam	16
Tenidap	122
Indomethacin	30
Diclofenac	2.2
Flurbiprofen	317



While a function of COX-1 (a house keeping enzyme) is to maintain homeostasis and synthesise prostaglandins that stimulate gastric mucous production and thereby protect the gastric mucosa, several studies have reported that meloxicam's predilection to preferentially inhibit COX-2 results in fewer side effects, e.g. reduced gastric ulceration than non-selective NSAIDs (Engelhardt et al., 1996, Dequeker et al., 1998). For example, Dequeker et al., (1998) performed a large scaled human clinical trial on osteoarthritis patients to compare GIT tolerance of meloxicam (4320 patients) to that of piroxicam (4336 patients), and a significant lower incidence of gastric ulceration (10.3% vs. 15.4%;  $p < 0.001$ ) was reported in the group on meloxicam. As a result, meloxicam has been a popular NSAID for humans and for veterinary use. For humans and certain domestic species, an efficacious dosage is well established. For humans, meloxicam is approved for the treatment of arthritic conditions and the therapeutic dosage is 0.1-0.2 mg/kg, oral, s.i.d. (Gates et al., 2005). Meloxicam is also registered for the relief of inflammation and pain for cats (0.05 mg/kg, PO, s.i.d.), dogs (0.1 mg/kg, PO, s.i.d.) and horses (recommended at 0.6 mg/kg, PO, s.i.d.) (EMA, 2015).

### **1.9.1. Meloxicam PK**

The PK of meloxicam has been investigated in many mammalian species (**Table 1.9.2**). Similar to other oxicam NSAIDs, meloxicam is highly lipophilic ( $\log p = 1.9_{\text{octanol/water}}$ ), thus it rapidly diffuses across the intestinal membrane *via* transcellular absorption resulting in favorable oral bioavailability (> 85 %) in many species. Meloxicam has a low  $V_d$  as a result of high plasma protein binding (96-99 %). Meloxicam is considered a low hepatic *Cl* drug, thus the prolonged elimination  $t_{1/2}$  is favorable for once daily

administration in humans and variety of other species, however  $Cl$  and consequently  $t_{1/2}$  is variable among mammalian species as documented in **Table 1.9.2**.

**Table 1.9.2)** Pharmacokinetic profiles of meloxicam in selected mammalian species.

Species	$Cl$ L/hr/kg	$Vd$ L/kg	$t_{1/2}$ H	$F_{oral}$ (%)	Ref
Humans	0.010		20.0	89%	(Turck et al., 1996)
Dogs	0.010	0.32	24.0	106%	(Busch et al., 1998b)
Cats	0.006*	0.27*	37.0		(Giraudel et al., 2005)
Rats (male)	0.015	0.27	13.4		(Busch et al., 1998b)
Mice	0.015	0.467	6.4	94%	(Busch et al., 1998b)
Piglet	0.061	0.19	2.7		(Fosse et al., 2008)
Horses	0.034	0.12	8.5	85%	(Toutain et al., 2004)
Ponies	0.042	0.16	2.7		(Lees et al., 1991)
Calves	0.006	0.155	27.5	100%	(Coetzee et al., 2009)
Sheep	0.016	0.25	10.8		(Shukla et al., 2007)
Goats	0.030	0.26	6.7		(Shukla et al., 2007)
Goats	0.018	0.24	10.9	79%	(Ingvast-Larsson et al., 2011)
Camels	0.019	0.09	40.2		(Wasfi et al., 2012)

\*apparent total  $Cl$  ( $Cl / F$ ) and apparent  $V_{ss}$  ( $V_{ss} / F$ ) scaled by bioavailability after SC injection.

### 1.9.2. Hepatic metabolism of meloxicam

Similar to other NSAIDs, meloxicam is reported to be extensively metabolised in the liver and CYP2C is suggested as the major drug metabolising enzyme involved in the hydroxylation of meloxicam in humans (Turck et al., 1996). Meloxicam is extensively biotransformed into 5-hydroxy methyl metabolite *via* CYP2C9 ( $K_m$ : 9.6  $\mu$ M) and with a lesser contribution by CYP3A4 ( $K_m$ : 475  $\mu$ M) (Chesne et al., 1998). Subsequently, Chesne et al., (1998) demonstrated *via* human hepatocytes cell cultures that the 5-hydroxy methyl metabolite was the initial product for further 5-carboxyl derivative.

Meloxicam metabolites have been isolated from the faeces of several other mammalian species (Schmid et al., 1995b, Busch et al., 1998, Tevell Aberg et al., 2009, Grude et al., 2010). Although the CYP isoform has not been determined in other species, similar metabolic patterns have been demonstrated among species. For example in rats, the major metabolites recovered from the excreta were the 5-hydroxy methyl metabolite (~55 %) and the 5-carboxyl derivative (65 %), indicating oxidation as the primary metabolic pathway (Schmid et al., 1995b). Similarly, variable amounts of 5-hydroxy methyl metabolite, likely due to CYP2C-like activity, was presented in the excreta of mice (Busch et al., 1998), cats (Grude et al., 2010), mini-pigs (Busch et al., 1998) and horses (Tevell Aberg et al., 2009).

## **2. Aims of the study**

Meloxicam is the most commonly administered NSAID to koalas for the purposes of providing analgesia (de Kauwe et al., 2014) and for controlling inflammatory conditions such as arthritis associated with shoulder and hip dysplasia in captive koalas (Pye, 2009, Pye et al., 2008). Although koalas, and other *E.* specialist feeders, are thought to have a highly specialised hepatic CYP mediated oxidation strategy to eliminate dietary toxins, the disposition of meloxicam (or other NSAIDs) has never been investigated in this group of animals prior to this research. Meloxicam is recognised as a low hepatic extractable drug in other species (Busch et al., 1998). Thus, the possibility of species differences in the hepatic CYP-mediated metabolism may result in difference of drug bioavailability in koalas. Furthermore, tolbutamide hydroxylation in koalas is reported to be extremely rapid compared to other mammalian species, e.g. rats or humans,

(tolbutamide being a representative CYP2C9 substrate in human) (Liapis et al., 2000). Therefore this research project was designed to evaluate the *in vivo* PK and compare to the *in-vitro*  $Cl_{int}$  of meloxicam. As  $Cl$  of lower or intermediate hepatic  $Cl$  drugs is difficult to estimate from other species, this research was designed to also investigate potential usefulness of *in vitro*  $Cl_{int}$  to predict *in vivo*  $Cl$  scaling. As CYP2C is important subfamily that involves metabolism of many therapeutic drugs, including NSAIDs in people, this research was extended to compare *in-vitro* metabolism of series of CYP2C9 substrates between the koalas and some selected species (brushtail possums, ringtail possums, and rats) to identify other NSAIDs that may have favorable PK characteristics for treating injured koalas.

Thus, specific aims of the work in this thesis are summarised below:

- 1) To develop the HPLC-PDA method to quantify plasma concentration of the meloxicam in koalas
- 2) To investigate meloxicam PK in koalas after intravenous, subcutaneous and oral administration
- 3) To compare *in-vitro* hepatic microsomal metabolism of meloxicam and predict *in-vivo* clearance in the koalas, brushtail possums, ringtail possums, rats and dogs.

4) To compare CYP2C like activity using series of human CYP2C9 substrates between koalas, brushtail possums, ringtail possums and rats as a screen to identify other NSAIDs that may have a favorable PK profile in these species.

# Chapter 2

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## **Quantitation of meloxicam in the plasma of koalas (*Phascolarctos cinereus*) by improved high performance liquid chromatography**

The following chapter is modified from the original article:

**Kimble, B., Li, K. M., Govendir, M.** (2013). Quantitation of meloxicam in the plasma of koalas (*Phascolarctos cinereus*) by improved high performance liquid chromatography. *Journal of Veterinary Science*, **141**, 7-14.

## 2.1. Abstract

A simple, sensitive and improved method using HPLC-PDA was developed and validated to determine meloxicam concentrations in koala plasma, applicable for in-vivo pharmacokinetic study. Prior to the analysis, the koala plasma sample was cleaned with hydrophilic-lipophilic copolymer SPE cartridge (Oasis HLB). Separations of meloxicam and the piroxicam (IS), from the interferences of the koala plasma endogenous matrix, were achieved using an isocratic mobile phase [MeCN and 50 mM potassium phosphate buffer (pH 2.15) (45:55, v/v)] on a Nova-Pak C<sub>18</sub> 4 µm (300 mm x 3.9 mm) column. The retention times for both meloxicam and the internal standard were approximately 8.03 and 5.56 min, respectively, with a flow rate of 0.8 mL/min. The total chromatographic run time was completed in 15 min and the peak area ratios of meloxicam to the internal standard were used for regression analysis of the calibration curve. The latter was linear from 10 to 1000 ng/mL ( $r^2 > 0.9998$ ) which detection wavelength of 355 nm was used. The average absolute recoveries, indicating extraction efficiencies, were 91 % and 96 % for meloxicam and the internal standard, respectively. Despite the complexity of koala plasma matrix, advantages of this method were that it had high selectivity and sensitivity which achieved LLOQ of 0.01 µg/mL and only small plasma volume (250 µL) was required for analysis. In addition to this, the developed method was shown to detect potential metabolites of meloxicam in koala plasma.

## 2.2. Introduction

A variety of analytical methods have been developed and established to measure the concentration of meloxicam; these include UV spectrophotometry (Joseph-Charles and Bertucat, 1999, Garcia et al., 2000), fluorimetry (Hassan, 2002), polarography (Altiokka et al., 2001), voltammetry (Wang et al., 2006), colorimetry (Zawilla et al., 2003), capillary zone electrophoretic method (Nemutlu and Kir, 2003) or thin layer chromatography with densitometry (Bebawy, 1998) etc. However, many of these are not suitable for measuring the concentration of meloxicam in biological samples for PK studies due to both poor sensitivity and selectivity. On the other hand, few studies demonstrated superior analytical sensitivity for measuring the concentrations of meloxicam in human plasma using LC-MS/MS (Wiesner et al., 2003, Ji et al., 2005, Yuan et al., 2007). Nevertheless, due to the high cost of the instrumentation which is beyond the reach of many laboratories, use of LC-MS/MS is not commonly achievable. Instead, HPLC equipped with either UV or PDA, which is more cost efficient than LC-MS/MS, are commonly applied in PK studies of meloxicam in humans (Velpandian et al., 2000, Dasandi et al., 2002, Bae et al., 2007, Ouarezki and Guermouche, 2010). However, several HPLC-UV (or PDA) methods shown to provide suitable sensitivity for PK studies (e.g. LLOQ: 0.01 µg/mL) (Bae et al., 2007, Ouarezki and Guermouche, 2010), these methods, unfortunately, require large sample volumes (0.5 to 1 mL) that may not be easily collected repeatedly from valuable, rare or non-domesticated animals such as adult koalas with an average body weight less than 10 kg. Furthermore, all of these HPLC methods have been developed with human plasma samples. During the initial optimisation of HPLC method, this study revealed two major challenges: 1) the



complexity of the endogenous koala plasma matrix and 2) limited sample volume. While one of the important aspects of PK studies is to, accurately, measure the concentration of a drug, this chapter was initially aimed to develop a suitable HPLC-PDA method using SPE to quantify plasma concentration of meloxicam in the koala plasma.

## **2.3. Materials and method**

### **2.3.1. Chemicals and materials**

Meloxicam, piroxicam (IS), potassium dihydrogen phosphate and *ortho*-phosphoric acid (H<sub>3</sub>PO<sub>4</sub>) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade MeOH and MeCN were supplied from Analytical Science (Sydney, NSW, Australia). Purified water was obtained from Milli-Q water system (Millipore, Milford, MA, USA) and Oasis HLB 1cc (30 mg) extraction cartridges were purchased from Waters (Milford, MA, USA).

### **2.3.2. Chromatographic condition**

The HPLC system consisted of a Shimadzu CBM-20A module equipped with a LC-20AT delivery unit with DGU-20As degassing solvent delivery unit, SIL-20AC auto injector, CTO-20AC column oven, and SPD-M20A diode array detector (Kyoto, Japan). As well, Shimadzu class VP data system (software version 7.4) (Kyoto, Japan) was used for chromatographic control, data collection, and data processing.

Chromatographic separation was initially tested with several C<sub>18</sub> columns in various sizes of column internal diameters [Apollo C<sub>18</sub>, 5 µm, 250 mm x 4.6 mm (Alltech, Hunting wood, NSW, Australia); Nova-Pak C<sub>18</sub>, 4 µm, 300 mm x 3.9 mm (Waters, Rydalmere, NSW,

Australia); Gemini C<sub>18</sub>, 5 µm, 150 mm x 2 mm (Phenomenex, Lane Cove, NSW, Australia)]. As well, organic modifiers (MeOH and MeCN) with different compositions with purified water (or buffer) were tested. Final chromatographic separation was performed by a Nova-Pak C<sub>18</sub>, 4 µm, 300 mm x 3.9 mm attached to a 1 mm Opti-guard C-18 column (Optimize Technologies, Alpha Resources, Thornleigh, Australia) with the column temperature maintained at 30 °C. The isocratic mobile phase was composed of 50 mM potassium phosphate buffer of pH 2.15 and acetonitrile (55: 45, v/v). The mobile phase was delivered at a flow rate of 0.8 mL/min. The eluent was monitored at 355 nm. The total run time for each sample was 15 min.

### 2.3.3. Sample preparation

A stock solution of meloxicam (0.1 mg/mL) was prepared in 50 % MeOH and was further diluted with 50 % MeOH to give a series of working solutions of 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µg/mL. A stock solution of IS (0.1 mg/mL) was also prepared in 50 % MeOH and diluted to 0.5 µg/mL in 50 % MeOH as the working solution. Both stock solutions were stored at -20 °C and working solutions were freshly prepared from the prepared stock solution whenever required. Blank koala plasma obtained from different animals was pooled together and stored at -20 °C prior to use for preparation of calibration standards and quality control (QC) samples. For the method validation, low, middle, and high concentrations of QC samples (0.01, 0.2, and 1 µg/mL) were prepared by spiking the working solutions (0.05, 1, and 5 µg/mL) of meloxicam into blank pooled koala plasma and stored at -20 °C. For the preparation of unknown plasma samples (IV dosed koala sample) (**Chapter 3**), heparinised whole blood samples (approximately 1-1.5 mL)

were centrifuged at 1,400 g for 10 min. The plasma was carefully removed and stored at -20 °C. Plasma calibration standards (ranging from 0.01 to 1 µg/mL) were freshly prepared for each analysis by spiking 50 µL of appropriate working solutions of meloxicam into 250 µL of blank pooled plasma which was pre-thawed at room temperature.

#### **2.3.4. Solid phase extraction**

Plasma samples (250 µL) were diluted with 750 µL of water and then spiked with 50 µL of working IS solution to give 0.1 µg/mL of final plasma concentration. The SPE cartridges were connected to a Vac. Elut. Vacuum manifold (Supelco, Bellefonte, PA, USA) and conditioned with 1 mL of MeOH followed by 1 mL of water. The samples were allowed to run through sorbents at a flow rate of less than 1 mL/min. Cartridges were then rinsed with 1 mL of 5 % MeOH and dried under vacuum for 2 to 3 min. Analytes were eluted with 1.5 mL of MeOH. The eluent was then dried under vacuum in a Speed Vac concentrator (Thermo Scientific, USA) at 40 °C for 2 h and the dried residue was reconstituted in 100 µL of mobile phase. The reconstituted sample was vortexed (15 sec), sonicated for 5 mins and then centrifuged at 14,000 x g for 10 mins to remove any particulates. The supernatant was then transferred into HPLC insert vials and 10 µL of reconstituted sample was injected into the HPLC system.

#### **2.3.5. Method validation**

**Selectivity:** The selectivity of the assay was established by analyzing blank koala plasma ( $n = 10$ ) to identify endogenous interference around the retention times of both

meloxicam and the IS. Meloxicam peak in the plasma was identified from the retention time and UV spectra of the reference standard.

**Linearity and sensitivity:** Meloxicam concentrations in plasma samples were quantified *via* calibration curves whereby seven concentrations, 0.01, 0.02, 0.04, 0.1, 0.2, 0.4 and 1 µg/mL, were used to establish a non-weighted least square linear regression of the curve ( $y = ax + b$ ). This was done by plotting the concentrations of meloxicam (x) versus the peak area ratios (y) of meloxicam to IS, where (a) and (b) indicate the slope and y-intercept of the curve, respectively. According to ICH guidelines (International Conference on Harmonisation, 2005), the lowest limit of quantification (LLOQ) was determined based on the calibration curves using below formula:

$$\text{LLOQ} = 10 \times \sigma/S$$

where  $\sigma$  is a standard deviation of the y-intercepts from the regression lines and S is the mean slope of calibration curves.

In this study, an acceptance criterion for LLOQ was defined as precision less than 15 % of CV and accuracy within  $\pm 20\%$  of nominal concentration with repeated analyses (International Conference on Harmonisation, 2005).

**Precision and accuracy:** Intra- and inter-day precision were analysed from triplicates of QC samples (0.1, 0.2 and 1 µg/mL), both within a day and on five consecutive days, respectively. The relative difference of the estimated concentrations were expressed as

$$\text{CV} = (\text{standard deviation} / \text{mean value}) \times 100.$$

Intra- and inter-day accuracy, expressed as bias was determined by a percentage difference between estimated value and the nominal value of meloxicam.

$$\text{Bias} = (\text{estimated value} - \text{nominal value}) / \text{nominal value} \times 100$$

**Recovery:** Absolute recovery of meloxicam was determined by comparing the peak area of pre-spiked plasma samples ( $n = 5$ ) at concentrations of 0.01, 0.2, and 1  $\mu\text{g}/\text{mL}$  with corresponding concentrations of meloxicam in mobile phase.

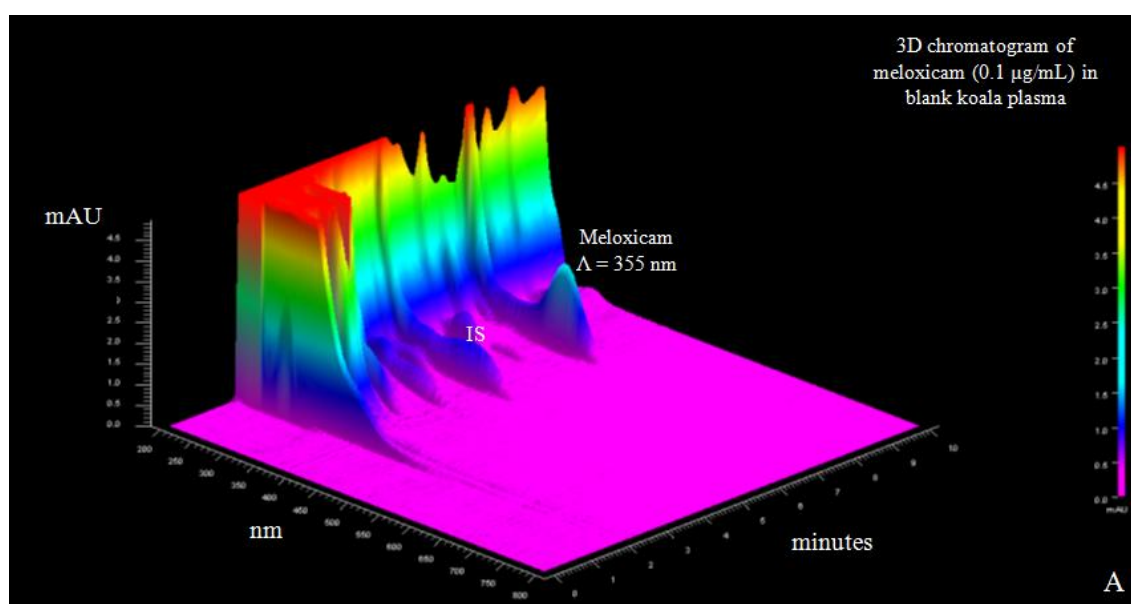
**Stability:** Stability, including three freeze/thaw cycles (over a 5-day period) and long term stability (up to 3 months), was assayed with pre-spiked samples at two concentrations (0.01 and 1  $\mu\text{g}/\text{mL}$ ) at  $-20\text{ }^{\circ}\text{C}$ . Each assay was conducted in triplicate and statistical data analysis was achieved by ANOVA and unpaired student t-tests using Graph Pad Prism software version 5.01 for Windows (Graph Pad Software, San Diego California USA), where the minimum significance level for all statistical tests was set at  $P < 0.05$ .

## 2.4. Results

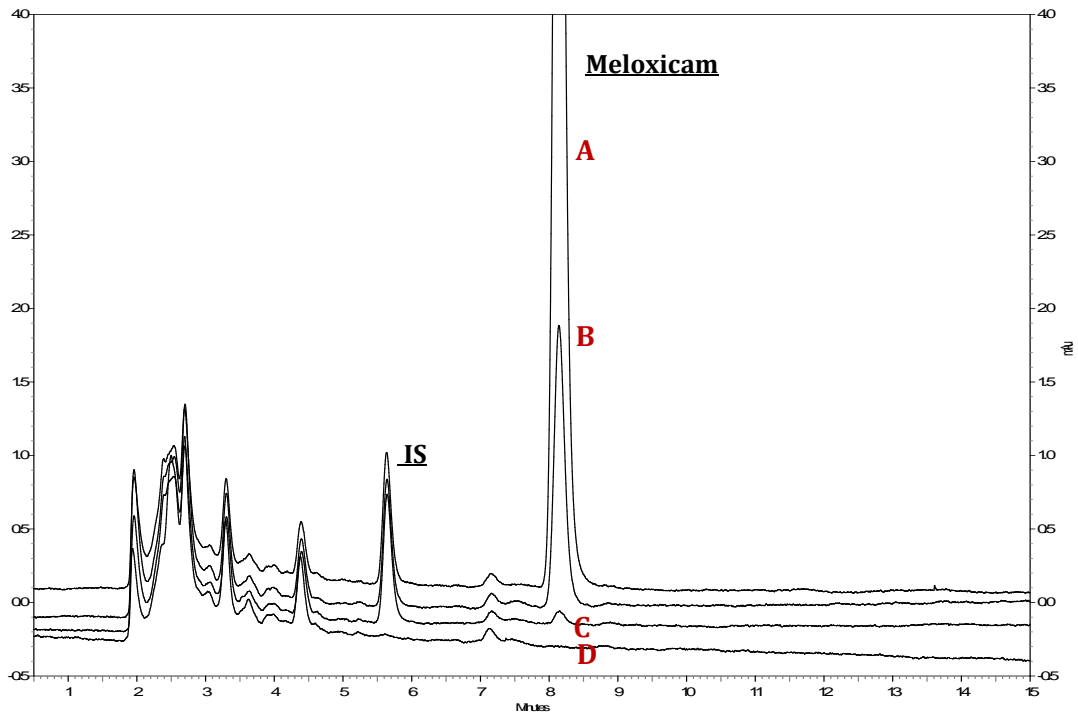
### 2.4.1. Chromatographic separation

With reference to the UV spectra (**Figure 2.1**), the optimal wavelength was set at 355 nm for quantitative analysis of meloxicam. Typical chromatograms of extracted double blank plasma and QC plasma samples (0.01, 0.2 and 1  $\mu\text{g}/\text{mL}$ ) are shown in **Figure 2.2**. Accordingly, the retention times of meloxicam and IS were approximately 8.03 and 5.56

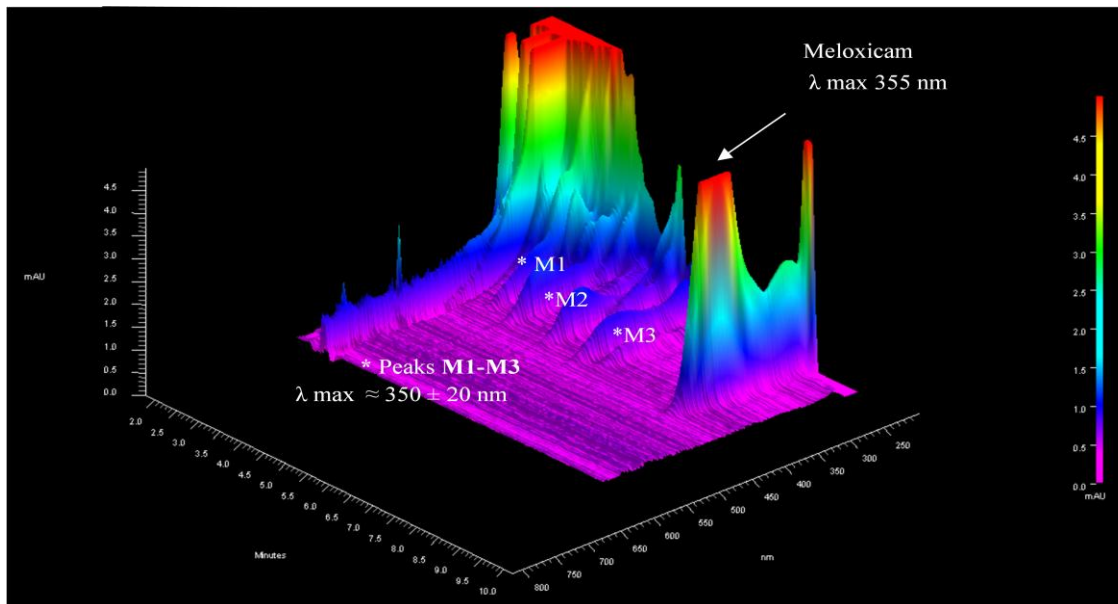
mins, respectively. **Figure 2.3** is the representative 3D-chromatogram of meloxicam and IS in the koala plasma sample obtained after 15 min of IV injection (0.4 mg/kg), where typical chromatograms (UV wavelength at 355 nm) of IV injected (0.4 mg/kg) koala plasma at  $t=0$ ,  $t=5$  and  $t=15$ ) are shown in **Figure 2.4**.



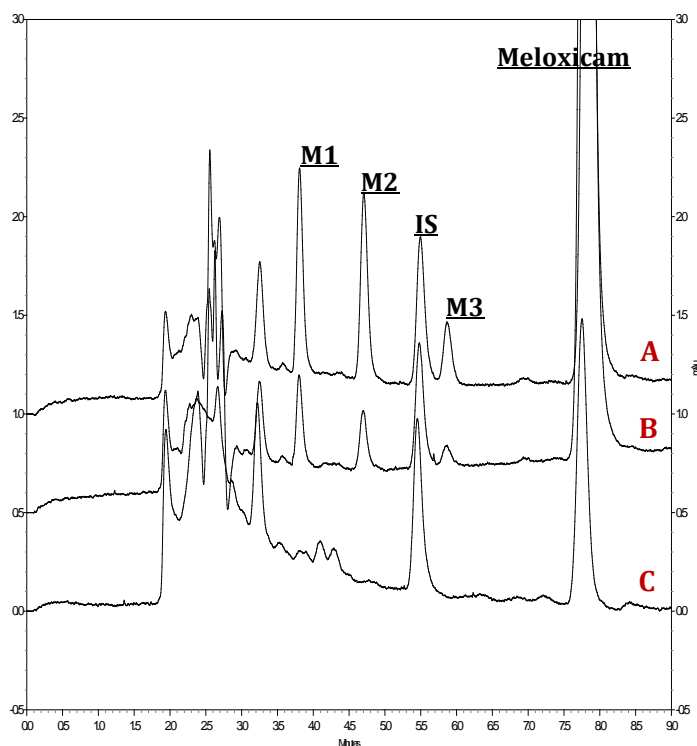
**Figure 2.1)** Representative 3D-chromatograms of meloxicam (0.1 µg/mL) and IS pre-spiked in blank koala (pooled) plasma.



**Figure 2.2)** Chromatograms of (A) high QC (1  $\mu\text{g}/\text{mL}$ ), (B) middle (0.2  $\mu\text{g}/\text{mL}$ ), and (C) low (0.01  $\mu\text{g}/\text{mL}$ ) QC samples contained IS; (D) double blank koala plasma (pooled); UV wavelength: 355 nm



**Figure 2.3)** Representative 3D-chromatograms of meloxicam and IS in koala plasma after 15 min of IV administration (0.4 mg/kg)



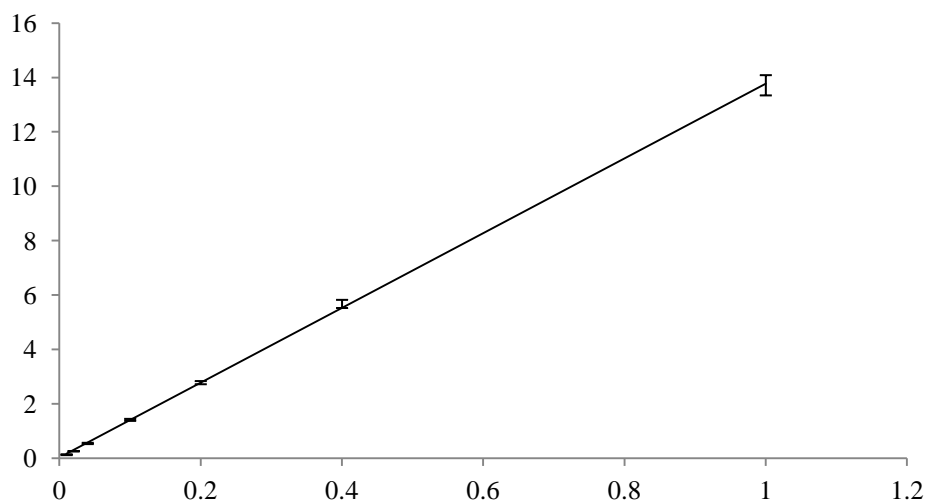
**Figure 2.4)**

Chromatograms of IV injected (0.4 mg/kg) koala plasma (A)  $t = 15$  min; (B)  $t = 5$  min; and blank plasma pre spike with  $0.1 \mu\text{g/mL}$  of meloxicam (C). UV wavelength: 355 nm. M1, M2, and M3: possible metabolites of meloxicam

## 2.4.2. Validation

**Linearity and sensitivity:** The mean regression calibration curves ( $n = 5$ ) were described as  $y = 13.9562 (\pm 0.2863, \text{SD}) x + 0.0003 (\pm 0.0162, \text{SD})$  with the  $r^2$  value for each curve greater than 0.9998 (**Figure 2.5**). The calculated Fisher ratio calibration curve in triplicate was 1.67 (2.96 at the 95% confidence level), and the curve was demonstrated to be linear.





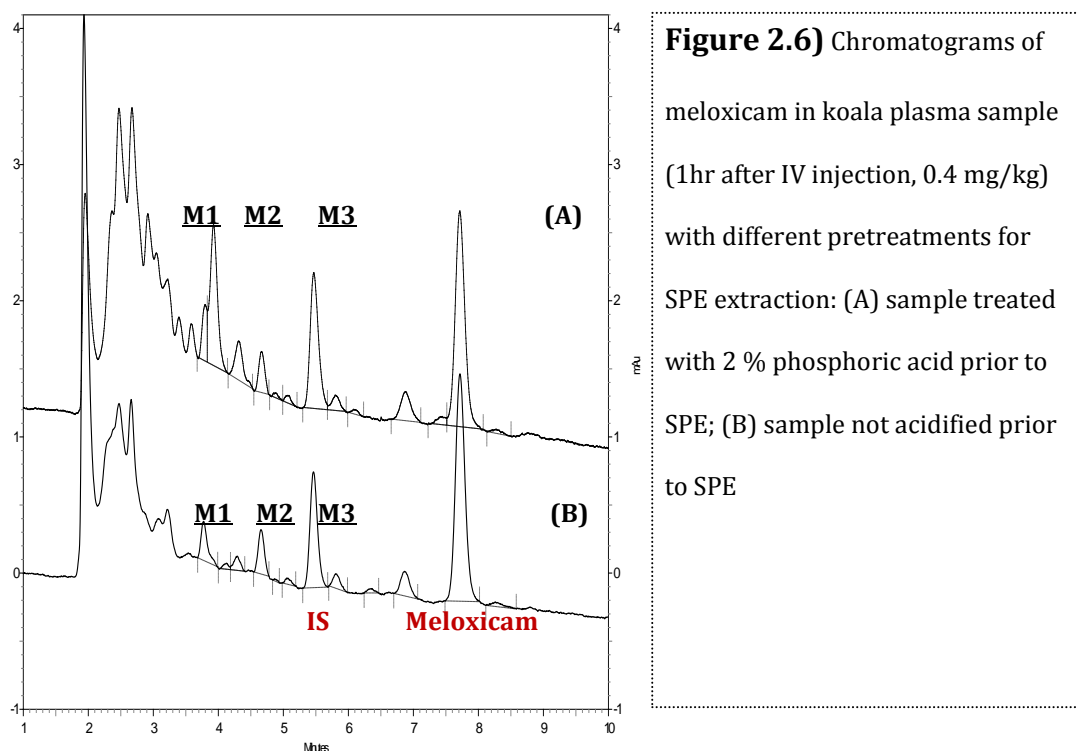
**Figure 2.5)** The mean regression calibration curve ( $y = 13.9562 (\pm 0.2863, \text{S.D.}) x + 0.0003 (\pm 0.0162, \text{S.D.}), n = 5$ ), ranged from 0.01 to 1  $\mu\text{g/mL}$ , where  $r^2$  value for each curve greater than 0.9998.

**Precision and accuracy:** Precision and accuracy of the LLOQ (0.01  $\mu\text{g/mL}$ ) were less than 15 % (CV) and within 20 % (bias) of nominal concentration, respectively (**Table 2.1**).

**Table 2.1)** (Intra- and interday) precision (% CV) and accuracy (% bias) of QCs.

<b>Nominal concentration</b> ( $\mu\text{g/mL}$ )	<b>Estimated concentration</b> ( $\mu\text{g/mL}$ )		<b>Precision</b> (% C.V.)	<b>Accuracy</b> (% bias)
<b>Intra day</b>				
<b>Day 1 (n = 3)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	1.001	0.014	1.351	0.078
0.200	0.200	0.001	0.596	0.035
0.010	0.010	0.001	12.673	0.021
<b>Day 2 (n = 3)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	1.004	0.007	0.662	0.423
0.200	0.195	0.003	1.608	0.486
0.010	0.010	0.000	1.853	0.013
<b>Day 3 (n = 3)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	1.001	0.019	1.865	0.098
0.200	0.204	0.007	3.616	2.011
0.010	0.011	0.001	9.640	19.420
<b>Day 4 (n = 3)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	0.996	0.016	1.599	0.418
0.200	0.203	0.008	4.087	1.288
0.010	0.010	0.001	9.934	3.420
<b>Day 5 (n = 3)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	1.008	0.029	2.828	0.752
0.200	0.211	0.008	3.590	5.452
0.010	0.012	0.001	9.468	17.580
<b>Interday</b>				
<b>(n = 5 days)</b>	<b>Mean</b>	<b>S.D.</b>		
1.000	1.002	0.004	0.435	0.186
0.200	2.203	0.006	2.858	1.200
0.010	0.011	0.001	8.163	5.600

**Recovery:** The absolute recovery of MEL QC samples of 10, 200 and 1000 ng/mL (n = 5), using SPE, were 89.50 ( $\pm$  6.79, S.D.), 93 ( $\pm$  1.75, S.D.) and 90.50 ( $\pm$  1.44, S.D.) %, respectively, and the absolute recovery of the IS was 96 % (n = 15).



## 2.5. Discussion

For quantitative determination of meloxicam, HPLC-UV methods have been commonly applied in human plasma. Velpandian et al. (2000) reported a fast HPLC-UV method to determine plasma concentrations of meloxicam in human plasma where its retention time was 2.7 min. However, although the method was straight-forward and rapid, the sensitivity of the method to quantify meloxicam (LLOQ 0.1  $\mu$ g/mL) was not sufficient to apply in koala PK study. Other studies have utilised HPLC-UV and HPLC-PDA methods to

quantify human plasma and serum concentration of meloxicam as low as 0.01 µg/mL either by LLE (Bae et al., 2007) and SPE (Ouarezki and Guermouche, 2010). But these methods used large volumes of plasma (0.5 to 1 mL) where obtaining multiple samples of this magnitude over an 8 hour period is not feasible from koalas as their average body weight is less than 10 kg. The HPLC-PDA method described here is optimised not only to provide sufficient sensitivity, but also to utilise less than half of the plasma volume required of previously reported methods.

The removal of interfering endogenous matrix in the koala plasma samples is an important step for HPLC analysis especially when the sample volume is limited. Liquid-liquid extraction (Ji et al., 2005, Bae et al., 2007) or PPE (Dasandi et al., 2002, Wiesner et al., 2003, Yuan et al., 2007) have been used as sample cleaning up procedures for meloxicam in biological fluids. Liquid-liquid extraction is labour intensive, time-consuming and requires large amounts of organic solvent(s) that are environmentally toxic and hazardous and often result in poor drug recovery. Although the procedure of PPE is simple, it is non-selective which often leads to low recovery rates for some drugs (Li et al., 2004). Alternatively, as general performance of SPE, including the recovery of many drugs, is known to be superior than either LLE or PPE (Li et al., 2004), this study utilised SPE. Meloxicam was initially reported to possess only one pKa (4.08) corresponding to the ionisation of enolic (OH) moiety of the molecule (Tsai et al., 1993). However, it was subsequently demonstrated that meloxicam exhibits a second pKa (1.08) corresponding to the protonation of thiazole ring nitrogen (Luger et al., 1996). Consequently, meloxicam exists as ionic species (anion-, cation or zwitterionic) in broad

pH ranges. Likewise, SPE of meloxicam from the plasma matrix based on conventional non-polar (C18) sorbents will be inadequate for the adsorption of the molecules. In this regard, Oasis HLB was employed to provide a wide spectrum of retention capacity for both polar and non-polar species with high pH stability. Our average absolute recovery value of QC samples (0.01, 0.2 and 1 µg/mL) was 91% ( $\pm$  1.80, S.D.) which was superior to both LLE (77.2 to 86.7 %) (Bae et al., 2007) and protein precipitation techniques (>85 %) (Dasandi et al., 2002). Our results are comparable to the method of Ouarezki and Guermouche, (2010), whereby SPE (Oasis HLB, 60 mg cartridges) were used for extraction of meloxicam from acidified human serum followed by HPLC-PDA analysis, which resulted in an average absolute recovery of 95.33% ( $\pm$  2.14, SD) from 0.25, 0.5 and 1 µg/mL (Ouarezki and Guermouche, 2010). Interestingly, in our study, acidification of koala plasma prior to SPE consistently interfered with the elution of meloxicam or IS peaks (no attempts were made to investigate the interfering components). Therefore, the plasma acidification step was removed during the clean-up process, which resulted in improved selectivity and greater reliability of peak resolution as illustrated in **Figure 2.6**.

In this study three unknown peaks, presumably metabolites of meloxicam were observed at retention times of 3.82, 4.72 and 5.89 min. The areas of these peaks were dependant on the peak area of meloxicam and observed in IV dosed samples. The wavelengths ( $\lambda$ ) max obtained from the UV spectrum of these unknown peaks were approximately at  $350 \pm 20$  nm, indicating possible metabolites formed from the parent drug during the kinetic phase (Naidoo et al., 2008). However further structural

elucidation of these unknowns (NMR or LC-MS-MS) is required to confirm the presence of these metabolites. The endogenous matrix in koala plasma was found to be complicated, causing greater interference when analyzing MEL as illustrated in Figure 2.4. This compositional difference may be due to the koala's unique physiology as they represent one of a few selective folivore marsupials which rely on certain species of *Eucalyptus* leaf as their sole dietary source. (Stupans et al., 2001).

This investigation highlights that HPLC conditions may require modifications when used to detect drug concentrations from different species, and methods require adaptation to improve sensitivity when small sample volumes are available and a simple, sensitive and specific HPLC-PDA method using SPE for the assay of plasma meloxicam was developed. To our knowledge, this is the first validated method for the quantification of meloxicam in koala plasma or any other marsupials. The developed method has been successfully applied to a PK study (**Chapter 3**) which demonstrates the unique drug disposition capacity of the koala.

# Chapter 3

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## **Pharmacokinetics of meloxicam in the koala (*Phascolarctos cinereus*) after intravenous, subcutaneous and oral administration**

The following chapter is modified from the original article:

**Kimble, B., Black, L.A., Li, K.M., Valtchev, P., Gilchrist, S., Gillett, A., Higgins, D.P., Krockenberger, M.B., Govendir, M., (2013),** Pharmacokinetics of meloxicam in koalas (*Phascolarctos cinereus*) after intravenous, subcutaneous and oral administration. *Journal of Veterinary Pharmacology and Therapeutics*, **36(5)**, 486-493

### 3.1. Abstract

The PK profile of meloxicam in clinically healthy koalas ( $n = 15$ ) was investigated. Single doses of meloxicam were administered intravenously (0.4 mg/kg;  $n = 5$ ), subcutaneously (0.2 mg/kg;  $n = 1$ ) and orally (0.2 mg/kg;  $n = 3$ ), and multiple doses were administered to two groups of koalas *via* the oral and SC routes ( $n = 3$  for both routes) with a loading dose of 0.2 mg/kg for day 1 followed by 0.1 mg/kg, s.i.d., for a further 3 days. Plasma meloxicam concentrations were quantified by HPLC-PDA. Following IV administration, meloxicam exhibited a rapid plasma  $Cl$  of  $0.44 \pm 0.20$  L/h/kg, a  $V_z$  of  $0.72 \pm 0.22$  L/kg and a  $V_{ss}$  of  $0.22 \pm 0.12$  L/kg (average  $\pm$  SD). Median plasma terminal elimination  $t_{1/2}$  was 1.19 h (range 0.71 to 1.62 h). Following oral administration either from single or repeated doses, only  $C_{max}$  ( $0.013 \pm 0.001$  and  $0.014 \pm 0.001$   $\mu\text{g/mL}$ , respectively) was measurable (LLOQ  $>0.01$   $\mu\text{g/mL}$ ) between 4 to 8 h. Oral bioavailability was negligible in koalas. Plasma protein binding of meloxicam was about 98%. Three metabolites of meloxicam (M1, M2 and M3) were detected in the koala plasma with one (M1) identified as the 5-hydroxy methyl metabolite. This study demonstrated that koalas exhibited rapid plasma  $Cl$  and extremely poor oral bioavailability of meloxicam compared with other eutherian species. Accordingly, the currently recommended dosage regimen of meloxicam for this species appears inadequate.



### 3.2. Introduction

Meloxicam is one of the widely prescribed NSAID where it is approved for administration to humans for the treatment of arthritic conditions (Gates et al., 2005) and for domestic animals, such as dogs, cats and horses, for the relief of inflammation and pain (EMA, 2015). Pharmacokinetics of meloxicam have been investigated in rodents [e.g. mice and rats (Busch et al., 1998)], companion animals [e.g. cats (Giraudel et al., 2005), dogs (Busch et al., 1998); donkeys and horses (Sinclair et al., 2006)], livestock [e.g. poultry (Baert & De Backer, 2003), piglets (Fosse et al., 2008), sheep (Shukla et al., 2007)], rabbits (Carpenter et al., 2009) and humans (Turck et al., 1996) demonstrating differences in *Cl* and elimination  $t_{1/2}$ . Meloxicam is also administered to other species, such as exotic animals and wildlife, for which the recommended dosing regimen is not based on species specific pharmacokinetic profiles but on anecdotal observations or extrapolation from other species (Kirchgessner, 2006).

The wild koala is an iconic Australian marsupial that frequently requires veterinary attention after being traumatised by feral or domestic carnivores or vehicle strikes, especially during the breeding season when they are most ambulatory (Griffith, 2010). Meloxicam is frequently used as an analgesic and anti-inflammatory drug for injured koalas and is also used to control arthritis associated with shoulder and hip dysplasia in captive koalas (Pye et al., 2008; Pye, 2009). The current recommended dosing regimen of meloxicam in koalas (Blanshard & Bodley, 2008) is extrapolated from that recommended for dogs (0.2 mg/kg loading dose orally or s.c. in day 1, followed by 0.1 mg/kg orally daily). Previous work has demonstrated that the antibacterial drug

enrofloxacin, when administered at conventional recommended dose rate for dogs, failed to reach adequate plasma concentrations in koalas (Griffith et al., 2010). The aim of this study was to investigate the pharmacokinetics of meloxicam after a single dose administered by the IV, SC and oral routes, to determine SC and oral bioavailability after sequential daily dosing, and to characterise the predominant plasma metabolites of meloxicam in this species.

### **3.3. Materials and method**

#### **3.3.1. Animals**

Eighteen clinically normal koalas (7 males and 11 females), ranging in age from 1.5 to 11 years ( $3.8 \pm 3.1$  yr, mean  $\pm$  SD) as determined by tooth wear (Martin, 1981) or previous admission data, and ranging in weight from 3.4 to 14 kg ( $6.7 \pm 3.2$  kg, mean  $\pm$  SD); were recruited opportunistically from the Australia Zoo Wildlife Hospital (Beerwah, QLD, Australia) and Sydney Wildlife World (Sydney, NSW, Australia). During the study, koalas were housed in pens, either singly or in groups, and supplied with food (various *Eucalyptus* spp.) and water *ad libitum*. This study was approved by The University of Sydney Animal Ethics Committee and the NSW Office of Environment and Heritage.

#### **3.3.2. Drug administration and blood collection**

An intravenous catheter for drug administration and serial blood collection was placed under general anesthesia *via* mask induction using isoflurane in 100 % oxygen. A 20-gauge, 1-inch catheter was placed into the cephalic vein and bandaged in place for the duration of blood collection. Each koala had a 1 to 2 hr of recovery after GA and prior to

meloxicam administration. Blood was collected prior to drug administration ( $t = 0$  hr) to determine baseline hematology and biochemistry values. **Single dose study** - A single dose of meloxicam (Metacam®, Boehringer Ingelheim, North Ryde, NSW, Australia) was administered to koalas either IV at 0.4 mg/kg ( $n = 6$ ); SC at 0.2 mg/kg ( $n = 3$ ) or *via* oral suspension (PO) at 0.2 mg/kg ( $n = 3$ ). After meloxicam administration, blood (1 to 1.5 mL) was collected at the following time points: IV administration:  $t = 2, 5, 10, 15, 30$  min, then 1, 1.5, 2, 3, 4, 6, 8, 12 and 24 hr; SC administration:  $t = 15, 30$  min, then 1, 2, 3, 4, 6 and 8 hr; PO administration:  $t = 1, 2, 4, 6, 8, 12$  and 24 hr. **Repeat dose study** - Two groups of koalas ( $n = 3$  for each group), after baseline blood collection ( $t = 0$  hr), were administered meloxicam either SC or PO, at loading dose of 0.2 mg/kg which was followed by a dose of 0.1 mg/kg once daily for a further 3 days for both routes. After the last dose, blood was collected at the following time points:  $t = 1, 2, 4, 8, 12$  and 24 hr for both SC and PO administration. All blood samples were centrifuged within 1 hr of collection and the plasma was removed and placed into plain tubes. Samples were stored at  $-20$  °C and protected from light until analysis.

### **3.3.3. Drug analysis**

Meloxicam concentrations in the plasma samples were quantified by HPLC-PDA as described in **Chapter 2, Section 2.3.2**.

### **3.3.4. Identification of metabolites**

The meloxicam metabolites in plasma were investigated using LC-MS consisting of a Shimadzu LC-MS 2010EV module (Shimadzu, Kyoto, Japan). The mobile phase consisted

of water and acetonitrile (65:35, v/v) with 0.1 % of formic acid, and the column used was a Phenomenex Gemini C<sub>18</sub> 5 µm (150 mm x 2 mm). The flow rate was 0.3 mL/min. Electrospray ionisation (ESI) ion source was operated in positive ion mode and the interface voltage was 2 KV. The interface temperature was maintained at 200 °C. The flow rate of nitrogen as a nebulising gas was 1.2 mL/min. Mass spectra were acquired over the mass range of 50 to 700 m/z in scan mode, with scan speed of 1000 amu/sec. Structure elucidation of unknown metabolites by in source collision-induced dissociation (CID), using meloxicam standard as a reference compound, were performed at optimized Q-array DC voltage of 62 V.

### **3.3.5. Plasma Protein binding**

The plasma protein binding of meloxicam in koala plasma at three concentrations (0.5, 2 and 6 µg/mL) was determined, in triplicate, by the modified ultra-filtration method (Ulrich Busch et al., 1998). Briefly, 200 µL of meloxicam (2.5, 10 and 30 µg/mL) in 50 % methanol were transferred to 2 mL Eppendorf tubes and evaporated to dryness using a speed vacuum (SPD 121P, Thermo Scientific, Australia) at 35 °C for 20 min. Drug free koala plasma (1 mL), adjusted to 7.4 pH was then added to each Eppendorf tube, vortexed and incubated in a water bath at 37 °C for 30 min. From each Eppendorf tube, 250 µL of plasma was removed for determination of the total drug concentration (Drug<sub>total</sub>) and the remaining plasma was transferred to the reservoir of the ultrafiltrate device (Amicon Corp., Beverly, MA, USA) which had a membrane of a molecular weight cut-off of 30.000 daltons. The ultrafiltrate device was centrifuged with a fixed 45 degree angle rotor and spun at 2000 g for 1 hr at 25 °C. After centrifugation, the filtrate portion was

used for determining the free drug concentration ( $\text{Drug}_{\text{free}}$ ). Both the ( $\text{Drug}_{\text{total}}$ ) and ( $\text{Drug}_{\text{free}}$ ) fractions were cleaned and analysed by SPE and HPLC-DAD, respectively, as described previously. Subsequently, the plasma protein binding of meloxicam in koala plasma was determined by the following equation.

$$\text{Plasma protein binding (\%)} = 100 - [(\text{Drug}_{\text{free}} / \text{Drug}_{\text{total}}) \times 100]$$

Non-specific binding of meloxicam in the ultrafiltrate device membrane, using phosphate buffer (pH 7.4) as a control, was less than 5 %.

### 3.3.6. Pharmacokinetic analysis

Pharmacokinetic parameters for IV, SC and PO administrations were determined as a non-compartmental analysis using PKSolver (Zhang et al., 2010). Peak plasma concentrations and  $T_{\text{max}}$  for SC and PO were determined by visual inspection of the plasma concentration vs. time curve. The  $k_{\text{el}}$  was estimated by semi-log linear regression of the terminal slope, and elimination  $t_{1/2}$  was estimated by  $\ln 2 / k_{\text{el}}$ . AUC and area under the first moment curves (AUMC) from 0 to last observed concentration ( $\text{AUC}_{0-t}$  and  $\text{AUMC}_{0-t}$ , respectively) were determined by the linear trapezoidal method. The AUC and AUMC from the observed concentration to infinity were determined by

$$\text{AUC}_{t-\infty} = C_{\text{last}} / k_{\text{el}}$$

$$\text{AUMC}_{t-\infty} = (C_{\text{last}} \times t_{\text{last}} / k_{\text{el}}) + C_{\text{last}} / k_{\text{el}}^2$$

The following pharmacokinetic parameters, total plasma  $Cl$ ,  $V_{ss}$ ,  $V_z$ ,  $MRT$  and  $F_{\text{specific route}}$  were determined by the following equations:

$$Cl = \text{Dose}_{IV} / AUC_{IV}$$

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

$$V_z = Cl / k_{el}$$

$$MRT = AUMC / AUC$$

$$F \% = (AUC_{\text{specific route}} / AUC_{IV}) \times (\text{Dose}_{IV} / \text{Dose}_{\text{specific route}}) \times 100$$

The fraction of elimination associated with the terminal phase was estimated according to the following equation (Rowland and Tozer, 1995, McLean et al., 2007):

$$(\beta / k_{el}) / AUC_{0-\infty}$$

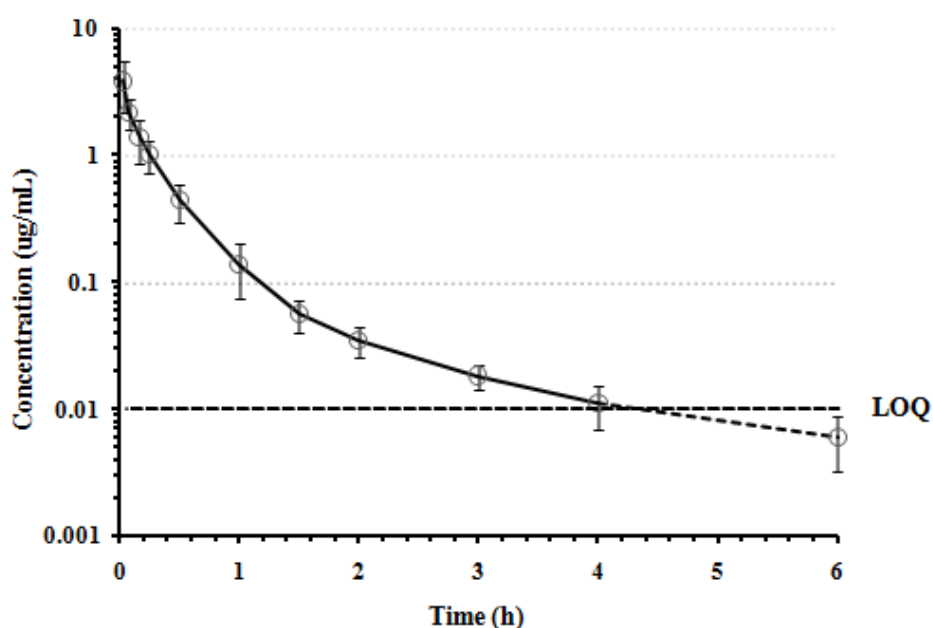
( $\beta$  = zero time intercept of the exponential terminal elimination line)

All pharmacokinetic parameters are expressed as mean  $\pm$  standard deviation, except for the  $t_{1/2}$  (harmonic mean  $\pm$  pseudo-standard deviation).

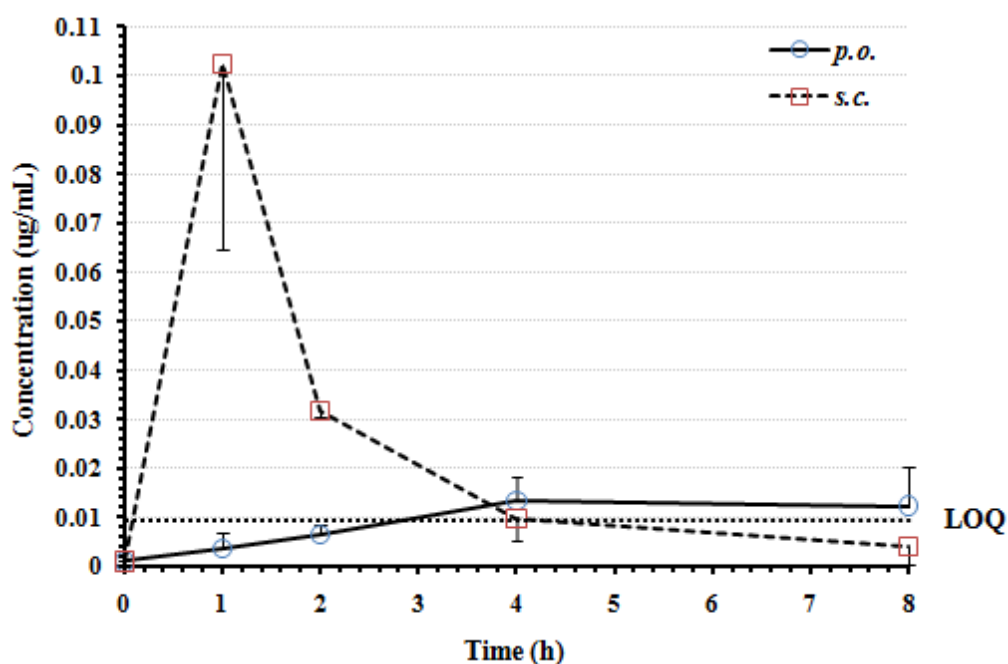
### 3.4. Results

A summary of the pharmacokinetic parameters of meloxicam, as determined by a non compartmental analysis, is presented in **Table 3.1**. In this study, one IV and two single SC dosed koalas were excluded from the analysis due to technical difficulties with blood collection. For the orally dosed koalas, it was not possible to undertake non compartmental analysis as plasma concentrations of most blood samples, apart from

$C_{max}$ , were below the LLOQ (0.01  $\mu\text{g/mL}$ ) and accordingly, only the  $C_{max}$  and  $T_{max}$  are reported. The  $T_{max}$  for single dosed (0.2 mg/kg, SC) koala was 30 min, whereas for koalas with repeated dose (0.1 mg/kg, SC), the  $T_{max}$  was 1 h; due to the study time-lines, it was not possible to collect the blood samples at  $t = 30$  min for the latter koalas. The plasma meloxicam concentration vs. time, presented as a semi-logarithmic curve, after a single IV administration (0.4 mg/kg) is presented in **Figure 3.1**. The plasma meloxicam concentration vs. time curves after repeated oral or SC administration (0.2 mg/kg day 1, followed by 0.1 mg/kg for 3 days) are presented in **Figure 3.2**.



**Figure 3.1)** Semi-logarithmic curve of mean  $\pm$  SD meloxicam plasma concentrations vs. time after a single IV administration (0.4 mg/kg) to clinically healthy koalas ( $n = 5$ )



**Figure 3.2)** Mean  $\pm$  SD meloxicam plasma concentrations vs. time, after repeated PO or SC administrations (both routes initially administered 0.2 mg/kg on the first day followed by 0.1 mg/kg qd for three days,  $n = 3$  both groups).

The overall plasma protein bindings of meloxicam, at concentrations of 0.5, 2 and 6  $\mu\text{g/mL}$ , in koalas were  $98.17 \pm 0.11 \%$  (average  $\pm$  SD) (**Table 3.2**).

**Table 3.2)** Plasma protein bindings of meloxicam (0.5, 2 and 6  $\mu\text{g/mL}$ ) in koalas

$\mu\text{g/mL}$	Average ( $n = 3$ )	SD ( $n = 3$ )
6	98.2860	0.133
2	98.0720	0.138
0.5	98.1520	0.103
overall	98.1700	0.108



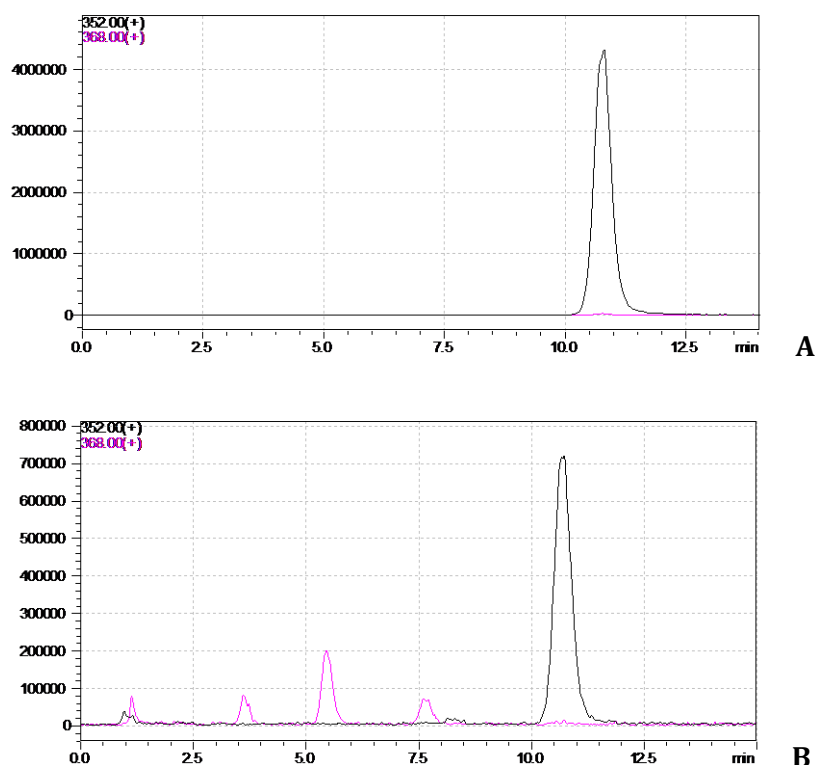
**Table 3.1)** Pharmacokinetic parameters (mean + SD) of meloxicam (determined by non compartmental model) in koalas after PO, SC and IV administrations.

	<b>Subcutaneous Repeated dose 0.1 mg/kg (n = 3)</b>	<b>Subcutaneous Single dose 0.2 mg/kg (n = 1).</b>	<b>Oral Repeated dose 0.1 mg/kg (n = 3)</b>	<b>Oral Single dose 0.2 mg/kg (n = 3)</b>	<b>Intravenous 0.4 mg/kg (n = 5)</b>
<b>C<sub>max</sub> (µg/mL)</b>	0.1 ± 0.04	0.19	0.014 ± 0.006	0.013 ± 0.001	-
<b>T<sub>max</sub> (min)</b>	60	30	320 ± 14	400 ± 14	-
<b>k<sub>el</sub> (min<sup>-1</sup>)</b>	0.01 ± 0.005	0.01	-	-	0.01 ± 0.002
<b>t<sub>1/2</sub> (min)*</b>	65.26 ± 28.36	61	-	-	70.04 ± 17.45
<b>AUC<sub>0-∞</sub> (µg min/L)</b>	10.74 ± 2.26	17.17	-	-	61.6 ± 19.69
<b>AUMC<sub>0-∞</sub> (µg min<sup>2</sup>/mL)</b>	1280.16 ± 360.22	1429.78	-	-	1856.9 ± 627.41
<b>MRT (min)</b>	114.04 ± 34.99	83.25	-	-	29.95
<b>Cl (L/h/kg)</b>	-	-	-	-	0.44 ± 0.2
<b>Vz (L/kg)</b>	-	-	-	-	0.72 ± 0.19
<b>Vss (L/kg)</b>	-	-	-	-	0.22 ± 0.12
<b>Bioavailability (F)</b>	69.74 %	55.74 %	Nil	Nil	-

\* Harmonic mean

The percentages of extrapolated AUC<sub>0-inf</sub>, except for one koala repeated dosed 0.1 mg/kg SC (25 %), were all less than 20 % (3.77 ± 3.66)

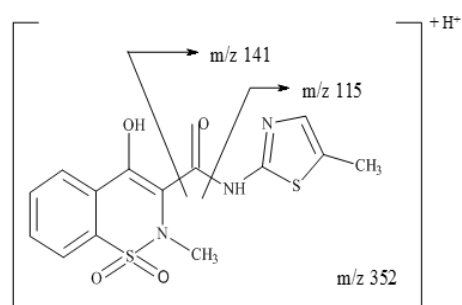
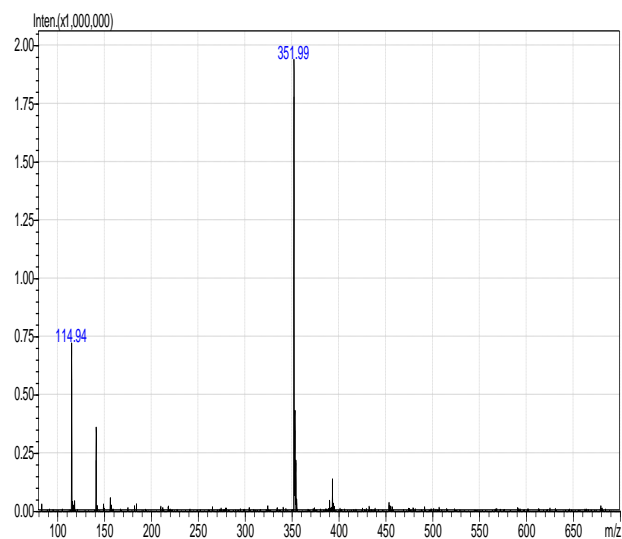
Three metabolites were detected in the plasma of IV and SC samples at 3.5, 5.4 and 7.6 min (Rt) based on LC-MS. A representative ESI<sup>+</sup> extracted ion chromatogram of plasma metabolites is shown in **Figure 3.3**. The molecular characteristic of metabolites was [M + H]<sup>+</sup> m/z 368 which corresponded to the addition of an oxygen atom to the parent drug meloxicam.



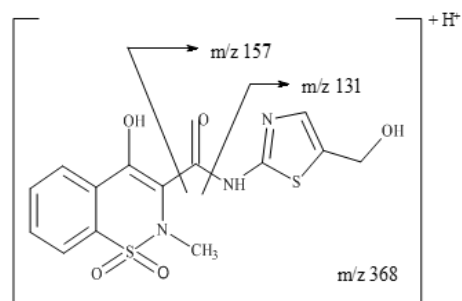
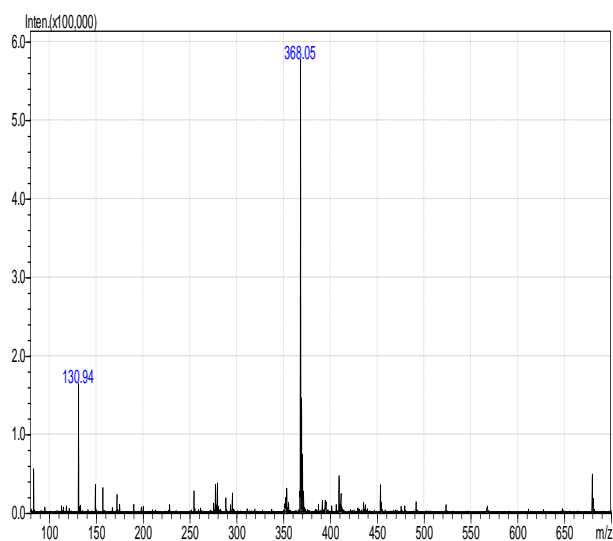
**Figure 3.3)** ESI<sup>+</sup> extracted ion chromatogram of (A): meloxicam standard; and (B) plasma metabolites of meloxicam in koalas after a single IV administration (0.4 mg/kg) (pooled sample from  $t = 15$  and 30 mins).

Fragmentation of the parent drugs in CID revealed that the characteristics of fragmented ions of meloxicam and M1 were m/z 115 and 141, and m/z 131 and 157, respectively (**Figure. 3.4**). The mass spectra of M1 indicated the oxidation was on the methylthiazole

moiety, and likely to be a 5-hydroxyl methyl derivative. There were no signs of adverse side effects in any koalas during or following administration of meloxicam by any route.



**A**



**B**

**Figure 3.4)** LC-MS in-source CID spectra of (A): meloxicam; (B): 5-hydroxymethyl metabolite (M1).

### 3.5. Discussion

This is the first PK study on meloxicam, or any NSAID, in Australian *E. feeders* (marsupials). One of the most interesting findings was that the estimated plasma *Cl* ( $0.44 \pm 0.2$  L/h/kg) of meloxicam following IV administration was more rapid than that reported for any eutherian species including rats (0.01-0.15 L/h/kg), dogs (0.01 L/h/kg), humans (0.01 L/h/kg) (Busch et al., 1998), donkeys (0.19 L/h/kg), and horses (0.03 L/h/kg) (Sinclair et al., 2006) and greater than that reported for birds except for ostriches (0.72 L/h/kg) (Baert and De Backer, 2003). In retrospect, this rapid plasma *Cl* may potentially be explained by what is known about koala hepatic metabolism. The koala is a herbivorous marsupial, also known as specialist *E. feeder*, with a normal diet consisting almost exclusively on some *Eucalyptus* spp. foliages which contain potential toxic PSM, such as phenolics and terpenes, at a concentration that would be fatal to many unadapted species (Stupans et al., 2001). Several studies have suggested that one of the strategies by which koalas detoxify their diet is *via* the elevated catalytic activity of some CYP enzymes (McLean and Foley, 1997, Boyle et al., 2000, Boyle et al., 2001, Stupans et al., 2001). The rate of hepatic metabolism in koalas, as estimated from bromosulphthalein clearance rates, is more efficient than other grass-eating herbivores (Pass and Brown, 1990) and catalytic activity of CYP2C-like enzymes, which was evaluated from tolbutamide hydroxylase activity, is ten and twenty times higher than that in rodents and humans, respectively (Liapis et al., 2000, Jones et al., 2008). As both tolbutamide and meloxicam are reportedly substrates for the CYP2C9 in humans (Chesne et al., 1998), it could be possible that the rapid plasma *Cl* of meloxicam in koalas is due to extensive hepatic metabolism.

The  $V_{ss}$  was low in koalas ( $0.23 \pm 0.12$  L/kg) and substantially different from that in dogs ( $0.32$  L/kg) (Busch et al., 1998), horses ( $0.19$ – $0.27$  L/kg) (Lees et al., 1991, Sinclair et al., 2006) or humans ( $0.17$  L/kg) (Turck et al., 1996). Generally, low  $V_d$  of meloxicam is demonstrated in many species, likely attributable to the high binding affinity to plasma proteins (e.g.  $> 96\%$  in dogs and humans) (Turck et al., 1996, Busch et al., 1998), which is typical of NSAIDs (Lees et al., 2004b). Similarly, observed plasma protein binding in koalas was high ( $98.17 \pm 0.11\%$ ) which is in accordance of low  $V_{ss}$  demonstrated in other species. The  $V_z$  ( $0.72 \pm 0.33$  L/kg) was approximately three times greater than the  $V_{ss}$ , which indicates that appreciable amount of the drug was eliminated during the distribution phase (or before pseudo-equilibrium) (Toutain and Bousquet-Melou, 2004c). To support this, fraction of the eliminated drug associated with the terminal elimination phase ( $f_z$ ) was calculated in IV dosed koalas ( $n = 5$ ). Accordingly,  $20.17 \pm 7.24\%$  of the drug was estimated to associate with terminal elimination phase, whereas approximately  $80\%$  of the drug was eliminated during distribution phase. A possible explanation for this extensive distributional elimination in koalas could be due to rapid  $Cl$  with relatively high concentration of the drug presented in organs of elimination (McLean et al., 2007).

In this study, the estimated terminal elimination  $t_{1/2}$  in koalas was  $1.17 \pm 0.29$  h. The terminal elimination  $t_{1/2}$ , reported here was shorter than for dogs ( $24$  h) (Busch et al., 1998b), horses ( $8.5$  h) (Lees et al., 2004b), ponies ( $2.7$  h) (Lees et al., 1991) and humans ( $\sim 13$  h) (Turck et al., 1996), and comparable with donkeys ( $\sim 1$  h) (Mahmood & Ashraf, 2011). In some birds such as ducks, turkeys and ostriches, the terminal elimination  $t_{1/2}$

is shorter (0.5–0.99 h) (Baert and De Backer, 2003) than that in koalas, and compared with koalas, it is likely attributable to the small  $V_z$  (0.07–0.08 L/kg) except for ostriches; however, the greater  $Cl$  may explain the shorter terminal  $t_{1/2}$  in koalas (similar to ostriches). Accordingly, the observed terminal elimination  $t_{1/2}$  suggests that accumulation of meloxicam is unlikely 12 h after administration to koalas.

The oral absorption of meloxicam, whether administered by single or repeated daily dose, was extremely low ( $C_{max}$   $0.013 \pm 0.001$  and  $0.014 \pm 0.001$   $\mu\text{g/mL}$ , respectively) especially when compared to  $C_{max}$  of the same dose rate (single oral administration 0.2 mg/kg) in rabbits (0.168  $\mu\text{g/mL}$ ) (Carpenter et al., 2009), dogs (0.46  $\mu\text{g/mL}$ ) (Busch et al., 1998) and humans ( $\sim 0.93$   $\mu\text{g/mL}$ ) (Turck et al., 1996); however, the  $T_{max}$  ( $6 \pm 2.3$  h) was comparable between species ( $\sim 4$  to 10 h). Meloxicam has been shown to provide an excellent oral bioavailability in dogs ( $\sim 100\%$ ) (Busch et al., 1998b), horses (85.3–95.9%) (EMA, 2015) and humans ( $\sim 89\%$ ) (Turck et al., 1996), and hence is the preferred route of administration for these species. However, the oral bioavailability in koalas was extremely low which may be due to one or more gastrointestinal and metabolic factors (Kararli, 1995). The koala is a hind-gut fermenter, like other herbivores such as horses and rabbits; however, what separates koalas from other herbivores is their specialist diet. The koala's stomach is usually full of masticated *Eucalyptus* spp. foliage, including macroparticles with a high content of lignified fibers ( $\sim 50\%$  of the foliage) (Blanshard & Bodley, 2008, Griffith, 2010) which could potentially bind to xenobiotics. This, in conjunction with a short small intestine transit time (0.1 h for particulate phase and 1 h for solute phase selectively) (Cork and Warner, 1983), is

likely to minimize xenobiotic absorption. Other potential contributing factors may be attributable to limited absorption through caecum and large colon or extensive presystemic metabolism.

After the repeated SC doses (0.1 mg/kg, qd),  $C_{\max}$  was  $0.10 \pm 0.04 \mu\text{g/mL}$  at  $\sim 1$  h of post administration. Compared with repeated p.o. administration, the  $C_{\max}$  was approximately 10 times higher with improved bioavailability which indicated this to be a superior route for koalas. With repeated doses up to 4 days, no significant drug concentration ( $>0.01 \mu\text{g/mL}$ ) was detected 4 h after s.c. administration, and as a possible result of rapid  $Cl$  ( $t_{1/2} 1.09 \pm 0.47$  h), plasma concentrations  $>0.1 \mu\text{g/mL}$  were only achieved at 1 h and declined to  $0.03 \pm 0.001 \mu\text{g/mL}$  2 h post administration. This study did not investigate the effective meloxicam plasma concentration for clinical response nor has this been established in koalas. Therefore, we were not able to suggest either suitable dose rates or dosing frequencies, for either analgesic or anti-inflammatory indications. It has been reported that the association between dose rate and efficacy is variable between species (Lees et al., 2004b). For example, effective plasma concentrations to ameliorate inflammation suggested for dogs, horses, and humans are 0.82, 0.13–0.2, and 0.57–0.93  $\mu\text{g/mL}$ , respectively (Turck et al., 1996, Montoya et al., 2004, Toutain and Cester, 2004). Compared with plasma concentrations determined for those species, the currently recommended SC dose rate (0.1 mg/kg qd) is not adequate to reach such concentrations in koalas.

Three metabolites were detected in the plasma after IV and SC administrations, of which, one of these was identified as a 5-hydroxy methyl derivative (M1). No metabolites were detected in the plasma of orally dosed koalas, which was probably due to incomplete absorption of the drug. In most species, the 5-hydroxy methyl derivative is the major metabolite and in humans, it is predominately catalysed by CYP2C9 and further metabolized to 5-carboxy methyl derivative *via* a non CYP-dependent pathway (Chesne et al., 1998). The presence of a 5-hydroxy methyl derivative in the plasma likely indicates the involvement of CYP isoforms in the biotransformation of meloxicam. We were unable to structurally assign the other two metabolites detected in the koala plasma, although they appeared as additional hydroxyl derivatives.

Extrapolating the dose regimen between species, especially to wild species, is commonly carried out due to the lack of PK studies and the difficulties in accessing sufficient numbers of a species not accustomed to domestication. There were a number of challenges at the time of the study as koalas are wild animals and listed as a 'vulnerable' species in Australia. These challenges included recruiting sufficient numbers of animals and undertaking an adequate sampling schedule for repeat dose groups (time points were reduced to minimize the stress of handling), therefore performing a pivotal study ideally utilizing a cross-over study, was difficult. As well potential for carryover effect was one of the limitations in this study, and was not controlled for in this study. Despite these limitations, this preliminary study demonstrated that koalas exhibit rapid *Cl* and poor oral bioavailability of meloxicam compared with dogs; accordingly, extrapolating current dog dose regimens of meloxicam to koalas appears inadequate for all routes of



administration. To achieve concentrations associated with adequate pharmacological response in this species, the SC route requires more frequent administration than once a day with a higher dose rate; however, this requires further clinical studies. We hypothesize rapid *Cl* is attributable to a superior rate of hepatic intrinsic metabolism; alternatively, further studies could consider the co-administration of a selective CYP enzyme modulator to improve sustained meloxicam concentrations; however, caution must be undertaken with this approach to avoid jeopardizing the metabolism of the naturally occurring toxic dietary constituents. This study also highlights the importance of performing *in-vivo* studies to understand the PK of drugs specific to the species of interest, to design efficacious dose regimens.

# Chapter 4

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***In-vitro* hepatic microsomal metabolism of meloxicam in koalas (*Phascolarctos cinereus*), brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*), rats (*Rattus norvegicus*) and dogs (*Canis lupus familiaris*)**

The following chapter is modified from the original article:

Kimble, B., Li, K.M., Valtchev, P., Higgins, D. P., Krockenberger, M. B., Govendir, M. (2014). *In-vitro* hepatic microsomal metabolism of meloxicam in koalas (*Phascolarctos cinereus*), brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*), rats (*Rattus norvegicus*) and dogs (*Canis lupus familiaris*). ***Comparative Biochemistry and Physiology, Part C: Toxicology & Pharmacology*, 161, 7-4**

## 4.1. Abstract

Quantitative and qualitative aspects of *in-vitro* metabolism of the NSAID meloxicam, mediated *via* hepatic microsomes of Australian marsupials (*E. specialist* feeders: koalas and ringtail possums; *E. generalist* feeder: brushtail possums), rats, and dogs, are described. Using a substrate depletion method, hepatic *in-vitro*  $Cl_{int}$  was determined. Significantly, rates of oxidative transformation of meloxicam, likely mediated *via* CYP enzymes, were higher in marsupials compared to rats or dogs. The rank order of apparent *in-vitro*  $Cl_{int}$  was brushtail possums ( $n = 3$ ) (mean: 394  $\mu\text{L}/\text{min}/\text{mg}$  protein) > koalas ( $n = 6$ ) (50  $\mu\text{L}/\text{min}/\text{mg}$  protein) > ringtail possums ( $n = 2$ ) (36  $\mu\text{L}/\text{min}/\text{mg}$  protein) (with no significant difference between koalas and ringtail possums) > pooled rats (3.2  $\mu\text{L}/\text{min}/\text{mg}$  protein) > pooled dogs (in which the rate of depletion, as calculated by the ratio of the substrate remaining was <20% and too slow to determine). During the depletion of meloxicam, at a first-order rate constant, 5-hydroxymethyl metabolite (M1) was identified in the brushtail possums and the rat as the major metabolite of meloxicam. However, multiple hydroxyl metabolites were observed in the koala (M1, M2, and M3) and the ringtail possum (M1 and M3) indicating that these *E. specialist* feeders have diverse oxidation capacity to metabolize meloxicam. Using a well-stirred model, the apparent *in-vitro*  $Cl_{int}$  of meloxicam for koalas and the rat was further scaled to compare with published *in vivo*  $Cl$ . The closest *in-vivo*  $Cl$  prediction from *in-vitro* data of koalas was demonstrated with scaled hepatic  $Cl_{(total)}$  (average fold error = 1.9) excluding unbound fractions in the blood and microsome values; whereas for rats, the *in-vitro* scaled hepatic  $Cl f_{u(blood, mic)}$ , corrected with unbound fractions in the blood and microsome values, provided the best prediction (fold error = 1.86). This study indicates

that eutherians such as rats or dogs serve as inadequate models for dosage extrapolation of this drug to marsupials due to differences in hepatic turnover rate. Furthermore, as *in-vivo* *Cl* is one of the pharmacokinetic indexes for determining therapeutic drug dosages, this study demonstrates the utility of *in-vitro* to *in-vivo* scaling as an alternative prediction method of drug *Cl* in koalas.

## 4.2. Introduction

Knowledge of PK properties of a drug, particularly  $Cl$ , is essential to estimate the dosage to sustain desired plasma concentration of the drug; however it is lacking for many wild and exotic species for which the dosage is usually extrapolated from that used for rats, dogs or humans (Blanshard and Bodley, 2008). In the previous chapter, it was demonstrated that koalas had low plasma concentration of meloxicam following oral and subcutaneous route administration due to an extremely rapid plasma  $Cl$  (0.44 L/h/kg) compared to other eutherian species such as rats (0.015 L/h/kg) (Busch et al., 1998), dogs and humans (both approximately 0.01 L/h/kg) (Busch et al., 1998). The rapid  $Cl$  in koalas was presumed to result from a superior intrinsic hepatic clearance rate ( $Cl_{int}$ ), especially *via* oxidative metabolism. To confirm it, the aim of the study described in **Chapter 4** was to investigate *in-vitro*  $Cl_{int}$  of meloxicam in koalas, utilizing hepatic microsomes, in order to confirm the *in-vivo*  $Cl$  (compared with *in vitro* to *in vivo* scaling value) and to compare koalas'  $Cl_{int}$  with that of other marsupials, such as common brushtail possums and common ringtail possums. As rats and dogs are conventional models for *in-vitro* and *in-vivo* drug metabolism studies for human pre-clinical studies (Zuber et al., 2002), meloxicam *in-vitro*  $Cl_{int}$  for these species were also investigated.

## **4.3. Materials and methods**

### **4.3.1. Chemicals and materials**

Meloxicam and piroxicam (the latter used as the internal standard, [IS] for liquid chromatography), NADP, glucose 6-phosphate dehydrogenase and glucose 6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Liquid chromatography (LC) grade solvents were obtained from Analytical Science (Sydney, NSW, Australia).

### **4.3.2. Preparation of hepatic microsomes**

Hepatic microsomes from pooled Sprague Dawley male rats and from pooled male beagle dogs were purchased from Sigma-Aldrich (St. Louis, MO, USA; product number M 9066) and BD Biosciences (Woburn, MA, USA; lot number 00269), respectively, and stored at -80 °C prior to use. Recently deceased (< 24 hrs) koalas ( $n = 6$ ) were transported chilled to our institution where livers were quickly removed and microsomes extracted. Liver harvesting for brushtail possums ( $n = 3$ ) and ringtail possums ( $n = 2$ ) occurred immediately after death and were transported to our institution in ice within 2 hrs, where microsome harvesting occurred and stored at -80 C. All livers were collected opportunistically from animals euthanised with pentobarbitone by veterinarians for reasons independent of this study (such as trauma from vehicle strikes or feral animal attacks) and with no clinical signs, or gross organ appearance, of hepatic disease. Microsomes from livers were extracted and prepared according to a method described previously (Hill, 2001), with some modifications. Briefly, hepatic tissues were homogenized at 4 °C in 3 volume of a buffer containing 0.1 M Tris·Cl of pH 7.4 with, 10 mM EDTA and 150 mM KCl. The homogenate was then separated by

differential centrifugation (12,500 × g for 15 min; 19,000 × g for 20 min; 105,000 × g for 70 min) at 4 °C. The final pellet, the microsomal fraction, was subsequently washed and re-suspended in a buffer of 0.05 M Tris·Cl of pH 7.4 with 10 mM EDTA and 20 % glycerol. Aliquots (~250 µl) of microsomal fractions were stored at -80 °C. Protein concentrations of the microsomal fractions were determined using the Bradford assay kit (Bio-Rad, Hercules, CA, USA) and standardised with bovine serum albumin.

#### **4.3.3. Microsomal experimentation**

Meloxicam (1.25 µM) was preincubated in 2 mL of 0.1 M phosphate buffer (pH 7.4) containing a NADPH regenerating system (1 mM NADP, 0.8 U glucose 6 phosphate dehydrogenase and 3 mM glucose 6 phosphate) and 3 mM MgCl<sub>2</sub>, in an open air shaking water bath at 37 °C for 5 min. The enzymatic reaction was then initiated by adding a predetermined concentration of microsomal protein (0.5 mg/mL for koalas and both species of possums; 1 mg/mL for rats and dogs). During the incubation, 200 µL aliquots were removed at time (*t*) = 0, 2.5, 5, 7.5 and 10 min for brushtail possums (*n* = 3); 0, 5, 10, 15, 20 (koalas only) and 30 min for koalas (*n* = 6) and ringtail possums (*n* = 2); 0, 5, 15, 30, 45 and 60 min for the rat and dog. Each extracted aliquot was mixed with 125 µL of ice-cold methanol (which also contained 5 µM of IS) to deactivate the reaction. The resultant mixture was vortexed and centrifuged at 14,000 × g for 10 min, and the supernatant was either stored at -80 °C or directly injected to the HPLC system for analysis. In addition, to determine stability of meloxicam, incubation of the drug without NADPH, in which the corresponding volume was substituted by buffer, was undertaken for time points up to and including 60 min for each species. All samples were prepared

and analysed in duplicate. To identify the structure of any metabolites, meloxicam concentrations of 1.25  $\mu\text{M}$  underwent additional incubation containing a NADPH regenerating system with pooled hepatic microsomes of 1 mg/mL of each species for 0, 30, and 60 min, and were analyzed by liquid chromatography-mass spectrometry (LC-MS).

#### **4.3.4. Microsomal binding and blood-plasma (B/P) ratio in the koala and rat**

Meloxicam (1.25  $\mu\text{M}$ ) was incubated with 0.5 mg/mL of pooled koala microsomes ( $n = 3$ ) or 1 mg/mL pooled rat microsomes in 1 mL of 0.1 M phosphate buffer (pH 7.4) containing 3 mM  $\text{MgCl}_2$ , in an open air shaking water bath at 37 °C for 30 min. The same mixtures without microsomal protein served as controls. After incubation, both samples and controls were transferred to the reservoir of the ultrafiltrate device (10 kDa) (Millipore, Billerica, MA) and centrifuged (1500 x g) for 15 min at 37 °C. Upon completion, the filtrate portion was analyzed via HPLC. The filtrate portion of the control was used for determination of recovery of meloxicam which was ~65 %. All experimentation was in triplicate. The unbound fraction of microsomes,  $f_u$  (*mic*), was expressed as concentration ratio between samples vs control ( $C_{\text{sample}} / C_{\text{control}}$ ).

For koalas, the meloxicam B/P ratio was determined according to method previously described (Yu et al., 2005). Briefly, final concentrations of 0.1 and 0.2  $\mu\text{g/mL}$  of meloxicam (comparable to the  $C_{\text{max}}$  obtained from **Chapter 3**) were added to 1 mL of fresh pooled whole blood of koalas ( $n = 2$ ) and incubated at 37 °C for 0, 10, 30, and 60 min. After incubation, the plasma was separated from whole blood and concentrations



of meloxicam in the separated plasma were measured ( $C_p$ ). Prior to the assay, the hematocrit (Hct) value of the koala blood was determined with an automated hematology analyzer, Sysmex XT-2000i (Kobe, Japan) by an accredited veterinary pathology lab. For a control that represented the whole blood concentration (ref  $C_p$ ) the same concentration of meloxicam (0.1 and 0.2  $\mu\text{g}/\text{mL}$ ) was added to 1 mL of blank koala plasma and incubated at 37 °C for 0, 10, 30, and 60 min. The B/P ratio was determined by ref  $C_p/C_p$ . In addition, the theoretical B/P ratio was calculated in koalas using following equation:

$$B/P = 1 + \text{Hct} \times (f_u(\text{plasma}) - 1)$$

where  $f_u(\text{plasma})$  is the unbound fraction in the plasma which is 0.0183 in koalas (**Chapter 3**) As < 10 % of meloxicam is recognized to penetrate rat red blood cells (Busch et al., 1998), the B/P ratio was calculated from above equation. Hct value and  $f_u(\text{plasma})$  used for rats were 0.46 L/L (Zou et al., 2012) and ~0.004 (Busch et al., 1998), respectively.

#### 4.3.5. Calculations

The  $Cl_{\text{int}}$  was estimated by the substrate depletion method using *in-vitro*  $t_{1/2}$  approach (Obach, 1999). Briefly, using the peak ratio of meloxicam/IS at  $t = 0$  as 100 % of substrate, the peak ratio of the other time points were converted to a percentage of the substrate remaining, plotted as natural log of remaining drug vs. incubation time and the slope of the regression line, represented as rate of constant ( $-k$ ), was used for estimation of the *in vitro*  $t_{1/2}$  by the following equation: *in vitro*  $t_{1/2} = -0.693 / k$ . Subsequently, *in-*

*in vitro*  $Cl_{int}$  was calculated by following formula:  $(0.693 / \textit{in vitro } t_{1/2}) \times (\mu\text{L incubation volume/mg protein})$ . *In-vitro*  $Cl_{int}$  was further estimated to *in vitro*  $Cl_{int}'$  based on kg per body weight (b.w.) using following equation:

$$\textit{In vitro } Cl_{int}' = \textit{in vitro } Cl_{int} \times \text{mg protein/g liver} \times \text{g liver/kg b.w.}$$

The standard value of 45 mg protein/g liver was used for all species (Laufer et al., 2009). For the rat and brushtail possums, reference values used for g liver/kg b.w. were 40 (Davies and Morris, 1993) and 30.5 (McManus and Ilett, 1977), respectively. Allometrically scaled values [Liver weight =  $0.0370 (\text{b.w.})^{0.849}$ ] (Boxenbaum, 1979) were used for g liver/kg b.w. for koalas (28.37) and ringtail possums (39.39), respectively. Based on the well stirred model (Gillette, 1971), the hepatic  $Cl$  was predicted with total drug concentration and unbound drug concentration in the blood, using following equation:

$$1) Cl_{(total)} = (Q \times \textit{in-vitro } Cl_{int}') / (Q + \textit{in-vitro } Cl_{int}')$$

$$2) Cl f_{u(blood)} = (Q \times f_{u(blood)} \times \textit{in-vitro } Cl_{int}') / (Q + f_{u(blood)} \times \textit{in-vitro } Cl_{int}')$$

$$3) Cl f_{u(blood, mic)} = [Q \times f_{u(blood)} \times (\textit{in-vitro } Cl_{int}' / f_{u(mic)})] / [Q + f_{u(blood)} \times (\textit{in-vitro } Cl_{int}' / f_{u(mic)})]$$

where  $Q$  is hepatic blood flow and  $f_{u(blood)}$  is unbound fraction in the blood. Equation 2 and 3 were applied to koalas and the rat only in which  $f_{u(blood)}$  was calculated from  $f_{u(plasma)} / (B/P)$ . Reference values for hepatic blood flow (mL/min/kg) in rat and brushtail possum are 55.2 (Davies and Morris, 1993) and 42.5, respectively (McDonald and Than,

1976). Interspecies, hepatic flow is correlated with b.w. according to following relationship  $[Q = 0.0554 (\text{b.w.})^{0.894}]$  (Boxenbaum, 1979). Hepatic flow (mL/min/kg) was calculated accordingly for koalas (45.89) and ringtail possums (56.88), respectively. For the koala and rat, where plasma *Cl* is available, accuracies of predicted values were estimated by following equation, in which actual blood *Cl* was calculated from plasma *Cl* / (B/P).

$$\text{Average fold error} = 10^{\{ \sum \log | (\text{hepatic } Cl \text{ (predicted)} / \text{blood } Cl) | \} / n}$$

Accordingly, average fold error value of  $\leq 2$  was considered as satisfactory prediction for an actual value.

#### **4.3.6. HPLC and LC-MS**

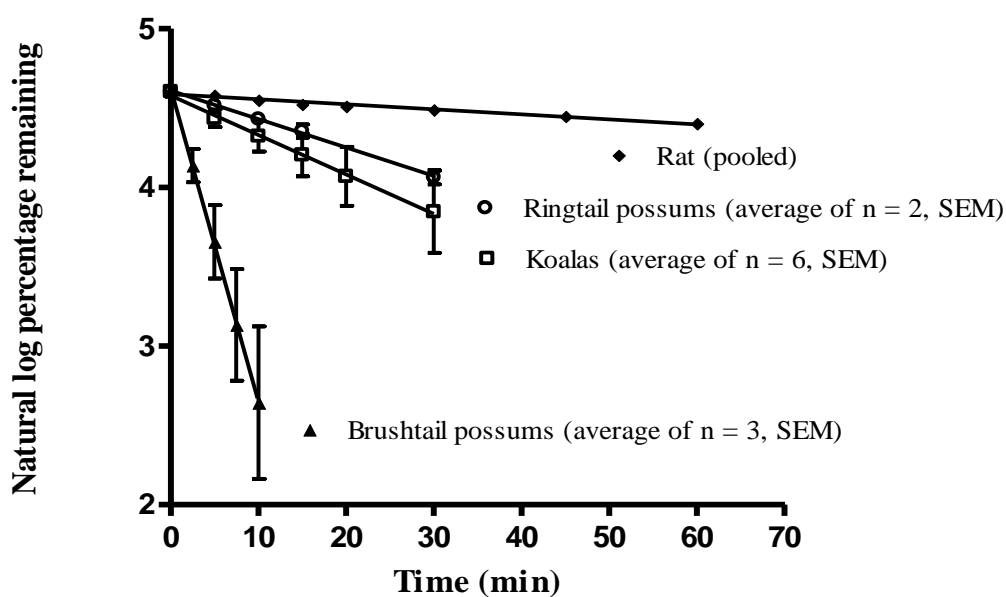
Meloxicam and its metabolites were analyzed by HPLC-UV or LC-MS as described previous Chapters (**Chapter 2, Section 2.3.2; Chapter 3, Section 3.3.4**).

#### **4.3.7. Data analysis**

A one-way ANOVA was used for the comparison of *in-vitro*  $Cl_{\text{int}}$  and *in-vivo*  $Cl_{\text{(predicted)}}$  among different species. Multiple comparisons were then performed using the Tukey-Kramer *post hoc* test. Results were considered statistically significant at  $p < 0.05$ .

#### 4.4. Results

The plot of meloxicam depletion (expressed as natural log percentage of substrate remaining) vs. incubation time for each species, except for dogs, is presented in **Figure 4.1**.



**Figure 4.1)** Meloxicam depletion concentration (expressed as log substrate remaining) vs. incubation time between species; microsome concentration of 0.5 mg/mL for possums and koalas, and 1 mg/mL for rat (pooled) incubated with 1.25  $\mu$ M of meloxicam; individual samples and pooled rats were average of duplicates; SEM: standard error;  $r^2$  values of the depletion slopes were within the range of 0.971 - 0.999

Meloxicam was stable and no associated metabolites were observed in any incubation mixture that lacked either the NADPH-regenerating system or microsomes. In conjunction with final concentrations of both meloxicam (1.25  $\mu$ M) and microsomes, the period of incubation determined for each species was based on the linearity of the slope

( $r^2$  values were within the range of 0.971 - 0.999). In all species except the dog, the substrate depleted at the final incubation times were approximately equal to, or greater than 20 % of the initial value (at  $t = 0$  min). The rank order of *in vitro*  $Cl_{int}$  of meloxicam between species was brushtail possums > koalas > ringtail possums > rat, as shown in **Table 4.1**.

**Table 4.1)** *In-vitro*  $t_{1/2}$  values and *in-vitro* intrinsic clearances (mean  $\pm$  SD) of meloxicam in investigated species.

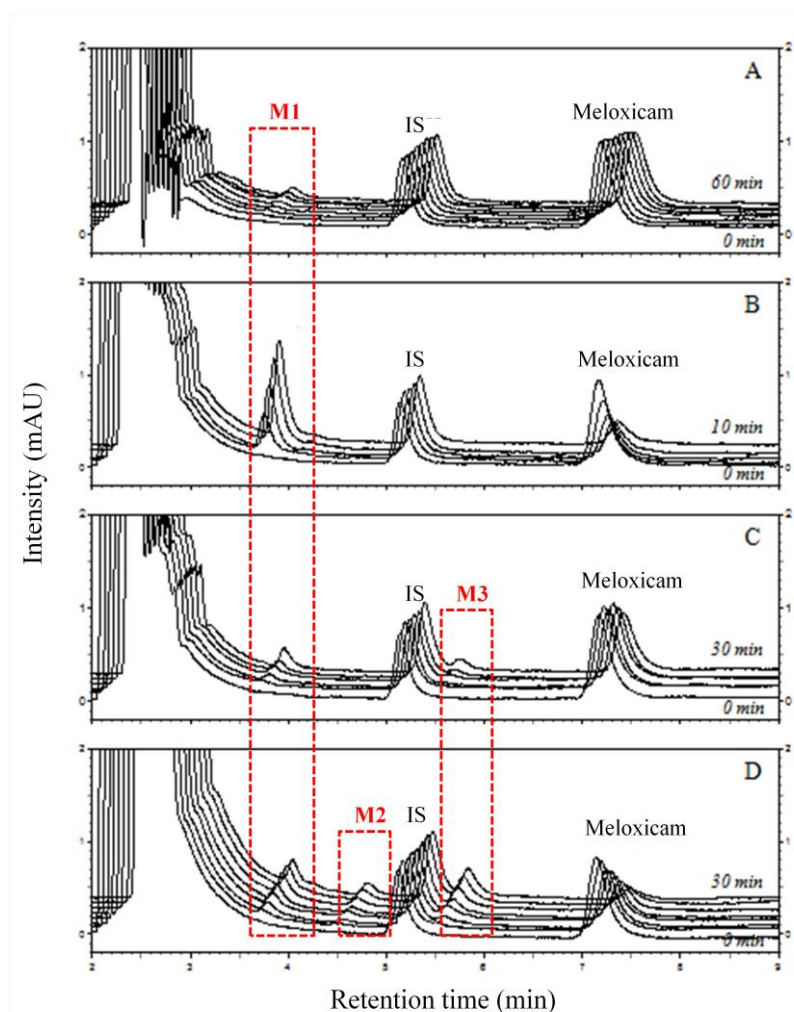
Species	m.c. mg/mL	<i>In-vitro</i> $t_{1/2}$ min	<i>In-vitro</i> $Cl_{int}$ $\mu$ L/min/protein mg	<i>In-vitro</i> $Cl_{int}'$ mL/min/kg
Koalas ( $n = 6$ )	0.5	38 $\pm$ 15	50 $\pm$ 41	63 $\pm$ 53
Brushtail possums ( $n = 3$ )	0.5	3.9 $\pm$ 1.5	394 $\pm$ 168	540 $\pm$ 231
Ringtail possums ( $n = 2$ )	0.5	39 $\pm$ 5	36 $\pm$ 5	62 $\pm$ 9
Rat (pooled) <sup>a</sup>	1	217 $\pm$ 19	3.2 $\pm$ 0.3	5.8 $\pm$ 0.5
Dog (pooled)	1	n.d.	n.d.	n.d.

m.c., microsomal protein concentration; <sup>a</sup> for rat, mean  $\pm$  SD values represent duplicated determination from the pooled microsomes; n.d., not determined.

The *in-vitro*  $Cl_{int}$  was extremely high in marsupials:  $\sim$ 10 fold (koalas and ringtail possums) and  $\sim$ 100 fold (brushtail possums) higher, compared to the rat. Within marsupials, brushtail possums exhibited highest activity,  $\sim$ 8 to 11 fold higher than

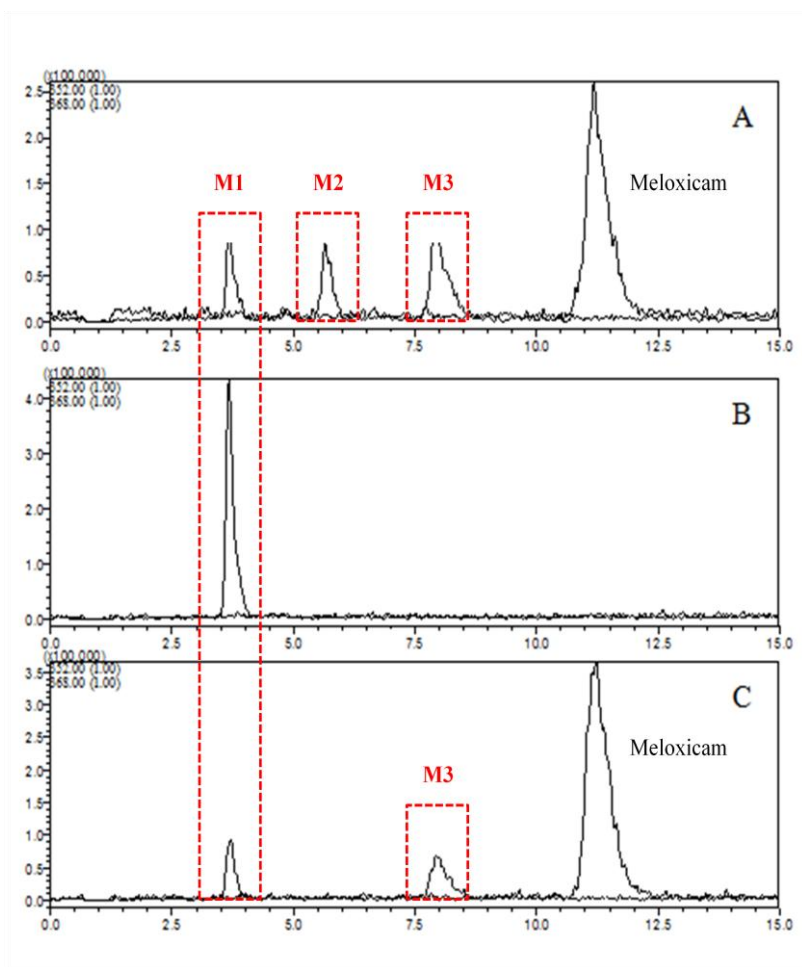
koalas and ringtail possums ( $p < 0.01$  for both), and no significant difference was observed between koalas and ringtail possums.

The metabolites generated during the substrate depletion experiment for each species are demonstrated in HPLC-UV chromatograms (**Figure 4.2**). Based on the condition used for HPLC analysis metabolite M1 was produced in all species studied excepting the dog, which was excluded. M1 was generated in brushtail possums and the rat during 10 min and 60 min of incubation, respectively. Additional metabolites were also observed in ringtail possums (M3) and koalas (M2 and M3) at 30 min of incubation.



**Figure 4.2)** Typical HPLC-UV chromatograms, monitored at wavelength of 355 nm, representing metabolites (M1, M2, and M3) generated during substrate depletion experiment for investigated species; A) rats (pooled) 0-60 min; B) brushtail possums 0-10 min; C) ringtail possums 0-30 min; D) koalas 0-30 min.

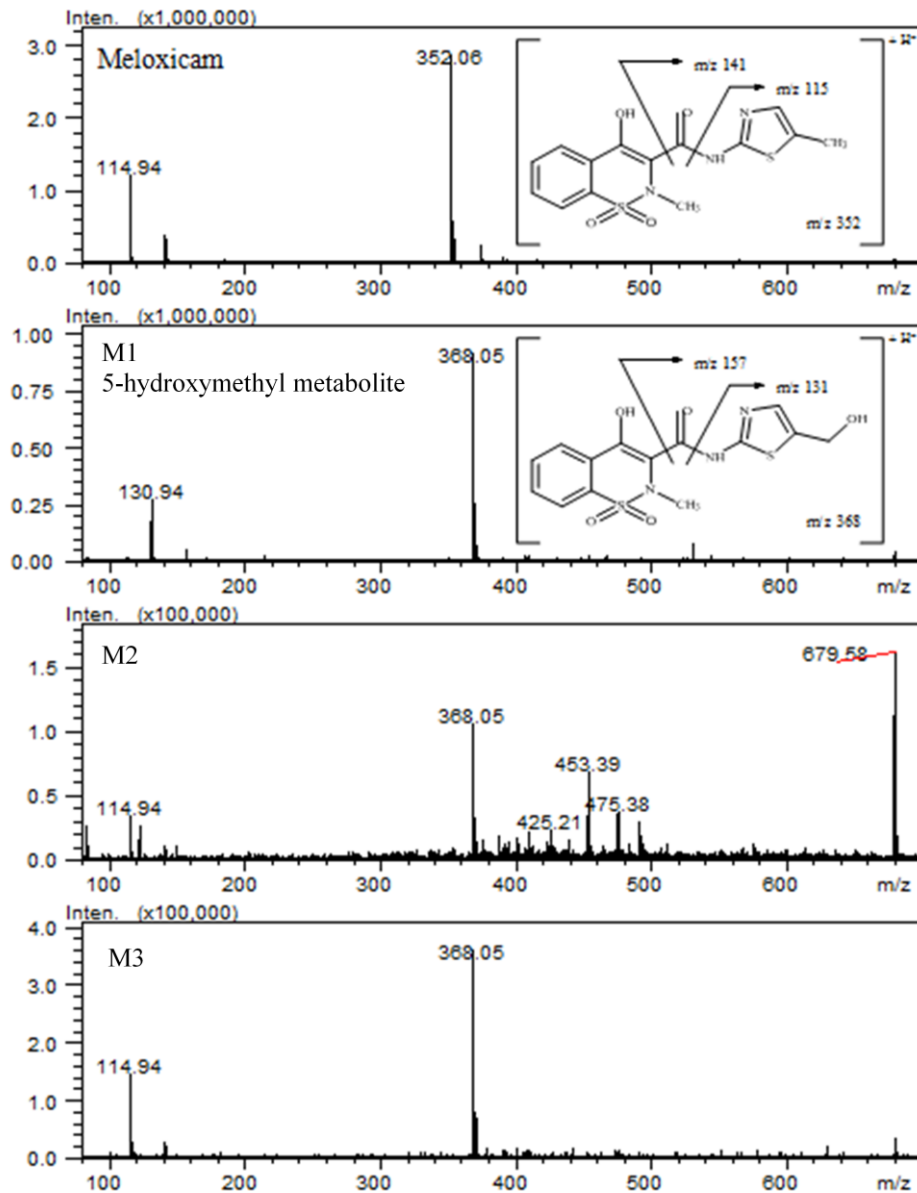
Molecular characteristics of metabolites (M1, M2, and M3), described by ESI+ extracted ion chromatogram, are depicted in **Figure 4.3**. Protonated molecular mass  $[M + H]^+$  of M1, M2 and M3 were all  $m/z$  368 indicating they are hydroxylated metabolites.



**Figure 4.3)** ESI<sup>+</sup> extracted (*m/z* of 352 and 368) ion chromatograms of metabolites (M1, M2, and M3) of meloxicam (1.25  $\mu$ M) generated in microsomes (1 mg/mL) of marsupials incubated for 30 min; A) koalas; B) brushtail possums; C) ringtail possums

The major fragments of meloxicam standard and M1 were  $[M + H]^+$  *m/z* of (115) and (131), respectively (**Figure 4.4**); indicating the addition of an oxygen atom on the methyl-thiazole moiety of the meloxicam to form 5-hydroxymethyl metabolite (M1). The exact location of oxidation of metabolites M2 and M3 were unable to be assigned in this study; however ion fragments of  $[M + H]^+ 115$  observed from both metabolites suggests that the oxidation occurred at other than the thiazole moiety.





**Figure 4.4)** In-source CID spectra of meloxicam and its associated metabolites (M1, M2, and M3); fragmentation,  $[M + H]^+$  m/z of 131, suggestion of 5-hydroxymethyl metabolite for M1 in ESI+

Scaled hepatic  $Cl$  from *in-vitro* data is provided in **Table 4.2**. Koalas and ringtail possums had comparable scaled hepatic  $Cl_{(total)}$  values, which were  $\sim 1.5$  fold less than brushtail possums but  $\sim 5$  fold higher than the rat. The  $f_u$  ( $mic$ ) determined in koalas and rats (mean

$\pm$  SD) were  $0.39 \pm 0.05$  and  $0.17 \pm 0.01$ , respectively. The Hct value determined from koala blood was 0.41 (L/L). The mean  $\pm$  S.D. B/P ratio observed from the experimentation in koalas was  $0.62 \pm 0.11$ . Subsequently, determined B/P ratio values from plasma protein binding in koalas and rats were 0.60 and 0.54, respectively.

**Table 4.2)** Predicted hepatic *Cl* (mL/min/kg) values (mean  $\pm$  SD) for each species (except dog) predicted from *in-vitro* data based on the well stirred model with, and without the unbound fraction in the blood.

Species	Actual		Predicted	
	Blood <i>Cl</i> <sup>b</sup>	<i>Cl</i> <sub>(total)</sub>	<i>Cl fu</i> <sub>(blood)</sub>	<i>Cl fu</i> <sub>(blood, mic)</sub>
Koalas ( <i>n</i> = 6) (fold error)	12.22	$24.2 \pm 6.3$ 1.9	$1.84 \pm 1.4$ 8	$4.29 \pm 3.04$ 3.3
Brushtail possums ( <i>n</i> = 3)	n.a	$39.1 \pm 1.2$	n.d	n.d
Ringtail possums ( <i>n</i> = 2)	n.a.	$29.6 \pm 2$	n.d.	n.d
Rat (pooled) <sup>a</sup> (fold error)	0.46	$5.2 \pm 0.4$ 11.3	$0.043 \pm 0.004$ 10.7	$0.24 \pm 0.02$ 1.86

<sup>a</sup> for rat, mean  $\pm$  SD values represent duplicated determination from the pooled microsomes; <sup>b</sup> blood *Cl* was calculated by plasma *Cl* / (B/P); plasma *Cl* (mL/min/kg) for the koala and rat is 7.33 (determined from **Chapter 3**) and 0.15 (Busch et al., 1998), respectively, where estimated (B/P), described in the material and method, for the koala and rat is 0.60 and 0.54, respectively; n.d., not determined; n.a., not available.

## 4.5. Discussion

This study demonstrated that the NADPH-dependent *in-vitro* hepatic microsomal metabolism of meloxicam differed, quantitatively and / or qualitatively, between species. Quantitatively, significantly higher apparent *in-vitro*  $Cl_{int}$  was observed in the foliage eating marsupials (koalas and possums) indicating that these species have a higher intrinsic hepatic rate to clear meloxicam. Qualitatively, while the formation of M1, as a single hydroxyl metabolite, was observed in the rat and brushtail possums during the initial depletion of meloxicam, additional hydroxyl metabolites were observed in koalas (M2 and M3) and in ringtail possums (M3) including M1. Furthermore, the results of this *in-vitro* study confirmed a previous observation (**Chapter 3**) of significantly higher plasma  $Cl$  of meloxicam in koalas compared to a number of eutherian species such as rats and dogs.

In this study, the overall *in-vitro*  $Cl_{int}$  was estimated using the substrate depletion method (Obach, 1999b) rather than the product formation method (Rane et al., 1977a) as there were multiple metabolic pathways involved for  $Cl$  of meloxicam in koalas and ringtail possums. Preliminary studies to determine the conditions for meloxicam depletion were performed to establish that a substrate concentration of 1.25  $\mu$ M was  $< K_m$  (Michaelis - Menten constant) and that the rate of depletion was linear (representing a first order rate constant) (**Appendix 1**). The conditions were further optimised with respect to protein concentrations and incubations for each species except for the dog (data not shown). Under these conditions, the depletion of meloxicam by dog

microsomes was insufficient (< 20 %); therefore *in-vitro*  $Cl_{int}$  was not estimated in this species.

The observation of the oxidative transformation of meloxicam, whereby 5-hydroxymethylation was a common metabolic pathway among the species studied, is consistent with previous reports for other species (Schmid et al., 1995a, Schmid et al., 1995b, Busch et al., 1998). In those studies, the oxidation of meloxicam is reported to be qualitatively similar between species, including rats and humans, and is the principle metabolic pathway by which the predominant metabolites produced are M1 and a 5-carboxylated metabolite. In another *in-vitro* study using human microsomes, hepatic CYPs, particularly CYP2C9, was demonstrated to catalyze meloxicam to the intermediate metabolite, M1, prior to undergoing further non-CYP dependent carboxylation (Chesne et al., 1998). We speculate that CYP2C mediated activity may contribute to the high apparent *in-vitro*  $Cl_{int}$  of meloxicam observed in marsupials, particularly for brushtail possums, and at least partly for koalas and ringtail possums, as demonstrated by relatively higher production of M1 in marsupials (**Figure 4.2**). These findings are consistent with another study that demonstrated enhanced CYP2C mediated metabolism, using tolbutamide as a substrate for CYP2C, in marsupials (koalas and brushtail possums) compared to eutherian species, including rats and humans (Pass et al., 2001). The *in-vitro*  $Cl_{int}$  of meloxicam in rats ( $3.2 \pm 0.3$   $\mu\text{L}/\text{min}/\text{mg}$  microsomal protein) was slightly higher than the *in-vitro*  $Cl_{int}$  (2.7) resulting from human hepatic microsomes (Obach et al., 2008), which is consistent with the pattern seen for plasma  $Cl$  (L/h/kg) in rats and humans (0.015 and 0.01, respectively) (Busch et al., 1998). As many

drug dosages used clinically in marsupials are the same as those used for dogs, the apparent slow rate of canine microsome activity was of interest. It is possible that shipping and/or conditions during the transportation of the microsomes may have been responsible for inactivity of these microsomes. However the canine microsomes were received on dry ice and kept in -80 °C prior to all experimentation and they were functional (producing 5-hydroxymethyl metabolite) when incubated with significantly higher doses of meloxicam. The authors hypothesize that inactivity of the canine hepatic microsome may be due to relative low affinity ( $K_m$ ) and low  $V_{max}$  for meloxicam. Other studies also support low CYP2C mediated activity in dog microsomes (Chauret et al., 1997, Graham et al., 2003).

The finding that the *in-vitro*  $Cl_{int}$  of meloxicam in koalas was less than that of brushtail possums, contrasts with previous findings that koalas demonstrate higher tolbutamide hydroxylation than brushtail possums, respectively (Pass et al., 2001). In this study, it was not feasible to compare CYP2C-like activity (if it is involved in the production of M1), between brushtail possums and koalas, as meloxicam undergoes multiple hydroxylation in koalas. Studies have indicated that variation in CYP isoforms influence the substrate affinity and subsequently alter the  $Cl_{int}$  of drugs, particularly for CYP dependent drugs (Guengerich, 1997). Thus, although tolbutamide hydroxylation is higher in koalas (Pass et al., 2001), it is possible that CYP2C mediated meloxicam oxidation (production of M1) could be lower in brushtail possums due to differences in their CYP isoforms (Jones et al., 2008, Pass et al., 1999).

Subsequently, differences in CYP isoforms and their activity may also account for the multiple hydroxylation pathways observed in koalas (M2 and M3) and ringtail possums (M3), suggesting that these species have diverse oxidation capacities for  $Cl_{int}$  of meloxicam. Studies have indicated that specialist *E. feeders* such as the koala and, to a slightly lesser extent, ringtail possums, have a greater capacity to oxidize (and detoxify) dietary terpene, compared to generalist *E. feeders*, such as brushtail possums, or rats (Boyle et al., 2001); although this is not necessarily as a result of higher  $Cl_{int}$  (Pass et al., 2001, Pass et al., 2002). It has been suggested that the specialist *E. feeders* rely on a strategy of poly-oxidation in order to avoid the conjugation phase, to conserve energy consumption (McLean et al., 2003, Foley and Moore, 2005).

Since it has been previously described that meloxicam is extensively metabolised in the liver in all species studied (Schmid et al., 1995a, Schmid et al., 1995b, Busch et al., 1998), we predicted hepatic  $Cl$  from scaling the *in-vitro*  $Cl_{int}$  with or without binding effects to plasma proteins and microsomes, and then compared with the *in-vivo*  $Cl$  reported previously for koalas and rats. In this study, the closest projection of *in-vivo*  $Cl$  for rats was demonstrated with scaled hepatic  $Cl_{fu(blood, mic)}$ , which includes binding effects from both plasma protein and microsomes (fold error = 1.86). On the other hand, hepatic  $Cl_{(total)}$  (mL/min/kg) in koalas of  $24.2 \pm 6.3$  was best correlated to *in-vivo*  $Cl$  (12.22 mL/min/kg) (average fold error = 1.9). One possible explanation for this difference is that  $Cl$  of a low extraction drug (such as meloxicam) is generally more dependent on plasma protein binding, but due to the relatively high *in-vitro*  $Cl_{int}$  in koalas compared to the rat, is likely to result in underestimation of actual *in vivo*  $Cl$  when incorporating

values of plasma protein binding (and/or microsomes). However in the koala  $Cl_{fu(blood, mic)}$  also reasonably predicted *in-vivo* clearance and whether  $Cl_{fu(blood, mic)}$  or  $Cl_{(total)}$  should be used to predict *in-vivo* clearance in humans is controversial (Obach, 1999). It is also acknowledged that some scaling factors for koalas were not available and were estimated by allometric scaling and could be a source of potential error.

Previously, we have demonstrated higher plasma  $Cl$  in koalas accompanied by the formation of multiple hydroxylated metabolites (M1, M2 and M3) after intravenous and subcutaneous administration of meloxicam, and accordingly, involvement of hepatic metabolism was hypothesised. The current findings confirm that diverse oxidation of meloxicam occurring within in the liver, likely via CYPs in koalas, further supports *in-vitro* to *in-vivo* scaling. In possums, estimated hepatic  $Cl_{(total)}$  was appeared to be higher than that of koalas and while meloxicam is anecdotally reported as being widely used in possums, the results of this study indicate that extrapolating the dosage of meloxicam from investigated eutherians is likely inadequate, due to significant differences in  $Cl_{int}$ , to achieve similar plasma concentrations. However, both further PK and PD studies are required to confirm this statement. For drugs undergo hepatic (and/or intestinal) metabolism,  $Cl$  is an important determinant of drug bioavailability and thus influences the dosage required to meet therapeutic concentrations. This study demonstrates the potential pitfalls of predicting therapeutic dosages without species specific pharmacokinetic studies. For many wild-life species such as koalas, conducting pharmacokinetic studies are an intensive and invasive procedure in a species that is generally not accustomed to regular handling. However relative *in-vitro* clearance can be

beneficial is assessing similarities and differences in rates and metabolism. Thus, establishing that there is good correlation between *in-vitro* and *in-vitro Cl* to predict drug PK profiles, demonstrates that *in-vitro* metabolism studies can serve as valuable pre *in-vivo* studies.



# Chapter 5

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**Stability of human CYP2C9 substrates in the  
hepatic microsomes of the koala, brushtail  
possum, ringtail possum and rat: a preliminary  
study**

## 5.1. Introduction

The catalytic activity mediated *via* CYP2C enzymes, particularly CYP2C9, is considered an important metabolic pathway for drug elimination in humans (Rettie and Jones, 2005). It is reported that approximately 15 % of therapeutic drugs that undergo phase-1 metabolism are catalysed by CYP2C9 in humans (Evans and Relling, 1999). Drugs metabolised in humans by CYP2C9 include many weakly acidic drugs that have a pKa range between 3.8 to 8.1 (Miners and Birkett, 1998), such as NSAIDs (Rodrigues, 2005), and some narrow therapeutic index drugs such as tolbutamide and warfarin (Rettie and Jones, 2005). Species differences in the catalytic activities of hepatic CYP enzymes, particularly CYP2C-like activities, have been demonstrated by several studies (Smith, 1991, Lin, 1995), and consequently there are differences between the rate of drug metabolism between species (Guengerich, 1997). While it is common that dosages are extrapolated from conventional veterinary species to others, such as exotic and wild animals, it is important to take the variability of disposition, especially the rate of drug elimination into account, when selecting a NSAID and optimising its dosage for a particular species. The previous chapters report the rapid plasma *Cl* of meloxicam in koalas, brushtail and ringtail possums. And a higher rate of metabolic conversion (*in vitro Cl<sub>int</sub>*) by koala hepatic microsomes, compared to those of rats or dogs (at least > 15 fold). This observation further confirms that a single bolus of the drug is likely to be metabolised too rapidly to be administered on a once daily basis in this species. As the *in vitro Cl<sub>int</sub>* of meloxicam is primarily metabolised *via* CYP2C9 in humans (Chesne et al., 1998), it was of interest to investigate whether a similar biotransformation pathway of NSAIDs was occurring in marsupials by exploring the rate of metabolism of known

human CYP2C9 substrates. Therefore the aims of this study were to investigate the stability of CYP2C9 substrates (particularly weakly acidic NSAIDs) in the hepatic microsomes of marsupials [specialist *E. feeders* (koala and ringtail possum) vs. a generalist *E. feeder* (brushtail possum)]. This *in vitro* study was designed as a preliminary screen to identify the most ideal NSAID candidate for further pharmacokinetic *in vivo* studies in marsupials. In addition, rat hepatic microsomes were used as a control and a comparison between the marsupials and a representative eutherian species.

## **5.2. Materials and Methods**

### **5.2.1. Chemicals**

The NSAIDs diclofenac, flurbiprofen, indomethacin, meloxicam and other CYP2C9 substrates: tolbutamide and fluvastatin were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Ethyl acetate was purchased from Thermo Fisher Scientific (Scoresby, VIC, Australia). The origin of all other chemicals and rat pooled hepatic microsomes, used in this study, are described in **Chapter 4, Section 4.3.1**.

### **5.2.2. Preparation of hepatic microsomes**

The details of microsomes extraction and preparation from the liver tissues of the koala and both possum species are described in **Chapter 4, Section 4.3.2**.

### 5.2.3. Microsomal experimentation

Microsomes, 0.5 mg/mL for the koala and both possum species ( $n = 1$  of each species); 1 mg/mL for the rat (pooled microsomes), were preincubated in 0.5 mL of 0.1 M phosphate buffer (pH 7.4) containing a NADPH regenerating system (1 mM NADP, 0.8 U glucose 6 phosphate dehydrogenase and 3 mM glucose 6 phosphate) and 3 mM  $MgCl_2$ , in an open air shaking water bath at 37 °C for 5 min. After preincubation, the enzymatic reaction was initiated by adding 1  $\mu$ M of substrate (dissolved in MeCN); and for rat, an additional substrate concentration of 4  $\mu$ M was also used. The percentages of organic solvent in the resultant mixture were less than 0.1 %. At ( $t$ ) = 0 min and after the incubation time designated for each species (rat = 15 min; koala and possums = 10 min for all drugs), a 200  $\mu$ L of aliquot was removed and mixed with 100  $\mu$ L of iced cold MeCN which contained IS (2  $\mu$ M) specific to the substrate (as documented in **Table 5.1**) to deactivate the enzymatic reaction. The resultant mixture was vortexed and centrifuged at 14,000  $\times$  g for 10 min. After that, the supernatant was extracted with 1 mL ethylacetate and dried under vacuum in a Speed Vac concentrator (Thermo Scientific, USA) at 35 °C for 1 h. The dried residue was reconstituted in 90  $\mu$ L of mobile phase and 10  $\mu$ L was injected into HPLC system. The extraction recovery for each of the drugs (0.25, 0.5, 0.75 and 1  $\mu$ M) were investigated in triplicate, and absolute recoveries were > 80%, except for fluvastatin (67 %), and the precision (C.V.) for all drugs was < 10 %. The drug concentration extraction was linear along the range of investigated concentrations ( $r^2 > 0.998$ ). In addition, 10  $\mu$ M of flurbiprofen was incubated with hepatic microsomes (1 mg/mL) of the koala and brushtail possum for 10 min.

**Table 5.1)** Internal standards used for microsomal experimentation

Substrate (1 $\mu\text{M}$ )	Internal standard (2 $\mu\text{M}$ )
Meloxicam	Piroxicam
Flurbiprofen	Meloxicam (marsupials); Diclofenac (rat)
Diclofenac	Meloxicam (marsupials); Indomethacin (rat)
Fluvastatin	Meloxicam (marsupials); Flurbiprofen (rat)
Indomethacin	Meloxicam (marsupials); Diclofenac (rat)
Tolbutamide	Meloxicam (marsupials); Flurbiprofen (rat)

#### 5.2.4. Calculation

To estimate the stability of a drug, the percentage of the peak area of drug that disappeared during the incubation (expressed as turnover rate), between ( $t$ ) = 0 min ( $C_0$ ) and ( $t$ ) = incubation time (min) designated for each species ( $C_x$ ), was used (Hill, 2001):

$$\text{Turnover rate (\%)} = [100 - (C_x / C_0)] \times 100$$

Additionally, turnover rates of drugs were converted into *in vitro*  $Cl_{\text{int}}$  (described in **Chapter 4, Section 4.3.5**) using the single time point ( $C_x$ ), in which the percentage of substrate remaining, was calculated from the peak area ratio of  $C_x/C_0 \times 100$ .

#### 5.2.5. HPLC analysis

The details of HPLC-PDA system are described in **Chapter 2, Section 2.3.2**. The column used was a Synergy MAX-RP-80A (4 $\mu$ , 150  $\times$  4.6 mm) (Phenomenex, Torrance, CA, USA) with a 1 mm Optic-guard C-18 pre-column (Optimize Technologies, Alpha Resources, Thornleigh, Australia), and the column oven temperature at 35  $^{\circ}\text{C}$ . The mobile phase used was 52.5 % MeCN containing 0.1 % of formic acid and the flow rate was 1 mL/min.

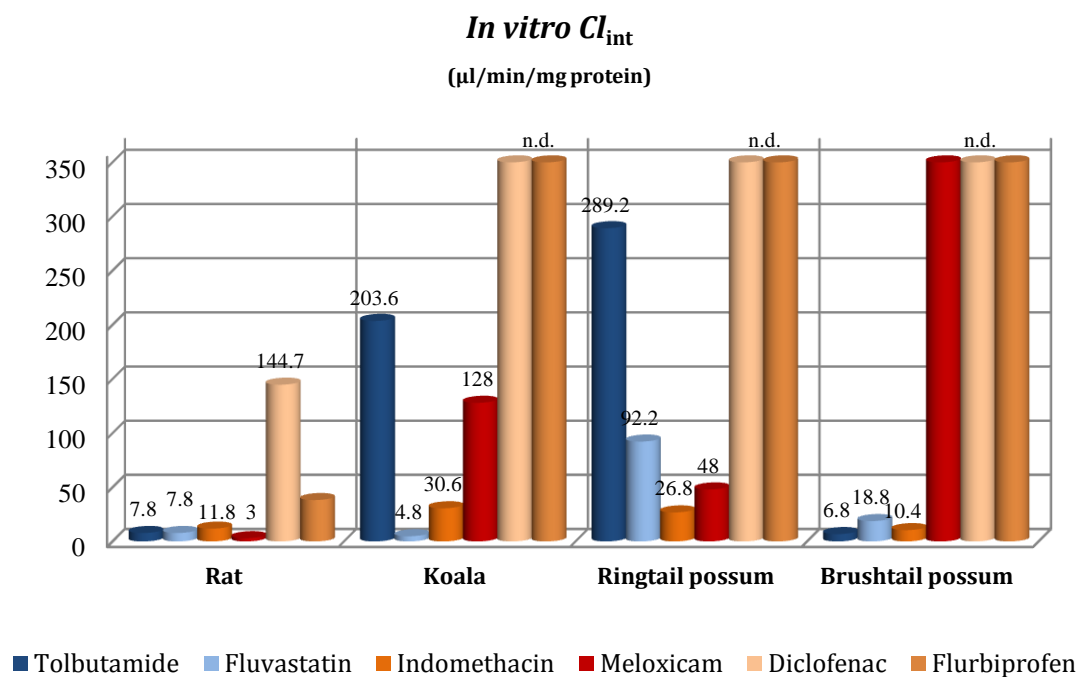
The specific UV wavelengths used for target drugs were: 355 nm (meloxicam), 245 nm (flurbiprofen), 276 nm (diclofenac), 318 nm (indomethacin), 228 nm (tolbutamide), and 304 nm (fluvastatin). Except for fluvastatin due to the lack of UV sensitivity, the contour plot (HPLC-UV) was used to compare the possible metabolites of diclofenac, flurbiprofen, indomethacin, meloxicam and tolbutamide; of which, samples of before and after the microsomal incubation at the concentrations of 1  $\mu$ M for marsupials and 1  $\mu$ M and 4  $\mu$ M for the rat were used. Additionally, metabolites of flurbiprofen (10  $\mu$ M) incubated with microsomes of the koala and brushtail possums were also screened at 270 nm.

### 5.3. Results

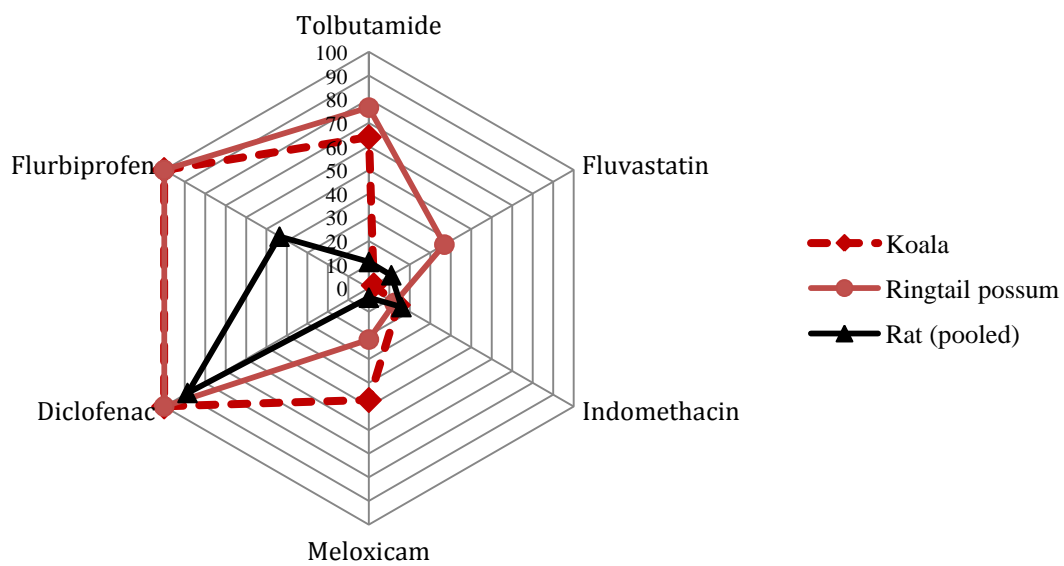
The *in-vitro*  $Cl_{int}$  of human CYP2C9 substrates investigated in this study, including NSAIDs, determined by the hepatic microsomes of rat (pooled) vs. *E. feeders* (estimated by the depletion method using a single incubation time point) is presented in **Figure 5.1**. For marsupials, *in-vitro*  $Cl_{int}$  of several substrates [diclofenac, flurbiprofen and meloxicam (in the case of brushtail possum)] were not determined accurately (e.g. *in-vitro*  $Cl_{int} \geq 350$   $\mu$ L/min/mg protein was not measured) as these substrates were completely depleted before 10 min of microsomal incubation.

The pattern of turnover rates on investigated CYP2C9 substrates in these marsupials and rat are presented in **Figure 5.2**. A similar pattern of turnover rates on these CYP2C9 substrates was demonstrated between specialist *E. feeders* (the koala and ringtail possum) and a higher turnover rate of tolbutamide was a typical characteristic in these

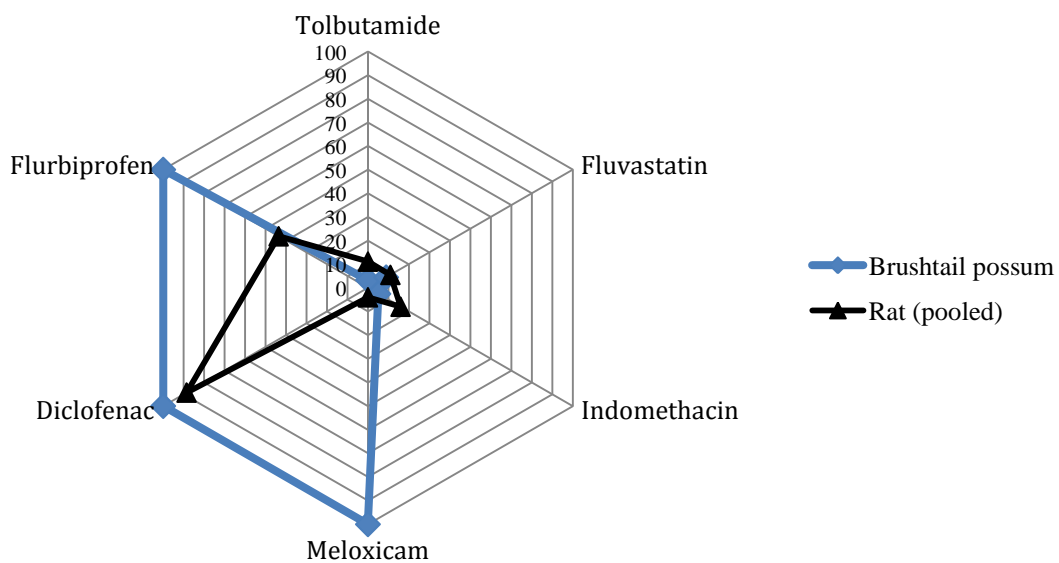
species compared to the generalist *E. feeder* (the brushtail possum); whereby the brushtail possum had a highest turnover rate of meloxicam.



**Figure 5.1)** *In-vitro*  $Cl_{int}$  of CYP2C9 substrates determined from the hepatic microsomes of the rat (pooled) and *E. feeders* ( $n = 1$ , each species); numbers indicate *in-vitro*  $Cl_{int}$  ( $\mu\text{l}/\text{min}/\text{mg}$  protein) estimated from single time point; n.d. (not determined): as the depletion of substrates was extremely fast, and the *in-vitro*  $Cl_{int}$  (at least  $> 350$   $\mu\text{l}/\text{min}/\text{mg}$  protein) was not determined.



**A**

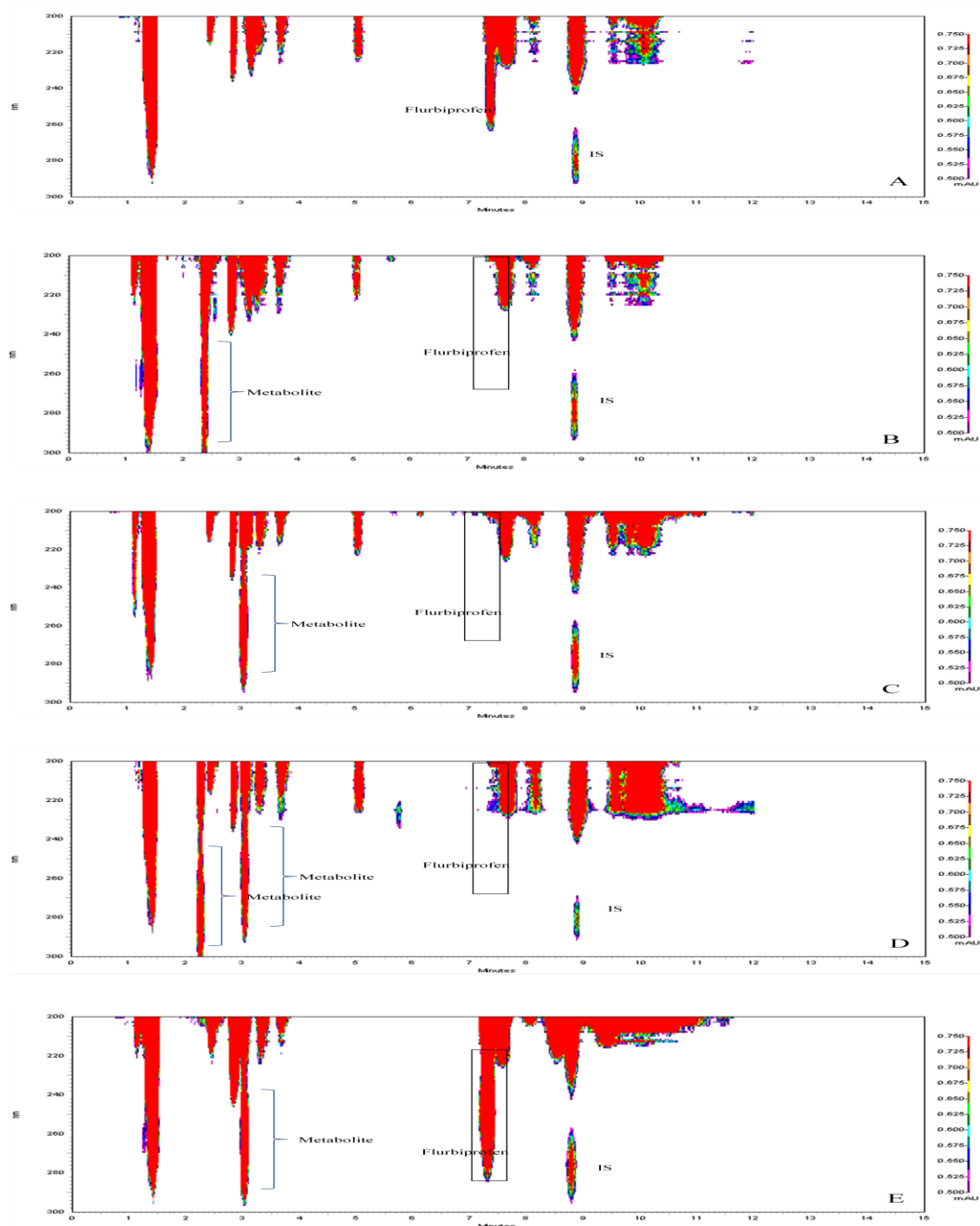


**B**

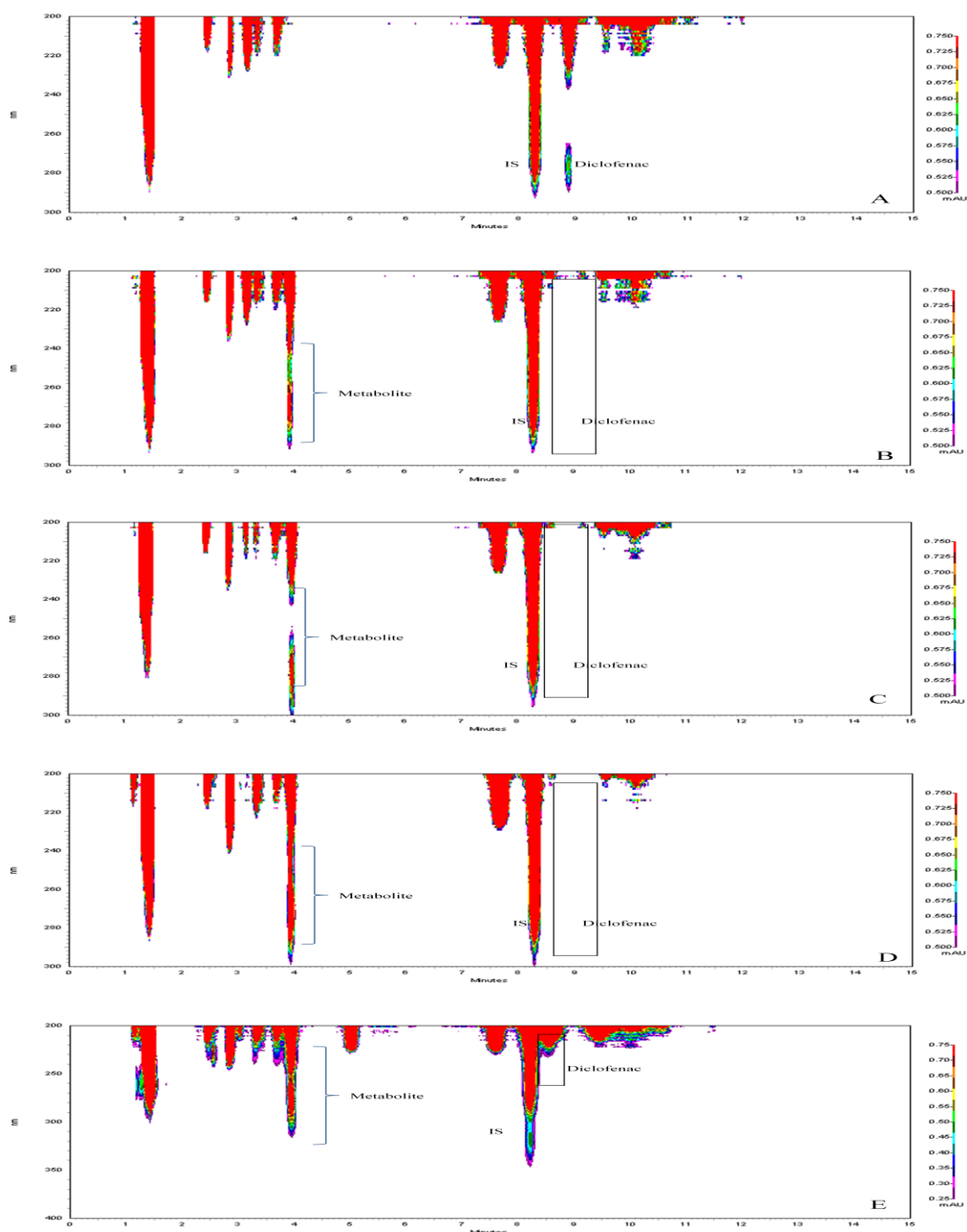
**Figure 5.2)** Patterns of turnover rates on investigated CYP2C9 substrates determined from hepatic microsomes of specialist *E. feeders* ( $n = 1$ , each species) **(A)** and generalist *E. feeder* ( $n = 1$ ) **(B)** compared with the rat; the number indicate % of turnover rate (e.g. 100 % indicates substrate has totally depleted with designated microsomal condition).



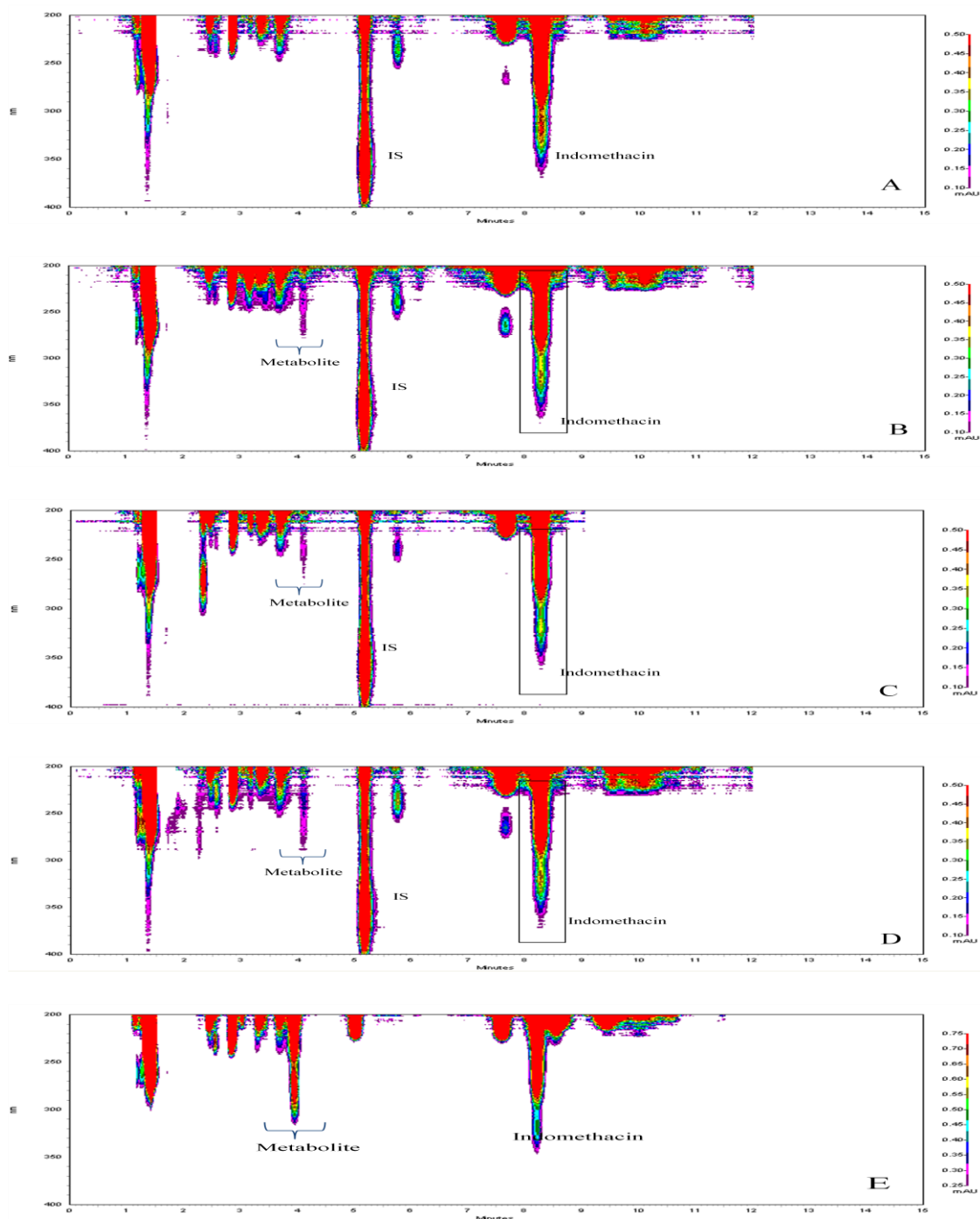
Possible metabolites of CYP2C9 substrates (diclofenac, flurbiprofen, indomethacin and tolbutamide), at substrate concentrations of 1  $\mu\text{M}$  for *E. feeders* and 4  $\mu\text{M}$  for the rat before and after microsomal incubation, are presented in **Figures 5.3 to 5.6**. The resultant metabolites of these substrates, except for flurbiprofen and meloxicam, were similar between the species. A single peak ( $\lambda_{\text{max}} = 258 \text{ nm}$ ) (FbM1), eluted at 3.02 min, was determined as the major product when rat microsomes were incubated with both 1 and 4  $\mu\text{M}$  of flurbiprofen. At the substrate concentration of 1  $\mu\text{M}$ , FbM1 was observed in both the koala and ringtail possum and an additional peak ( $\lambda = 258 \text{ nm}$ ) (FbM2) was observed at retention time of 2.25 min in the koala (**Figure 5.3**). In contrast, FbM1 was not detected in the brushtail possum with 1  $\mu\text{M}$ , instead a peak ( $\lambda_{\text{max}} = 261 \text{ nm}$ ) (FbM3) eluted at 2.36 min was the major product (**Figure 5.3**). However, at a higher substrate concentration (10  $\mu\text{M}$ ), the major product in both the brushtail possum and koala was FbM1 (**Figure 5.7**).



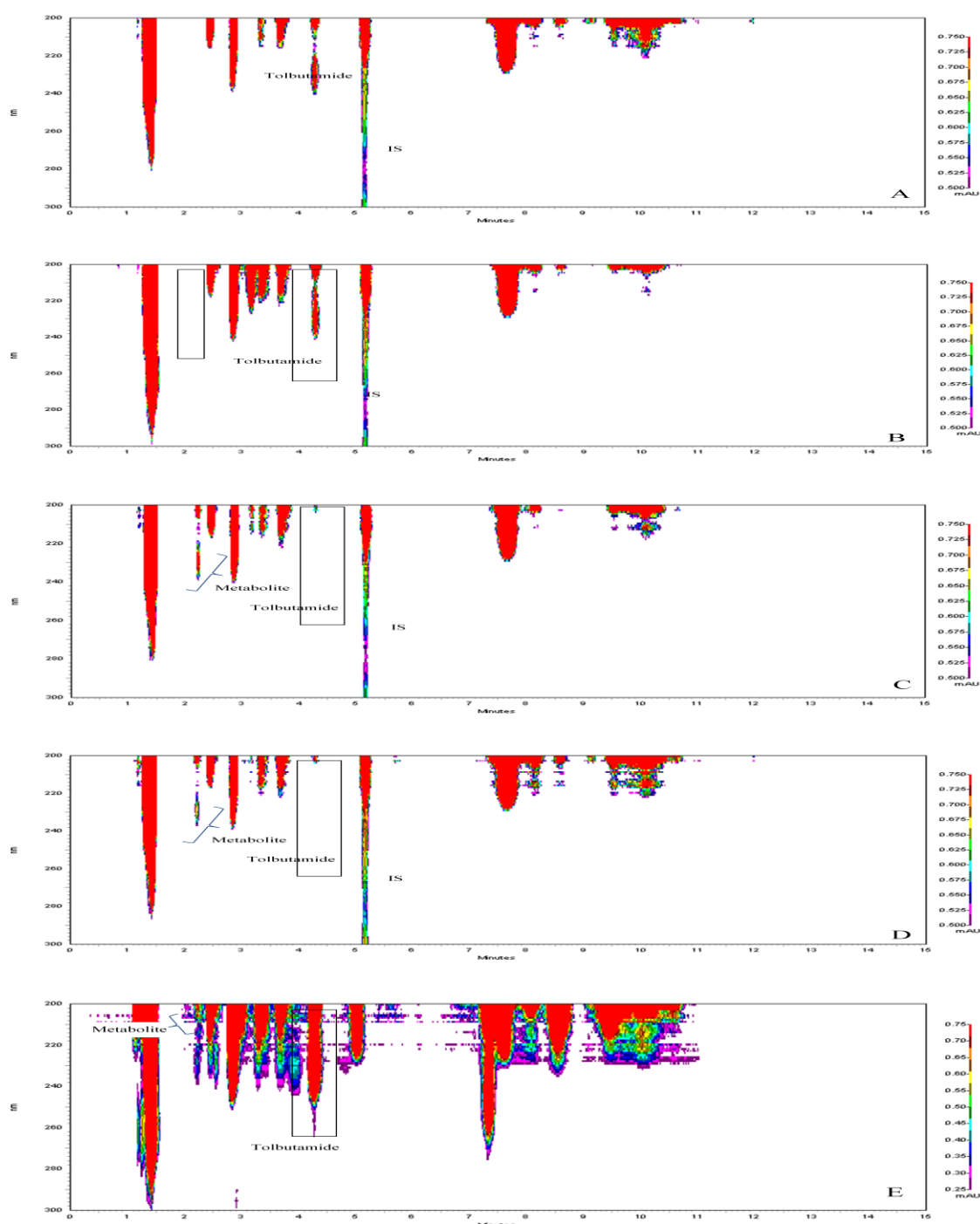
**Figure 5.3)** HPLC contour plot (UV scanned range: 200 to 300 nm) of flurbiprofen and its metabolite(s) before and after the microsomal incubation; 0.5 mg/mL and 1 mg/mL of microsomal concentrations were used for *E. feeders* and the rat, respectively; A: typical  $t = 0$  (brushtail possum); B:  $t = 10$  min (brushtail possum); C:  $t = 10$  (ringtail possum); D:  $t = 10$  min (koala); E:  $t = 15$  min (rat). Substrate concentrations were  $1 \mu\text{M}$  for *E. feeders* and  $4 \mu\text{M}$  for the rat.



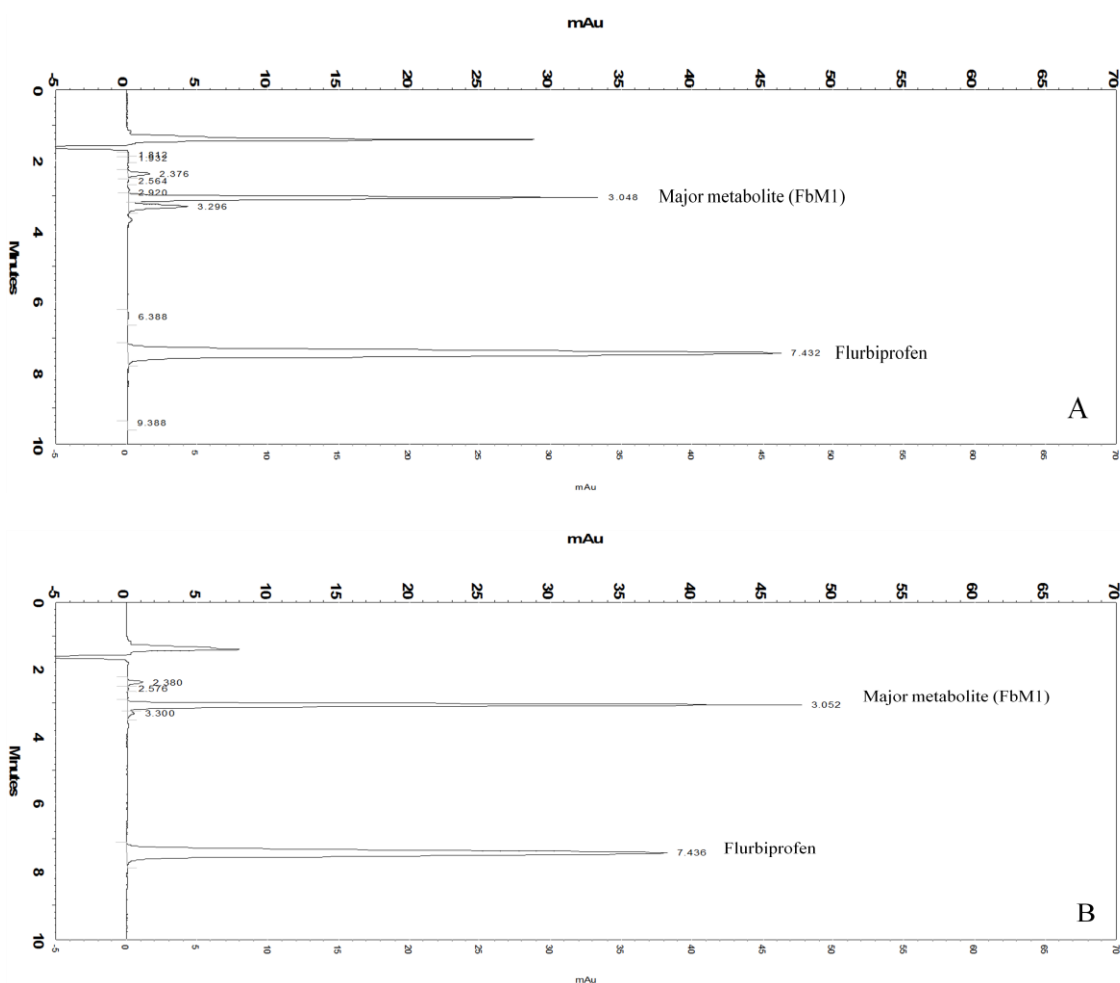
**Figure 5.4)** HPLC contour plot of diclofenac and its metabolite(s) before and after the microsomal incubation; 0.5 mg/mL and 1 mg/mL of microsomal concentrations were used for *E. feeders* and the rat, respectively; A: typical  $t = 0$  (brushtail possum); B:  $t = 5$  min (brushtail possum); C:  $t = 5$  min (ringtail possum); D:  $t = 5$  min (koala); E:  $t = 15$  min (rat). Substrate concentrations were  $1 \mu\text{M}$  for *E. feeders* and  $4 \mu\text{M}$  for the rat.



**Figure 5.5)** HPLC contour plot (UV scanned range: 200 – 400 nm) of indomethacin and its metabolite(s) before and after the microsomal incubation; 0.5 mg/mL and 1 mg/mL of microsomal concentrations were used for *E. feeders* and the rat, respectively; A: typical  $t = 0$  (brushtail possum); B:  $t = 10$  min (brushtail possum); C:  $t = 10$  min (ringtail possum); D:  $t = 10$  min (koala); E:  $t = 15$  min (rat). Substrate concentrations were  $1 \mu\text{M}$  for *E. feeders* and  $4 \mu\text{M}$  for the rat.



**Figure 5.6)** HPLC contour plot (UV scanned range: 200 – 300 nm) of tolbutamide and its metabolite(s) before and after the microsomal incubation; 0.5 mg/mL and 1 mg/mL of microsomal concentrations were used for *E. feeders* and the rat, respectively; A: typical  $t = 0$  (koala); B:  $t = 10$  min (brushtail possum); C:  $t = 10$  min (ringtail possum); D:  $t = 10$  min (koala); E:  $t = 15$  min (rat). Substrate concentrations were  $1 \mu\text{M}$  for *E. feeders* and  $4 \mu\text{M}$  for the rat.



**Figure 5.7)** HPLC-UV ( $\lambda = 245 \text{ nm}$ ) chromatograms on possible metabolite of flurbiprofen, after incubating flurbiprofen ( $10 \mu\text{M}$ ) with  $1 \text{ mg/mL}$  of microsomes of brushtail possum **(A)** and koala **(B)** for 10 min. Retention time of the major metabolite (FbM1) was approximately 3.02 min for both the koala and brushtail possum.

## 5.4. Discussion

In humans, the CYP2C subfamily, particularly CYP2C9 isoenzymes, tend to have a high selectivity for weakly acidic compounds such as NSAIDs (Lewis et al., 2004). While those hepatic CYP isoenzymes specifically involved in the degradation of these CYP2C9 substrates in marsupials are currently unknown; this study utilised hepatic microsomes,

which consist of a variety of CYP isoenzymes, to characterise the stability of different human CYP2C9 substrates.

The apparent *in-vitro*  $Cl_{int}$  of CYP2C9 substrates (diclofenac and tolbutamide), determined from the depletion approach, in the rat (**Figure 5.1**) were comparable to that documented previously (diclofenac: 110  $\mu\text{l}/\text{min}/\text{mg}$  protein; tolbutamide: 6.8  $\mu\text{l}/\text{min}/\text{mg}$  protein) (Ito and Houston, 2004). In this study the *in-vitro*  $Cl_{int}$  of diclofenac, flurbiprofen and meloxicam (brushtail possum only) were not determined in *E. feeders* as the NSAIDs were rapidly depleted even with relatively lower microsomal concentrations (0.5 mg/mL vs. 1 mg/mL: rat) and shorter incubation times (10 min vs. 15 min: rat) (**Figure 5.1**). Ideally, determination of the optimal conditions for microsomal experimentation (such as microsome concentration and incubation time) are required (as demonstrated in the previous chapter) to measure the rate of initial depletion and to facilitate accurate calculation of *in-vitro*  $Cl_{int}$ . Such optimisation was not undertaken here; instead this preliminary study compared metabolic turnover, which by measuring percentage of substrate depletion, of different human CYP2C9 substrates between *E. feeders* under identical microsomal experimental conditions (e.g. substrate concentration: 1  $\mu\text{M}$ ; microsomal protein 0.5 mg/mL; incubation time: 10 min). Therefore further study is necessary to confirm actual *in-vitro*  $Cl_{int}$  of NSAIDs in these species. Moreover, the metabolic mechanism of substrate depletion, e.g. whether due to the selectivity of CYP isoenzymes ( $K_m$ : Michaelis-Menten constant) or CYP content ( $V_{max}$ ), was not determined by this study.

Despite that the *in-vitro*  $Cl_{int}$  of investigated CYP2C9 substrates was not accurately determined, nor available, the results indicate that these weakly acidic NSAIDs are generally not stable in the hepatic microsomes of *E. feeders* compared to the rat. As many NSAIDs are frequently used to minimise inflammation or provide analgesia in koalas and other marsupials (de Kauwe et al., 2014), this *in-vitro* result has clinical implications. Accordingly, it demonstrates that many weakly acidic NSAIDs, except for indomethacin, are unlikely to have pharmacokinetic applicability due to rapid hepatic  $Cl_{int}$ , thus resulting in a faster  $Cl$  and shorter  $t_{1/2}$ , in these species. This result is supported by a previous study where tolbutamide hydroxylase activity (a common CYP2C9 marker) in the koala and brushtail possum was considerably more active than in the rat (or human) (Liapis et al., 2000) and also exemplified by the rapid 5-hydroxy-methylation of meloxicam in *E. feeders*, as described in the previous chapter of thesis, and this pathway is recognised to be metabolised by CYP2C9 in humans (Chesne et al., 1998).

In contrast to the other weakly acidic NSAIDs, there was relatively less difference in the *in-vitro*  $Cl_{int}$  of indomethacin between the rat and the specialist *E. feeders* [approximately two fold (ringtail possum) and three fold (koala) differences compared to the rat], whereas a similar *in-vitro*  $Cl_{int}$  in the brushtail possum vs. rat (10.4 vs. 11.8  $\mu\text{l}/\text{min}/\text{mg}$  protein, respectively), was demonstrated. Indomethacin is an indole acetic derivative (pKa of 4.5) where CYP2C9-catalysed *O*-demethylation is the major phase-1 metabolic pathway in the human, resulting in about 40-55 % of the drug being eliminated *via O*-desmethylin-domethacin into the urine (Nakajima et al., 1998), and the



plasma half life of indomethacin, after the oral administration, is reportedly 2 and 4 hr in human and rat, respectively (Hucker et al., 1966). For opossums (*Didelphis virginiana*), a generalist marsupial, the severity of radiation oesophagitis was significantly limited by indomethacin pretreatment through the inhibition of prostaglandin synthesis (Northway et al., 1980). Accordingly, a further PK (or disposition) study on indomethacin is warranted to evaluate its potential benefit on investigated marsupials. Moreover, as oral bioavailability of several drugs (e.g. enrofloxacin, marbofloxacin, and fluconazole), including meloxicam, appears poor in koalas (Griffith et al., 2010, Black et al., 2014), it is compelling to investigate rectally administered indomethacin as an alternative for oral administration for koalas and the marsupial species studied here, as it avoids rectal absorption bypasses hepatic first pass metabolism.

Although the percentage of substrate depletion of most CYP2C9 substrates was relatively similar between all *E.* feeders, a significantly higher turnover rate of tolbutamide was observed in the koala and ringtail possum compared to the brushtail possum (**Figure 5.6**). In contrast, meloxicam was rapidly depleted in brushtail possum. Consequently this study demonstrated that the patterns of depletion on the CYP2C9 substrates are different between these *E.* feeders (specialist vs. generalist). Tolbutamide is considered a low affinity substrate for CYP2C9 ( $K_m$  97-200  $\mu$ M), and is thus slowly metabolised in humans, and other species, including rats (Chauret et al., 1997a, Liapis et al., 2000). A previous study reported that the *in-vitro*  $Cl_{int}$  of tolbutamide in the koala was approximately ten fold higher than the brushtail possum or rat, where it is reported that 1,8-cineole (a constituent of *Eucalyptus* spp. foliage), induces tolbutamide

hydroxylation in brushtail possums (Liapis et al., 2000). Subsequently, the  $V_{\max}$  value for koalas was 5895-6403 nmol/mg per min compared to brushtail possums with a  $V_{\max}$  of 1406-1484 nmol/mg per min indicating that the amount of CYP involved in tolbutamide hydroxylation in koalas is higher than brushtail possums (Liapis et al., 2000). Therefore, as the diet of both koalas and ringtail possums is almost exclusively *Eucalyptus* spp. foliage, it is possible that foliage constituents, such as 1,8-cineole, influence the rapid depletion of tolbutamide in these specialist *E.* feeders. Of course another explanation for the high rates of tolbutamide hydroxylation in these species could be due to differences in CYP isoenzymes specific for tolbutamide metabolism (Jones et al., 2008). Despite tolbutamide hydroxylation being considered a common probe substrate to define CYP2C9 activity in humans and other species, it is unlikely to fulfill this role in the brushtail possum.

Overall, the metabolites of the CYP2C9 substrates were similar between the species, except for flurbiprofen (**Figure 5.3**) and meloxicam. Flurbiprofen ( $pK_a = 4.14$ ) is a lipophilic NSAID that belongs to the 2-arypropionic acid class (Risidall et al., 1978). It is reported that the major oxidative metabolite of flurbiprofen is 4-hydroxy flurbiprofen (FbM1), mostly metabolised by CYP2C9 in humans and CYP2C11 in rats, and can undergo further oxidation into 3', 4'-dioxxygenated- or 3'-hydroxy, 4'-methoxy-flurbiprofen (Shimizu et al., 2003). Another study indicated that the majority of flurbiprofen is metabolised into FbM1 in rats (Risidall et al., 1978). Although structural identification of metabolites was not performed, the UV spectra of FbM1 ( $\lambda_{\max} = 258$  nm, in MeCN) was similar to that reported previously for FbM1 which was detected in

methanol (MeOH) (Shimizu et al., 2003). It is therefore likely that the metabolite in the rat is 4-hydroxy flurbiprofen (**Figure 5.3**). Compared to the rat, with the substrate concentration of 1 $\mu$ M, the same metabolite was detected in marsupials, an additional metabolite was detected in the koala, and a different metabolite was produced in the brushtail possum. However, with the higher substrate concentration (10  $\mu$ M), the major metabolite produced was similar to that of the rat in both koala and brushtail possum (**Figure 5.8**). Thus, it is possible that those metabolites detected in the koala and brushtail possum are further oxidised products of FbM1.

This study demonstrated that degradation of NSAIDs and other CYP2C9 substrates by hepatic microsomes of *E. feeders* were generally at a greater rate than those of the rat. Subsequently, this study illustrates that species will affect the pharmacokinetic profile of xenobiotics such as NSAIDs, and the rate of metabolism must be considered with respect to dosage formulation. While *in-vitro*  $C_{int}$  of investigated substrates, e.g. diclofenac and tolbutamide, in the rat are comparable to other eutherians, such as humans or dogs (Nishimuta et al., 2013), this study suggests caution when extrapolating the dosage of CYP2C9 substrates, particularly weakly acidic NSAIDs, from eutherian species to other mammalian species such as marsupials. Instead, as similar metabolic patterns on depletion of CYP2C9 substrates were demonstrated between specialist *E. feeders*, this study speculates that exchanging drug dosages (CYP2C9 substrates) between these species is likely to have a more accurate outcome than those used from eutherians. Upon successful characterisation of general pattern of CYP2C-like activity, this study suggests using multiple substrates when comparing metabolic activity of CYP isoenzymes

between different species provides a more meaningful result compared to using a single CYP substrate.

There are several limitations in this study. As this was a preliminary study for screening, this study used hepatic microsomes extracted from a single animal per species and percentages of substrates depleted with an identical microsomal experimentation condition. Subsequently, this study used a single time point (marsupials = 10 min and rat = 15 min) to estimate *in-vitro*  $Cl_{int}$ . However, despite these limitations, this study has demonstrated that the *in-vitro* microsome assay is a valuable tool as a screen to select or omit candidate drugs, known to undergo hepatic phase 1 metabolism, prior to more invasive to *in-vivo* clinical trials. For example, the present result suggests indomethacin may be a potential therapeutic drug candidate for further investigating in these marsupials, particularly koalas.

# Chapter 6

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## General discussion and future directions

Prior to this research, no disposition studies of meloxicam (nor any other NSAID) had been conducted in koalas (a *E.* specialist feeder) despite being readily administered to this species, in the field (de Kauwe et al., 2014). Compared to the studied eutherians such as rats (Busch et al., 1998b), dogs (Montoya et al., 2004), horses (Toutain et al., 2004) and humans (Turck et al., 1996), the rapid *in-vivo* plasma *Cl* of meloxicam demonstrated in koalas (**Chapters 3**) was previously unknown and surprising. Subsequently, in order to investigate possible mechanism/s for this rapid *Cl* of meloxicam, an *in-vitro* hepatic microsomal metabolism model was optimised; indicating that meloxicam was rapidly eliminated likely *via* hepatic CYP mediated oxidation in koalas and as well as other *E.* feeders (**Chapter 4**). This study further identified that other weakly acidic NSAIDs were also rapidly depleted by hepatic microsomes of *E.* feeders (both by specialists and a generalist) compared to those of rats (**Chapter 5**).

This project used HPLC to evaluate the meloxicam concentration in koala plasma for PK studies (**Chapter 2**). A major challenge was the unique endogenous matrix in the koala plasma. Unexpected peaks on the chromatogram interfered with the detection of the drug and metabolite peaks and thus required the LC conditions to be customised for this species. Furthermore, a highly sensitive method was necessary to quantify low plasma drug concentrations from a restricted sample volume collected from koalas. Koalas are relatively small animals, with a body weight of less than 10 kg, therefore extensive withdrawal of blood over 24 to 48 h to conduct PK studies was not possible. The HPLC-PDA method developed and described in **Chapter 2** was highly sensitive, providing accurate quantification as low as 0.01 µg/mL (LLOQ) in the 250 µL of plasma sample.

Furthermore, as the established HPLC condition could detect and separate the hydroxyl metabolites of meloxicam, a comparative study of the *in-vitro* hepatic metabolism of meloxicam and metabolites between selected species was undertaken (**Chapter 4**).

The *in-vivo* PK study described in **Chapter 3** demonstrated that the currently registered (or suggested) oral and injectable dosages for dogs of 0.1 to 0.2 mg/kg (Montoya et al., 2004), or for other eutherians (such as cats, humans or horses) of 0.075 to 0.4 mg/kg (Turck et al., 1996, Toutain et al., 2004, Giraudel et al., 2005), were insufficient to provide bioequivalent plasma drug concentrations when administered to koalas due to rapid plasma *Cl* ( $0.44 \pm 0.2$  L/h/kg; average  $\pm$  SD); approximately 30 fold faster than that reported in male rats (0.015 L/h/kg) (Busch et al., 1998). Another important finding was the poor oral bioavailability demonstrated in koalas. This could be attributable to poor oral absorption and/or the significant hepatic first pass metabolism. This observation is an important revelation as oral administration is the preferential route to administer meloxicam due to its high bioavailability as seen in other eutherian species such as rats (Busch et al., 1998), cats (Grude et al., 2010), dogs (Busch et al., 1998), horses (Toutain and Cester, 2004) and humans (Turck et al., 1996). Administration of a meloxicam SC injection seemed more clinically relevant for koalas as this route has an improved bioavailability (approximately 56-70 % of the IV dose achieved in this species). In other eutherian species, meloxicam's SC bioavailability is almost complete in cats (Grude et al., 2010), dogs (Busch et al., 1998) and humans (Turck et al., 1996) and between 64-166 % in calves (Coetzee et al., 2009). However as SC administration with the canine dosage is unlikely to achieve steady state conditions due to the rapid plasma

*Cl* resulted in a short terminal  $t_{1/2}$  (median 1.19 h; range 0.71 to 1.62 h) in the koala, further investigation of a species-specific dosage of SC injection for koalas is necessary. Recently, a modified-release (that is delayed release) SC formulation of meloxicam has been studied which shown to provide both higher plasma drug concentrations and a steady state of two to three days in non-human primates resulting in a preferable PK profile than multiple IM administration or oral dosing (Bauer et al., 2014). Therefore it is warranted to investigate whether modified-release SC formulation may be beneficial to ameliorate prolonged pain or inflammation in this species.

The appearance of hydroxylated metabolites (M1, M2 and M3) of meloxicam in koala plasma provided evidence that oxidative pathways (likely hepatic phase-1 reactions) were involved in the elimination of meloxicam in this species. This observation was further confirmed in the *in-vitro* study, where multiple hydroxylated metabolites (M1, M2 and M3) of meloxicam were identified when incubated with koala hepatic microsomes (**Chapter 4**). Hepatic CYP mediated oxidation is reported as the initial elimination pathway of meloxicam, and M1 (5-hydroxymethyl metabolite) is the predominant metabolite identified in many other eutherian species (Busch et al., 1998). In humans, a CYP2C9 enzyme is recognised to facilitate 5-hydroxymethylation of meloxicam (Chesne et al., 1998). Accordingly, this study demonstrated that similar metabolic pathway, which likely facilitated *via* CYP2C-like enzyme, is involved in the elimination of meloxicam, and further additional hydroxylations of meloxicam (M2 and M3) were identified in koalas. Similar to koalas, this study further identified that in the other *E. specialist* feeder (ringtail possums) meloxicam undergoes multiple



hydroxylation (M2 and 3) but not in other eutherians (rats and dogs) or in the *E.* generalist (brushtail possums). In contrast, the metabolites of the other investigated NSAIDs and human CYP2C9 substrates were not greatly different between these species. Other studies have demonstrated that *E.* specialist feeders tend to rely on CYP mediated multiple oxidations to detoxify dietary terpenes (McLean et al., 2003, Foley and Moore, 2005). While essential oils, such as dietary terpenes, are highly concentrated in *Eucalyptus* spp. foliage, it was speculated that specialist feeders have evolved a more efficient phase-1 reactions to detoxify this group of PSMs (McLean and Foley, 1997). It is understandable that biotransformation pathway activity is driven by dietary composition, and this has been observed in other closely related species; such as the big-eared wood rat (*Neotoma macrotis*), an oak specialist feeder, that has greater phase-2 reaction activity to detoxify PSMs, which are mostly polyphenolics, compared with the desert wood rat (*Neotoma lepida*) a generalist feeder (Haley et al., 2007). Whether the qualitative difference in the hepatic oxidation (*via* CYP enzymes) of meloxicam in *E.* specialist feeders is a direct consequence of their dietary adaptation remains unproven. However, this study provides some insight that these *E.* specialist feeders may qualitatively possess a different metabolic strategy to eliminate some xenobiotics such as drugs.

Meloxicam was shown to be highly unstable in the liver of koalas as the *in-vitro*  $Cl_{int}$  was at least 16 fold faster than that of the rat or dog. This finding suggests that the fast rate of the phase-1 reaction, which is likely due to a greater catalytic activity of CYP2C-like enzyme(s) and possibly other CYP enzyme(s) involved, is the major factor associated

with the rapid plasma *Cl* of meloxicam seen in koalas. Other *E.* feeders were also shown to metabolise meloxicam faster than rats (*in-vitro*  $Cl_{int}$ :  $3.2 \pm 0.3$   $\mu\text{L}/\text{min}/\text{protein mg}$ ) or dogs; particularly between *E.* specialist feeders, the *in-vitro*  $Cl_{int}$  of meloxicam was comparable [ $50 \pm 41$  (koalas) vs.  $39 \pm 5$  (ringtail possums)  $\mu\text{L}/\text{min}/\text{protein mg}$ ]. Furthermore, this study demonstrated that the depletion of weakly acidic NSAIDs in *E.* feeders were generally faster than the rat, with the exception of indomethacin in the brushtail possum (for *E.* specialist feeders, indomethacin was relatively stable compared to the other NSAIDs). While the *in-vitro*  $Cl_{int}$  of meloxicam was much faster in brushtail possums ( $394 \pm 168$   $\mu\text{L}/\text{min}/\text{protein mg}$ ) than *E.* specialist feeders, this study speculates that CYP mediated metabolism of weakly acidic NSAIDs or CYP2C9 substrates has greater similarity between *E.* specialists compared to *E.* generalist feeder. Consequently, this research suggests that extrapolating drug dose rates of human CYP2C9 substrates between *E.* specialist feeders are likely to provide greater pharmacokinetic conformity than when compared to *E.* generalist feeders or other eutherians.

The *in-vivo* *Cl* of meloxicam in koala and the rat were reasonably well predicted from the relevant *in-vitro* data (rate of drug depletion) where the difference between actual *Cl* and predicted *Cl* were within an acceptable range (< two fold). For drugs that are predominately eliminated by hepatic metabolism, this IVIVE approach is frequently applied when deciding the initial dosage for human clinical drug trials (Houston, 1994, Obach et al., 1997, Ito and Houston, 2005) but has rarely been used for veterinary species. For many wild, rare, valuable and/or dangerous animal species, conducting an

*in-vivo* PK study, such as recruiting adequate numbers of animals or collecting blood samples at multiple time points etc, may be extremely challenging and difficult. While the *Cl* of drugs is an important PK parameter which affects the plasma drug concentration, this study demonstrated a successful application of *in-vitro* hepatic  $Cl_{int}$  to predict, or evaluate *in-vivo* elimination of meloxicam. This exemplified that *in-vitro* models may be useful to evaluate other therapeutic drugs for koalas (or other exotic and wild species) and thereby minimise the number of *in-vivo* studies. While collecting blood sampling at multiple time points for conventional *in-vivo* PK studies can be an extremely challenging procedure for some species, population PK modeling may be considered as a potential alternative to traditional PK study as it can estimate population PK parameters from a conservative number of individuals or alternatively it may estimate population PK parameters from 'sparse' blood sampling (that is predict PK concentration vs. time curves from amalgamating one or a few time points from each individual), although many more individuals are required (Wright, 1998).

In contrast to the other weakly acidic NSAIDs, indomethacin was relatively stable in hepatic microsomes of all the *E. feeders* which may have longer lasting anti-inflammatory or analgesic effects. Particularly for the *E. generalist* (brushtail possum), the stability of indomethacin was comparable to that in the rat. In the case of the koala, where the oral absorption of drugs are relatively unpredictable as seen from enrofloxacin and fluconazole oral absorption studies (Griffith et al., 2010, Black et al., 2013a) and meloxicam; indomethacin may warrant further *in-vivo* investigation due to its availability as a suppository for rectal (in the case of marsupials, cloacal)

administration, although its drug permeability *and* whether it bypasses the portal vein and consequent first pass metabolism is not known in koalas. In humans, drugs delivered by suppositories are transported *via* the middle and inferior rectal veins into the inferior vena cava, with the drug easily transported into the systemic circulation likely to avoid hepatic first pass metabolism (Prasanna et al., 2012). The bioavailability of indomethacin delivered by suppositories in humans is approximately 80 % (Jensen and Grenabo, 1985).

Hepatic microsomal stabilities of human CYP2C9 substrates were observed to be similar between *E. feeders*; however, tolbutamide was exceptionally unstable in the *E. specialist feeders*. Although, induction of tolbutamide hydroxylation was reported when 1, 8-cineole was fed to brushtail possums (Liapis et al., 2000), it is warranted to further justify whether this is also a typical metabolic characteristic of CYP2C-like isoform(s) between all *E. specialist feeders*, such as the greater glider (*Petauroides volans*) as a result of their diet. On the other hand, tolbutamide is considered a marker substrate (as well as diclofenac) to represent human CYP2C9 activity (Miners and Birkett, 1998) and has been used to evaluate CYP2C-like activity in other species (Veronese et al., 1990, Liapis et al., 2000). In the case of the brushtail possum, tolbutamide stability (as well as that of indomethacin) did not correlate with other human CYP2C9 substrates (meloxicam, diclofenac and flurbiprofen). This study highlights that the use of multiple probe substrates and examination of metabolic patterns provided a more accurate picture of the potential activity of CYP2C-like enzymes in the brushtail possum. Creation of metabolic 'patterns' sourced from multiple substrates as demonstrated in **Chapter 5**

page 142, provides further information to estimate and compare CYP activity between different species. Multiple CYP2C isoforms [CYP2C47 and CYP2C48 (Jones et al., 2008a)] have been identified, however their involvement in the metabolism of meloxicam (or other NSAIDs) have not been studied as yet. CYP isoforms will be required to confirm whether one or more CYP2C isoforms are responsible for the multiple hydroxylations on meloxicam seen in koalas.

This study focused on aspects of the PK of meloxicam in koalas. However, it is acknowledged that integrating PD information with the PK profile is mandatory for efficacious dosage formulation. This research evolved to confirm and explain the PK observations of rapid intrinsic *Cl* seen in koalas and other marsupials. Thus, future studies examining the plasma concentration for therapeutic efficacy of this drug in koalas, and perhaps other marsupials, will be required to establish an accurate dosages specific for these species. The plasma drug concentrations required to ameliorate inflammation (and/or pain) varies between eutherians (e.g. dogs, horses and humans are 0.82, 0.13 to 0.2, and 0.57 to 0.93 µg/mL, respectively) (Montoya et al., 2004, Toutain and Cester, 2004, Turck et al., 1996). The common method to evaluate pharmacological activity of NSAIDs is to determine the selectivity of COX inhibition of prostaglandin formation in whole blood *in-vitro* (Blain et al., 2002). But *in-vitro vs ex-vivo* results are often highly variable for some NSAIDs including meloxicam (Blain et al., 2002). Therefore, the clinical relevance of an *in-vitro* assay using whole blood has not been clarified for meloxicam (Blain et al., 2002). In this regards, an *in-vivo* study, utilising an *ex-vivo* whole blood, should be considered cautiously to determine the *in-*

*vivo* pharmacological activity of meloxicam. This raises an interesting issue: several dietary terpenes, such as 1, 8-cineole or others, abundant in *Eucalyptus* spp. foliage are also known to possess inherent anti-inflammatory- and analgesic activities (Silva et al., 2003), therefore it is interesting to speculate whether the inflammatory or pain threshold in koalas, and other *E.* feeders, is higher due to the anti-inflammatory mediators present in their diet? Another study indicated that an oral dose (400 mg/kg) of 1, 8-cineole reduced carrageen induced paw oedema in rats by 46% (vs. 64 % inhibition when 5 mg/kg of indomethacin was administered orally). The analgesic effect was of an amplitude similar to SC administration of morphine (10 mg/kg) when the same dose of 1, 8-cineole was administered to mice (Santos and Rao, 2000). As koalas consume up to 10 kg of *Eucalyptus* spp. foliage every day, and likely exposed to substantial amounts of 1, 8-cineole (or other dietary terpenes), it is of great future interest to investigate the role of the almost exclusive *Eucalypt* spp. diet has on baseline pharmacological analgesic and inflammatory activity.

Limitations of this study were the unknown hydroxyl metabolites (M2 and M3) were not structurally identified, the metabolic pathway responsible for their formation was not identified, and the penultimate metabolites in urine and faeces were not identified, due to lack of access to equipment with greater sophistication and capacity such as LC-MS/MS or nuclear magnetic resonance (NMR).

Generation of M1, M2 and M3 initiated additional small pilot studies in order to appreciate their significance. When meloxicam was co-incubated with an essential oil or its constituent (such as 1, 8-cineole and palmitate) in hepatic microsomes of koalas,

some inhibition on the formation of hydroxylated metabolites (M1-M3) was apparent, however the pattern of inhibition varied according to the co-substrate (**Appendix 2**). For example, 1, 8-cineole tended to inhibit formation of M3; whereas M1 was specifically inhibited by palmitate (inhibition concentration (IC) of 50 % on M1 was achieved at 50  $\mu$ M). Both 1, 8-cineole and palmitate are known to inhibit CYP3A4 activity (additionally CYP3A5 activity for 1, 8-cineole) in humans (Dresser et al., 2002, Duisken et al., 2005). These pilot studies are mentioned here as the factors that influence the transformation of xenobiotics such as meloxicam, 1, 8-cineole or palmitate concentrations etc are a key to understanding how koalas eliminate plant secondary metabolites (PSMs) in their natural diet which is not only important from evolutionary and comparative pharmacology perspectives but also in relation to koalas' capacity to cope with environmental toxins such as spray-drift from agricultural chemicals and changes in foliage composition as a result of land use practices and global climate change (ecotoxicology).

In conclusion, findings from this research demonstrate that meloxicam is eliminated rapidly in koalas compared to conventional veterinary species and the dosages of other eutherians are inadequate for koalas (probably other ringtail possums and brushtail possums as well), and it is imperative that this is kept in mind when extrapolating the dosages of other NSAIDs (or human CYP2C9 substrates) from eutherians to these *E.* feeders. Instead, it is more likely to be appropriate to relate the dosages between similar species (e.g. between *E.* specialist feeders), if relevant PK data is unavailable. This study demonstrates species differences on CYP2C-like activity in koalas (also for other *E.*

feeders), which is the major source for rapid  $Cl$  of meloxicam, and accordingly this research introduces the benefit of utilising *in-vitro* data (*in-vitro*  $Cl_{int}$ ) to evaluate PK profile (e.g.  $Cl$ ) for meloxicam which also applicable for drugs where hepatic phase 1 reaction is the predominant elimination pathway.



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## **Appendix One)**

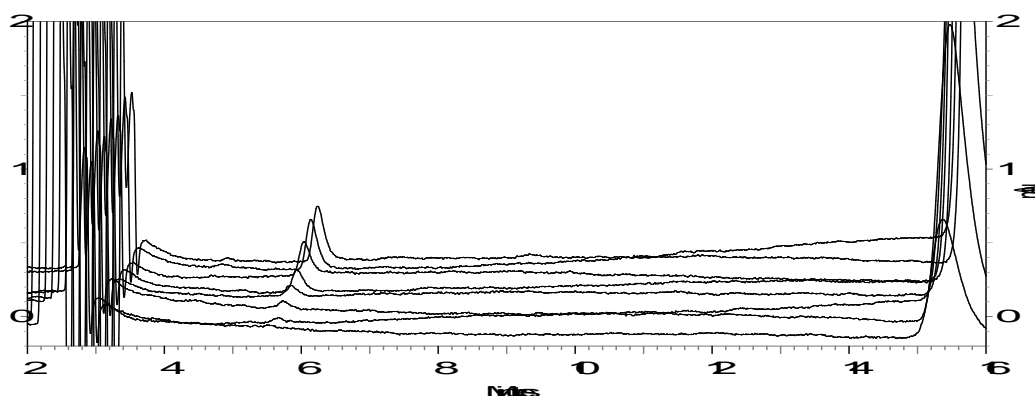
### **Determination of $K_m$ values of meloxicam metabolite(s) for individual species: a preliminary study.**

In order to extrapolate *in-vitro*  $Cl_{int}$  data to *in-vivo*  $Cl$  by substrate depletion method (**Chapter 4**), it was essential to use substrate concentration at less than overall  $K_m$  value to determine appropriate depletion rate (as intrinsic  $Cl$  is determined by  $[S] < K_m$  approximate  $V_{max} / K_m$ ). Therefore the study was conducted, with both product formation and/or multiple substrate depletion methods, to confirm whether 1.25  $\mu\text{M}$  of meloxicam was  $< K_m$  for each species.

#### **1) Determination of approximate $K_m$ values of 5-hydroxymethyl metabolite (M1) and as well as M3 in koala and ringtail possum.**

The final incubation volume (phosphate buffer including NADPH regenerating system described in **Chapter 4, Section 4.3.3**) was 0.5 mL and was executed with eight meloxicam concentrations (2.5 to 400  $\mu\text{M}$ ). The reaction was terminated by adding 250

$\mu\text{L}$  of iced-cold methanol after incubating 5 min with liver microsomes of pooled brushtail possum ( $n = 3$ ; 0.125 mg/mL); 10 min with pooled koalas ( $n = 6$ ; 0.25 mg/mL) and ringtail possums ( $n = 2$ ; 0.25 mg/mL) and 30 min with pooled rats (0.5 mg/mL) and dogs (0.5 mg/mL) (**Figure A.1**), respectively.

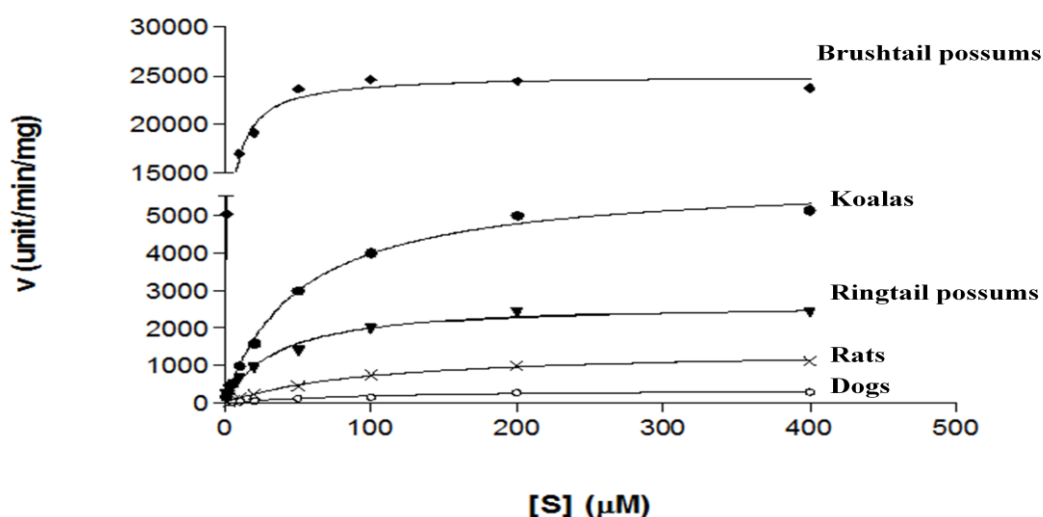


**Figure A.1)** Representative HPLC-UV chromatograms (described in **Chapter 2, Section 2.3.2** except that composition of MeCN in the mobile phase was 55 %), monitored at wavelength of 355 nm, of 5 hydroxymethyl metabolite (M1) generated during the incubation (30 min) of meloxicam with liver microsomes of dogs (0.5 mg/mL); substrate concentrations of 1.25  $\mu\text{M}$  (bottom) to 400  $\mu\text{M}$  (top); retention time for M1 and meloxicam are  $\sim 5.6$  min and  $\sim 15$  min, respectively.

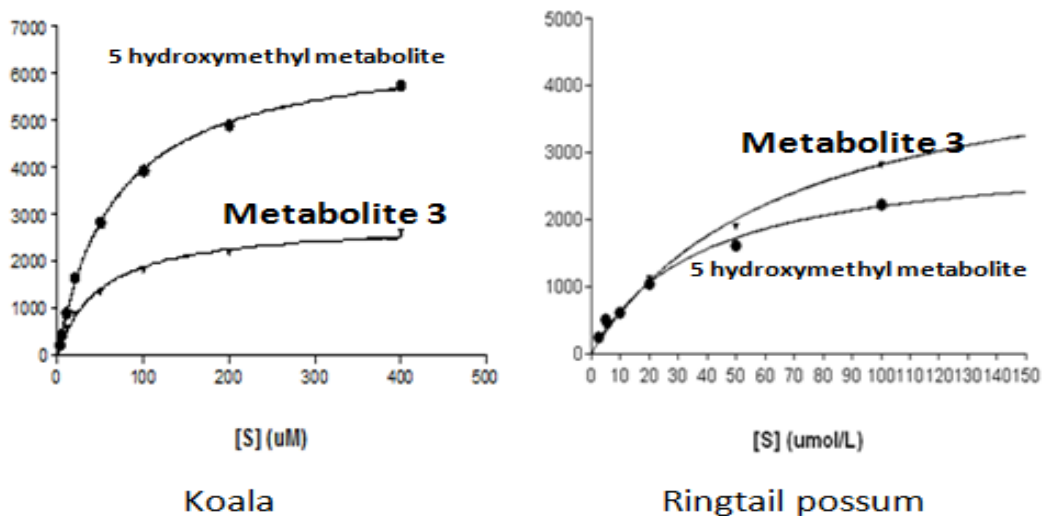
From the pilot study, metabolite(s) formed in relation to designated incubation time and microsome concentrations for each species were linear and  $< 20\%$  of substrate was depleted. Estimation of apparent  $K_m$  ( $\mu\text{M}$ ) and  $V_{\max}$  (expressed as arbitrary units) of metabolite(s) were determined by Graph Pad Prism, 6.01 (Graph Pad Software, Inc., CA, USA) using one-enzyme Michaelis-Menton model using nonlinear regression analysis.

Apparent  $K_m$  values ( $\mu\text{M}$ ) of M1 for each species (**Figure A. 2**) were: 1) Dog:  $102 \pm 19.67$   $\mu\text{M}$  (Mean  $\pm$  S.E; 95% confidence intervals: 53-150  $\mu\text{M}$ ); 2) Rats:  $95.89 \pm 6.57$   $\mu\text{M}$ ; 79-

112  $\mu\text{M}$ ); 3) Koalas:  $49.74 \pm 3.75 \mu\text{M}$ ; 40-58  $\mu\text{M}$ ); 4) Ringtail possums:  $35.16 \pm 6.13 \mu\text{M}$ ; 20-49  $\mu\text{M}$ ); 5) Brushtail possums:  $5.23 \pm 0.42 \mu\text{M}$ ; 4-6  $\mu\text{M}$ ). Apparent  $K_m$  values of M3 in koalas and ringtail possums (**Figure A. 3**) were: 1) Koalas:  $34.56 \pm 3.27 \mu\text{M}$ ; 26-42  $\mu\text{M}$ ); 2) Ringtail possums:  $86.63 \pm 9.21 \mu\text{M}$ ; 64-108  $\mu\text{M}$ ). Thus, the results indicated that the meloxicam concentration of 1.25  $\mu\text{M}$  was adequate to use for the measurement of depletion rates in possums, rats and dogs. However, using the product formation method, the study was unable to approximate  $K_m$  for M2 in koalas.



**Figure A.2)** Formation of 5-hydroxymethyl metabolite (M1) in investigated species' microsomes.



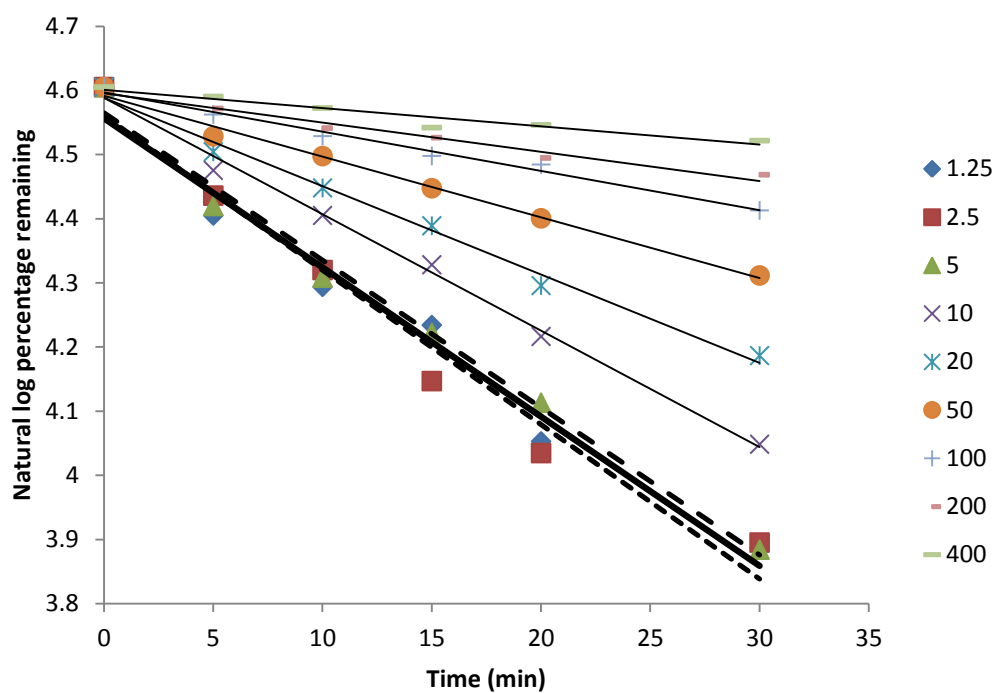
**Figure A.3)** Formation of M1 and 3 in koala and ringtail possum microsomes.

## 2) Determination of depletion rate constant of meloxicam in koalas by substrate depletion method.

Using one koala (liver microsome, 0.5mg/mL), the study further investigated if 1.25  $\mu\text{M}$  of meloxicam was adequate to represent the rate constant for this species. This was done *via* measurement of depletions of multiple substrate concentrations (0.15-400  $\mu\text{M}$ ). Meloxicam depletion concentrations (expressed as log substrate remaining) vs. incubation times (up to 30 min) is depicted in (**Figure. A. 4.**), whereas measured depletion rate constant ( $K_{\text{dep}}$ ) for each substrate concentrations is depicted in **Table A.**

1. Accordingly, a theoretical maximum depletion constant ( $K_{\text{dep}} ([s] = 0)$ ) was determined using linear log plot ( $K_{\text{dep}}$  vs. substrate concentrations) (**Figure A. 5**); substrate concentrations up to 100  $\mu\text{M}$  were used as substrate depletion higher than this (100  $\mu\text{M}$ ) was < 20 % (compared to initial  $t = 0$  min). According to the preliminary study, result ( $K_{\text{dep}} ([s] = 0) = 0.0235$ ) indicates that use of 1.25  $\mu\text{M}$  (depletion rate = 0.234)

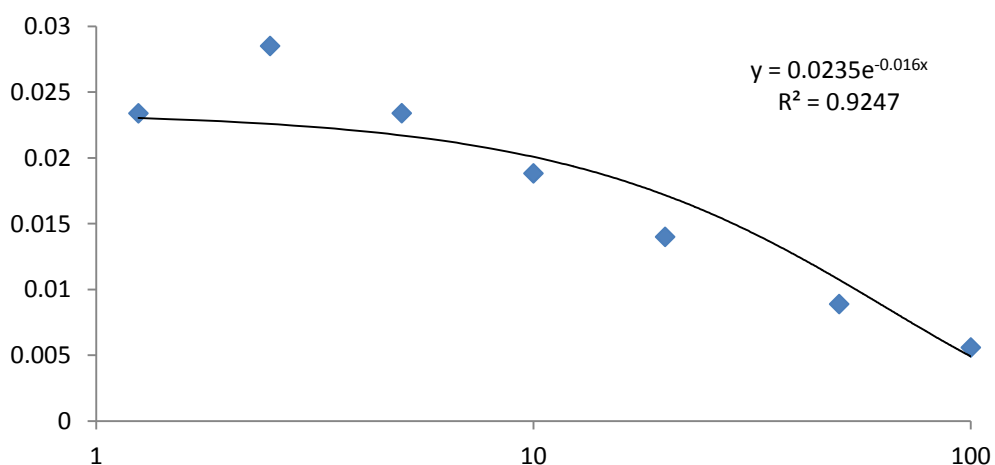
was likely to represent rate constant value. Therefore, the study (**Chapter 4**) used 1.25  $\mu\text{M}$  of meloxicam which likely to be  $< K_m$  or adequate to represent rate constant for investigated species.



**Figure A. 4)** Meloxicam depletion concentrations (expressed as log substrate remaining) vs. incubation time; Method: Substrate (meloxicam) concentrations used for depletion assay were 1.25, 2.5, 5, 10, 20, 50, 100, 200 and 400  $\mu\text{M}$ , and 0.5 mg/mL of koala microsomes ( $n = 1$ ) and incubation time was up to 30 min. Other methodology is same as described in the **Chapter 4, Section 2.3**.

**Table A. 1)** Measured depletion rate constants (K dep) for each substrate concentrations.

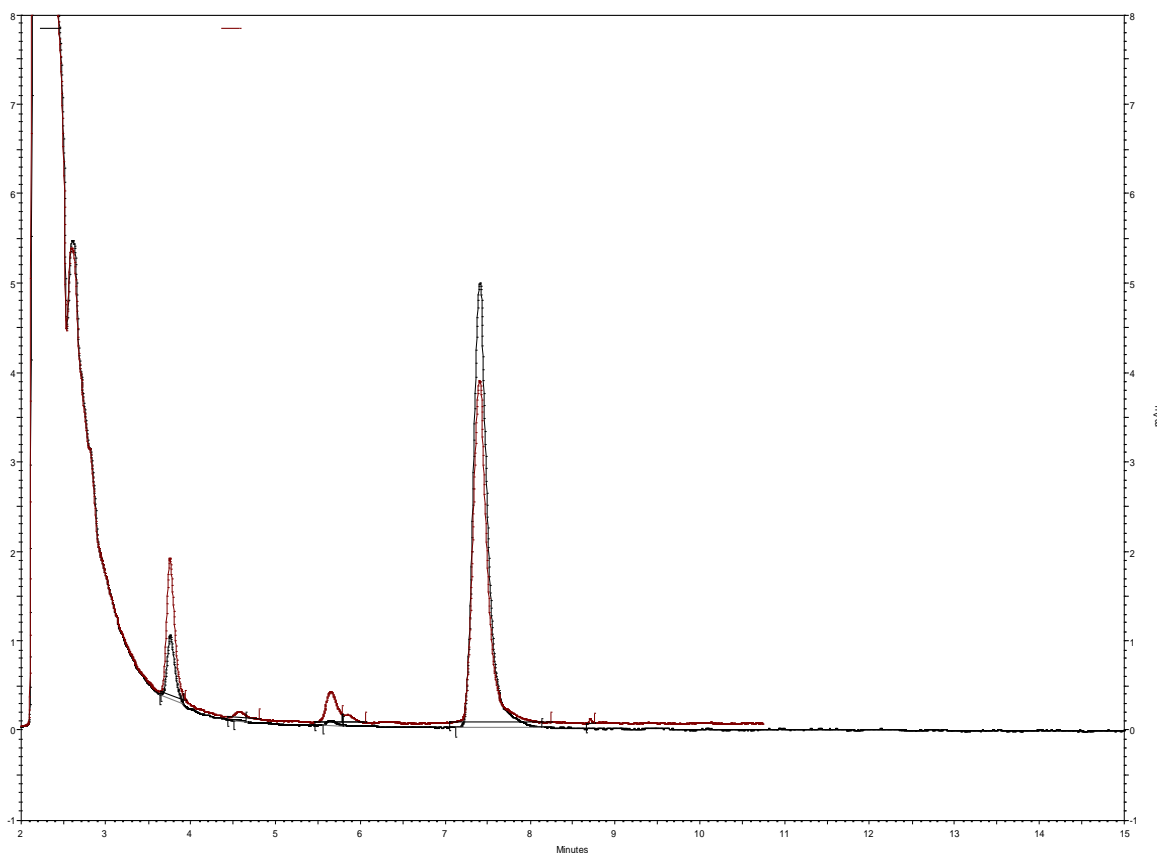
Substrate Concentrations ( $\mu\text{M}$ )	Depletion rate constant ( $\text{min}^{-1}$ )
1.25	0.0234
2.5	0.0285
5	0.0234
10	0.0188
20	0.0140
50	0.0089
100	0.0056
200	0.0036
400	0.0022



**Figure A. 5)** Linear log plot (K dep vs. substrate concentrations); X axis: K dep ( $\text{min}^{-1}$ ); y axis: substrate concentrations ( $\mu\text{M}$ ); substrate concentrations up to 100  $\mu\text{M}$  were used as substrate depletion higher than this (100  $\mu\text{M}$ ) was < 20 % (compared to initial  $t = 0$  min).

## **Appendix Two) Inhibition of the formation of meloxicam metabolites in the koala microsomes by 1, 8-cineole and palmitate**

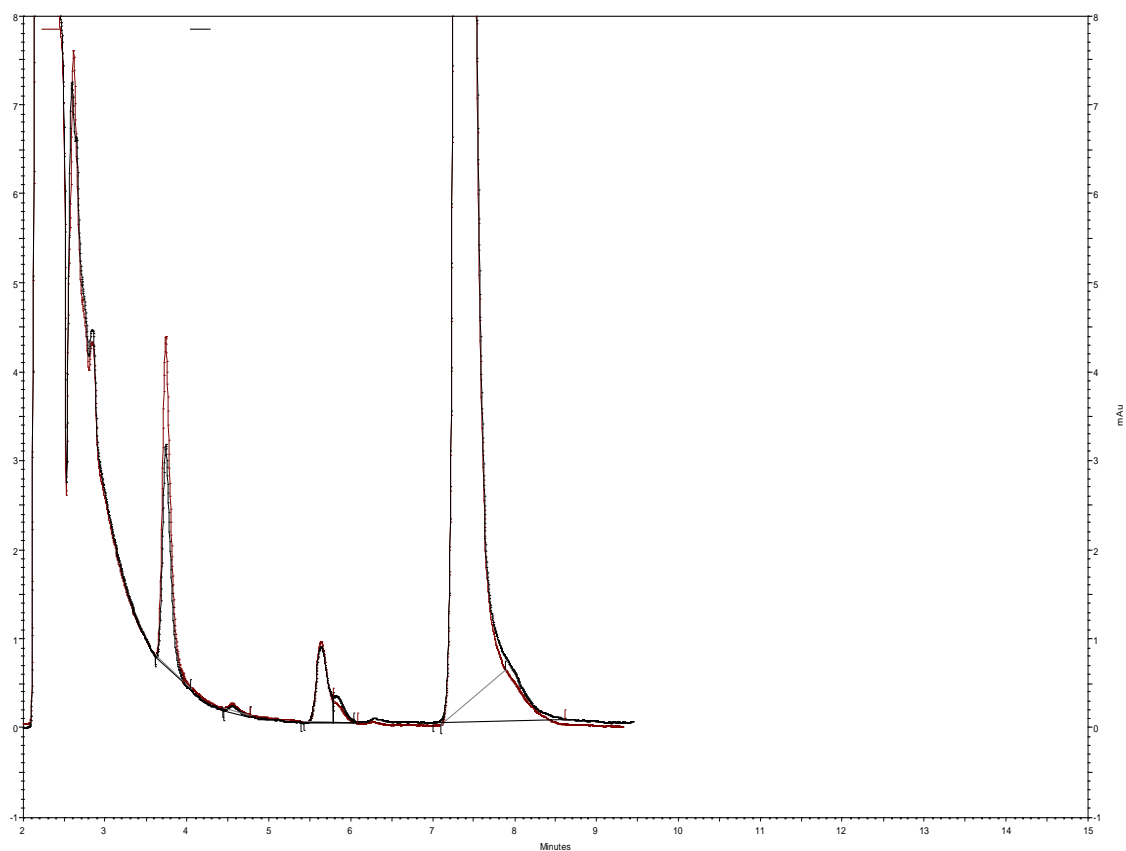
In the preliminary study, inhibition of meloxicam metabolites in the koala microsomes ( $n = 1$ , 1 mg/mL) was investigated. The final incubation volume (phosphate buffer including NADPH regenerating system described in **Chapter 4, Section 4.3.3**) was 0.5 mL consisted of 5  $\mu$ M of meloxicam (control) or also co-incubated with 1, 8-cineole (40, 200 and 1000  $\mu$ M) for 30 min. After the incubation, the reaction was terminated by adding 250  $\mu$ L of iced-cold methanol and analysed by HPLC-DAD (described in **Chapter 2, Section 2.3.2**). Compared to the control, the production of M2 and M3 was inhibited by presence of 1, 8-cineole (40 and 200  $\mu$ M), approximately 36 % and 54%, respectively. On the other hand, production of M1 was not altered with these 1, 8-cineole concentrations (inhibition of approximate 50 % was achieved at 1000  $\mu$ M) (**Figure A. 6**).



**Figure A. 6)** HPLC-UV chromatograms of meloxicam metabolites in the koala microsomes (1 mg/mL) after incubating 5  $\mu$ M of meloxicam (Brown) or co-incubated with 1000  $\mu$ M of 1, 8-cineole (Black); M1 (Rt: 3.6 min); M2 (Rt: 4.5 min); M3 (Rt: 5.6); meloxicam (Rt: 7.4 min).

The inhibition of meloxicam metabolites in the koala microsomes ( $n = 1$ , 1 mg/mL) with the presence of palmitate (12.5, 25 and 50  $\mu$ M) was also investigated. The condition was same as above, except 50  $\mu$ M of meloxicam was used. Compared to the control, about 50 % of M1 production was inhibited when co-incubated with 50  $\mu$ M of palmitate. However, production of M2 or M3 was not altered with these palmitate concentrations (IC 50 was achieved at 1000  $\mu$ M) (**Figure A. 7**).





**Figure A.7)** HPLC-UV chromatograms of meloxicam metabolites in the koala microsomes (1 mg/mL) after incubating 50  $\mu$ M of meloxicam (Brown) or co-incubated with 50  $\mu$ M of 1, 8-cineole (Black); M1 (Rt: 3.6 min); M2 (Rt: 4.5 min); M3 (Rt: 5.6); meloxicam (Rt: 7.4 min).

## Appendix Three) Acknowledgment of contribution to the research project and/or authorship

### Acknowledgment of contribution to the research project and/or authorship

Re the publication entitled 'Quantitation of meloxicam in the plasma of koalas (*Phascolarctos cinereus*) by improved high performance liquid chromatography'

I, Benjamin Kimble (PhD candidate), was the chief investigator of the research documented in this publication. I was the primary liaison among all co-authors; I performed the study design, analysis and writing of this publication.

Drs Merran Govendir and Kong Li assisted the project and provided assistance finalising the manuscript prior to publication.

Benjamin Kimble  Date 4 June 2015

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

Merran Govendir  Date 2 June 2015

Kong Li  Date 1-Jun-2015

**Acknowledgment of contribution to the research project and/or authorship**

Re the publication entitled “Pharmacokinetics of meloxicam in koalas (*Phascogale cinereus*) after intravenous, subcutaneous and oral administration”



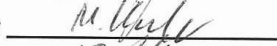

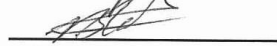

I, Benjamin Kimble (PhD candidate), was the chief investigator of the research documented in this publication. I was the primary liaison among all co-authors, I performed all the sample analyses, data analyses, was the primary author of all drafts of the manuscript, and was the chief respondent to the journal referees’ comments.

Drs Merran Govendir, Kong Li, Mark Krockenberger and Damien Higgins assisted the project as supervisors and finalising the manuscript for publication.

Lisa Black, Sam Gilchrist and Amber Gillett assisted the project with treating koalas with the drug and collecting blood samples.

Peter Valtchev assisted the project with liquid chromatography-mass spectrometry (LC-MS) work.

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

Merran Govendir		(sign)	<u>2 June 2015</u> (Date)
Kong Li		(sign)	<u>1-Jun-2015</u> (Date)
Mark Krockenberger		(sign)	<u>3/6/15</u> (Date)
Damien Higgins		(sign)	<u>4/6/15</u> (Date)
Lisa Black		(sign)	<u>14/1/13</u> (Date)
Sam Gilchrist	_____	(sign)	_____ (Date)
Amber Gillett	_____	(sign)	_____ (Date)
Peter Valtchev		(sign)	<u>21/01/2015</u> (Date)

**Acknowledgment of contribution to the research project and/or authorship**

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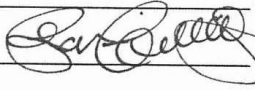
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Mark Krockenberger	_____	(sign)	_____	(Date)
Damien Higgins	_____	(sign)	_____	(Date)
Lisa Black	_____	(sign)	_____	(Date)
Sam Gilchrist		(sign)	3/6/15	(Date)
Amber Gillett	_____	(sign)	_____	(Date)
Peter Valtchev	_____	(sign)	_____	(Date)

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Damien Higgins	_____	(sign)	_____	(Date)
Lisa Black	_____	(sign)	_____	(Date)
Sam Gilchrist	_____	(sign)	_____	(Date)
Amber Gillett		(sign)	15/1/13	(Date)
Peter Valtchev	_____	(sign)	_____	(Date)

**Acknowledgment of contribution to the research project and/or authorship**

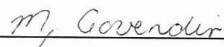
Re the publication entitled 'In-vitro hepatic microsomal metabolism of meloxicam in koalas (*Phascolarctos cinereus*), brushtail possums (*Trichosurus vulpecula*), ringtail possums (*Pseudocheirus peregrinus*), rats (*Rattus norvegicus*) and dogs (*Canis lupus familiaris*)'

I, Benjamin Kimble (PhD candidate), was the chief investigator of the research documented in this publication. I was the primary liaison among all co-authors; I performed the study design, analysis and writing of this publication.

Drs Merran Govendir and Kong Li, Mark Krockenberger and Damien Higgins assisted the project and provided assistance finalising the manuscript prior to publication. Peter Valtchev assisted the project with liquid chromatography-mass spectrometry (LC-MS) work.

Benjamin Kimble  Date 4 June 2015

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

Merran Govendir  Date 1 June 2015

Kong Li  Date 1 June 2015

Mark Krockenberger  Date 3/6/15

Damien Higgins  Date 4/6/15

Peter Valtchev  Date 11/06/2015