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# EFFECT OF CYTOCHROME P450 INHIBITION ON PHARMACOKINETICS AND TOXICITY OF DICLOFENAC IN CHICKENS: UNRAVELLING TOXICITY IN GYPS VULTURES

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**EFFECT OF CYTOCHROME P450 INHIBITION ON PHARMACOKINETICS AND TOXICITY OF DICLOFENAC IN CHICKENS: UNRAVELLING TOXICITY IN GYPS VULTURES**

BY

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**SUPERVISOR: PROFESSOR VINNY NAIDOO**

## DECLARATION OF ORIGINALITY

By submitting this thesis, I declare that the entirety of the work contained herein is my own original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by the University of Pretoria will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

The experimental work reported in this dissertation was carried out in the section of Pharmacology and Toxicology of the Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the expert supervision of Professor Vinny Naidoo.

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DR. SARA LINDSEY LOCKE

_____

PROF. VINNY NAIDOO (SUPERVISOR)

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## ABSTRACT

The nonsteroidal anti-inflammatory drug (NSAID) diclofenac was responsible for the decimation of *Gyps* vulture species on the Indian subcontinent over the last two decades of the 20$^{th}$ century. For an unknown reason, *Gyps* vultures were extremely sensitive to diclofenac ($LD_{50}$ ~ 0.1-0.2 mg/kg), with toxicity appearing to be linked to a metabolic deficiency, demonstrated by the long $T_{1/2}$ (~12-17 h) and low Cl (0.0001-0.0002 L/h*kg). This was in striking comparison to other bird species such as the domestic chicken (*Gallus gallus domesticus*), where the $LD_{50}$ is ~10 mg/kg, the $T_{1/2}$ is ~1 h and the Cl values are ~0.1-0.2 ml/h*kg. The aim of this study was to determine if Cytochrome P450 2C9 (CYP2C9) homolog pharmacogenomic differences among avian species is driving diclofenac toxicity in *Gyps* vultures. For this evaluation, we exposed each of 10 CYP-inhibited (fluconazole) test group domestic chickens to a unique dose of diclofenac, centred on the $LD_{50}$ of 9.8 mg/kg, as per OECD toxicity testing guidelines. The toxicity and pharmacokinetic results were compared to control group birds that received no fluconazole.

The birds showed typical clinical and post mortem signs of diclofenac toxicity; depression, lethargy and anorexia within 48 -56 h and visceral gout with varying degrees of nephrosis. Though no differences were noted in the $LD_{50}$ values for each group (11.92 mg/kg in the CYP-inhibited test group and 11.58 mg/kg in the control group), the pharmacokinetic profile of the test group was suggestive of partial inhibition of CYP metabolism. This was evident in the geomean values for $C_{max}$ (0.61 vs. 0.41 µg/ml), $AUC_{last}$ (0.5 µg/ml*h vs. 0.4 µg/ml*h) and clearance (1.52 L/h*kg vs. 1.59 L/h*kg), despite CYP-inhibited birds at the two highest doses succumbing without a definable pharmacokinetic curve. In contrast both birds dosed at the two highest doses from the control group demonstrated high $T_{1/2}$ and MRT values, consistent with expectations.

Evaluation of the metabolite peaks produced also suggested partial inhibition of CYP enzymatic metabolism in test group birds as they produced lower amounts of metabolites for one of the 3 peaks demonstrated and had higher diclofenac exposure. Furthermore, though the general trend was that birds that produced less metabolites and that died tended to be those dosed towards the higher end of the dose range, the results were not consistent. One bird in the test group, dosed at a much lower dose, exhibited very low metabolite production compared to birds in both treatment groups. This bird also exhibited pharmacokinetic data suggestive of metabolic constraint. These findings, coupled with the high variation in levels of metabolites produced across both treatment groups, indicates that there is a degree of natural variation in metabolism which is independent of dose in chickens, and which would also explain the higher $LD_{50}$ in the chicken in comparison to the vulture.

This pilot study supports the hypothesis that CYP metabolism is varied among bird species and may explain the higher resilience to diclofenac in the chicken vs. *Gyps* vultures. Further studies using a larger sample size and a single dose of diclofenac may provide more conclusive results.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| **AA** | Arachidonic acid |
| **ANOVA** | Analysis of variance |
| **ATP** | Adenosine triphosphate |
| **AWBV** | African white-backed vulture |
| **BNHS** | Bombay Natural History Society |
| **BW** | Body weight |
| **CGV** | Cape griffon vulture |
| **CINOD** | COX-inhibiting nitric oxide donor |
| **COX** | Cyclooxygenase |
| **CV** | Coefficient of variation |
| **CYP P450** | Cytochrome P450 |
| **DDT** | Dichlorodiphenyltrichloroethane |
| **EGV** | Eurasian griffon vulture |
| **EPA** | Environmental Protection Agency |
| **EPV** | Eco-pharmacovigilance |
| **ERA** | Environmental risk assessment |
| **EH** | Epoxide hydrolase |
| **FCR** | Feed conversion ratio |
| **FMO** | Flavin monooxygenase |
| **GI** | Gastrointestinal |
| **H&E** | Haematoxylin and eosin |
| **HETE** | Hydroxyeicosatetraenoic acid |

| | |
|---|---|
| **HPLC** | High performance liquid chromatography |
| **$K_m$** | Michaelis-Menton constant |
| **LBV** | Long-billed vulture |
| **LCMSMS** | Liquid chromatography-mass spectrometry-tandem mass spectrometry |
| **$LD_{50}$** | Lethal Dose that kills 50% of a population or median lethal dose |
| **LOD** | Limit of detection |
| **LOQ** | Limit of quantification |
| **LOX** | Lipooxygenase |
| **LTs** | Leukotrienes |
| **LXs** | Lipoxins |
| **NSAID** | Nonsteroidal anti-inflammatory drug |
| **OAT** | Organic anion transporter |
| **OECD** | Organisation for Economic Co-operation and Development |
| **OWBV** | Oriental white-backed vulture |
| **PAH** | p-amino-hippuric acid |
| **PCT** | Proximal convoluted tubule |
| **PG** | Prostaglandin |
| **$PGI_2$** | Prostacyclin |
| **PIE** | Pharmaceuticals in the environment |
| **ROS** | Reactive oxygen species |
| **RTE** | Renal tubular epithelium |
| **SAVE** | Saving Asian Vultures from Extinction |
| **SFS** | Supplementary feeding station |

| | |
|---|---|
| **SBV** | Slender-billed vulture |
| **$TXA_2$** | Thromboxane $A_2$ |
| **UDP** | Uridine Disphosphate |
| **UGT** | Uridine 5'-diphospho-glucuronosyltransferase |
| **UPBRC** | University of Pretoria Biological Research Centre |
| **VICH** | International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products. |
| **VSZ** | Vulture Safe Zone |
| **$T_{max}$** | Time to maximum plasma concentration |
| **$C_{max}$** | Maximum plasma concentration |
| **$AUC_{last}$** | Area under the curve to the last point quantified |
| **$AUC_{inf}$** | Area under the curve extrapolated to infinity |
| **$AUCM_{last}$** | Area under the moment curve to the last time point quantified |
| **$T_{1/2}$** | Terminal half-life of elimination |
| **MRT** | Mean residence time |
| **Cl** | Clearance |
| **$V_z$** | Volume of distribution during the terminal phase |
| **$V_{ss}$** | Volume of distribution during the steady phase |

# 1 INTRODUCTION

## 1.1 Background

The use of chemicals to exert a biological effect has long been understood and utilised by man. One of the earliest archaeological suggestions of plant use as a pharmacological remedy stems back almost 60 000 years, and medicinal scripts regarding plant use dating back to around 5000 years ago have been retrieved from India, China and Egypt (Fatemeh et al., 2018).

Today, it can be little disputed that the use of modern chemical compounds in the human and veterinary sector has had profound influence on health and disease management, agriculture and the economy at large. But the impact of pharmaceuticals on the environment has, on several occasions, been the price we have paid for these advancements. A historical, yet infamous example, is the pesticide DDT, dichlorodiphenyltrichloroethane. While controlling crop-destroying insects in the US and assisting with mitigating the spread of diseases like malaria in Africa, DDT had devastating effects on other wildlife species, most notably raptorial birds of prey such as the peregrine falcon (*Falco peregrinus*) and the bald eagle (*Haliaeetus leucocephalus*) (Rattner, 2009; Köhler & Triebskorn, 2013). More recently, modern pharmaceuticals used in human medicine, such as the frequently prescribed blood-lipid lowering statin and fibrate drugs have demonstrated adverse effects on aquatic species under laboratory conditions (Santos et al., 2010), bringing into question their threat to aquatic environments.

In the veterinary context, animal pharmaceuticals have not been exempted from their share of impact on the environment. More so a problem in food producing animals, and as an example, the organophosphate ectoparasiticide diazinon is highly toxic to earthworms and beneficial insects such as bees (Woodward, 2005). One of the biggest impacts of any veterinary drug has been that caused by the non steroidal anti-inflammatory drug (NSAID) diclofenac. Used for the relief of pain and inflammation in domestic ruminants on the Indian sub-continent, diclofenac decimated populations of *Gyps* vulture species in the region over a period of just 20 years, the so-called ‘Asian Vulture Crisis’ (Prakash et al., 2007).

Though the crises is not fully resolved, the combined efforts of governmental and non-governmental organisations, researchers and the general livestock-owning public have demonstrated that it is possible to reverse some environmental disasters; a recent publication indicated a partial recovery in numbers of birds for two vulture species (*Gyps bengalensis* and

*Gyps tenuirostris)* in an especially designated 'Vulture Safe Zone' in Nepal (Galligan et al., 2019).

The Asian Vulture Crisis highlighted environmental risk as an important aspect of drug and pesticide regulation. Unfortunately, even in developed economies, the environmental risk assessment (ERA) required for registration of both human and veterinary pharmaceutical products extends mainly to effects on soil and water organisms and does not cover possible effects on non-target higher life forms such as mammals and birds (VICH, 2000; VICH, 2004; EPA, 2012). Two organisations, the Environmental Protection Agency (EPA) and the Organisation for Economic Co-operation and Development (OECD, 1984; OECD, 1984; OECD) have developed guidelines for acute, dietary and reproductive toxicity testing in assessing pesticide risk to birds, but even these have not been shown to be predictive of safety to all birds, most likely because the indicator species used in toxicity testing do not accurately reflect the entire avian clade (Hassan et al., 2018). Indeed for drugs like diclofenac, marked differences in Median Lethal Dose ($LD_{50}$) values across bird species have been demonstrated; *Gyps* vultures are extremely sensitive with a $LD_{50}$ of 0.1-0.2 mg/kg, while the domestic chicken (*Gallus gallus domesticus*) is far more resilient with a $LD_{50}$ of 9.8 mg/kg, and finally the pigeon (*Columba livia domestica*) is relatively insensitive at doses as high as 616 mg/kg. The difference in $LD_{50}$ also appears to be correlated with the pharmacokinetic parameter, half-life of elimination ($T_{1/2}$), suggesting deficient metabolism of diclofenac as a cause of increased sensitivity. The $T_{1/2}$ is long in *Gyps* vultures (> 7 hrs) and short in the chicken, pied crow and pigeon (<7 hrs) (Naidoo et al., 2007; Naidoo et al., 2011; Hassan et al., 2018).

A plausible explanation for these striking inter-species differences could lie in the avian clade evolutionary history. While birds appear to have evolved from only 3 major groups (the Palaeognathae, the Galloanserae and the Neoaves) (Watanabe et al., 2013), they diverged greatly over the millennia into the thousands of species present today, and their ability to metabolise xenobiotic compounds (biologically active compounds that are foreign to the body) most likely diverged concurrently in accordance with difference of diet and habitat (Thomas, 2007; Watanabe et al., 2013; Hutchinson et al., 2014). Xenobiotic metabolism is divided into phase 1 and phase 2 reactions. These enzymatic processes serve, for the most, to reduce biological activity of a compound and facilitate its excretion by the body (Brunton et al., 2018). In examining the groups of enzymes responsible for these phases, the Cytochrome P450 (CYP) superfamily of the phase 1 reactions is by far the largest and most diverse across animal species (Hutchinson et al., 2014; Konstandi et al., 2014; Brunton et al., 2018).

A useful feature of CYP enzymes is that they are subject to inhibition of activity by chemical means. This feature can be exploited; inhibition of a specific enzyme by one drug

should alter the pharmacokinetics of another drug that is a substrate for the same enzyme. In humans, diclofenac is largely metabolised by phase 1 CYP2C9 activity (Leemann et al., 1993; Daly et al., 2007; Brunton et al., 2018). Homologous CYP2C9 enzymes have been found to be present in the domestic chicken (Kawalek et al., 2006; Watanabe et al., 2013; Shafi et al., 2015). In the case of *Gyps* vultures, it is hypothesized that their sensitivity to diclofenac may be as a result of a homologous CYP2C9 deficiency (Naidoo et al., 2010; Naidoo et al., 2011; Hutchinson et al., 2014).

## 1.2 Hypothesis

A deficiency in CYP2C9 functionality is the underlying mechanism for the poor metabolism of diclofenac in *Gyps* vultures.

## 1.3 Aim

To determine if pharmacogenomic differences among avian species is driving metabolism of diclofenac and its toxicity in *Gyps* vultures by evaluating the effect of a known CYP2C9 inhibitor on the $LD_{50}$ of the drug in the domestic chicken, a validated surrogate model.

## 1.4 Objectives

- To compare the $LD_{50}$ of diclofenac in the chicken when dosed alone or in combination with a CYP2C9 inhibitor.
- To compare the plasma pharmacokinetics of diclofenac in the chicken when dosed alone or in combination with a CYP2C9 inhibitor.
- To compare the uric acid plasma concentrations in the chicken when dosed with diclofenac alone or in combination with a CYP2C9 inhibitor**.**
- To evaluate and compare clinical and gross post mortem signs in chickens when dosed with diclofenac alone or in combination with a CYP2C9 inhibitor.

# 2 LITERATURE REVIEW

## 2.1 Non Steroidal Anti-Inflammatory Drugs (NSAIDs)

### *2.1.1 Overview*

The NSAIDs are a diverse group of chemical medicines that play an important role in the management of patients in various clinical situations. The NSAIDs have revolutionised the management of pain and fever in both humans and animals. From the use of willow bark tea (containing the natural compound from which aspirin is derived) for childbirth pain by Hippocrates in c400BC (Elwood, 2001), to the investigation of acetaminophen as an antipyretic medicine by Mering in 1893 (Brune et al., 2015), to the current coxib group of NSAIDs which achieve anti-inflammatory efficacy with less gastrointestinal (GI) side effects than their predecessors, the NSAIDs are a perfect example of the progression in pharmacological understanding and subsequent development of drugs to manage pain and inflammation.

### *2.1.2 Mechanism of Action*

The NSAIDs work largely through inhibition of the prostaglandin G/H synthase enzyme, commonly referred to as the cyclooxygenase or COX enzyme. This enzyme converts arachidonic acid (AA), formed from the esterification of phospholipids in the cellular membrane, to unstable intermediates such as prostaglandin $G_2$ or $H_2$. The ultimate products of the COX pathway are the prostanoids, including prostacyclin ($PGI_2$), thromboxane $A_2$ ($TXA_2$) and a variety of prostaglandins (PGs). The COX enzyme exists in 3 isoforms;

- COX-1 enzymes are expressed in most cells and are the major source of homeostatic prostanoids, responsible for normal physiological function such as haemostasis and vascular tone. They also stimulate mucous production in the gastric mucosa, protecting the stomach from auto-digestion and ulceration (Brunton et al., 2018).
- COX-2 enzymes are mainly inducible following injury and are the major source of prostanoids released during inflammation (Brunton et al., 2018).
- A splice variant of the COX-1 enzyme has been found in canines, rodents and humans, largely in neuronal and cardiac tissue. It has been named COX-3, COX-1b, or COX-1v and its role in both physiological and pathological responses remains controversial, though it has been postulated by some authors to be the site of action of some NSAIDs that lack traditional anti-inflammatory properties, such as acetaminophen (Chandrasekharan et al., 2002). Different NSAIDs appear to have different inhibition potencies for COX-3 (Kam & So, 2009).

There is also a second pathway of AA metabolism. This is the lipooxygenase (LOX) pathway, leading to the formation of hydroxyeicosatetraenoic acids (HETEs), leukotrienes and lipoxins (LXs). In a pathological setting, the COX and LOX pathways together are often termed the inflammatory cascade (figure 2.1).

Because of their use in pain and inflammation control, the NSAID industry is a progressive one. Newer generation NSAIDs, such as the coxib group mentioned above and drugs such as meloxicam and diclofenac, were postulated to achieve a safer 'adverse effect' profile through preferential inhibition of the inducible COX-2 enzyme. In human medicine, this has been somewhat disputed by the alternative side-effects of COX-2 inhibition; reduced PGI production and consequent potential prothrombotic events (Brunton et al., 2018). Perhaps more clinically appropriate use of the COX-2 inhibitors is therefore in the management of pain, fever and inflammation in patients at risk of GI complications (Brunton et al., 2018). One has also seen the development of drugs such as zileuton, a LOX-5 inhibitor and tepoxalin, a dual COX and LOX inhibitor (Agnello et al., 2005) (figure 2.1). Furthermore, novel drugs like the CINODs (COX-inhibiting nitric oxide donors) offer the anti-inflammatory benefits of COX inhibition, but also the protective benefits of nitric oxide donation, particularly in the kidney (Marcelo & John, 2006).

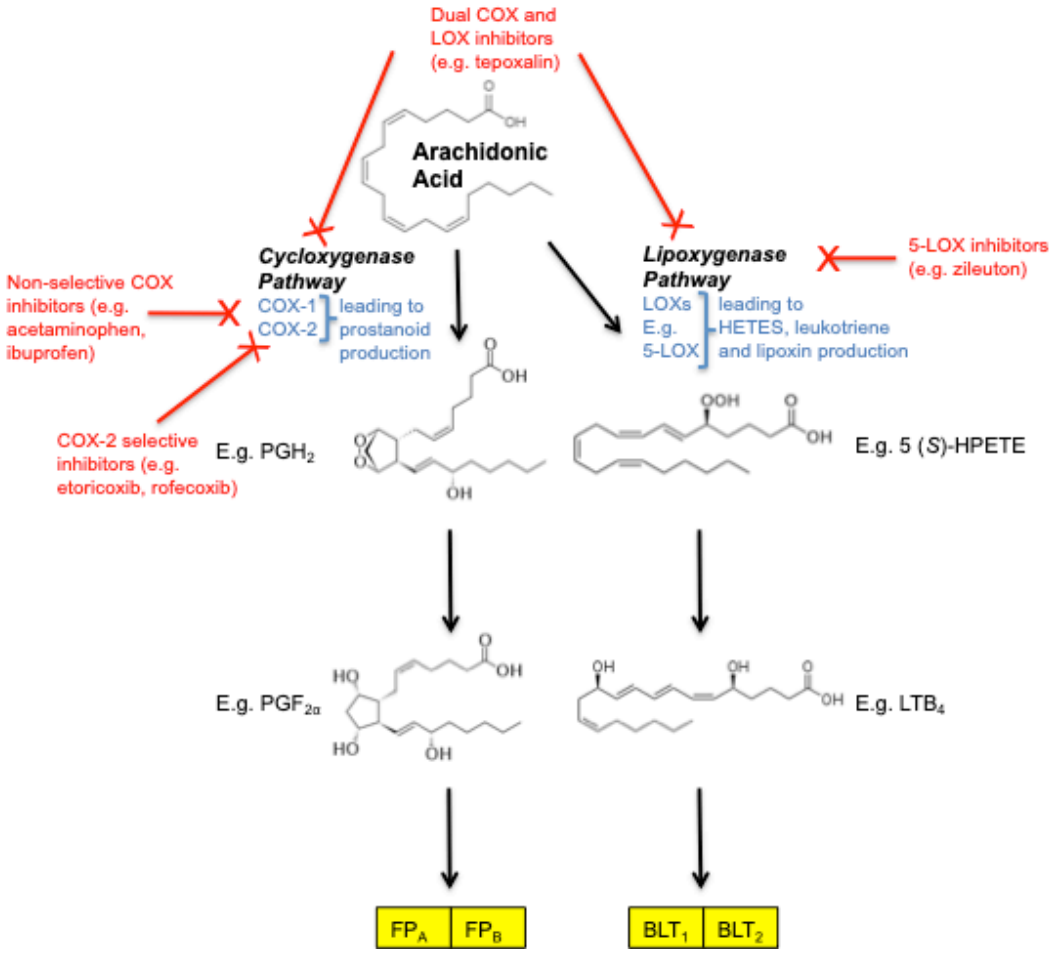


**Figure 2.1 The arachidonic acid cascade involved in inflammation, demonstrating the different selectivity of NSAIDs.**

Acetaminophen and other traditional NSAIDs are non-selective inhibitors of COX-1 and COX-2 enzymes. The coxibs such as etoricoxib and rofecoxib are more COX-2 selective inhibitors, in certain species. While neither of these affects LOX activity, zileuton inhibits LOX-5 activity and the dual 5-LOX–COX inhibitors such as tepoxalin interfere with both COX and LOX pathways.

Enzymes are shown in blue, pharmaceuticals and their site of enzyme inhibition are shown in red, receptors are shown in yellow [Adapted from (Brunton et al., 2018)].

$BLT_{1/2}$: Leukotriene $B_4$ ($LTB_4$) receptors, COX: Cyclooxygenase, $FP_{A/B}$: Prostaglandin $F_{2\alpha}$ receptors, HETE: Hydroxyeicosatetraenoic acid, HPETE: Hydroxyperoxyeicosatetraenoic acid, LOX: Lipoxygenase, $LTB_4$: Leukotriene $B_4$, $PGF_{2\alpha}$: Prostaglandin $F_{2\alpha}$, $PGH_2$: Prostaglandin $H_2$.

### *2.1.3 Adverse Effects*

NSAIDs are associated with adverse effects that relate directly to the inhibition of COX enzymes. Therefore, depending on the drug-specific COX-1 or -2 selectivity, GI ulceration, renal ischaemia and necrosis, and even cerebral and cardiovascular thromboembolic episodes may occur (Brunton et al., 2018). In addition, NSAIDs or their metabolites can be directly cytotoxic. This has been documented in both human and veterinary medicine:

- Phenylbutazone is toxic to humans, with the drug known to induce bone marrow hypoplasia, aplastic anaemia and pancytopaenia (Dewse & Potter, 1975).
- Acetaminophen is toxic to cats; a species-specific deficiency in phase 2 glucuronyl transferase metabolism allows accumulation of reactive oxygen species (ROS) metabolites produced during phase 1 Cytochrome (CYP) P450 metabolism, with resultant hepatotoxicity (Boothe, 2012). The CYPs are a good example of phase 1 enzymes, important for metabolism and excretion of xenobiotics. Phase 2 of metabolism follows CYP metabolism and is responsible for the attachment of an adduct onto the phase 1 molecule (please refer to section 2.6 for more detail on drug metabolism).

The example of acetaminophen being toxic to cats at doses that do not typically harm healthy dogs, introduces the concept of drugs having a species-specific safety profile. The environmental impacts of this would become apparent from the veterinary use of the NSAID diclofenac in the 1990s on the Asian sub-continent (please refer to section 2.4 and 2.5).

## 2.2 The NSAID Diclofenac

### *2.2.1 Overview*

Diclofenac is a phenylacetic acid derivative, best known under the trade name Voltaren®, and one of the most widely used NSAIDs in people because of its anti-inflammatory potency, which is substantially greater than other NSAID drugs (Brunton et al., 2018). Another reason for its common use is that it is a more COX-2 selective NSAID than the preceding generation of drugs, making is relatively safer to use.

In humans, the pharmacokinetics of diclofenac are fairly well understood. It is rapidly absorbed, with a $T_{max}$ of 1 h, extensively protein-bound (99%) and has a short half-life of elimination ($T_{1/2}$) of 1-2 h. Diclofenac also demonstrates a high first-pass effect; only 50% is available systemically after oral administration (Small, 1989; Brunton et al., 2018). It is predominantly metabolized in the liver (Aithal, 2004; Daly et al., 2007) and excreted in the urine and the bile (Brunton et al., 2018).

### 2.2.2 *Adverse Effects in Humans*

In humans, despite its relatively good safety profile, diclofenac can produce gastro-intestinal, hypertensive and myocardial adverse effects through inhibition of COX enzymes. Of equal concern is that at therapeutic doses, treatment may be complicated by severe hepatotoxicity, mainly due to the formation of the highly reactive metabolite diclofenac acylglucuronide, which forms hepatocellular protein adducts and elicits an immune-mediated reaction in the liver. There is evidence that genetic variation in humans can cause higher activity of the phase 2 UGT2B7 pathway. This produces correspondingly higher levels of diclofenac acylglucuronide in some individuals, which can predispose towards hepatotoxicity (Daly et al., 2007). Additionally, studies by Ng *et al.* in 2006 and 2008 report potential nephrotoxicity risk due to mitochondrial damage in elderly patients (Ng et al., 2006; Ng et al., 2008).

### 2.2.3 *Diclofenac in Veterinary Medicine*

Diclofenac remains one of the most widely used drugs in human medicine (Brunton et al., 2018). In contrast, its use in veterinary medicine is limited to the following geographic areas:

- In Asia, diclofenac was used in India and Pakistan for the alleviation of pain, fever and inflammation in animals with illness or disease. The drug was cheap, highly effective and available without veterinary prescription.
- In Africa, diclofenac is regionally registered for veterinary use and is available in human formulations (Woodford *et al.*, 2008, as cited by (Henriques et al., 2018)).
- In Europe, diclofenac is authorized for manufacture and sale in Spain and Italy, for use in cattle, pigs and horses. (BirdlifeInternational, 2014; Margalida et al., 2014).

## 2.3 The Eco-Pharmacological Impacts of the NSAIDs and Diclofenac

### 2.3.1 *The Impact of NSAIDs in the Environment*

As a group, NSAIDs have one of the highest potentials for environmental contamination and are often cited as an example for why eco-pharmacovigilance (EPV) should be instigated globally. This relatively new branch of pharmacology, which is only regulated in developing economies, is concerned with the effect that biologically active compounds may have on terrestrial and aquatic non-target species due to acute or long-term exposure (He et al., 2017).

Due to the large consumption of NSAIDs, both in the medical and veterinary sector, the potential for these drugs to enter the environment through carcasses, urine and faeces excretion, manufacturing emissions, elicit dumping of unused medicines and agricultural run-

off is high. Indeed, most especially in aquatic systems, the hydrophilicity and stability of the NSAIDs has meant they have potential to be widely distributed here (Wang et al., 2018). The biggest impact of these drugs may therefore extend past side effects in treated individuals.

As a widely used NSAID, the importance of diclofenac has been fairly recently highlighted by the European Union when, 7 years ago, they included this pharmaceutical on the first watch list of 'Priority Hazardous Substances' in order to gather Union-wide monitoring data (EU, 2013). For this drug in particular, the eco-pharmacological impact on aquatic systems, mammals and most dramatically, certain species of birds, has been alarming to say the least.

### *2.3.2 Diclofenac Toxicity in Aquatic Ecosystems; Fish*

Several studies have documented the effects of diclofenac on various fish species. As an example, Bickley *et al.,* 2017, demonstrated that the drug accumulated in the plasma of exposed fathead minnows (*Pimephales promelas*) in a concentration- and time- dependent manner and modulated genes associated with kidney repair and regeneration (Bickley et al., 2017). Ribas *et al., 2017,* showed that total blood leukocyte count and carrageenan-induced (a seaweed-derived polysaccharide) leukocyte migration (particularly polymorphonuclears) to the peritoneal cavity was significantly reduced in diclofenac-exposed South American catfish (*Rhadia quelen*) (Ribas et al., 2017). The study presented the theory that diclofenac could lead to inhibition of the innate immune system in this and potentially other fish species. Furthermore, diclofenac has been classified by some authors as having the greatest chronic risk score for threat to aquatic systems, when using a chronic prioritization process (Guo et al., 2016).

### *2.3.3 Diclofenac Toxicity in Terrestrial Ecosystems; Plants and Mammals*

Use of wastewater and biosludge for agriculture can transfer pharmaceuticals from aquatic to terrestrial ecosystems, potentially affecting agriculture and persisting for several months (Kinney et al., 2006). The effects of 15 pharmaceuticals, including 4 NSAIDs, on Queen of May variety lettuce seeds (*Lactuva sativa*) and unicellular green algae (*Chlamydomonas reinhardtii*) were investigated. Of those pharmaceutical groups studied, the NSAIDs (including diclofenac) were found to be the second most toxic group of pharmaceuticals to lettuce root and hypocotyl elongation, and also inhibited photosynthesis in unicellar green algae (Pino et al., 2016).

In another study the authors noted the effects of diclofenac in non-agricultural aquatic plant and lichen species. This study showed dose- and time-dependent toxicity of diclofenac in both the aquatic fern (*Azolla filiculoides*) and the lichen *Xanthoria parietina* (Vannini et al., 2018).

In contrast to the number of studies conducted in plants, there is little data on diclofenac impact in higher order wildlife mammals. One study by Richards and Scott, 2011, detected diclofenac and ibuprofen residues in the hair of 18% of sampled Eurasian otters (*Lutra lutra*), one of the UK's top aquatic predators. As the diet of this species is almost exclusively small aquatic animals such as fish, amphibians and crustaceans, the authors postulated chronic oral ingestion of NSAIDs through the food chain as a possible cause for the presence in the fur. The impact of these residues has yet to assessed (Richards & Scott, 2011).

### *2.3.4 Diclofenac Toxicity in Avian Species; Predatory and Scavenging Birds*

Toxicity under field conditions has been reported in predatory and scavenging bird species since the early 1990's. In predatory birds, toxicity has thus far only recently been reported in the steppe eagle (*Aquila nipalesis*) (Sharma et al., 2014). But the most severe environmental effect of diclofenac in any group of animals has been seen in the Old World vultures, belonging to the Family Accipitridae. So dramatic were the consequences of this drug, that diclofenac has been heralded by some authors as having the same magnitude of effect on vulture populations as the DDT derivative, dichlorodiphenyldicloroethylene had on raptors and other bird species in the 1960s (Anderson et al., 2005).

Most notable in Asia, populations of *Gyps bengalensis*, the oriental white-backed vulture (OWBV), were most severely affected. Their numbers were decimated by 99.9% on the Indian sub-continent between 1992 and 2007 (Prakash et al., 2007). In 2000, the World Conservation Union listed the OWBV, the long-billed vulture (LBV; *Gyps indicus*) and the slender-billed vulture (SBV; *Gyps tenuirostris*) as critically endangered (Vié et al., 2009). A year later Birdlife International added these three *Gyps* species to their critically endangered list as well (Collar, 2002).

## 2.4 The Impact of Diclofenac on Global Vulture Populations

### *2.4.1 Diclofenac and the Threat to Vultures; a Brief Review*

In 2000, the Peregrine Fund (a non-profit organization dedicated to saving birds of prey from extinction) and the late Dr Lindsey Oaks (a virologist from Washington State University) were requested to investigate the deaths of thousands of vultures across Pakistan and Nepal, a phenomenon which had also been in occurrence on the Indian sub-continent for almost a decade and had decimated populations of birds in this country (Prakash et al., 2003). The findings of the so-named 'Asian Vulture Crisis Project' indicated a consistent pattern in affected OWBVs; renal failure and visceral gout on post-mortem examination, with thick deposits of uric acid coating the internal organs (figure 2.2). Failing to demonstrate an infectious cause of the pathology, the team began investigating the primary food source of the vultures for toxicities.

After significant study, diclofenac, used to treat many infectious and inflammatory conditions in domestic cattle and water buffalo, was implicated following residue detection in affected tissue (Watson, 2003; Oaks et al., 2004).

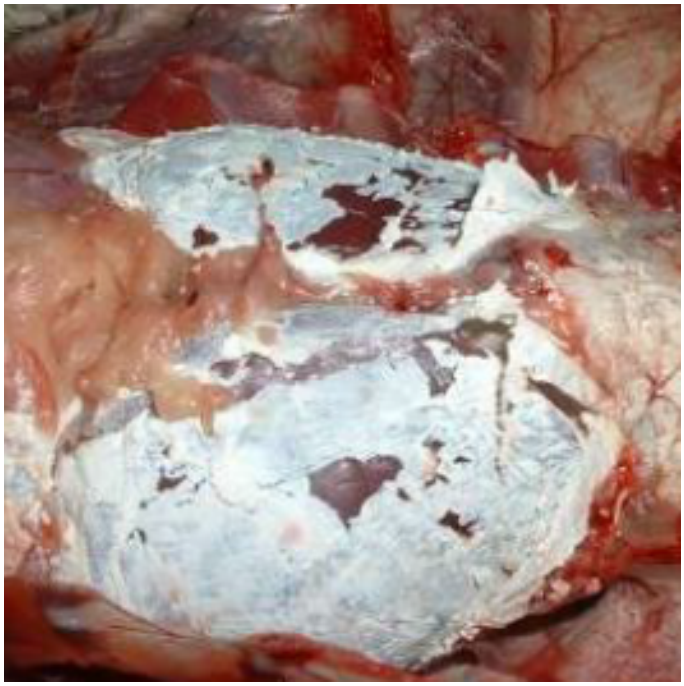

**Figure 2.2 Typical necropsy findings in *Gyps* vultures affected by diclofenac toxicity, showing severe visceral gout (accumulation of thick white uric acid crystals on the abdominal organs) (Oaks et al., 2004).**

Further studies revealed the extent of the diclofenac threat. Schultz *et al.,* 2004, in the same year, extended the association between diclofenac and vulture mortality to both a larger geographical region and to the LBV, following a study in India and Nepal (Shultz et al., 2004). Cuthbert *et al.,* 2016 demonstrated population declines in a further 2 vulture species in India; the Egyptian vulture (*Neophron percnopterus*) and the red-headed vulture (*Sarcogyps calvus*), also postulated to be caused by diclofenac (Cuthbert et al., 2016). And Acharya *et al.,* 2009 revealed dramatic declines in bird counts and nest numbers of the Himalayan griffon (*Gyps himalayensis*) between 2002 and 2005 in Nepal. These declines were again thought most likely due to veterinary diclofenac use (Acharya et al., 2009).

The findings and publications from Asia prompted increased awareness of another already known problem; declining endemic and migratory *Gyps* vulture species across the African continent. Unlike in Asia, there was and remains a multifactorial aetiology to the African vulture situation; victimisation (superstition), decreased food supply, reduced availability of breeding sites, electrocution, unintentional poisoning (predator control by

farmers), fetishism, food (hunting) and the "muti" trade (traditional medicines) have all been implemented (Koenig, 2006; Ogada et al., 2012).

Despite this, NSAIDs other than diclofenac may have played a role. Though diclofenac was not, nor is currently used as a veterinary drug in South Africa, concerns were raised here over the use of other NSAIDs in livestock and companion animals, including at the time, phenylbutazone, flunixin meglumine, eltenac, carprofen, meloxicam and vedaprofen (Anderson et al., 2005) and more recently from ketoprofen, aclofenac and nimesulide (Naidoo et al., 2010; Sharma, 2012; Fourie et al., 2015). This prompted research into the vulture crisis from South Africa.

A South African diclofenac toxicology study was conducted in one Asian vulture species [Eurasian griffon vulture (EGV), *Gyps fulvis*] and one African vulture species [African white-backed vulture (AWBV), *Gyps africanus*] (Swan et al., 2006). The study elucidated that at doses of 0.8 mg/kg, diclofenac was at least as toxic to these species as to *G. bengalensis*, and a median lethal dose ($LD_{50}$) of ~0.1-0.2 mg/kg was calculated. With the urgent need to research why diclofenac was so toxic to *Gyps* vulture species and the requirement for surrogate models for this purpose (given the critical levels of Asian vulture populations), Naidoo *et al.,* 2009 demonstrated that the sensitivity of the Cape griffon vulture (CGV; *Gyps coprotheres*) was similar to that of their Asian counter parts and the AWBV, and indicated that diclofenac susceptibility was probably the same for all *Gyps* vultures (Naidoo et al., 2007).

Most recently, and of great threat to European vultures and other opportunistic scavenging birds such the *Aquila* genus of eagles, was the EU authorisation of diclofenac manufacture and sale in Italy and Spain in 2013, despite the UK Veterinary Medicines Directorate (VMD) taking measures against the drug. This was most concerning in light of simultaneous findings in Asia, which suggested diclofenac toxicity in the steppe eagle (*Aquila nepalesis*), a predatory but facultative scavenging bird (Sharma et al., 2014). The study investigated dead birds found near a cattle carcass dumpsite; eagle carcasses demonstrated necropsy signs of visceral gout and renal tubular nephrosis, together with renal tissue diclofenac residue levels of 0.051 ug/g. The major reason for European concern in respect of vulture populations is that, of the total number of European vultures, 95% are found in Spain and include the Egyptian vulture (*Neopron percnopterus*), the bearded vulture (*Gypaetus* barbatus) and the cinereous vulture (*Aegypius monachus*) (Margalida et al., 2014).

### *2.4.2 The Importance of the Vulture; a World without Scavenging Birds*

The loss of vulture populations on the Indian sub-continent has and will continue to have huge ramifications for these areas:

- Most notably, without vultures to dispose of carcasses, populations of facultative scavengers such as feral dogs, cats and even rats have taken over the primary scavenging role, resulting in increased risk of the spread of diseases such as rabies and perhaps even the bubonic plague (Markandya et al., 2008; Buechley & Şekercioğlu, 2016).
- Vultures rapidly dispose of infected carcasses. In a review by Houston and Cooper, 1975, the authors discuss the role their very low gastric pH may play in livestock disease prevention, such as tuberculosis and brucellosis. Even the fairly environmentally resilient bacteria *Bacillus anthracis* is likely to demonstrate reduced environmental spore formation due to carcass consumption by vultures.
- The aesthetic value of carcass disposal by vultures should not be under-estimated; the smells and appearance of rotting carcasses are unpleasant and require time and financial resources to dispose of. Hill *et al.,* 2018 concluded that under warm and humid conditions, carcasses would persist longer in the environment, despite an increase in facultative scavengers, and potentially having profound effects on nutrient recycling in an ecosystem (Hill et al., 2018).
- The impact on rural, lower income communities in India has been severe. 'Bone collectors', who have traditionally relied on vultures to strip carcasses of flesh before harvesting bone for sale as fertiliser, can now no longer do so (Markandya et al., 2008).
- The Parsi communities in India and others in Nepal, which have traditionally relied on 'sky-burials' or vultures to dispose of their dead, will have to rely on a different method of body disposal, with large impacts on their cultural systems (Buechley & Şekercioğlu, 2016).

### *2.4.3 Solutions to the Asian Vulture Crisis*

With the aetiology, extent and consequences of the Asian Vulture Crisis established, there remained an urgent need to develop a solution. The threat of extinction of the 3 major Asian vulture species (the OWBV, LBV and SBV) was a distinct possibility, prompting the following actions:

- Meetings were held with appropriate stakeholder organisations (governmental and non-governmental) to understand educational needs, drive awareness and achieve consensus on an appropriate action plan.
    - Early surveys indicated that the majority of livestock owners, though dependent on diclofenac for use in their animals, also recognised the socio-ecological importance of the vulture and supported the notion of vulture conservation. Gaining public support for any change implementation was an important first step (Baral & Gautam, 2007).
    - A Vulture Safe Zone (VSZ) was gradually implemented over most of the geographical range of the two vulture species in Nepal (OWBV and LBV), to raise awareness about diclofenac, provide vultures with NSAID-free food and encourage the veterinary use of a vulture-safe alternative NSAID (meloxicam – see below).
    - In 2011, Saving Asian Vultures from Extinction (Arya et al.) was created. It consisted mainly of 20 NGO parties and had the priority of creating and reviewing a 'Blueprint Regional Vulture Recovery Plan'.
- Investigations into finding a safe, equally cost-effective substitute drug for use in livestock began in 2006.
    - A team of South African and European researchers conducted an investigation into the use of meloxicam as an alternative to diclofenac. This toxicology study involved several phases;
        - Gavage of a surrogate species (the AWBV) in increasing concentrations until an estimated maximum level of exposure was achieved.
        - Simulated-exposure in the AWBV to investigate whether residues in livestock meat could affect a toxic response.
        - Gavage of 10 birds of the highly endangered OWBV and LBV.

    No birds in the study died or even demonstrated an increase in uric acid levels, giving hope to the veterinary profession that meloxicam, already registered and used in India, might become a safe substitute for diclofenac (Swan et al., 2006).
- Diclofenac was banned for manufacture, sale and use.

- On May 11th 2006, the Drug Controller General India ordered the withdrawal of all licenses granted for the manufacture of diclofenac for veterinary use within India. Nepal and Pakistan instituted a similar ban later in 2006, with Bangladesh following suit. Unfortunately, an article released in 2007 indicated that the ban on diclofenac was still not entirely in force, with some manufacturers still producing the product and illegal 'smuggling' into India and Nepal occurring from across the border (Marchant et al., 2007). India and Pakistan have consequently placed a critical ban on diclofenac (July 2008), such that it is no longer legal to produce, import or sell the product for veterinary use.

- A captive breeding programme was developed.
  - The Bombay Natural History Society (BNHS) undertook initiatives for captive breeding programmes, together with support from the Royal Society for the Protection of Birds (UK), the Zoological Society of London and the International Centre for Birds of Prey (UK) (Mahapatro & Arunkumar, 2014). Unfortunately the process of recovery following captive breeding will be prolonged, as vultures breed slowly i.e. only one chick is hatched per year and each takes nearly 4 - 6 years to reach maturity (Mahapatro & Arunkumar, 2014). Furthermore, it is potentially unlikely that captive breeding contains sufficient birds to combat the natural decline in allelic diversity and heterozygosity (Johnson et al., 2008).
- Supplementary feeding stations have been established.
  - The establishment of supplementary feeding stations (SFSs) or 'vulture restaurants' was first highlighted as an important conservation strategy in 2007 (Gilbert et al., 2007). It was established that providing safe feeding stations for the OWBV could modify foraging behaviour to an extent, and potentially slow, but not completely prevent the decline in bird numbers. Though effective, care should be taken with establishing SFSs as subsequent research has shown that there can be negative consequences, such as aggregation of predators in focused areas (Cortés-Avizanda et al., 2016). SFSs also require good carcass management as one could inadvertently introduce another food-based toxin, which could be equally detrimental.

### *2.4.4 Recovery Trends*

Still not fully resolved, the conclusion on the story of the Asian vulture crisis has been punctuated by both positive and negative news since its origin in the 1990's. In 2014, the results of a survey into attitudes surrounding the ban on diclofenac and the alternatively proposed NSAID meloxicam were released (Cuthbert et al., 2014). The authors broadly concluded that conservation efforts *had* altered human behavior and palpably impacted on declining vulture numbers but emphasized the need for continued improvement in understanding 'Pharmaceuticals In the Environment' (PIE).

Unfortunately in 2015, a necropsy and tissue residue analysis study of vultures found dead in India between the years of 2002 and 2012 demonstrated only a small and non-significant decline in proportion of carcasses containing diclofenac following the ban in 2006 (Cuthbert et al., 2016). These results coincided with earlier survey results by the same primary author, released in 2011 (Cuthbert et al., 2011). Additionally, nimesulide residues were found in 4 carcasses not containing diclofenac residues, suggesting a new emerging threat from this NSAID.

On this note, and of equal concern is the emerging literature on further NSAIDs which are also toxic to *Gyps* vultures, such as flunixin, ketoprofen, the already mentioned nimesulide and aclofenac, which undergoes biotransformation to diclofenac in the bovine liver (Naidoo et al., 2010; Cuthbert et al., 2011; Fourie et al., 2015; Galligan et al., 2016; Eleni et al., 2019).

More positively, a recent study showed that between 2013 and 2018, there has been a partial recovery in both OWBV and SBV numbers within the VSZ in Nepal (Galligan et al., 2019). Furthermore, it appears that awareness campaigns have been effective in reducing the availability of diclofenac in a large part of the range of these species. The study indicates that the combination of education, regulation and recovery procedures can be effective in reversing the population declines of these birds.

While this news from Nepal is welcomed, understanding the pharmacological mechanisms of NSAID drug toxicity in *Gyps* vulture species will remain important in assisting with improved and hopefully globally legislated pharmaco-environmental vigilance for non-target wild bird species.

## 2.5 The Toxicology of Diclofenac in *Gyps* Vultures

### *2.5.1 Diclofenac Toxicity in Avian Species*

Several NSAIDs, including diclofenac, have demonstrated toxicity in bird species under laboratory conditions (Swan et al., 2006; Swan et al., 2006; Hussain et al., 2008; Naidoo et al., 2010; Fourie et al., 2015; Hassan et al., 2018; Naidoo et al., 2018).

Because of its infamous association with the decline of the Asian vultures, diclofenac has been most extensively studied. Naidoo *et al.,* 2007 demonstrated that the domestic chicken was an appropriate surrogate species for studies investigating the mechanism of diclofenac toxicity in *Gyps* vultures. Affected birds showed clinical signs similar to that of vultures; depression, anorexia and eventually death. Clinical pathology, necropsy and histopathological findings were also similar to that seen in vultures (Naidoo et al., 2007). The chicken was however, less sensitive to the drug; the median lethal dose ($LD_{50}$) in the chicken was 9.8 mg/kg by intra-muscular (i/m) injection, compared to 0.1-0.2 mg/kg in *Gyps* vultures (Swan et al., 2006).

Diclofenac toxicity has also been demonstrated in other avian species. Hussain *et al.,* 2008 investigated its toxicity in 4 bird species selected for their representation of farming, wild and ornamental birds; broiler chicks (*Gallus domesticus*), pigeons (*Columbia livia domestica*), Japanese quail (*Coturnix japonica*) and Indian mynas (*Acridotheres tristis*). There were similar but varied intensity of typical diclofenac-associated clinical signs, serum chemistry and pathological changes in each species, with a dose-dependent response in toxicity. The severity of toxicity appeared greatest in the domestic chicken, followed by the pigeon and quail, and least in the Indian mynah (Hussain et al., 2008). Hassan *et al.,* 2018 reported toxicity of diclofenac in Muscovy ducks (*Cairina moschata*) and Japanese quail (*Coturnix japonica*), but at lower sensitivity compared to *Gyps* vultures. Additionally, the domestic pigeon (*Columba livia domestica*) was relatively insensitive in this study (Hassan et al., 2018). These studies demonstrated definite differences in susceptibility to diclofenac across bird species.

### *2.5.2 The Proposed Mechanism of Diclofenac Toxicity in Gyps Vultures*

In a pathological study by Meteyer *et al.,* 2005, lesions in the kidneys of OWBV were described as ‘acute and severe’, there being no evidence of chronic illness in the carcasses and kidney lesions being absent of inflammation or evidence of repair (figure 2.3). The proximal convoluted tubular (PCT) renal tubular epithelial (RTE) cells are the major source of energy-dependent transporters of uric acid in birds and this was validated by the demonstration of accumulating levels of uric acid crystals in this tissue. However the acute necrotic changes to the PCT, in the absence of uric acid crystals in some samples, indicated that uric acid

accumulation was as a result and not the cause of PCT epithelial death (Meteyer et al., 2005).

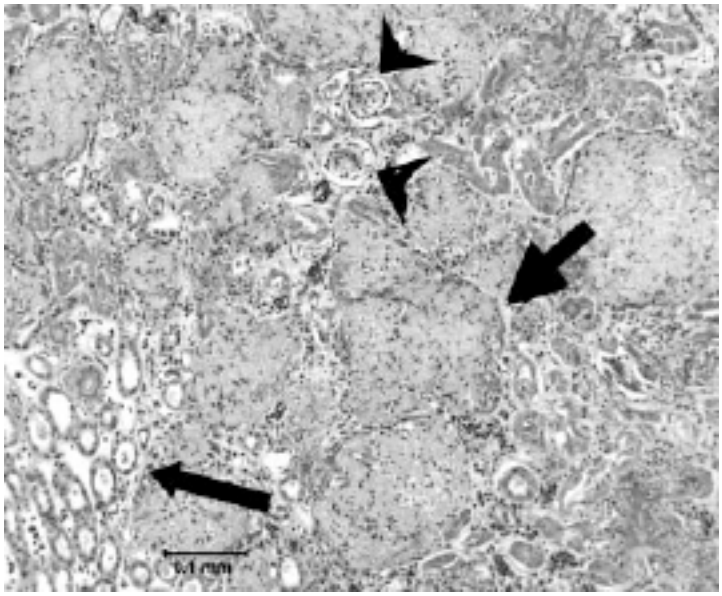

**Figure 2.3 Histopathology findings in *Gyps* vultures following diclofenac toxicity.**

Kidney from a wild OWBV which was found dead in Pakistan in 2002, as reported by Meteyer et al., 2005. The slide shows urate tophi accumulation distorting normal renal tissue. Of note is that the inflammatory response appears minimal. (Meteyer et al., 2005).

The authors thus advanced the theory that diclofenac altered renal blood flow to the cortex, this being supplied by the renal portal system (via the renal portal veins), (Lierz, 2003; Burgos-Rodríguez, 2010). It was proposed that diclofenac inhibited COX-2 and therefore decreased the production of $PGI_2$ and $PGE_2$, prostanoids thought to have played a role in maintaining adrenergic-controlled patency of the renal portal valves. The resultant closure or partial closure of these valves was thought to have interfered with cortical blood flow and caused PCT necrosis.

In 2006, Ng *et al.* exposed rodent cells to diclofenac and advanced a second theory. The authors proposed that diclofenac inhibited transport of mitochondrial malate and glutamate into the kidney, liver and heart cells, leading to decreased ATP production and increased production of ROSs. Uric acid accumulation within PCT RTE cells was therefore thought to be due to energy-dependent Multi-drug Resistant Protein (MDR4 or MRP4) transporter inhibition on the apical cell membrane (figure 2.4) (Ng et al., 2006). In 2008, a canine kidney cell culture study by the same authors demonstrated that both diclofenac and meloxicam (now demonstrated as safe in *Gyps* vultures) induced cellular apoptosis, though meloxicam was less toxic (Ng et al., 2008). Unfortunately, the studies did not allow for inter-species extrapolation from mammals to birds. Additionally, the striking clinical difference in toxicity between diclofenac and meloxicam in *Gyps* vultures could not be explained.

Naidoo *et al.,*2007 noted that pharmaceutically, the only difference between the two drugs was the molecular structure, suggesting that either differences in receptor affinity or pharmacokinetics was the cause of the difference in toxicity (Naidoo et al., 2007). Naidoo *et al.,* 2007 and Naidoo and Swan, 2009 therefore investigated diclofenac toxicity in a validated surrogate model, the domestic fowl (*Gallus domesticus*), and a modification of the Meteyer *et al.,* 2005 theory was put forward (Naidoo et al., 2007; Naidoo & Swan, 2009). The authors proposed that diclofenac, through inhibition of PGI production, could induce vasoconstriction of the renal portal vein itself. As this vein supplies 75% of blood to the renal cortex, subsequent PCT necrosis could ensue. However, they found that neither diclofenac nor meloxicam caused renal portal vein constriction in bird tissue; in fact both drugs caused vasodilation, perhaps through inhibition of $PGF_{2a}$, which has a known vasoconstrictory effect in renal vascular beds in rats (Peredo, 2003).

Subsequently a third hypothesis into the mechanism of diclofenac toxicity was investigated, using an organ bath *in vitro* toxicity study in broiler chickens and a uric acid clearance study in a single AWBV. As seen in the Ng *et al.,* 2008 study, both meloxicam and diclofenac were directly toxic to avian RTE cells in a time- and concentration-dependent manner and as a result of ROS formation, which peaked at 12 hrs. But whilst RTE cells exposed to meloxicam for only 2 hours demonstrated no signs of toxicity, those exposed to diclofenac for the same length of time did, indicating an additional mechanism of toxicity for diclofenac and a prolonged pharmacodynamic effect post drug withdrawal.

This effect was hypothesized to be caused by intra-cellular RTE uric acid depletion, validated when both chicken and vulture renal cell cultures incubated concurrently with uric acid and diclofenac were devoid of toxicological changes. This suggested that uric acid provided an endogenous buffering system against ROS production. It also suggested that diclofenac inhibited the renal avian baso-lateral Organic Anion Transporter (OAT), also called the p-amino-hippuric acid (PAH) transporter, which transports uric acid into the RTE cells from the efferent blood vessels (figure 2.4). As birds are net excretors of uric acid, with limited capacity to reabsorb it, inhibition of this transporter would quickly deplete the RTE cells of buffering capability (figure 2.4).

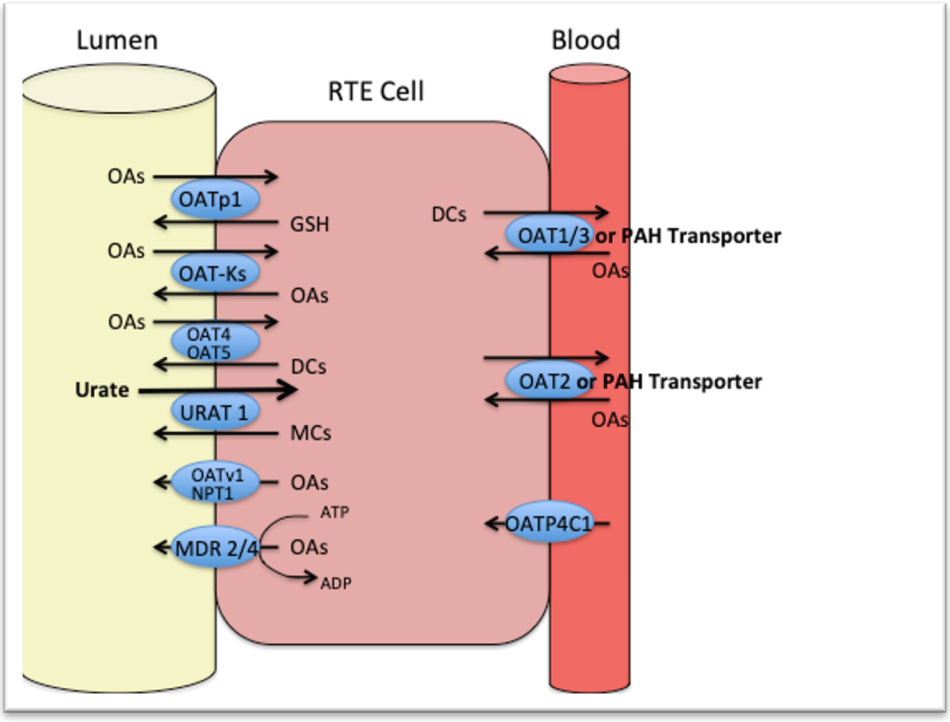


**Figure 2.4 The RTE renal transport channels involved in excretion and reabsorption of uric acid in humans.**

In man, uric acid is actively transported via OAT 1/3 and 2 transporters into the RTE cells. It is then further actively excreted into the tubular lumen via the MDR 2 or 4 transporters, or filtered via the glomerulus. Uric acid can be conserved through reabsorption via the URAT1 channel.

In birds, while there are OAT transporters on the basolateral membrane and MDR2/4 transporters on the apical membrane, there is no URAT1 transporter on the apical membrane. Consequently birds are net excretors of uric acid, having little capacity to reabsorb it (Dudas et al., 2005). Reproduced from (Naidoo, 2013).

### *2.5.3 The Significance of the Half-Life of Elimination*

While the mechanism of diclofenac toxicity in birds appears to be due to a combination of NSAID-induced production of ROSs and inhibition of the PAH transporters in the PCT RTE cells of the kidney, the reason for the specific sensitivity of *Gyps* vultures to diclofenac is still not fully understood. The most strikingly difference is found in the pharmacokinetics of the drug in the specific species; the $T_{1/2}$ of diclofenac in AWBVs was 14 and 18 h for the 2 birds studied by Naidoo *et al.,* 2009, which was significantly longer that the 1 h seen in the domestic chicken (Naidoo et al., 2007; Naidoo et al., 2009). Furthermore, the $T_{1/2}$ of meloxicam, which had been shown to be safe for *Gyps* vultures (Swan et al., 2006) was 0.42 ± 0.1 h by the i/m route and 0.32 ± 0.17 h per os (Naidoo et al., 2008).

This highlighted the importance of both long-term exposure to certain NSAIDs and the on-going production of ROSs as being important in toxicity. In the case of *Gyps* vultures, whilst

meloxicam is directly toxic to *Gyps* vulture PCT RTE cells in an *in vitro* setting, toxicity is not demonstrated clinically because of the short $T_{1/2}$ (Naidoo et al., 2007; Naidoo & Swan, 2009), whilst diclofenac, with a relatively long $T_{1/2,}$ is extremely toxic in these birds both *in vitro* and *in vivo*.

This relationship between a longer $T_{1/2}$ and toxicity in *Gyps* vultures was also a consistent finding for other NSAIDs such as carprofen, flunixin and ketoprofen during investigations (Naidoo et al., 2010; Naidoo et al., 2010; Fourie et al., 2015; Naidoo et al., 2018). These studies showed that for these NSAIDs, a longer $T_{1/2}$ was associated with higher doses (5 mg/kg for ketoprofen and 64 mg/kg for carprofen) and death.

The findings suggest that zero-order metabolism or saturation of metabolic capacity is occurring for the NSAIDs ketoprofen, carprofen and most likely diclofenac in *Gyps* vultures. Furthermore, the point at which metabolism became saturated or non-linear for ketoprofen and carprofen is variable between birds and is therefore likely linked to an enzyme system which displays pharmacogenetic variation. Understanding xenobiotic metabolism in *Gyps* vultures could therefore demonstrate why diclofenac and many other NSAIDs are toxic to these birds, but meloxicam is not.

## 2.6 The CYP P450 Enzyme System and Implication thereof in Diclofenac Toxicity in *Gyps* Vultures

With a few exceptions, most xenobiotics, including drugs, are subjected to one or more enzymatic reactions that fall within the following phases;

- Phase 1 oxidation, reduction or hydrolysis reactions, conducted by the Cytochrome P450 (CYP), Flavin Monooxygenase (FMO) and Epoxide Hydrolase (EH) enzymes systems (Brunton et al., 2018).
- Phase 2 conjugations, comprised of glucuronidation, sulfation, glutathione, N-acetylation and methylation reactions (Brunton et al., 2018).

These reactions generally serve to convert hydrophobic to hydrophilic compounds that can more easily be eliminated from the body, and to convert xenobiotics to less biologically active derivatives (Brunton et al., 2018).

Because of their role in the elimination of many drugs, CYPs are of key importance to the pharmaceutical industry and in eco-pharmacology, CYP-related differences in susceptibility to pharmaceuticals between animal species may be an important cause of

environmental toxicity in species other than those for which a compound was originally intended.

This is suspected to be the case in the Asian Vulture Crisis and the phase 1 CYP enzyme system has been cited by several authors to be the most plausible component of xenobiotic metabolism implicated in diclofenac toxicity in these birds, for reasons outlined in section 7 below (Naidoo et al., 2010; Naidoo et al., 2010; Fourie et al., 2015; Hassan et al., 2018; Naidoo et al., 2018).

The CYP enzymes belong to a super family of metabolising enzymes that are evolutionarily related. CYPs are involved in both the synthesis of endogenous compounds (such as steroids) and the majority of phase 1 metabolic reactions. They metabolise many structurally diverse chemicals and are transcribed from what are thought to be among the fastest evolving genes (Konstandi et al., 2014).

Of the different and diverse CYP families in mammals, the enzymes involved in the metabolism of most drugs fall into the CYP 1, 2 and 3 families. Subfamilies, denoted by a letter, exist within these 3 major groups. The final number in the CYP nomenclature represents the gene locus. In humans, the CYP3A4, CYP3A5, CYP2D9, CYP2C8 and CYP2C9 enzymes are estimated to be the largest metabolisers of xenobiotics (figure 2.5) (Preissner et al., 2013; Brunton et al., 2018). The CYP2C subfamily is considered one of the largest and most complex and CYP2C9 accounts for 60% of the total human CYP2C subfamily (Martignoni et al., 2007).

Among the diverse reactions carried out by the major 3 families are N-dealkylation, O-dealkylation, aromatic hydroxylation, N-oxidation, S-oxidation, deamination, and dehalogenation reactions. Because most drugs must pass through these reaction pathways to be excreted, and because CYPs are promiscuous in their substrates (a single enzyme may metabolise many different drugs, which may therefore compete with each other for the active site), much is known about the human CYPs, the genome sequences which encode for them and which drugs they metabolise.

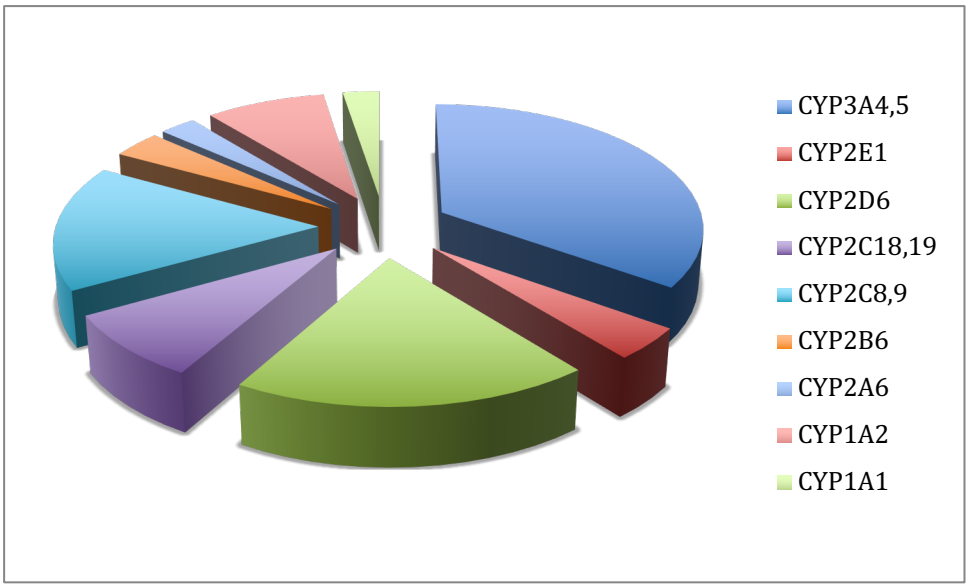


**Figure 2.5 The fraction of clinically used drugs metabolized by the major phase 1 CYP enzymes in humans.**

The relative size of each pie section represents the estimated percentage of drugs metabolized by each enzyme group. Adapted from (Preissner et al., 2013; Brunton et al., 2018).

By contrast, the specific CYP enzymes remain virtually unknown in *Gyps* vultures, but that the CYP system is active is supported by the work of Naidoo and Swan, 2009 who investigated the pharmacokinetics of meloxicam in CGVs. This study illustrated that both phase 1 and phase 2 enzyme systems appear to be active, as evidenced by the presence of hydroxyl meloxicam metabolites typical of CYP phase 1 reactions and a glucuronide meloxicam metabolite typical of Uridine Diphosphate (UDP) phase 2 reactions (figure 2.6) (Naidoo & Swan, 2009). Therefore potential deficiencies in either of the enzyme systems as opposed to absolute absence, could account for the evident zero-order metabolism noted for ketoprofen (Naidoo et al., 2010) and the long half-life of diclofenac (Naidoo & Swan, 2009).

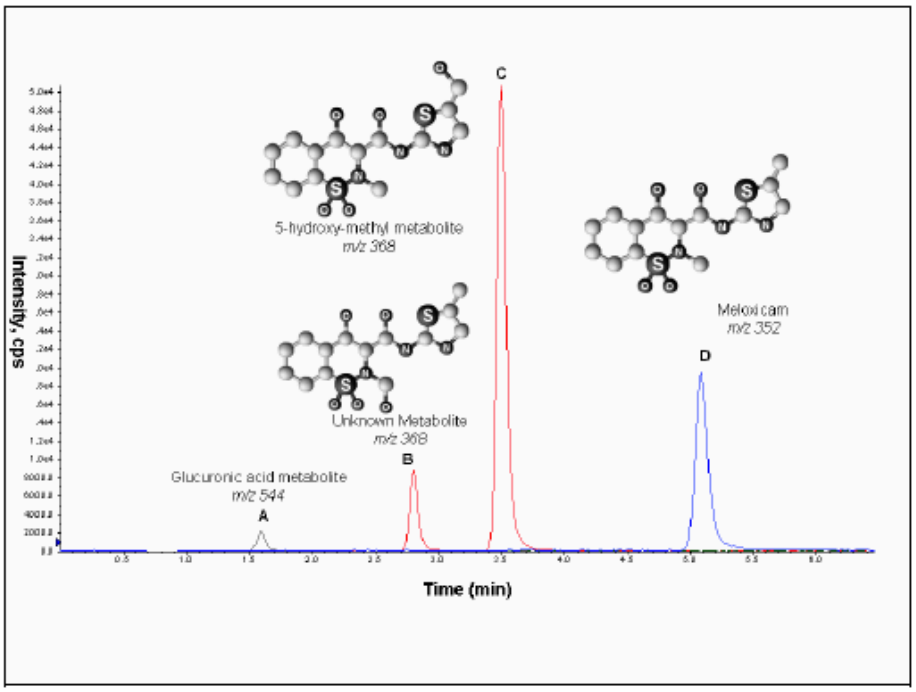


**Figure 2.6 Meloxicam metabolites produced in *Gyps* vultures**.

Meloxicam and its metabolites as determined by LCMSMS (Liquid Chromatography - Mass Spectrometry and Liquid Chromatography - Tandem Mass Spectrometry) by Naidoo and Swan, 2009.

A: Glucuronic acid metabolite, B: unknown metabolite, potentially a second hydroxyl metabolite, C: hydroxyl metabolite, D: meloxicam. Borrowed from Naidoo and Swan, 2009.

Despite the rapid metabolism of meloxicam, the long half-life for the metabolism of diclofenac still points to the vulture being metabolically constrained at the cytochrome enzyme level. Certain characteristics of the CYP enzyme group, trends in other animals and the pharmacokinetic trends from NSAID research in *Gyps* vultures, have favoured them over phase 2 enzymes as being more likely deficient in these birds.

### *2.6.1 Current Evolutionary Understanding in Support of Phase 1 CYP Involvement*

Current evolutionary understanding is supportive of phase 1 CYP involvement. It is estimated that the final major expansion of several CYP P450 families involved in xenobiotic metabolism (including CYP2, CYP3, CYP4 and CYP6) began about 400 million years ago. Much of this genetic diversification is considered to have coincided with the transition of some life forms from aquatic to terrestrial environments and the beginning of plant consumption as a food source. Predatory and scavenging birds and animals, by nature of their diet containing very little plant material (containing xenobiotic compounds), did not face the same evolutionary drive to develop as diverse xenobiotic metabolising CYP systems as their omnivorous or herbivorous counterparts (Gonzalez & Nebert, 1990; Nelson et al., 2013; Hutchinson et al., 2014).

### 2.6.2 CYP Genetic Instability

Further to this, CYP enzyme genes are renowned for exhibiting instability. Thomas, 2007 describes how genes encoding for xenobiotic-metabolising CYPs, including the CYP2 and 3 subfamilies, are relatively unstable (Thomas, 2007). They are influenced by evolutionary pressures to a larger degree than their more stable endogenous compound-forming counterparts within the P450 family, such as the CYP19 and 21 subfamilies. Unstable genes undergo a more frequent birth-death evolutionary process by means of duplication and loss, meaning they are more diverse across species. This is consistent with the later work of Watananbe *et al.,* 2013, which demonstrated that CYP2C avian genes had undergone frequent duplication events (Watanabe et al., 2013).

### 2.6.3 Inter- and Intra-species CYP Diversity

The CYP enzyme system also displays remarkable diversity across the animal kingdom; there are 37 P450 families across many species (Nelson et al., 2013) and they are renowned for their interspecies pharmacogenetic differences in drug-metabolising capability. Best studied in veterinary science, the major obstacle to understanding and predicting drug metabolism across the mammalian species treated is due to differences between CYP activity and substrate specificity (Fink-Gremmels, 2008). For example, the dog has several unique CYP isoenzymes such as canine CYP1/2, CYP2B11 and CYP2C21 (Eguchi et al., 1996; Trepanier, 2006), for which the specific substrates are relatively unknown (Bogaards et al., 2000). In another example, it was demonstrated that making cross-species pharmacokinetic extrapolations from one species of farm animal to another (cattle, sheep and pigs), would be challenging because of differences in CYP1A1/2, CYP3A and also likely CYP2C and CYP4A functioning. Key highlights from this study showed very high 7- ethoxyresorufin-*O*-deethylase activity, reflective of CYP1A1/2 functioning, in bovines compared to the other species. There was 2-3 times higher 7-methoxy-4-trifluoromethyl-coumarin demethylase and 4-10 times higher 12-lauric acid hydroxylases activity (probably corresponding to CYP2C and CYP4A functioning respectively) in ovines. The highest 6ß-testosterone hydroxylase activity, which is usually considered to be a CYP3A activity marker, was found in the pig (Szotáková et al., 2004).

Furthermore, CYP enzymes also demonstrate inter-individual variation. This has been best studied in humans, where the cause is usually due to genetic polymorphisms (more than 1 allele at a specific gene locus). For example, CYP2A6, CYP2C9, CYP2C19 and CYP2D6 all exhibit polymorphisms in humans, whereby a slight difference in the enzyme genetic sequence may be present (Brunton et al., 2018). This has clinical application; increased rates of organ transplant rejection in subjects of African descent are seen because of decreased plasma concentration levels of the anti-rejection drug tacrolimus. This is because these groups of

people have higher activity levels of CYP3A5, the enzyme responsible for phase 1 metabolism of this drug (Birdwell et al., 2012).

There is quite remarkable difference in susceptibility of bird species to diclofenac (Naidoo et al., 2011; Hassan et al., 2018). Furthermore, *Gyps* vultures appear to display a degree of inter-individual variation in metabolism of some NSAIDs (Naidoo et al., 2010; Fourie et al., 2015; Naidoo et al., 2018).

## 2.7 Chemical Inhibition as a Methodology for Inferring CYP Functionality

For this study I propose using a commonly used pharmacokinetic technique to ascertain whether the CYP2C9 enzyme system is involved in the metabolism of diclofenac in *Gyps* vultures. The domestic chicken has a short $T_{1/2}$ for diclofenac, indicating CYP enzyme metabolism is most likely functioning and the 2C subfamily has been demonstrated as present in this species (Watanabe et al., 2013). In contrast, in *Gyps* vultures, the longer $T_{1/2}$ for diclofenac suggests deficiency of a functioning enzyme system for this drug.

Though it is not possible to genetically inhibit CYP metabolism in the chicken to demonstrate that a change in half-life enhances toxicity, this may be achieved through the use of chemical surrogates, whereby the enzyme can be artificially inhibited following pharmacological exposure. This methodology of ascertaining the functioning of specific CYP enzymes has been commonly employed in the literature across human and animal species (Pelkonen et al., 1998; Szotáková et al., 2004; Takanohashi et al., 2010; Zhai et al., 2013; Hasegawa et al., 2017). For CYP2C enzymes, there are over 150 listed pharmacological inhibitors of wide and varied indication (Wishart et al., 2008). Some of the more common examples include the azole antifungals (e.g. ketoconazole, miconazole, fluconazole), calcium-channels blockers (e.g. clevidipine, felodipine and nicardipine), sulphonamide antibiotics (e.g. sulphamethazole, sulphapyridine and sulphadiazine) and antiviral drugs (e.g. delavirdine, lopinavir and nevirapine. We elected to use fluconazole for CYP2c9 homolog inhibition for reasons of availability and formulation compatibility with intra-peritoneal dosing (see section 3.3 below).

## 2.8 Conclusion

Whilst NSAIDs have important clinical applications in the management of inflammatory and painful conditions in veterinary medicine, the devastation that diclofenac had on Old World vulture populations in Asia emphasizes the importance of regulated Environmental Risk Assessment (ERA) of new compounds submitted for marketing

authorisation. Despite this as a measure to prevent ecological disasters, as emphasized by Hassan *et al.,* 2018, it is not always easy to predict the impact of a drug in the environment.

A major component of this difficulty in predicting drug effects in non- target species is the diversity of metabolising capability across the animal kingdom. Particularly in birds, the functioning of CYP enzymes is little understood. For this study it is intended that by inhibiting the functioning of the CYP2C enzymes in a species where it is known to function and recording the subsequent pharmacokinetic profile, the deficiency thereof in *Gyps* vultures can be proved.

# 3 MATERIALS AND METHOD

## 3.1 Study Design

The study design followed the guidelines for the $LD_{50}$-only component of the OECD guidelines for Acute Avian Oral Toxicity Testing (OECD, 2016). The $LD_{50}$-only test consists of 2 stages that are performed sequentially in an effort to minimise the number of birds used (figure 3.1). The sample size of 10 per group is the minimum required to provide statistically viable results with a 95% confidence level (OECD, 2016).

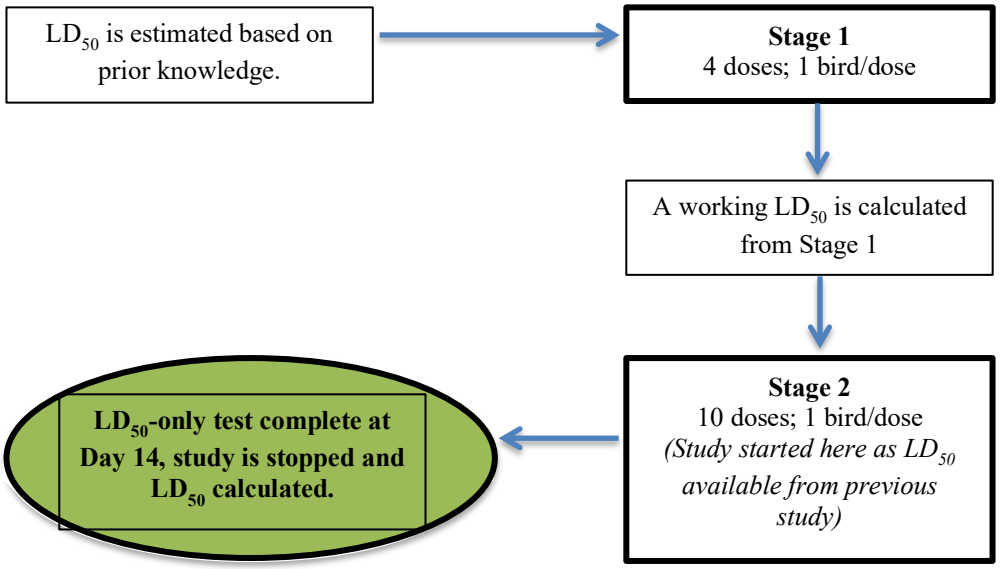


**Figure 3.1 The 'Sequential Design Procedure' for Acute Toxicity Testing in Avian (OECD Test Guideline 223), not including control birds.**

As a working $LD_{50}$ was available from a previous study (Naidoo et al., 2007), only Stage 2 of figure 3.1 was required. The range of doses used was calculated using the Maximum Likelihood Estimation method (Section 8.1) (OECD, 2016).

## 3.2 Animals

### *3.2.1 Animal Ethics and Approval*

The study protocol was approved by the University of Pretoria Animal Ethics Committee and the Faculty of Veterinary Science Research Ethics Committee under project number V078-18.

### *3.2.2 Animal Procurement and Acclimatisation*

Thirty day-old Ross broiler chickens (*Gallus gallus domesticus)* weighing an average of 45.13 g were purchased from Eagle's Pride Hatchery in Roodeplaat Pretoria and transported to the University of Pretoria Biomedical Research Facility (UPBRC), Faculty of Veterinary

Science. The chicks were checked for clinical abnormalities on arrival. The 14-day acclimatisation period was included in the rearing period of 5 weeks.

### *3.2.3 Housing and Care*

The chickens were housed in the UPBRC Aviary for the full duration of rearing and until completion of the study. This facility is a mimic of a standard poultry house and has automated fan driven ventilation. The room is thus under intermittent positive pressure. The light-dark phasing for the study was 10/14 hrs. The room temperature and relative humidity ranged from 19 to 30.6 ˚C and 20 to 60 % respectively.

Birds were raised from chicks, on the floor, with wood shavings as bedding. The birds had *ad lib* access to food (poultry standard ration, according to age) and municipal potable water and were kept as a group for socialisation needs. The birds were attended to twice daily by UPBRC staff members. The facility makes use of a simple biosecurity protocol, whereby persons entering change into surgical scrubs (provided and laundered on site) and wear impermeable boots. Footbaths with F10 disinfectant are provided on entry and gloves are worn at all times.

Birds were individually identified with unique wing-tags at 14 days. They were weighed on arrival and weekly until 5 weeks of age, the commencement of the study.

### *3.2.4 Mortalities*

Mortality was the primary endpoint in the study and background mortality was negligible, with only one healthy control group bird euthanased during the study (see below). All chickens were euthanased, where unscheduled death did not occur as a consequence of treatment, and post mortem examinations completed on Day 15 of the study. During the test, animals obviously in pain or showing signs of severe distress were euthanized immediately.

Five birds were lost during the rearing period; 2 at approximately 2 weeks, 1 at approximately 3 weeks and 2 at approximately 4 weeks of age. The birds showed reluctance to move, lethargy, poor appetite and poor growth. An additional bird, 5840, was euthanased on day 6 of the study due to lameness and difficulty in reaching food. Necropsy of these birds revealed soft bones and beaks, synonymous with primary nutritional osteopathy, most likely calcium, phosphorus or vitamin D3 deficiency. Interestingly the breast muscle was also affected in some of these birds, showing widespread myolysis. The likely aetiology was the poultry starter ration and the ration was immediately changed after the first death. Except for bird 5840, no further lameness or death occurred prior to the start of the study.

### 3.3 Treatment Groups and Dosing

Prior to the study, at 5 weeks of age, the birds checked and deemed to be healthy were randomly allocated to 3 groups (table 3.1). Test and negative control group birds were placed into individual cages to allow for ease of monitoring and identification. The healthy control birds remained on the floor.

**Table 3.1 Bird treatment groups**

| **Group** | **Number of Birds** | **Intravenous Treatment** |
|---|---|---|
| Test | 10 | Diclofenac Sodium + CYP2C9 inhibitor. |
| Negative Control | 10 | Diclofenac Sodium. |
| Healthy Control | 5 | No treatment. Provision of untreated blood for analytical phase of the study. |

The test group birds were dosed with a known potent CYP2C9 inhibitor (Brunton et al., 2018), fluconazole (Diflucan®, Pfizer). Birds were dosed at 15 mg/kg BW (Carpenter & Marion, 2018), intra-peritoneally (i/p), for 3 days prior to the start of the study.

The negative control and test group were subsequently both dosed with diclofenac sodium (Panamor 75®, Aspen Pharmacare Holdings). Each bird within a group was dosed with a unique dose, spaced around the working $LD_{50}$ of 9.8 mg/kg BW (Naidoo et al., 2007) on a log scale, by intravenous (i/v) injection, according to the OECD Maximum Likelihood Estimation method (OECD, 2016) (section 8.1 and table 3.2).

**Table 3.2 Individual bird doses per treatment group.**

| | **Diclofenac dose per bird (1 dose per bird) in** mg/kg BW | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **1*** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** | **10*** |
| **Test Group** *Diclofenac + fluconazole(15 mg/kg BW)* | 3.36 | 4.26 | 5.42 | 6.88 | 8.74 | 11.10 | 14.10 | 17.90 | 22.74 | 28.61 |
| **Negative Control*** *Diclofenac* | 3.36 | 4.26 | 5.42 | 6.88 | 8.74 | 11.10 | 14.10 | 17.90 | 22.74 | 28.61 |

* 1= 'ldose' = low dose, 10 = 'hdose' = high dose.

Table does not show pre-treatment of negative control group with CYP2C9 inhibitor for 3 days prior to study start.

The healthy control group was required to monitor the health and husbandry of test birds to ensure that the ability of the study to provide reliable results was not compromised and to provide blank (untreated) plasma samples for the analytical phase of the study. If there had been more than 10% mortality in the healthy control group from the start of the study, the study would have been considered invalid (OECD, 2016).

## 3.4 Blood Sampling and Monitoring of Birds

Sampling and sample handling were conducted in the UPBRC. Blood samples were drawn from the *V. cutaneous ulnaris* (wing vein) using heparinised 21 G needles and 3 ml syringes. Blood samples were collected from the test and negative control groups at 0 h, 15 min, 30 min, 1 h and 2 h after dosing or as soon as possible after death (where birds died during the bleeding period, see section 4.1 below). Blood volumes collected were 2.5 ml per bleed. The healthy control group was bled once at 2 h and blood volumes collected were 12.5 ml per bird.

Samples were transferred into pre-labelled plasma tubes and centrifuged within 1 h of sampling at 1660 x g and 25 °C for 15 min. Samples were split for uric acid and diclofenac analysis and were then stored at -80 °C.

The birds were continuously monitored for the first 2 h post dosing, and then again at 4 h and 6 h post bleeding. Thereafter the birds were monitored twice daily until mortality or the study termination on day 14. All birds that did not die were euthanased on day 15 by isofluorane sedation and $CO_2$ in a chamber.

## 3.5 Observations

### *3.5.1 Clinical Observation*

Birds were observed for mortality and estimated time of death, onset and clinical signs association with intoxication (abnormal behaviour, anorexia, regurgitation and lameness), remission from intoxication and changes in body weight and Feed Conversion Ratio (FCR). Because of variation in pre-study weights, a Chi Square analysis (SPSS Version 1.0.0.95 statistical software, IBM, New York, NYS, US) was performed to assess whether there was a relationship between mortality and bird weight being below average for the group.

### *3.5.2 Pathological Examination*

Bird carcasses were sent to the Department of Paraclinical Sciences' veterinary pathology section for gross necropsy examination. Where indicated by gross pathology, tissues samples were taken and placed in 10% buffered formalin. These were examined histologically following sectioning and H&E staining.

## 3.6 Blood Sample Data Analysis

### *3.6.1 Uric Acid Analysis*

Uric acid analysis was conducted in the University of Pretoria Department of Companion Animal Clinical Studies, at the clinical pathology laboratory. Analysis was conducted by a Roche Cobas Integra®-400 plus analyser (Roche diagnostics GmbH, Mannheim, Germany), using photometric, fluorescence polarisation and electrolyte analysis. More specifically, the uricase enzyme colorimetric test used measures the intensity of quinone-diimine dye red colour absorbance formed at reaction termination, using a wavelength of 552 nm. Uric acid was completed across all time points and results reported in mmol/L.

Plasma concentration-time profiles for uric acid were generated to compare trends following treatment. The reference range used for normal chicken uric acid concentration was based on Ross et al., 1978 and Wilson & Miles, 1988. The linear trapezoidal rule was used to calculate the $AUC_{last}$ (area under the curve to the last quantifiable time point) via the following equation, $\mathbf{(AUC_{last} = \sum_{i=1}^{n} 0.5\ x((C_i + C_{i+1})\ x\Delta t)}$, where $\mathbf{C}$ is uric acid concentration and $\mathbf{t}$ is time. The $AUC_{last}$ was used to compare total exposure to uric acid. Baseline corrections per scheduled time points (difference between 0 h baseline reading and each time point for an individual bird) were also calculated. The Independent Samples Students t-test was used to compare the means of the $AUC_{last}$ and baseline corrections per time point of the test and negative control group, using SPSS Version 1.0.0.95 statistical software (IBM, New York, NYS, US).

### *3.6.2 Drug Analysis*

#### *3.6.2.1 Calibration Curve Standard Preparation*

Diclofenac sodium, 4'-hydroxydiclofenac, 5'-hydroxydiclofenac and fluconazole analytical standards were purchased from Sigma Aldrich (Merck).

- **Diclofenac:** Calibration curves were achieved by first preparing stock and working solution standards. Two (2) mg of diclofenac sodium was dissolved in 5 ml of water to produce a 0.4 mg/ml stock solution. Five hundred (500) µl of the stock solution was then diluted by adding 500 µl of water to give a working solution of 200 µg/ml. The working solution (200 µg/ml diclofenac) was then added to each of 10, 2 ml microcentrifuge tubes, in volumes of 0.976, 1.953, 3.906, 7.813, 15.625, 31.25, 62.5, 125 and 250 µl. Two hundred (200) µl of blank plasma was then added to each microcentrifuge tube and this provided calibration standards of concentrations 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25 and 50 µg/ml. A vortex mixer with multi-tube capacity (Heathrow Scientific, Illinois, USA) was used to agitate the contents of each tube for 30 sec and the calibration standards were then treated as below, in a manner identical to the test samples, (Hassan et al., 2018), (Naidoo et al., 2007).
- **4'- and 5'-hydroxydiclofenac:** It was not possible to separate the chromatographic peaks for the 4'- and 5'-hydroxydiclofenac metabolites. Consequently the metabolites were treated as a unit, to confirm separation of retention times from diclofenac, and to compare the ratio of their combined peak to the diclofenac peak. A stock solution was created by adding 0.1 mg of each metabolite to a total of 1 ml of methanol and combining 1 ml of each solution to give a 50 µg/ml 4' hydroxydiclofenac and a 50 µg/ml 5'-hydroxydiclofenac combined metabolite solution. This was dilute enough to also serve as a working solution, which was then added to each of 7, 2 ml microcentrifuge tubes, in volumes of 1.953, 3.906, 7.813, 15.625, 31.25, 62.5 and 125 µl. Two hundred (200) µl of blank plasma was then added to each microcentrifuge tube and this provided calibration standards of concentrations 0.097, 0.195, 0.391, 0.781, 1.563, 3.125 and 6.25 µg/ml. A vortex mixer with multi-tube capacity (Heathrow Scientific, Illinois, USA) was used to agitate the contents of each tube for 30 sec and the calibration standards were then treated as below, in a manner identical to the test samples (Hassan et al., 2018), (Naidoo et al., 2007).
- **Fluconazole:** Fluconazole did not produce chromatographic peaks using the below method of extraction, therefore it was concluded that there was no interference from this drug and consequently no calibration curves were conducted.

### 3.6.2.2 *Plasma Sample Preparation*

Two hundred (200) µl of plasma from each sample was placed in a 2 ml microcentrifuge tube. Four hundred (400) µl of diethyl ether and 400 µl of potassium dihydrogen phosphate (0.3M and pH 3.5) were added sequentially to each tube. The tubes were capped and agitated for 2 min using a vortex mixer with multi-tube capacity (Heathrow Scientific, Illinois, USA). The tubes were then centrifuged for 20 min at 5878 x g and 4 °C. Following this, the tubes were placed in an ice-bath of methanol and $CO_2$ for 3 min. This facilitated the solidified aqueous phase to separate from the organic layers. The latter were then decanted into 10 ml tubes and evaporated until dry using a mild nitrogen flow for 30 min at 50 °C. Residues remaining in the tubes were frozen at -25 °C until HPLC analysis. For HPLC analysis, 400 µl of the mobile phase, consisting of a 42.5: 57.5 ratio of sodium dihydrogen phosphate (0.05M and pH 4.86-4.88): acetonitrile, was added to dissolve the sample residue. The liquid was transferred into HPLC carousel tube inserts that were capped with crimp tops (Hassan et al., 2018), (Naidoo *et al.,* 2007) and transferred to the analyser.

### 3.6.2.3 *Separation and Quantification of Diclofenac Sodium and Metabolites using HPLC*

Samples were analysed at the University of Pretoria Veterinary Paraclinical Department toxicology and pharmacology laboratory. A Beckman System Gold HPLC analyser system and a gradient methodology was used. The analyser consists of the following components; a 32 Karat™ software package, a diode array detector (DAD) 168, an autosampler module 508 and a programmable solvent module 126 (Beckman Instruments, Fullerton, California, USA). A 250 x 4.6 mm, 5 µ BDS HYPERSIL Phenyl column was used and, initially (see below), a mobile phase of 42.5: 57.5 ratio of sodium dihydrogen phosphate (0.05M and pH 4.86-4.88): acetonitrile was used for both diclofenac and metabolite detection. Thirty microliters (30 µl) of reconstituted sample was injected into the HPLC column. The flow rate and detection wavelength used were 1 ml/min and 275 nm respectively. The gradient method of analysis, with a total run time of 8 min, was used to gradually decrease the ratio of sodium dihydrogen phosphate: acetonitrile from its starting ratio to 20: 80 over a time of 3 min. Data was collected over the first 6 minutes of the run and the method ended at 7 minutes. The machine returned the ratio of mobile phase constituents to the start ratio over the remaining 1 min of the run.

The mean retention time for diclofenac and the 4'- and 5'-hydroxydiclofenac metabolite combination was 4.51 and 3.45 min respectively (see figure 3.2 and 3.3). The standard calibration curve showed an $r^2$ value > 0.99 for each run for diclofenac and the 4'- and 5'-hydroxydiclofenac metabolites. The LOD and LOQ values for diclofenac were 0.195 µg/ml and 0.396 µg/ml respectively. The LOD and LOQ were both 0.095 µg/ml for the 4'- and 5'-

hydroxydiclofenac metabolites. Please see Addendum 8.2 for full details of the validation report.

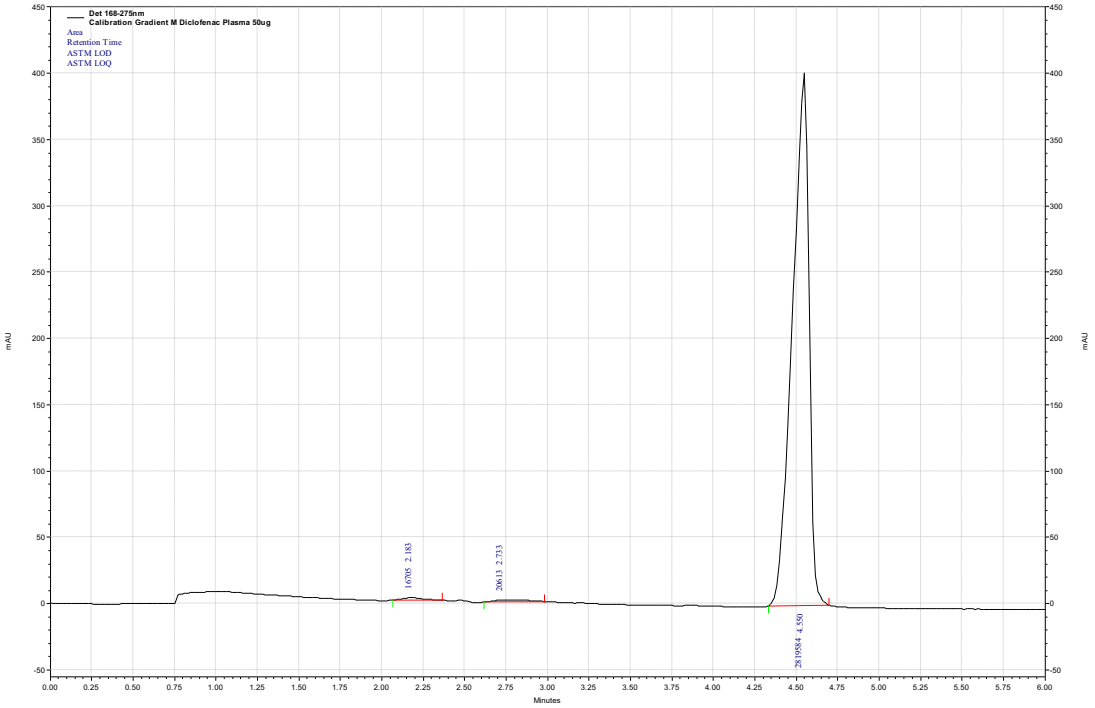


**Figure 3.2 Diclofenac in plasma at 6.25 µg/ml.**

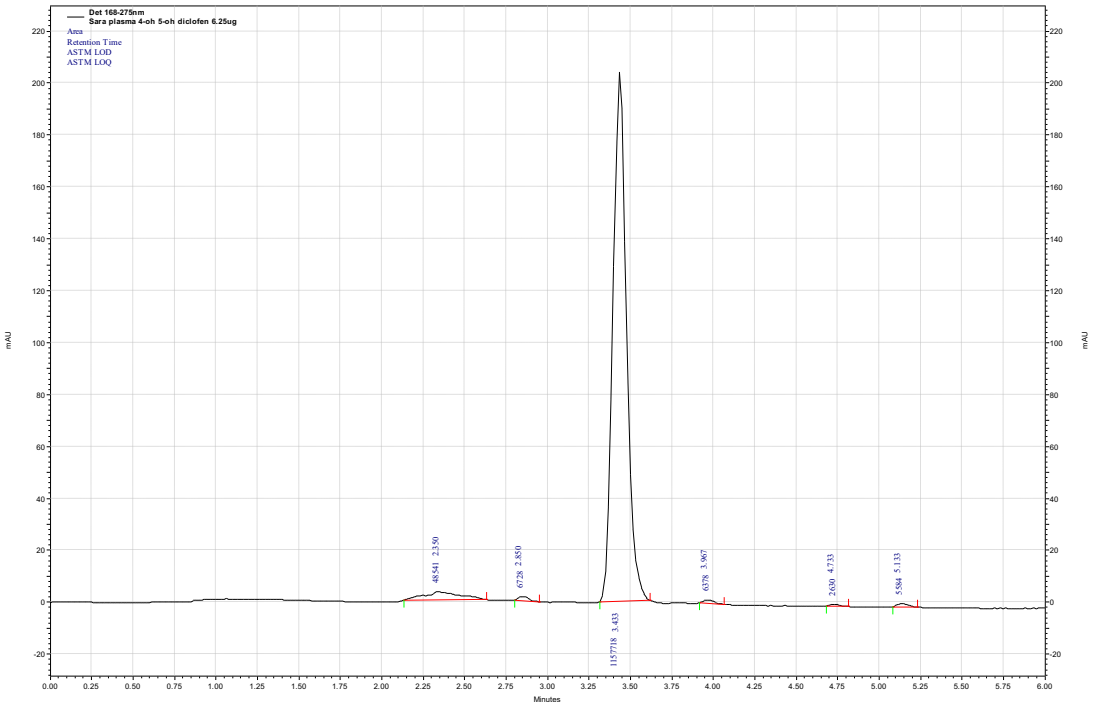


**Figure 3.3 Metabolites 4'- and 5'-hydroxydiclofenac in plasma at concentration 6.25 µg/ml.**

### 3.6.3 *Diclofenac Pharmacokinetic Analysis*

Concentration-time data generated from the HPLC analysis was dose equalised to 1mg/kg and evaluated using non-compartmental modelling and Kinetica 5.0 software (ThermoFisher Scientific, Waltham, Massachusetts, US). The pharmacokinetic parameters reported were; $T_{max}$, $C_{max}$, AUC, AUMC, $T_{1/2}$, MRT, Cl, $V_z$ and $V_{ss}$. Values for these parameters were determined or calculated according to table 3.3.

**Table 3.3 Kinetic 5.0 methods and equations used to calculate PK parameters.**

| **PK Parameter** | **Kinetica 5.0 Equation** |
|---|---|
| **$T_{max}$** (time to maximum plasma concentration) | Read directly from the concentration versus time plasma curve |
| **$C_{max}$** (maximum plasma concentration) | Read directly from the concentration versus time plasma curve |
| **$AUC_{last}$** (the area under the curve to the last time point quantified) | By use of the linear trapezoidal rule: $\mathbf{AUC_{last} = \sum_{i=1}^{n} 0.5\ x((C_i + C_{i+1})\ x\Delta t)}$ |
| **$AUC_{inf}$** (the total area under the curve, extrapolated to infinity) | $\mathbf{AUC_{tot} = AUC_{last} + AUC_{extra}}$, where $\mathbf{AUC_{extra} = C_{Last}/\lambda.}$ $C_{last}$ is the last measured concentration and λ is the terminal elimination rate constant |
| **$AUCM_{last}$** (the area under the moment curve to the last measured time point) | $\mathbf{AUMC_{last} = \sum_{i=1}^{n} 0.5\ x(t_i\ xC_i + t_i^{+1}\ x\ C_{i+1})\ x\Delta t.}$ |
| **$T_{1/2}$** (terminal half-life of elimination) | $\mathbf{T_{1/2} = Ln(2)/\lambda}$ |
| **MRT** (mean residence time) | $\mathbf{MRT = AUMC_{tot}/AUC_{tot}}$ |
| **Cl** (Clearance) | $\mathbf{Cl = Dose/AUC_{tot}}$ |
| **$V_z$** (Volume of distribution during the terminal phase) | $\mathbf{V_z = Cl/\lambda = Dose/(AUC\ x\ \lambda).}$ |
| **$V_{ss}$** (volume of distribution during the steady phase) | $\mathbf{V_{ss} = (Dose\ x\ MRT)/AUC}$ |

### 3.6.4 *Diclofenac Statistical Analysis*

The means of the test and negative control groups for the PK data $AUC_{last}$, $C_{max}$ ,$T_{1/2}$, MRT and Cl were subjected to univariate ANOVA analysis, using SPSS Version 1.0.0.95 statistical software (IBM, New York, NYS, US);

### *3.6.5 Metabolite Analysis*

Three distinct peaks, believed to be metabolites (not visible in blank plasma), were seen on HPLC analysis. Due to inability to separate 4'- and 5'-hydroxydiclofenac on calibrations and extreme variation in profiles produced between birds, metabolite peaks were analysed and compared by first calculating the dose equalised $AUC_{last}$ values for each peak, based on the peak height readings, over time. These values were compared to the diclofenac peak dose equalised $AUC_{last}$ values, per treatment group and mortality status. The metabolite $AUC_{last}$ results were further subject to binary logistic regression, using SPSS Version 1.0.0.95 statistical software (IBM, New York, NYS, US), to check for relationship and significance thereof between extent of exposure and mortality. The treatment groups were compared by subjecting the means of the ratio of diclofenac: metabolite $AUC_{last}$ peak to the Independent Samples Students t-test, also using SPSS Version 1.0.0.95 statistical software (IBM, New York, NYS, US).

## 3.7 Median Lethal Dose ($LD_{50}$) Determination

The $LD_{50}$ for the test and negative control group of birds was determined using the MLE probit model (OECD, 2016). The OECD guideline 223 for avian acute oral toxicity testing uses the model as follows, "The philosophy underlying tolerance distributions is that an individual bird will die if it receives a dose above a certain value but will survive if the dose is equal to or less than this value. The specific value is called a tolerance and is assumed to be fixed for an individual bird, but to vary among birds. Thus, for a population of birds one can speak of a distribution of tolerances, or a tolerance distribution.

In order to estimate the tolerance distribution from a sample of birds, a statistical model is fitted. If it is assumed that the tolerances follow a normal distribution a probit model is fitted which takes the form

**Probit(p) = a + b*log(d),**

where: **'p'** is the probability that the tolerance of an individual bird is less than dose **'d'** – i.e., the probability that a bird receiving dose d will survive, and **'a'** and **'b'** are parameters representing the intercept and slope of a straight line relationship between probit **(p)** and **log(d)**. The probit model is fitted to test data in order to obtain estimates of the parameters **'a'** and **'b'**. The estimate of the mean of the tolerance distribution of the population of birds (called the $LD_{50}$) can then be determined from the equation:

**Estimate [log($LD_{50}$) = -a/b,**

and the variance of the tolerance distribution can be estimated by:

**Variance = 1/b.”** (OECD, 2016, p 21)

Microsoft® Excel workbook SEquential DEsign Calculator (SEDEC) was used for these calculations.

# 4 Results

## 4.1 Clinical Signs and Mortalities

### *4.1.1 Test Group*

Two birds, 5877 and 5833, died during diclofenac i/v dosing. These birds received the highest (28.61 mg/kg) and second highest (22.55 mg/kg) dose respectively (table 4.2). The animals collapsed, from what was suspected to be acute cardiovascular collapse, with mucous membrane pallor, apnoea and asystole. Resuscitative techniques were not successful. A further 2 birds, 5836 (dosed at 14.00 mg/kg) and 5842 (dosed at 6.86 mg/kg) succumbed within approximately 48 hours of i/v dosing. Affected birds showed expected typical signs of diclofenac toxicity; lethargy and depression, anorexia and unwillingness to move. Birds were found dead on Day 3 of the study at the 08:00 morning check. The remaining 6 birds showed no obvious signs of intoxication. There were no weight losses for the birds that survived diclofenac treatment and only a mild decrease in Feed Conversion Ratio (FCR) over the first 3 days post treatment. The latter was probably partly due to treatment and partly due to stress and handling on day 1 of the study.

### *4.1.2 Negative Control Group*

One bird, 5840 (dosed at 5.40 mg/kg), died during collection of the first blood sample at 15 minutes after dosing (table 4.2). As for birds from the test group, the animal collapsed, with mucous membrane pallor, apnoea and asystole. Resuscitative techniques were also not successful in this animal. A second bird, 5847 (dosed at the highest dose of 28.61 mg/kg), died approximately 7 hours after dosing. The animal was found dead during the afternoon 16:00 check and died acutely, with minimal clinical signs. A third bird, 5999, succumbed within approximately 28 hours. This bird received a dose of 17.70 mg/kg and also showed expected typical signs of diclofenac toxicity; lethargy and depression, anorexia and unwillingness to move. It was found dead on Day 2 of the study at the 12:30. A fourth bird, 5845, was euthanased at approximately 56 hours after dosing. The animal was collapsed, anorexic and showing respiratory distress for most of day 3 and it was deemed to be in distress. This bird had been dosed at 22.55 mg/kg. The remaining 6 birds showed no obvious signs of intoxication. There were no weight losses over the study period for the birds that survived diclofenac treatment and

only a mild decrease in FCR over the first 3 days post treatment. The latter was probably partly due to treatment and partly due to stress and handling on day 1 of the study.

Overall mortalities per group are shown in table 4.1, including bird 5850, euthanased for musculoskeletal developmental abnormalities (section 3.2.4) from the healthy control group. Weight trends for both groups treated are shown in table 4.2. Chi Square analysis revealed no statistical significance (p = 0.582) between birds being below average weight (both for the group and broiler birds at 5 weeks of age) and mortality (section 8.3, figure 8.8).

**Table 4.1 Overall mortalities per treatment group after diclofenac dosing.**

| | **Test group** *(n=10)* | **Negative control group** *(n=10)* | **Healthy control group** *(n=5)* |
|---|---|---|---|
| **Alive** | 6 | 6 | 4 |
| **Dead** | 4 | 4 | 1 (5850)* |

*5850 euthanased on day 6 of study due to musculoskeletal abnormalities.

**Table 4.2 Individual weight trends per treatment group.**

| **Bird ID** | **Dose** (mg/kg) | **Mortality** | **Weight** (g) | | | |
|---|---|---|---|---|---|---|
| | | | **Day -3** | **Day 1** | **Day 8** | **Day 15** |
| **TEST GROUP** | | | | | | |
| **5838** | 3.36 | No | 1803 | 2030 | 2738 | 3723 |
| **5848** | 4.26 | No | 1972 | 2265 | 2894 | 3856 |
| **5876** | 5.40 | No | 1849 | 2041 | 2600 | 3334 |
| **5842** | 6.86 | Yes | 1282 | 1426 | - | - |
| **5841** | 8.70 | No | 1604 | 1814 | 2407 | 3164 |
| **5849** | 11.04 | No | 1187 | 1430 | 1895 | 2617 |
| **5836** | 14.00 | Yes | 1129 | 1268 | - | - |
| **5844** | 17.70 | No | 1380 | 1620 | 2230 | 3136 |
| **5833** | 22.55 | Yes | 1272 | 1450 | - | - |
| **5877** | 28.61 | Yes | 1098 | 1229 | - | - |
| **NEGATIVE CONTROL GROUP** | | | | | | |
| **5843** | 3.36 | No | 1051 | 1118 | 1535 | 2754 |
| **5839** | 4.26 | No | 1191 | 1373 | 1808 | 2604 |
| **5840** | 5.40 | Yes | 1112 | 1287 | - | - |
| **5846** | 6.86 | No | 1427 | 1626 | 2062 | 2698 |
| **5834** | 8.70 | No | 1469 | 1701 | 1990 | 2506 |
| **5878** | 11.04 | No | 1652 | 1920 | 1813 | 1827 |
| **5835** | 14.00 | No | 1498 | 1749 | 2207 | 2761 |
| **5999** | 17.70 | Yes | 2156 | 2548 | - | - |
| **5845** | 22.55 | Yes | 1476 | 1674 | - | - |
| **5847** | 28.61 | Yes | 2023 | 2322 | - | - |

Rows highlighted in yellow are birds that died.

## 4.2 Pathology

### *4.2.1 Acute Deaths*

#### *4.2.1.1 Birds 5877, 5833 (test group) and 5840 (negative control group)*

No gross or histopathological lesions were observed for the two birds from the test group which died immediately after i/v diclofenac dosing, nor the bird from the negative control group which died at the 15 min bleed point.

### *4.2.2 Subacute Deaths*

#### *4.2.2.1 Test group – 5836 (figure 4.1)*

Gross pathology: There was marked urate deposition on all serosal surfaces, the pericardium, the air sacs, the liver and the spleen. The kidneys were markedly enlarged, bulging from their fossae, with prominent tubules and soft in texture. Urates were also present in the joints and tendon sheaths of the legs.

Histopathology: There was marked widespread cell injury and necrosis of the renal tubular epithelium with dilatation of the damaged tubules and marked deposition of globule urates within the lumens of the damaged tubules. Cell changes ranged from increased eosinophilia to cell membrane disruption and sloughing into the lumen, with spicules of uric acid visible within the eosinophilic masses.

#### *4.2.2.2 Test group – 5842*

Gross pathology: Bird 5842 exhibited similar necropsy findings to 5836 but there was milder urate deposition and the kidneys only showed a lightening in colour.

Histopathology: Similarly, the histopathology was as for 5836 but milder, with many of the tophi showing giant cell aggregates at the periphery.

#### *4.2.2.3 Negative control group – 5999*

Gross pathology: There was urate crystal deposition on all serous surfaces and air sacs, as well as in the epicardium and pericardium. Marked pulmonary oedema and congestion was present as well as a mild hydropericardium. The kidneys were swollen and bulged from their fossae, with the pattern of the tubules accentuated.

Histopathology: The findings for 5999 were similar to 5836 and of the same severity.

#### *4.2.2.4 Negative Control group – 5845 (figure 4.2)*

Gross pathology: There were mild urate deposits on the serosa, pericardium, liver and spleen. The kidneys were enlarged, bulging from fossae with a prominent tubule pattern.

Histopathology: The findings for 5845 were similar to 5836 but milder, with many of the tophi showing giant cell aggregates at the periphery.

#### *4.2.2.5 Negative control group – 5847*

The pathology report for this bird could not be found by the Pathology Department.

### *4.2.3 Birds Euthanased on Day 15 of Study*

Birds 5838, 5848, 5876, 5841, 5849, 5844 (test group) and birds 5843, 5839, 5846, 5834, 5878, 5835 (negative control group) showed no gross or histopathological lesions.

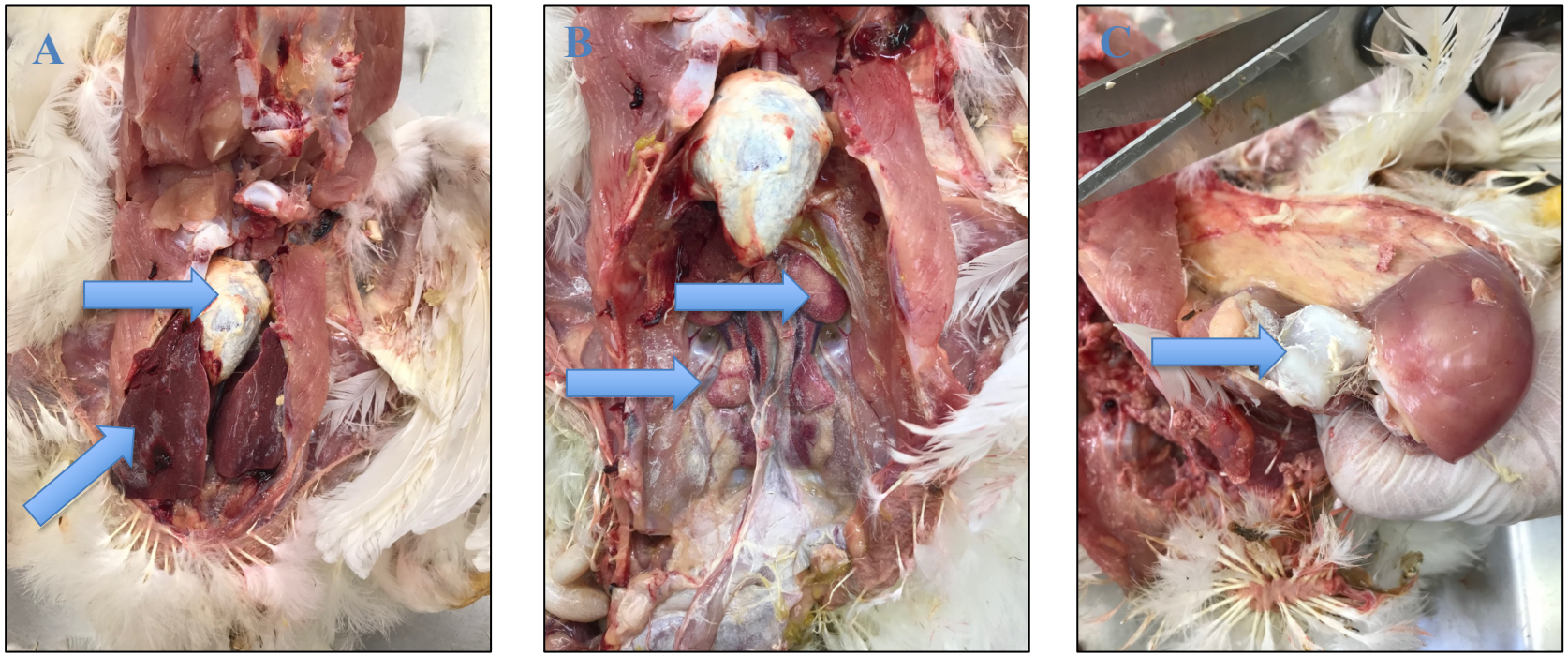


**Figure 4.1 Gross pathology findings for test group birds.**

Bird 5836 showing visceral gout with uric acid accumulation on the serosal surface of the liver and pericardium (A), nephrosis of the kidneys (B) and uric acid accumulation in the tibeo-tarsal joint (C).

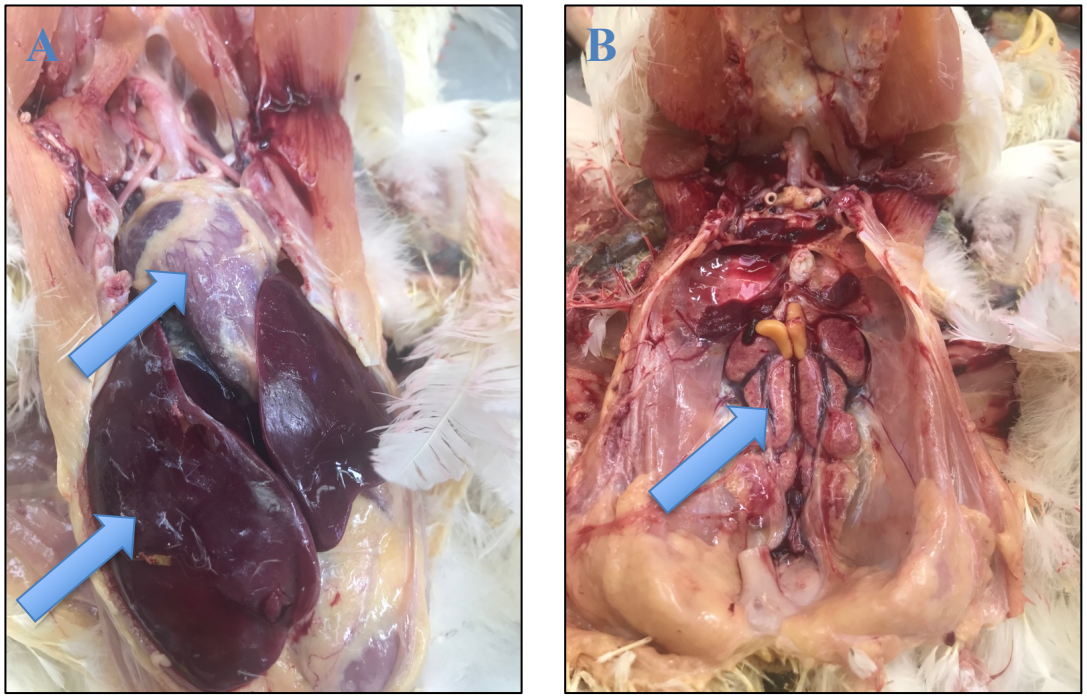


**Figure 4.2 Gross pathology findings for control group birds.**

Bird 5845, showing visceral gout and uric acid accumulation on the serosal surface of the liver and pericardium (A) and nephrosis of the kidneys (B).

## 4.3 Uric Acid Analysis

The uric acid concentration trends over the scheduled time points and total exposure ($AUC_{last}$) are shown per bird in each group (table 4.3, figure 8.9 and figure 8.10) and per treatment group in figure 4.3. The t-test output results for comparing the mean $AUC_{last}$ values per group are shown in figure 8.11. There were no significant differences between the treatment groups ($p = 0.103$). Baseline correction of uric acid values obtained over the scheduled time points are presented in table 4.4. As previously, no significant difference was evident between the treatment groups (all p values >0.05).

Both test and negative control group birds showed similar 0 h (prior to treatment with diclofenac) uric acid readings and within the reference range as described by Ross et al., 1978 and Wilson & Miles.,1988. Over the scheduled sampling points of 0.25, 0.5, 1 and 2 h post-dosing, there were no remarkable differences between groups. Only birds 5999 (dosed at 17.70 mg/kg), 5835 (dosed at 14.00 mg/kg) and 5847 (dosed at 28.61 mg/kg) showed plasma uric acid concentrations above the reference range (underlined in table 4.3) at any time point. All 3 of these birds were from the negative control group and only 5999 and 5847 died.

**Table 4.3 Uric acid trends per treatment group.**

| Bird ID | Dose (mg/kg BW) | Scheduled Bleed Time points (hours) | | | | | AUC (mmol*min/L) |
|---|---|---|---|---|---|---|---|
| | | 0 | 0.25 | 0.5 | 1 | 2 | |
| **TEST GROUP** | | | | | | | |
| **5838** | 3.36 | 0.27 | 0.4 | 0.4 | 0.33 | 0.34 | 22.27 |
| **5848** | 4.26 | 0.47 | 0.43 | 0.43 | 0.36 | 0.29 | 25.34 |
| **5876** | 5.4 | 0.38 | 0.33 | 0.24 | 0.21 | 0.13 | 16.64 |
| **5842** | 6.86 | 0.34 | 0.16 | 0.23 | 0.25 | 0.33 | 14.17 |
| **5841** | 8.7 | 0.28 | 0.22 | 0.21 | 0.29 | 0.34 | 14.77 |
| **5849** | 11.04 | 0.37 | 0.36 | 0.39 | 0.39 | 0.35 | 23.09 |
| **5836** | 14 | 0.3 | 0.26 | 0.28 | 0.43 | 0.44 | 19.19 |
| **5844** | 17.7 | 0.23 | 0.38 | 0.41 | 0.54 | 0.51 | 25.04 |
| **5833** | 22.55 | 0.16 | - | - | - | - | - |
| **5877** | 28.61 | 0.24 | - | - | - | - | - |
| **Mean** | | 0.30 | 0.32 | 0.32 | 0.35 | 0.34 | 20.06 |
| **Geomean** | | 0.29 | 0.30 | 0.31 | 0.34 | 0.32 | 19.60 |
| **SD** | | 0.09 | 0.09 | 0.09 | 0.11 | 0.11 | 4.50 |
| **SE** | | 0.03 | 0.03 | 0.03 | 0.04 | 0.04 | 1.59 |
| **NEGATIVE CONTROL GROUP** | | | | | | | |
| **5843** | 3.36 | 0.67 | 0.54 | 0.39 | 0.19 | 0.29 | 25.04 |
| **5839** | 4.26 | 0.41 | 0.41 | 0.41 | 0.39 | 0.36 | 24.44 |
| **5840** | 5.4 | 0.33 | - | - | - | - | - |
| **5846** | 6.86 | 0.33 | 0.37 | 0.38 | 0.35 | 0.27 | 22.12 |
| **5834** | 8.7 | 0.33 | 0.23 | 0.24 | 0.31 | 0.27 | 16.27 |
| **5878** | 11.04 | 0.39 | 0.46 | 0.5 | 0.53 | 0.47 | 29.32 |
| **5835** | 14 | 0.39 | 0.54 | 0.76 | 0.69 | 0.41 | 38.77 |
| **5999** | 17.77 | 0.41 | 0.71 | 0.82 | 0.97 | 0.99 | 105.5 |
| **5845** | 22.55 | 0.38 | 0.38 | 0.34 | 0.35 | 0.27 | 40.1 |
| **5847** | 28.61 | 0.35 | 0.41 | 0.84 | 2.83 | - | 180.2 |
| **Mean** | | 0.40 | 0.45 | 0.52 | 0.73 | 0.42 | 53.53 |
| **Geomean** | | 0.39 | 0.43 | 0.48 | 0.52 | 0.38 | 38.61 |
| **SD** | | 0.10 | 0.14 | 0.23 | 0.82 | 0.24 | 54.51 |
| **SE** | | 0.03 | 0.05 | 0.08 | 0.29 | 0.08 | 18.17 |

Rows highlighted in yellow are birds that died. Underlined values are those above the reference range.

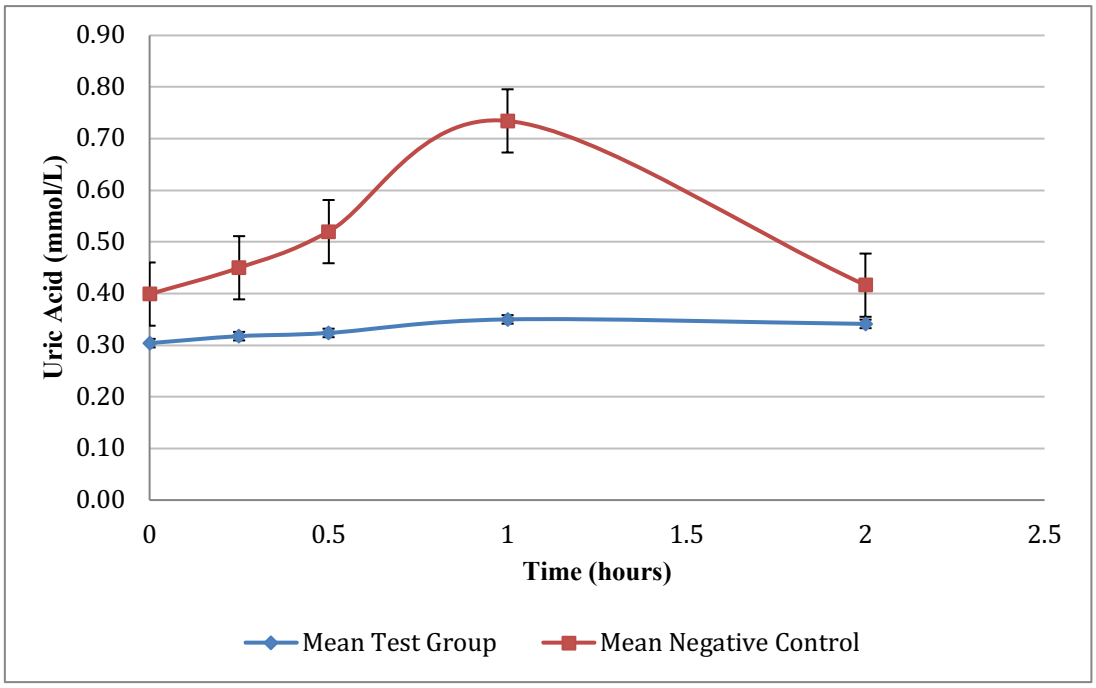


**Figure 4.3 Mean uric acid levels over scheduled time points per treatment group.**

**Table 4.4 Uric acid concentrations at scheduled bleed time points, following correction for the baseline (0 h) concentration.**

| Bird ID | Dose (mg/kg) | Scheduled Bleed Time Points (hours) | | | | |
|---|---|---|---|---|---|---|
| | | **0.00** | **0.25** | **0.50** | **1.00** | **2.00** |
| | | | **Baseline Correction per Scheduled Time Point*** | | | |
| **TEST GROUP** | | | | | | |
| **5838** | 3.36 | 0.27 | 0.13 | 0.13 | 0.06 | 0.07 |
| **5848** | 4.26 | 0.47 | -0.04 | -0.04 | -0.11 | -0.18 |
| **5876** | 5.40 | 0.38 | -0.05 | -0.14 | -0.17 | -0.25 |
| **5842** | 6.86 | 0.34 | -0.18 | -0.11 | -0.09 | -0.01 |
| **5841** | 8.70 | 0.28 | -0.06 | -0.07 | 0.01 | 0.06 |
| **5849** | 11.04 | 0.37 | -0.01 | 0.02 | 0.02 | -0.02 |
| **5836** | 14.00 | 0.30 | -0.04 | -0.02 | 0.13 | 0.14 |
| **5844** | 17.70 | 0.23 | 0.15 | 0.18 | 0.31 | 0.28 |
| | | **Mean** | **-0.01** | **-0.01** | **0.02** | **0.01** |
| **NEGATIVE CONTROL GROUP** | | | | | | |
| **5843** | 3.36 | 0.67 | -0.13 | -0.28 | -0.48 | -0.38 |
| **5839** | 4.26 | 0.41 | 0.00 | 0.00 | -0.02 | -0.05 |
| **5846** | 6.86 | 0.33 | 0.04 | 0.05 | 0.02 | -0.06 |
| **5834** | 8.70 | 0.33 | -0.10 | -0.09 | -0.02 | -0.06 |
| **5878** | 11.04 | 0.39 | 0.07 | 0.11 | 0.14 | 0.08 |
| **5835** | 14.00 | 0.39 | 0.15 | 0.37 | 0.30 | 0.02 |
| **5999** | 17.77 | 0.41 | 0.30 | 0.41 | 0.56 | 0.58 |
| **5845** | 22.55 | 0.38 | 0.00 | -0.04 | -0.03 | -0.11 |
| **5847** | 28.61 | 0.35 | 0.06 | 0.49 | - | 2.48 |
| | | **Mean** | **0.04** | **0.11** | **0.06** | **0.28** |

*Baseline correction is calculated as: Uric acid value at a scheduled time point - 0 h uric acid value, for the same animal. Rows highlighted in yellow are birds that died.

## 4.4 Diclofenac Pharmacokinetic Analysis

The pharmacokinetic parameters per treatment group are presented in tables 4.5 to 4.7 with the mean plasma concentration versus time profile presented in figure 4.4 (individual bird plasma concentration versus time profiles are presented in section 8.4, figure 8.12 and 8.13). The majority of the birds showed the expected i/v curve of linear depletion from a maximum concentration at the first point of sampling. Bird 5834 (dosed at 8.70 mg/kg) and 5878 (dosed at 11.04 mg/kg) from the negative control group and 5842 (dosed at 28.61 mg/kg) from the test group showed what appear to be an absorptive, or mixed absorptive/ intravenous curves (figure 8.12 and 8.13), most likely due to subcutaneous or partial subcutaneous dosing respectively. Bird 5841 from the test group (dosed at 8.70 mg/kg) had a 2 h outlier reading which was impossibly high with the result that we omitted the data point from the PK and statistical calculations. Finally, bird 5842 from the test group had a very high 15 min reading. This result could be either indicative of an outlier in analysis or the normal profile for a bird that is metabolically constrained, as indicated by the higher than average $AUC_{last}$ (1.87 µg/ml*h), $T_{1/2}$ (1.46 h) and MRT (1.83 h) values and lower than average Cl value (0.36 L/h*kg). This bird was consequently omitted from the ANOVA calculations but included in the descriptive statistics in table 4.6.

Though there was some variation in the curves, when excluding bird 5842, the mean $C_{max}$ and $T_{max}$ values were 0.51 µg/ml and 0.50 h across all birds, respectively. The mean $T_{max}$ value is skewed by the birds that demonstrated either absorptive or partially absorptive curves, with the median $T_{max}$ time being 0.25 h, as expected. Though the ANOVA analysis (figures 8.14 to 8.16) showed no significant difference between groups for $C_{max}$ ($p = 0.28$), $AUC_{last}$ ($p = 0.43$) and $T_{1/2}$ ($p = 0.59$), the values for $C_{max}$ and $AUC_{last}$ are on average higher for the test group. Though the mean $T_{1/2}$ value for the test group is lower than that of the negative control, the birds from the test group that received the 2 highest doses (22.55 and 28.61 mg/kg) both died on dosing. The absence of their PK curves from the data set may obscure differences between the groups for this parameter. This is evident for the corresponding birds in the negative control group that received the highest doses (5845 and 5847), as their respective $T_{1/2}$ values were 7.01 and 2.75 h. These values, which were expected from higher dosed birds, may have contributed to the higher mean $T_{1/2}$ value for this group, reflected in the higher %CV. The median $T_{1/2}$ values for the test and negative control group were 0.74 and 0.70 respectively, comparable with those from (Naidoo et al., 2007).

**Table 4.5 Pharmacokinetic data following i/v diclofenac dosing for test group of birds, excluding bird 5842.**

| **Bird** | **Dose** | **$T_{max}$** | **$C_{max}$** | **$AUC_{last}$** | **$AUC_{tot}$** | **$AUMC_{last}$** | **$L_z$** | **$T_{1/2}$** | **MRT** | **Cl** | **$V_z$** | **$V_{ss}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg | h | µg/ml | µg/ml*h | µg/ml*h | µg/ml*(h)$^2$ | 1/h | h | h | L/h*kg | L/kg | L/kg |
| 5838 | 3.36 | 0.25 | 0.52 | 0.44 | 0.53 | 0.32 | 0.94 | 0.74 | 1.13 | 1.89 | 2.01 | 2.14 |
| 5848 | 4.26 | 0.25 | 0.96 | 0.70 | 0.93 | 0.54 | 0.76 | 0.91 | 1.40 | 1.08 | 1.42 | 1.51 |
| 5876 | 5.40 | 0.25 | 1.06 | 0.84 | 1.34 | 0.67 | 0.46 | 1.50 | 2.04 | 0.75 | 1.61 | 1.53 |
| 5842 | 6.86 | | | | | | | | | | | |
| 5841 | 8.70 | 2.00 | 0.24 | 0.27 | 0.46 | 0.34 | 0.36 | 1.94 | 2.71 | 2.19 | 6.15 | 5.94 |
| 5849 | 11.04 | 0.25 | 0.22 | 0.20 | 0.20 | 0.13 | 2.15 | 0.32 | 0.68 | 4.90 | 2.28 | 3.32 |
| 5836 | 14.00 | 0.25 | 0.51 | 0.41 | 0.46 | 0.29 | 1.09 | 0.64 | 0.96 | 2.17 | 1.99 | 2.09 |
| 5844 | 17.70 | 0.25 | 0.42 | 0.40 | 0.44 | 0.29 | 1.17 | 0.59 | 0.96 | 2.25 | 1.93 | 2.16 |
| N | | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| **Mean** | | 0.50 | 0.56 | 0.46 | 0.62 | 0.37 | 0.99 | 0.95 | 1.41 | 2.18 | 2.49 | 2.67 |
| **Harmomean** | | 0.29 | 0.41 | 0.38 | 0.46 | 0.29 | 0.73 | 0.70 | 1.16 | 1.61 | 2.03 | 2.19 |
| **GeoMean** | | 0.34 | 0.48 | 0.42 | 0.53 | 0.33 | 0.85 | 0.81 | 1.27 | 1.87 | 2.20 | 2.39 |
| **SEM** | | 0.25 | 0.12 | 0.09 | 0.14 | 0.07 | 0.23 | 0.22 | 0.27 | 0.51 | 0.62 | 0.59 |
| **SD** | | 0.66 | 0.33 | 0.23 | 0.38 | 0.18 | 0.60 | 0.57 | 0.72 | 1.34 | 1.64 | 1.56 |
| **Median** | | 0.25 | 0.51 | 0.41 | 0.46 | 0.32 | 0.94 | 0.74 | 1.13 | 2.17 | 1.99 | 2.14 |
| **Min** | | 0.25 | 0.22 | 0.20 | 0.20 | 0.13 | 0.36 | 0.32 | 0.68 | 0.75 | 1.42 | 1.51 |
| **Max** | | 2.00 | 1.06 | 0.84 | 1.34 | 0.67 | 2.15 | 1.94 | 2.71 | 4.90 | 6.15 | 5.94 |
| **%CV** | | 74.30 | 25.75 | 20.64 | 26.98 | 20.65 | 26.45 | 26.49 | 21.35 | 27.04 | 28.21 | 24.77 |

$T_{max}$ = Time to maximum plasma concentration. $C_{max}$ = Maximum plasma concentration. $AUC_{last}$ = Area under the curve to last measured (quantifiable) time point. $AUC_{tot}$ = Area under the curve extrapolated to infinity. $AUCM_{last}$ = Area under the moment curve to last measured (quantifiable) time point. $L_z$ = Terminal elimination phase rate constant. $T_{1/2}$ = Half-life of elimination or terminal half-life. MRT = Mean residence time. Cl = Clearance. $V_z$ = Volume of distribution during the terminal phase. $V_{ss}$ = Volume of distribution during steady state.

Yellow highlighted cells are birds which succumbed.

**Table 4.6 Pharmacokinetic data following i/v diclofenac dosing for test group of birds, including bird 5842.**

| Bird | Dose | $T_{max}$ | $C_{max}$ | $AUC_{last}$ | $AUC_{tot}$ | $AUMC_{last}$ | $L_z$ | $T_{1/2}$ | MRT | Cl | $V_z$ | $V_{ss}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg | h | µg/ml | µg/ml*h | µg/ml*h | µg/ml*(h)$^2$ | 1/h | h | h | L/h*kg | L/kg | L/kg |
| 5838 | 3.36 | 0.25 | 0.52 | 0.44 | 0.53 | 0.32 | 0.94 | 0.74 | 1.13 | 1.89 | 2.01 | 2.14 |
| 5848 | 4.26 | 0.25 | 0.96 | 0.70 | 0.93 | 0.54 | 0.76 | 0.91 | 1.40 | 1.08 | 1.42 | 1.51 |
| 5876 | 5.40 | 0.25 | 1.06 | 0.84 | 1.34 | 0.67 | 0.46 | 1.50 | 2.04 | 0.75 | 1.61 | 1.53 |
| 5842 | 6.86 | 0.25 | 3.00 | 1.87 | 2.78 | 1.37 | 0.48 | 1.46 | 1.83 | 0.36 | 0.75 | 0.66 |
| 5841 | 8.70 | 2.00 | 0.24 | 0.27 | 0.46 | 0.34 | 0.36 | 1.94 | 2.71 | 2.19 | 6.15 | 5.94 |
| 5849 | 11.04 | 0.25 | 0.22 | 0.20 | 0.20 | 0.13 | 2.15 | 0.32 | 0.68 | 4.90 | 2.28 | 3.32 |
| 5836 | 14.00 | 0.25 | 0.51 | 0.41 | 0.46 | 0.29 | 1.09 | 0.64 | 0.96 | 2.17 | 1.99 | 2.09 |
| 5844 | 17.70 | 0.25 | 0.42 | 0.40 | 0.44 | 0.29 | 1.17 | 0.59 | 0.96 | 2.25 | 1.93 | 2.16 |
| N | | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| **Mean** | | 0.47 | 0.87 | 0.64 | 0.89 | 0.49 | 0.93 | 1.01 | 1.46 | 1.95 | 2.27 | 2.42 |
| **Harmomean** | | 0.28 | 0.46 | 0.42 | 0.51 | 0.32 | 0.68 | 0.75 | 1.22 | 1.12 | 1.68 | 1.70 |
| **GeoMean** | | 0.32 | 0.61 | 0.50 | 0.66 | 0.39 | 0.79 | 0.88 | 1.33 | 1.52 | 1.92 | 2.03 |
| **SEM** | | 0.22 | 0.32 | 0.19 | 0.30 | 0.14 | 0.21 | 0.20 | 0.24 | 0.49 | 0.58 | 0.57 |
| **SD** | | 0.62 | 0.91 | 0.54 | 0.84 | 0.39 | 0.58 | 0.56 | 0.68 | 1.40 | 1.64 | 1.61 |
| **Median** | | 0.25 | 0.52 | 0.42 | 0.49 | 0.33 | 0.85 | 0.82 | 1.26 | 2.03 | 1.96 | 2.11 |
| **Min** | | 0.25 | 0.22 | 0.20 | 0.20 | 0.13 | 0.36 | 0.32 | 0.68 | 0.36 | 0.75 | 0.66 |
| **Max** | | 2.00 | 3.00 | 1.87 | 2.78 | 1.37 | 2.15 | 1.94 | 2.71 | 4.90 | 6.15 | 5.94 |
| **%CV** | | 67.47 | 53.26 | 37.91 | 45.32 | 35.04 | 25.93 | 22.53 | 18.11 | 32.43 | 30.10 | 28.08 |

$T_{max}$ = Time to maximum plasma concentration. $C_{max}$ = Maximum plasma concentration. $AUC_{last}$ = Area under the curve to last measured (quantifiable) time point. $AUC_{tot}$ = Area under the curve extrapolated to infinity. $AUCM_{last}$ = Area under the moment curve to last measured (quantifiable) time point. $L_z$ = Terminal elimination phase rate constant. $T_{1/2}$ = Half-life of elimination or terminal half-life. MRT = Mean residence time. Cl = Clearance. $V_z$ = Volume of distribution during the terminal phase. $V_{ss}$ = Volume of distribution during steady state.

Yellow highlighted cells are birds which succumbed.

**Table 4.7 Pharmacokinetic data following i/v diclofenac dosing for negative control group of birds.**

| **Bird** | **Dose** | **$T_{max}$** | **$C_{max}$** | **$AUC_{last}$** | **$AUC_{tot}$** | **$AUMC_{last}$** | **$L_z$** | **$T_{1/2}$** | **MRT** | **Cl** | **$V_z$** | **$V_{ss}$** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mg/kg | h | µg/ml | µg/ml*h | µg/ml*h | µg/ml*(h)$^2$ | 1/h | h | h | L/h*kg | L/kg | L/kg |
| 5843 | 3.36 | 0.25 | 0.60 | 0.32 | 0.36 | 0.19 | 0.99 | 0.70 | 0.87 | 2.81 | 2.83 | 2.44 |
| 5839 | 4.26 | 0.25 | 0.71 | 0.41 | 0.46 | 0.25 | 1.05 | 0.66 | 0.87 | 2.16 | 2.05 | 1.88 |
| 5846 | 6.86 | 0.25 | 0.31 | 0.35 | 0.38 | 0.26 | 1.29 | 0.54 | 0.90 | 2.60 | 2.01 | 2.35 |
| 5834 | 8.70 | 1.00 | 0.34 | 0.44 | 0.61 | 0.40 | 0.61 | 1.13 | 1.65 | 1.63 | 2.66 | 2.69 |
| 5878 | 11.04 | 1.00 | 0.55 | 0.62 | 0.65 | 0.52 | 1.40 | 0.50 | 0.93 | 1.55 | 1.11 | 1.44 |
| 5835 | 14.00 | 0.25 | 0.83 | 0.79 | 1.43 | 0.67 | 0.40 | 1.73 | 2.48 | 0.70 | 1.75 | 1.74 |
| 5999 | 17.70 | 0.25 | 0.41 | 0.35 | 0.37 | 0.24 | 1.60 | 0.43 | 0.77 | 2.71 | 1.70 | 2.08 |
| 5845 | 22.55 | 0.25 | 0.12 | 0.19 | 1.14 | 0.20 | 0.10 | 7.01 | 10.22 | 0.88 | 8.88 | 8.98 |
| 5847 | 28.61 | 1.00 | 0.30 | 0.42 | 1.03 | 0.41 | 0.25 | 2.75 | 3.95 | 0.97 | 3.87 | 3.84 |
| N | | 9 | 9.00 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 | 9 |
| **Mean** | | 0.50 | 0.46 | 0.43 | 0.71 | 0.35 | 0.86 | 1.72 | 2.52 | 1.78 | 2.98 | 3.05 |
| **Harmomean** | | 0.33 | 0.34 | 0.38 | 0.56 | 0.29 | 0.40 | 0.81 | 1.26 | 1.40 | 2.17 | 2.33 |
| **GeoMean** | | 0.40 | 0.41 | 0.40 | 0.63 | 0.32 | 0.64 | 1.08 | 1.63 | 1.59 | 2.48 | 2.59 |
| **SEM** | | 0.13 | 0.08 | 0.06 | 0.13 | 0.05 | 0.18 | 0.71 | 1.03 | 0.28 | 0.78 | 0.78 |
| **SD** | | 0.38 | 0.23 | 0.17 | 0.39 | 0.16 | 0.54 | 2.12 | 3.08 | 0.83 | 2.35 | 2.33 |
| **Median** | | 0.25 | 0.41 | 0.41 | 0.61 | 0.26 | 0.99 | 0.70 | 0.93 | 1.63 | 2.05 | 2.35 |
| **Min** | | 0.25 | 0.12 | 0.19 | 0.36 | 0.19 | 0.10 | 0.43 | 0.77 | 0.70 | 1.11 | 1.44 |
| **Max** | | 1.00 | 0.83 | 0.79 | 1.43 | 0.67 | 1.60 | 7.01 | 10.22 | 2.81 | 8.88 | 8.98 |
| **%CV** | | 31.50 | 18.51 | 14.46 | 20.69 | 17.17 | 27.84 | 65.56 | 63.02 | 17.32 | 31.63 | 30.00 |

$T_{max}$ = Time to maximum plasma concentration. $C_{max}$ = Maximum plasma concentration. $AUC_{last}$ = Area under the curve to last measured (quantifiable) time point. $AUC_{tot}$ = Area under the curve extrapolated to infinity. $AUCM_{last}$ = Area under the moment curve to last measured (quantifiable) time point. $L_z$ = Terminal elimination phase rate constant. $T_{1/2}$ = Half-life of elimination or terminal half-life. MRT = Mean residence time. Cl = Clearance. $V_z$ = Volume of distribution during the terminal phase. $V_{ss}$ = Volume of distribution during steady state.

Yellow highlighted cells are birds which succumbed.

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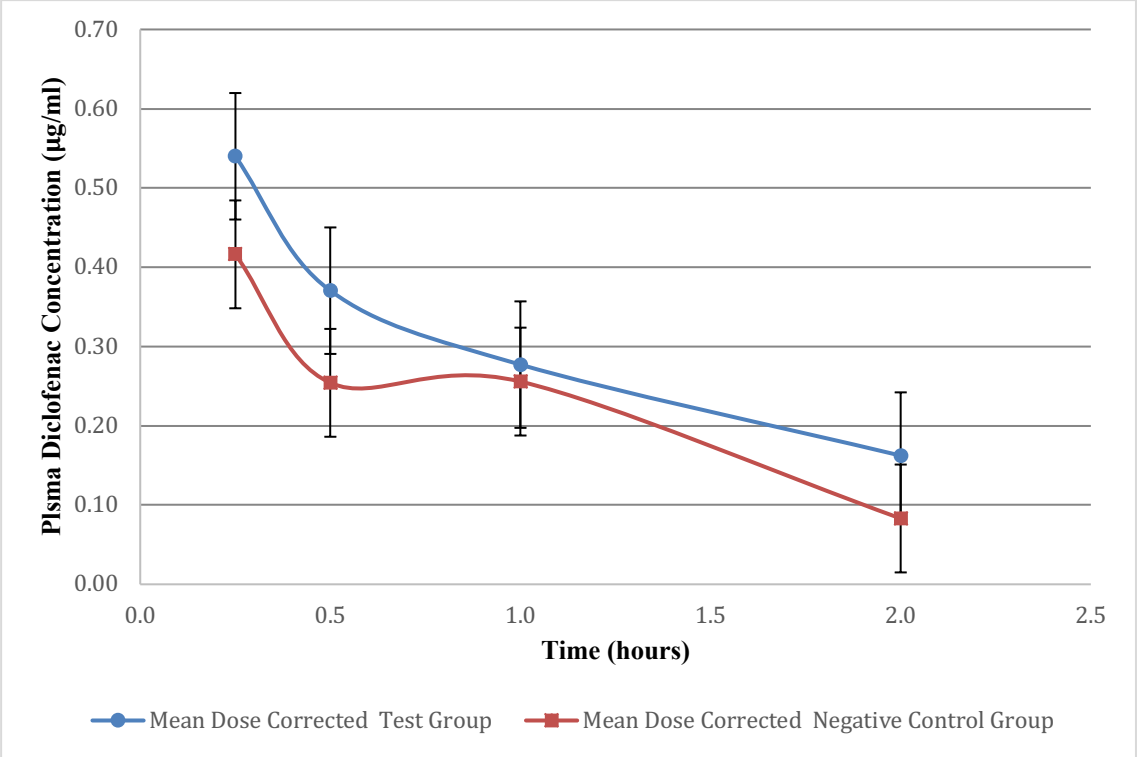


**Figure 4.4 Mean dose corrected diclofenac plasma concentration-time curves by treatment group.**

## 4.5 Metabolite Analysis

Three additional peaks were seen on HPLC evaluation of all blood samples. With these peaks absent from blank plasma samples, we interpreted them as likely diclofenac metabolites (figure 4.5). Unfortunately, individual peaks could not definitively identified due to metabolite overlap on the calibration chromatograms. The dose equalised HPLC peak $AUC_{last}$ diclofenac to metabolite ratio for each individual animal was calculated and the descriptive statistics are presented in table 4.8. Table 4.9 represents the dose corrected HPLC peak $AUC_{last}$ metabolite and diclofenac values split per treatment group and mortality status. There was no statistically significant relationship between extent of metabolite exposure and mortality. There was also no statistically significant difference in exposure between treatment groups. The results of the statistical analyses are presented in figure 8.17 to 8.21.

For peak 3 metabolites, though not significant, the overall mean ratios of diclofenac to each metabolite peak were higher in the test group compared to the control group. This was not the case for the ratio of diclofenac to peak 1 or 2 metabolites (table 4.8). Though not a consistent trend, individual data from animals that died tended to show higher diclofenac to metabolite

peak ratios than from animals that survived, such as for bird 5842 and 5836, both from the test group. Bird 5844, which did not die, but received the third highest diclofenac dose in the test group, also demonstrated higher diclofenac: metabolite peak ratios. Though less obvious in the control group, bird 5999 and 5845 showed high peak 2 and bird 5847 high peak 2 and 3 diclofenac to metabolite ratios (table 4.8). There is notable variation in values for the diclofenac: metabolite peaks, as evident by the %CV values, and variation is higher in the test group than the control group.

When comparing birds that died with those that survived across both treatment groups (table 4.9), the birds that died produced lower $AUC_{last}$ metabolite values for all 3 metabolite peaks, across both groups. When comparing the two treatment groups (both dead and alive birds), the test birds actually produced higher $AUC_{last}$ metabolite values compared to the control birds. The exception to this was peak 3 in the birds that died, which was notably lower in the test birds (value highlighted in red in table 4.9). The mean dose corrected diclofenac peak $AUC_{last}$ reading was also considerably higher in the dead birds (value highlighted in red in table 4.9).

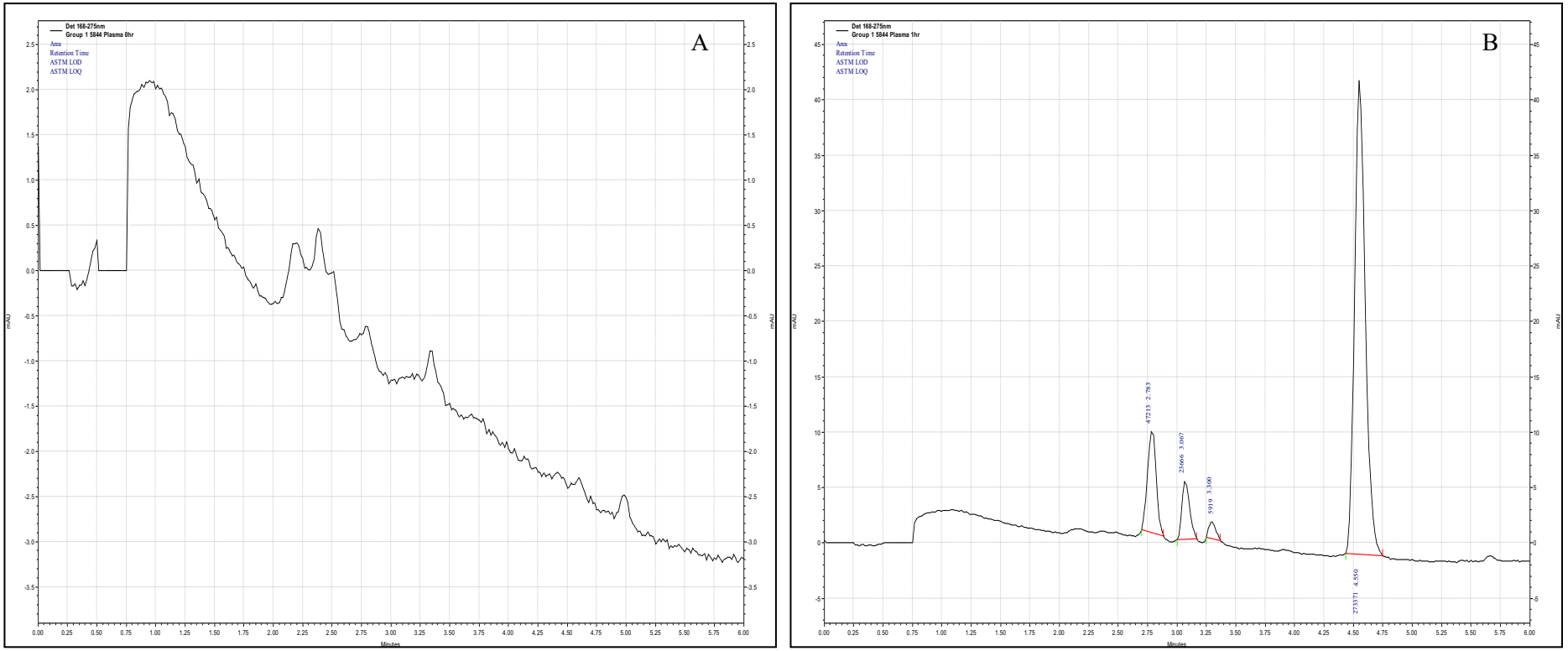


**Figure 4.5 HPLC Chromatograms for bird 5844 showing (A) blank plasma and (B) 2 h plasma sample.**

**Table 4.8 Individual animal dose corrected diclofenac to metabolite HPLC peak $AUC_{last}$ ratio, per metabolite peak.**

| TEST GROUP | | | | |
|---|---|---|---|---|
| **Bird ID** | **Dose** (mg/kg BW) | **$AUC_{last}$ Diclofenac: Metabolite Peak Ratio** | | |
| | | **Peak 1** (m Au) | **Peak 2** (m Au) | **Peak 3** (m Au)* |
| **5838** | 3.36 | 40.88 | 114.86 | 20.52 |
| **5848** | 4.26 | 57.41 | 178.66 | 220.66 |
| **5876** | 5.40 | 54.73 | 301.26 | 136.04 |
| **5842** | 6.86 | 701.03 | 1481.88 | 805.30 |
| **5841** | 8.70 | 65.21 | 74.47 | 175.71 |
| **5849** | 11.04 | 67.06 | 84.36 | 157.04 |
| **5836** | 14.00 | 104.87 | 232.10 | 718.28 |
| **5844** | 17.70 | 134.98 | 324.91 | 508.11 |
| | **Mean** | **153.27** | **349.06** | **342.71** |
| | **Geomean** | **92.95** | **213.64** | **217.43** |
| | **SD** | **223.43** | **467.39** | **294.27** |
| | **SE** | **78.99** | **165.25** | **104.04** |
| | **%CV** | **145.77** | **133.90** | **85.87** |
| **NEGATIVE CONTROL GROUP** | | | | |
| **5843** | 3.36 | 92.79 | 185.22 | 97.34 |
| **5839** | 4.26 | 43.25 | 104.44 | 62.72 |
| **5846** | 6.86 | 87.20 | 180.06 | 54.89 |
| **5834** | 8.70 | 91.02 | 159.54 | 373.04 |
| **5878** | 11.04 | 167.75 | 325.44 | 277.69 |
| **5835** | 14.00 | 204.59 | 396.01 | 311.62 |
| **5999** | 17.77 | 167.74 | 634.83 | 175.99 |
| **5845** | 22.55 | 160.26 | 905.55 | 351.62 |
| **5847** | 28.61 | 418.49 | 1446.71 | 493.75 |
| | **Mean** | **159.23** | **481.98** | **244.30** |
| | **Geomean** | **132.16** | **339.27** | **190.51** |
| | **SD** | **110.19** | **445.97** | **154.55** |
| | **SE** | **36.73** | **148.66** | **51.52** |
| | **%CV** | **69.20** | **92.53** | **63.26** |

*Peak 3 is thought to be 4'-, 5'- and possibly 3'-hydroxydiclofenac. Rows highlighted in yellow are birds that died.

**Table 4.9 Mean dose corrected $AUC_{last}$ values for each metabolite peak and diclofenac, per treatment group.**

| Mortality | | TEST GROUP | | | NEGATIVE CONTROL | | |
|---|---|---|---|---|---|---|---|
| | Peak | Mean $AUC_{last}$ Dose Corrected to 1 mg/kg | SD | %CV | Mean $AUC_{last}$ Dose Corrected to 1 mg/kg | SD | %CV |
| Alive | 1 | 676.00 | 432.09 | 63.92 | 383.81 | 248.19 | 64.67 |
| | 2 | 256.68 | 97.44 | 37.96 | 180.95 | 109.04 | 60.26 |
| | 3 | 470.09 | 630.59 | 134.14 | 243.40 | 200.68 | 82.45 |
| | Diclofenac | 39598.43 | 18550.89 | 46.85 | 38338.77 | 12625.82 | 32.93 |
| Dead | 1 | 276.79 | 17.97 | 6.49 | 191.75 | 106.17 | 55.37 |
| | 2 | 127.87 | 4.16 | 3.25 | 73.74 | 64.44 | 87.38 |
| | 3 | 136.08 | 132.67 | 97.49 | 243.90 | 212.00 | 86.92 |
| | Diclofenac | 107745.64 | 84770.90 | 78.68 | 31769.86 | 5537.38 | 17.43 |

## 4.6 Median Lethal Dose ($LD_{50}$)

### *4.6.1 Test Group*

The $LD_{50}$ for diclofenac was calculated at 11.92 mg/kg BW, with the 95% confidence intervals of 3.87 and 61.87 mg/kg BW and a probability of 0.56 (figure 4.6 and 8.22).

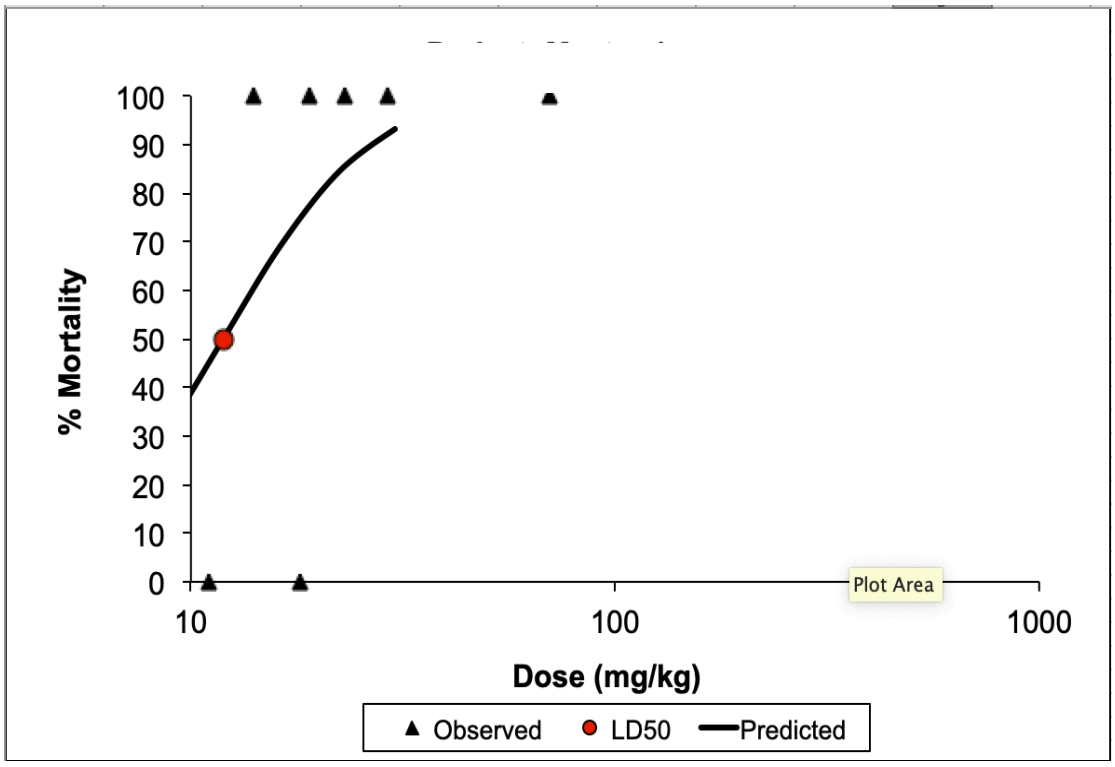


**Figure 4.6 Diclofenac oral $LD_{50}$ plot for the test group of chickens.**

### *4.6.2 Negative Control Group*

The $LD_{50}$ for diclofenac was calculated at 11.58 mg/kg BW, with the 95% confidence intervals of 3.31 and 78.62 mg/kg BW and a probability of 0.78 (figure 4.7 and 8.23).

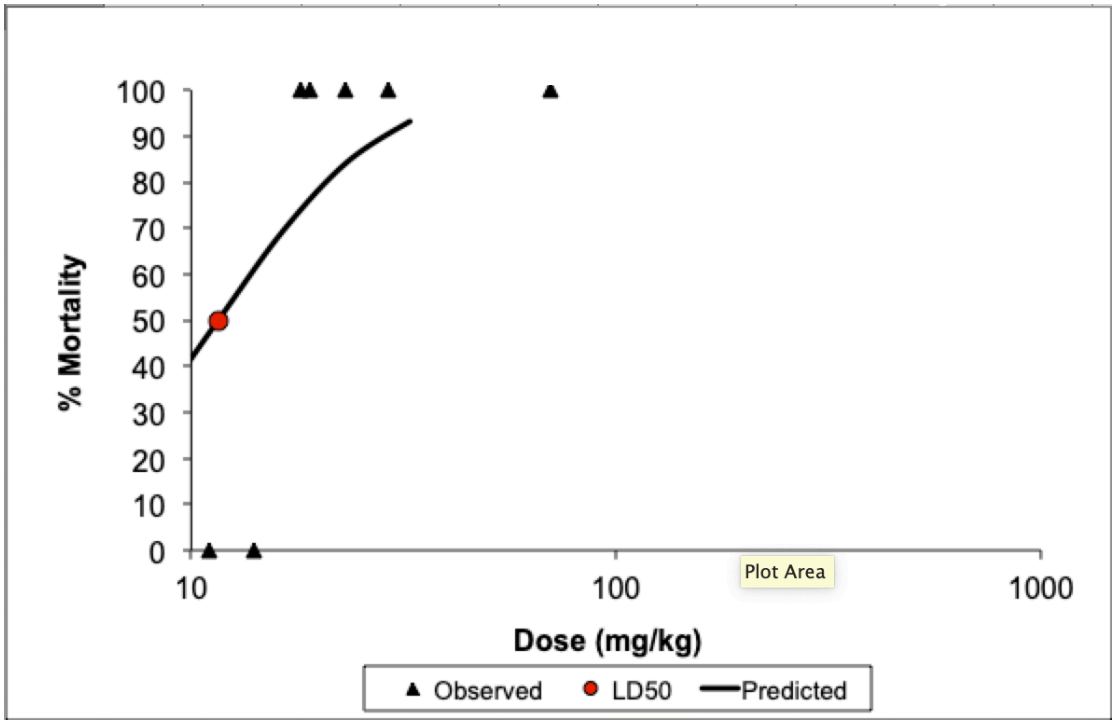


**Figure 4.7 Diclofenac oral $LD_{50}$ plot for the negative control group of chickens.**

# 5 Discussion

## 5.1 Diclofenac Toxicity due to Zero-order Metabolism

Diclofenac has been responsible for the mass mortality of three species of Asian vultures. Despite the causality being well established, much still needs to be understood on the mechanism of toxicity and why *Gyps* vultures were so susceptible. At present there is a fair body of literature that suggest a pharmacokinetic and metabolic reason as the underlying driver. When one looks at the trends in PK parameters from studies involving diclofenac administration in various species of birds (table 5.1) the data suggests an association between mortality and PK parameters that reflect length of exposure; $T_{1/2}$ and MRT. In most cases, birds with longer $T_{1/2}$ and MRT values died compared to birds from the same study that had shorter $T_{1/2}$ and MRT values. This correlation is most striking in *Gyps* vulture species studied, where the $T_{1/2}$ values are 12.24 and 16.78 h and the MRT values are 15.11 and 26.10 h for the CGV and the AWBV respectively. These values are despite a dose equal to that given to the chickens in the study by Naidoo et al., 2007 and much lower than those given to most of the other bird species studied; all vultures from these studies died. Other notable differences in their pharmacokinetics are in the $AUC_{last}$ values, which are much higher, and the Cl values, which are much lower, than in other bird species. The PK picture is suggestive of zero-order kinetic metabolism in these *Gyps* vultures, or saturation of the intrinsic enzyme metabolising capability.

Other NSAIDs studies in *Gyps* vultures show a corroborative picture and are summarised in table 5.2. Meloxicam, which demonstrates higher Cl, and lower $T_{1/2}$ and MRT values, is not toxic (Naidoo et al., 2008). Conversely, lower Cl and higher $T_{1/2}$ and MRT values are seen in some birds that died from ketoprofen and carprofen dosing (Naidoo et al., 2010; Naidoo et al., 2018); the $T_{1/2}$ and MRT values were markedly increased at the higher doses (5 mg/kg for ketoprofen and 64 mg/kg for carprofen), and in both cases was associate with mortality. Thus, from the studies involving NSAIDs other than diclofenac, there is also suggestion that zero-order metabolism occurs for ketoprofen, carprofen and therefore most likely diclofenac in *Gyps* vultures.

It should be noted that there are other factors that play a role in the toxicity of diclofenac. For instance, the Muscovy ducks that died exhibited a shorter $T_{1/2}$ than those that survived and much lower than $T_{1/2}$ values causing mortality in other species (1.58 h). The turkey vultures all exhibited a fairly long $T_{1/2}$, despite no mortalities. As such the aim of this study is to ascertain if metabolism plays a major role in the progression and susceptibility to diclofenac toxicity in *Gyps* vultures.

**Table 5.1 Summary of PK parameters for diclofenac exposure in bird species.**

| **Species** | **Sample Size** | **Dose** (mg/kg) | **$C_{max}$** (ug/ml) | **$AUC_{last}$** (ug/ml*h) | **Cl** (L/hr*kg) | **$V_d$** (L/kg) | **$T_{1/2}$** (h) | **MRT** (h) | **Mortality** | **Reference** |
|---|---|---|---|---|---|---|---|---|---|---|
| Chicken *Test* | 6 | * | 0.57 | 0.47 | 2.18 | 2.57 | 1.00 | 1.49 | No | Present study |
| Chicken *Test* | 2 | * | 1.76 | 1.14 | 1.26 | 1.37 | 1.05 | 1.40 | Yes | Present study |
| Chicken *Con.* | 6 | * | 0.56 | 0.49 | 1.98 | 2.07 | 0.87 | 1.28 | No | Present study |
| Chicken *Con.* | 3 | * | 0.28 | 0.30 | 1.52 | 4.82 | 3.40 | 4.98 | Yes | Present study |
| Quail | 11 | * | 0.03 | 0.19 | 7.29 | 30.74 | 3.41 | 3.82 | No | (Hassan et al., 2018) |
| Quail | 2 | * | 0.02 | 0.18 | 8.47 | 80.12 | 6.68 | 8.45 | Yes | (Hassan et al., 2018) |
| Muscovy duck | 7 | * | 0.21 | 0.88 | 2.16 | 4.88 | 1.65 | 2.21 | No | (Hassan et al., 2018) |
| Muscovy duck | 5 | * | 0.14 | 0.65 | 4.38 | 10.33 | 1.58 | 2.60 | Yes | (Hassan et al., 2018) |
| Pigeon | 6 | * | 0.04 | 0.33 | 3.48 | 14.92 | 3.42 | 3.48 | No | (Hassan et al., 2018) |
| Chicken | 18 | 0.8 i/m | 6.79 | 8.51 | Cl/F 0.1 | $V_d$/F 0.09 | 0.66 | - | No | (Naidoo et al., 2007) |
| Chicken | 18 | 0.8 oral | 2.11 | 4.33 | Cl/F 0.2 | $V_d$/F 0.24 | 0.89 | - | No | (Naidoo et al., 2007) |
| Chicken | 1 | 5 | - | 1.26 | 0.65 | - | 14.34 | - | Yes | (Naidoo et al., 2007) |
| CGV | 2 | 0.8 | - | 77.44 | 0.00001 | 0.18 | 12.24 | 15.11 | Yes | (Naidoo et al., 2009) |
| AWBV | 2 | 0.8 | - | 100.35 | 0.00002 | 0.30 | 16.78 | 26.10 | Yes | (Swan et al., 2006; Naidoo et al., |
| Pied crow | 6 | 10 | 0.01 | 0.05 | 17.33 | 58.35 | 2.33 | 6.11 | No | (Naidoo et al., 2011) |
| Turkey vulture | 2 | 25 | - | 141.15 | 0.26 | - | 6.29 | - | No | (Rattner et al., 2008) |
| Turkey vulture | 2 | 8 | - | 13.86 | 0.79 | - | 6.43 | - | No | (Rattner et al., 2008) |

*Chickens in the present study each received a unique dose centred on a working $LD_{50}$ of 9.8 mg/kg. For the quail, Muscovy ducks and pigeons used in the study by (Hassan et al., 2018), 4 birds received doses around an initial $LD_{50}$, then 10 birds received doses centred around a working $LD_{50}$. These two studies operated according to the OECD Acute Toxicity in Avians (Test No. 223) (OECD, 2016). Where n>1, figures are mean values for number of birds assessed in each study. Birds that died are highlighted in yellow.

**Table 5.2 Summary of PK parameters for NSAID exposure in *Gyps* vulture species.**

| Species | NSAID | Sample Size | Dose (mg/kg) | $C_{max}$ (ug/ml) | $AUC_{last}$ (ug/ml*h) | Cl (L/hr*kg) | $V_d$ (L/kg) | $T_{1/2}$ (h) | MRT (h) | Mortality | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| CGV | Meloxicam | 6 | 2 (Olson | 5.25 | 6.29 | Cl/F 56.82 | $V_d$/F 0.15 | 0.32 | - | No | (Naidoo et al., 2008) |
| CGV | Meloxicam | 6 | 2 (i/m) | 3.58 | 5.86 | 130.20 | 0.26 | 0.42 | - | No | (Naidoo et al., 2008) |
| CGV | Carprofen | 2 | 11.5 | 1.05 | 21.27 | 0.88 | 13.62 | 13.26 | 19.90 | No | (Fourie et al., 2015) |
| CGV | Flunixin | 2 | 1 | 0.33 | 0.78 | 1.38 | 3.29 | 1.84 | 2.17 | No | (Fourie et al., 2015) |
| CGV | Phenyl-butazone | 2 | 1.7 | 11.15 | 263.35 | 0.005 | 0.13 | 18.72 | 28.99 | No | (Fourie et al., 2015) |
| CGV | Ketoprofen | 5 | 1 | 3.08 | 9.79 | 0.05 | 0.13 | 0.46 | 0.83 | No | (Naidoo et al., 2010) |
| CGV | Ketoprofen | 4 | 5 | 10.77 | 50.31 | 0.10 | 0.45 | 3.24 | 4.97 | No | (Naidoo et al., 2010) |
| CGV | Ketoprofen | 7 | 5 | 21.00 | 156.51 | 0.01 | 0.32 | 7.38 | 28.0 | Yes | (Naidoo et al., 2010) |
| AWBV | Carprofen | 2 | 5 | 16.03 | 230.12 | Cl/F 0.02 | $V_z$/F 0.33 | 11.25 | 17.85 | No | (Naidoo et al., 2018) |
| AWBV | Carprofen | 1 | 64 | 40.37 | 650.16 | 0.01 | 0.08 | 8.74 | 14.48 | No | (Naidoo et al., 2018) |
| AWBV | Carprofen | 1 | 64 | 33.70 | 1231.27 | 0.02 | 1.32 | 37.75 | 54.48 | Yes | (Naidoo et al., 2018) |

Birds that died are highlighted in yellow. Where n>1, figures are mean values for number of birds assessed in each study. CGV; Cape griffon vulture (*Gyps coprotheres*), AWBV; African white-backed vulture (*Gyps africanus*).

## 5.2 Diclofenac and Cytochrome Metabolism

Diclofenac has a relatively short $T_{1/2}$ of 1-2 h in all mammalian species studied and the metabolic pathways are best understood in man (Brunton et al., 2018). The specific phase 1 CYP enzyme that metabolises the majority of diclofenac in the human liver is CYP2C9, producing the major and minor metabolites, 4'- and 3'-hydroxydiclofenac, respectively (Leemann et al., 1993; Bort et al., 1999). A further minor 5'-hydroxylation metabolite is produced through additional CYP enzymes, including CYP3A4, CYP2C8, CYP2C18 and CYP2C19 (Bort et al., 1999; Shen et al., 1999). From the evidence available, it is reasonable to conclude that metabolism in other mammals is also due to the activity of a member of the CYP2C subfamily. For instance, in the rat and dog the homologous (enzymes of the same ancestry and which catalyse the same reactions) CYP2C9 enzymes are CYP2C21 and CYP2B11 in the dog (Shou et al., 2003) and CYP2C11 in the rat (Shen et al., 1997). In the rat, the same metabolites are produced as in humans, i.e. 3'-, 4'- and 5'-hydroxydiclofenac. In the other species listed, hydroxylation and decarboxylation metabolites of diclofenac also suggest the presence of CYP-related metabolism (Oberle et al., 1994; Bogaards et al., 2000; Wasfi et al., 2003; Sarda et al., 2012), even though the specific enzymes had not been identified at the time of publication.

Unfortunately even less is known about avian CYP enzymes and their role in diclofenac metabolism (Hunter et al., 2008), with the domestic chicken remaining the best studied species. Early studies in chickens showed the inducibility of some CYP P450 forms by phenobarbitone, suggesting that they resembled members of the CYP2 family in mammals (Ronis & Walker, 1989). It is now known that, as in humans, the CYP2 family is predominant in the chicken (figure 5.1) and the CYP2C homologs are currently known as CYP2C8/9, CYP2C18, CYP2C23a, CYP2C23b and CYP2C45 (Joseph et al., 2006; Shang et al., 2013; Watanabe et al., 2013). Furthermore, the CYP2C8/9 homolog has been shown to produce 3'-, 4'- and 5'-hydroxydiclofenac in broiler chickens (Joseph et al., 2006).

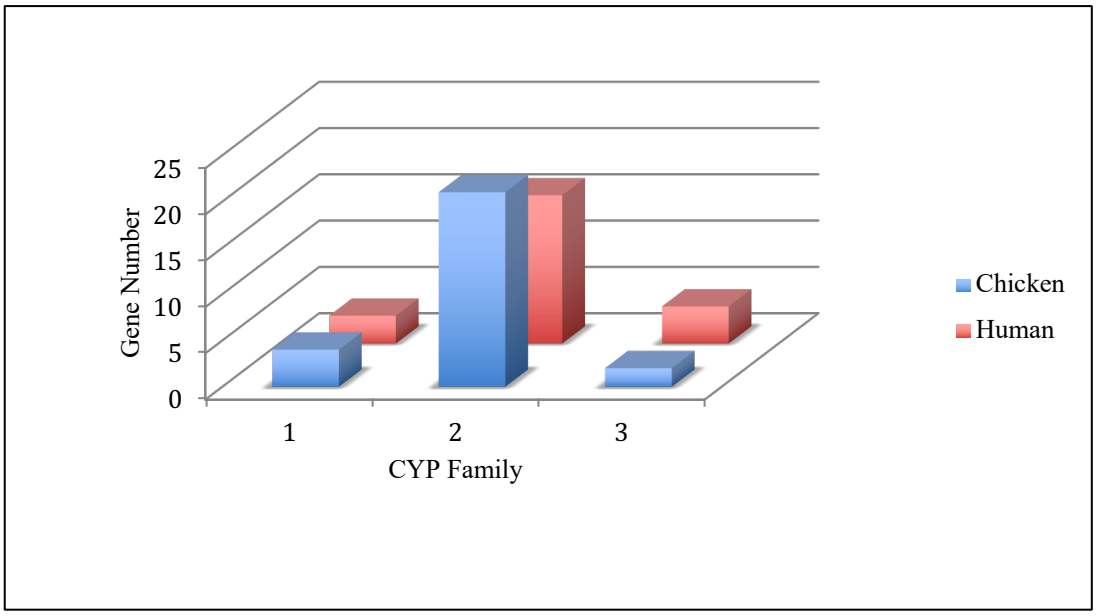


**Figure 5.1 Number of genes per CYP family in chicken and human, adapted from (Watanabe et al., 2013).**

CYP enzymes have a well-recognised feature in pharmacology; they are subject to induction or inhibition of activity by chemical means. In this PK study, we made use of this feature in an attempt to gain more understanding on CYP enzymology in *Gyps* vultures. By inhibiting CYP2C9 homologs in a test group of domestic chickens (a species where the enzyme is known to function in diclofenac metabolism) with an azole inhibitor, we compared the PK parameters obtained with those from control birds which received only diclofenac, in attempt to ascertain if the length of exposure and related toxicity in vultures may be because of CYP2C subfamily metabolic deficiency. Since the drug is a phase 1 inhibitor, it is expected that it would result in an increase in exposure to diclofenac and reduction in exposure to metabolites. The chicken was specifically chosen since it has a defined $LD_{50}$, which at 10 mg/kg, while lower than other bird species, is still higher than for old world vultures.

## 5.3 Mortality and Toxicity

The peracute deaths observed on or immediately post dosing by 2 birds in the test group and 1 bird in the negative control group have been reported in previous studies in animals when dosed i/v. Two of these deaths, occurring in birds from the test group, occurred in birds receiving the highest and second highest doses (22.55 and 28.61 mg/kg respectively). With no obvious gross pathological changes evident, the cause of death is possibly attributable to normal cardiac electrical disturbance such as atrial fibrillation or flutter; this has been documented in humans, even at low doses of diclofenac (Schmidt et al., 2018). There has also been a report of acute death following i/m administration of diclofenac in a human; this was attributed to

hypoxic brain damage following an anaphylactic reaction (Schäbitz et al., 2001). As the brain of these birds was not examined, this is also a plausible cause of death.

The remaining birds that died showed a typical and expected toxicological picture, which did not differ obviously between treatment groups. The clinical signs associated with toxicity (depression and anorexia) were consistent with previous studies in *Gyps* vultures, chickens and other bird species (Oaks et al., 2004; Swan et al., 2006; Naidoo et al., 2007; Hassan et al., 2018). All birds that succumbed, died within an expected timeframe of 48 h after dosing, within the exception of 5845 from the negative control group, which was euthanased for humane reasons at 56 h post dosing. Gross pathological signs were also typical, being serosal surface deposition of uric acid crystals and severe varying degrees of nephrosis.

Given the difference in pathological picture, it is likely that the initial 3 mortalities that occurred on or immediately after dosing shared a different mechanism of toxicity, linked to dose and route of administration. Nonetheless with the total number of birds that died being 4 in both the test and control group, there was no obvious difference in toxicity between the groups when comparing the calculated oral median lethal dose ($LD_{50}$); 11.92 mg/kg BW and 11.52 mg/kg BW respectively. The birds that died acutely on dosing were included in the $LD_{50}$ calculations because their deaths remained attributable to diclofenac, despite a potential difference in mechanism of toxicity. These findings were consistent with the clinical and pathological findings of no striking differences between treatment groups and perhaps also of non-significance in kinetic and metabolite findings, despite important trends in the latter data sets. The $LD_{50}$ values obtained in this study are also consistent with the values obtained in chickens from previous studies; 9.8 mg/kg BW (Naidoo et al., 2007).

The non-difference in the $LD_{50}$ between two groups was an unexpected finding, as fluconazole is well-described CYP enzyme inhibitor. Based on the absence of results, it is possible that a higher dose of fluconazole may be needed to suppress metabolic activity in the chicken. The recommended dosage from the literature varied from 2-5 to 100 mg/kg BW (Rochette et al., 2003; Carpenter & Marion, 2018). Due to the difficulty administering the volume associated with higher doses, the pscittacine dose of 15 mg/kg BW was used. Furthermore, research using a larger sample size per treatment group and a single dose of diclofenac may also induce a change in the $LD_{50}$ value in metabolically suppressed birds due to possible large intersubject variability in response.

### 5.4 Chicken Diclofenac Pharmacokinetics and Diclofenac Metabolites

Though there were no statistical differences between treatment groups, either for the PK parameters or for comparison of the diclofenac: metabolite ratio peaks, this is likely as a

result of sample size used; only 8 birds from the test group and 9 from the control group. In this respect, trends in the descriptive statistical data are considered by the authors to be more important. We also acknowledge a shortcoming in the study design in that different individuals were used between treatment groups, and therefore inter-subject variation is a complicating factor. As this was a $LD_{50}$ toxicity study, it was not possible to use a cross over study design.

The test group of chickens, which received a CYP inhibitor, demonstrated a mean PK curve suggestive of metabolic inhibition when compared to the control group (figure 4.4); evident in the geometric mean values for $C_{max}$ (0.61 vs. 0.41 µg/ml), $AUC_{last}$ (0.5 µg/ml*h vs. 0.4 µg/ml*h) and clearance (1.52 L/h*kg vs. 1.59 L/h*kg). As diclofenac was dosed i/v, the parameter $AUC_{last}$ provides a good measure for comparing the extent of exposure in each group, as the absorptive process has been excluded, with the result that distributive and excretory processes are being evaluated. Birds with suppressed metabolism would therefore be expected to have a higher $AUC_{last}$ than birds that do not, as is evident in this study. Another important parameter is clearance, which can be expressed per clearing organ through the following equation; $\mathbf{Cl = Q \textit{ x } E}$, where $\mathbf{Q}$ is the blood flow to the organ and **E** represents the 'Extraction Ratio' or percentage of drug removed from the blood by that organ during a single passage (Toutain & Bousquet-Mélou, 2004). As it was not expected that changes in blood flow to metabolising organs would impact clearance in this study (Naidoo & Swan, 2009), clearance would be related to **E**. With the extraction ratio being dependent on the intrinsic metabolising capability of an organ system, the lower geometric mean clearance for the CYP-inhibited test group of bird suggests capacity limited metabolism, as a function of CYP enzyme inhibition, as a plausible cause in support of the $AUC_{last}$ values seen.

It should be noted that the higher mean $AUC_{last}$ and lower mean Cl value for the test group of birds was evident as a trend despite the fact that the 2 birds that died on i/v dosing and therefore did not produce a PK curve from the test group (5877 and 5833) were the highest and second highest dosed birds respectively. Both birds dosed at these doses from the negative control group (5845 and 5847) died later and demonstrated high $T_{1/2}$ and MRT values, consistent with expectations. The geometric mean $T_{1/2}$ values of 1.08 h for the control and 0.88 h for the test group, and the geometric mean MRT values of 1.63 h for the control group and 1.03 h for the test group, should therefore also be interpreted with caution as the two highest dosed test group birds did not have analysable data.

Further proof of an inhibitory effect being present is evident from the evaluation of the metabolite peaks as the CYP-inhibited test group birds produced lower responses for the peak 3 metabolites (table 4.8 and 4.9) and those which died had higher diclofenac exposure (table

4.9). This is suggestive that peak 3 may represent a combination of 4'- and 5'-hydroxydiclofenac, corresponding to the retention times from the calibration curves. Though the CYP-inhibited birds actually produced more of peak 1 and 2 metabolites, it is possible these peaks represent alternative pathways of either phase 1 metabolism not impacted by fluconazole inhibition, or phase 2 metabolism, such as direct glucuronidation by a UGT2B7 homolog, producing diclofenac acylglucuronide.

While the general trend was for birds that produced lower metabolite responses (and therefore a higher diclofenac: metabolite ratio) and died to be from the higher end of the dose range, bird 5842, dosed at a much lower dose (6.86 mg/kg BW) also exhibited very low metabolite production compared to birds in both treatment groups. This supports the PK data for this animal as the bird had a very high 15 min diclofenac plasma concentration, suggesting that the animal was metabolically constrained rather than that the reading was an outlier. What is also notable is the large variation in the diclofenac: metabolite peak ratios, evident in the CV values, and which are higher in the test group birds. These two findings indicate that there is a degree of natural variation in metabolism which is independent of dose in chickens, and supports the higher resilience to diclofenac in the chicken than *Gyps* vultures, i.e. the higher degree of natural variation in CYP metabolism would explain the higher $LD_{50}$ in the chicken in comparison to the vulture.

## 5.5 Uric acid

There were no remarkable differences between treatment groups when comparing uric acid values, either for total exposure ($AUC_{last}$) or per scheduled time point vs. the baseline for each individual. Only two birds showed a steadily increasing uric acid trend and with readings out of the reported reference ranges for chickens; 5999 and 5847, both from the control group. These birds were the third highest and highest dosed birds respectively. This is in agreement with the clinical picture as these animals died. One other bird, 5835 from the control group, showed 30 min and 1 h readings out of the reference range but this bird survived and the 2 h uric acid reading was with normal range (table 4.4). Unfortunately, the two highest dosed birds from the test group (5877 and 5833) died on dosing, so it is unknown whether their uric acid trend would have been similar. The pattern of plasma uric acid accumulation and pathological gout findings appears to be fairly consistent in other bird species studied for diclofenac toxicity. (Naidoo et al., 2007; Hassan et al., 2018)

This was primarily a pharmacokinetic study and there were ethical limitations with respect to blood sampling volume. Consequently, uric acid levels were evaluated as secondary variables only. Correlation between uric acid level and mortality (steadily increasing levels over

time in birds which died) did not occur until 24 h post dosing in domestic chickens, Muscovy ducks, Japanese quail, domestic pigeons and OWBVs from other studies (Swan et al., 2006; Naidoo et al., 2007; Hassan et al., 2018). As the last bleed point from this study was at 2 h post dosing and most birds which succumbed only showed clinical signs from the earliest of 7 h (5847), it is not unexpected that no difference in uric acid levels were noted between birds that survived and birds that died, from the sampling points obtained. If future studies make use of pooled samples and can provide later sample points, it will be interesting to assess the impact of a CYP2C inhibitor on uric acid exposure in tested birds compared to control birds.

# 6 Conclusion

When exposed to diclofenac, though there was no significant difference in the $LD_{50}$ for the chickens given a known CYP2C9 inhibitor vs. those that were not, the mean PK curve and ratio of HPLC diclofenac: metabolite peaks for the birds dosed with the inhibitor were suggestive of partial inhibition of CYP2C functioning.

It is possible that the lack of statistical difference between the results obtained for this study could indicate that the chicken CYP2C homolog is either not inhibited by fluconazole or inhibited at a higher dose. The possibility of differences in inhibition across CYP2C9 isoforms in animal species has been documented; Boogaards et al., 2000 showed that rat liver microsomes did not show inhibition of diclofenac metabolism in response to sulphenazole, as is the case in man, dogs and monkeys. Sulphenazole is known to be one of the most potent inhibitors of CYP2C9 activity (Baldwin et al., 1995; Bogaards et al., 2000). Under-dosage is also plausible given that, due to volume limitations, the pscittacine dose was used.

It may also be the case that there are other reasons for the difference in sensitivity to diclofenac between the chicken and *Gyps* vultures, such as that the underlying driver is not a metabolic deficiency or that the deficiency exists at another level, such as the phase 2 conjugation reactions. In some species, such as the dog, phase 2 glucuronidation is the major metabolic pathway for diclofenac (Stierlin et al., 1979). It is also possible that CYP enzyme systems in the chicken are much more divergent than in the vulture than was previously thought. Whilst the %CV values for metabolite production are high in the chicken, there appear to be only two studies, including the present, where the median lethal dose in the chicken was calculated, so it is unknown if there is variation in this measure of toxicity as a means to assess variation in susceptibility.

Whilst identification of metabolites produced proved difficult within the constraints of methodology and funding available at the time, future studies could consider HPLC individual spiking of diclofenac-exposed and untreated bird plasma with standards of different hydroxy-

diclofenac metabolites and comparison of peak retention times. Liquid Chromatography with tandem mass spectrometry (LC-MS-MS) or nuclear magnetic resonance spectroscopy would also potentially differentiate metabolites.

Further research using more birds and a single larger dose of fluconazole, or another inhibitor which may be dosed without limitation at the recommended poultry dose, may provide more definitive answers on the functioning of CYP2C homologs in *Gyps* vulture species. It is the opinion of the authors that deficiency in phase 1 CYP metabolism remains the most likely cause for toxicity in these birds.

# 7 References


Acharya, R., Cuthbert, R., Baral, H. & Shah, K. (2009). Rapid population declines of Himalayan Griffon Gyps himalayensis in Upper Mustang, Nepal.

Agnello, K.A., Reynolds, L.R. & Budsberg, S.C. (2005) In vivo effects of tepoxalin, an inhibitor of cyclooxygenase and lipoxygenase, on prostanoid and leukotriene production in dogs with chronic osteoarthritis. *American journal of veterinary research*, **66**(6), 966-972.

Aithal, G.P. (2004) Diclofenac-induced liver injury: a paradigm of idiosyncratic drug toxicity. *Expert opinion on drug safety*, **3**(6), 519-523.

Anderson, M.D., Piper, S.E. & Swan, G.E. (2005) Non-steroidal anti-inflammatory drug use in South Africa and possible effects on vultures. *South African Journal of Science*, **101**(3-4), 112-114.

Arya, S., Robert, H.P., Pesavento, J.B. & Francisco, A.U. (2018). *Pathology of carbon monoxide poisoning in two cats*. In Pathology of carbon monoxide poisoning in two cats pp. 1-6.

Baldwin, S.J., Bloomer, J.C., Smith, G.J., Ayrton, A.D., Clarke, S.E. & Chenery, R.J. (1995) Ketoconazole and sulphaphenazole as the respective selective inhibitors of P4503A and 2C9. *Xenobiotica*, **25**(3), 261-270.

Baral, N. & Gautam, R. (2007) Socio-economic perspectives on the conservation of Critically Endangered vultures in South Asia: an empirical study from Nepal. *Bird Conservation International*, **17**(2), 131-139.

Bickley, L.K., van Aerle, R., Brown, A.R., Hargreaves, A., Huby, R., Cammack, V., Jackson, R., Santos, E.M. & Tyler, C.R. (2017) Bioavailability and Kidney Responses to Diclofenac in the Fathead Minnow (Pimephales promelas). *Environmental science & technology*, **51**(3), 1764-1774.

BirdlifeInternational (2014) Ban Veterinary Diclofenac - Technical Summary April 2014.

Birdwell, K.A., Grady, B., Choi, L., Xu, H., Bian, A., Denny, J.C., Jiang, M., Vranic, G., Basford, M., Cowan, J.D., Richardson, D.M., Robinson, M.P., Ikizler, T.A., Ritchie, M.D.,

Stein, C.M. & Haas, D.W. (2012) The use of a DNA biobank linked to electronic medical records to characterize pharmacogenomic predictors of tacrolimus dose requirement in kidney transplant recipients. *Pharmacogenetics and Genomics*, **22**(1), 32-42.

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., van Bladeren, P.J. & Walther, B. (2000) Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. *Xenobiotica; the fate of foreign compounds in biological systems*, **30**(12), 1131-1152.

Boothe, D.M. (2012). Small animal clinical pharmacology & therapeutics. Elsevier Saunders, St. Louis, Mo.

Bort, R., Macé, K., Boobis, A., Gómez-Lechón, M.a.-J., Pfeifer, A. & Castell, J. (1999) Hepatic metabolism of diclofenac: role of human CYP in the minor oxidative pathways. *Biochemical Pharmacology*, **58**(5), 787-796.

Brune, K., Renner, B. & Tiegs, G. (2015) Acetaminophen/paracetamol: A history of errors, failures and false decisions. *European Journal of Pain (London, England)*, **19**(7), 953-965.

Brunton, L.L., Hilal-Dandan, R. & Knollmann, B.r.C. (2018). Goodman & Gilman's the pharmacological basis of therapeutics. McGraw-Hill Education, New York.

Buechley, E.R. & Şekercioğlu, Ç.H. (2016) The avian scavenger crisis: Looming extinctions, trophic cascades, and loss of critical ecosystem functions. *Biological Conservation*, **198**, 220-228.

Burgos-Rodríguez, A.G. (2010) Avian renal system: clinical implications. *The veterinary clinics of North America. Exotic animal practice*, **13**(3), 393-411.

Busch, U., Schmid, J., Heinzel, G., Schmaus, H., Baierl, J., Huber, C. & Roth, W. (1998) Pharmacokinetics of meloxicam in animals and the relevance to humans. *Drug Metabolism and Disposition: The Biological Fate of Chemicals*, **26**(6), 576-584.

Carpenter, J.W. & Marion, C.J. (2018). Exotic animal formulary. Elsevier, St. Louis, Missouri.

Chandrasekharan, N.V., Roos, K.L.T., Dai, H., Evanson, N.K., Tomsik, J., Elton, T.S. & Simmons, D.L. (2002) COX-3, a Cyclooxygenase-1 Variant Inhibited by Acetaminophen and Other Analgesic/Antipyretic Drugs: Cloning, Structure, and Expression. *Proceedings of the National Academy of Sciences of the United States of America*, **99**(21), 13926-13931.

Collar, N.J. (2002). Threatened birds of Asia : the BirdLife International red data book. Birdlife international, Cambridge.

Cortés-Avizanda, A., Blanco, G., DeVault, T.L., Markandya, A., Virani, M.Z., Brandt, J. & Donázar, J.A. (2016) Supplementary feeding and endangered avian scavengers: benefits, caveats, and controversies. *Frontiers in Ecology and the Environment*, **14**(4), 191-199.

Cuthbert, R., Green, R.E., Ranade, S., Saravanan, S., Pain, D.J., Prakash, V. & Cunningham, A.A. (2006) Rapid population declines of Egyptian vulture (Neophron percnopterus) and red-headed vulture (Sarcogyps calvus) in India. *Animal Conservation*, **9**(3), 349-354.

Cuthbert, R., Taggart, M.A., Prakash, V., Saini, M., Swarup, D., Upreti, S., Mateo, R., Chakraborty, S.S., Deori, P. & Green, R.E. (2011) Effectiveness of action in India to reduce exposure of gyps vultures to the toxic veterinary drug diclofenac. *PloS One*, **6**(5).

Cuthbert, R.J., Galligan, T., Green, R.E., Taggart, M.A., Prakash, V., Chakraborty, S.S., Deori, P., Kulkarni, M., Ranade, S., Shringarpure, R., Saini, M. & Sharma, A.K. (2014) Avian scavengers and the threat from veterinary pharmaceuticals. *Philosophical Transactions of the Royal Society B: Biological Sciences*, **369**(1656).

Cuthbert, R.J., Taggart, M.A., Saini, M., Sharma, A., Das, A., Kulkarni, M.D., Deori, P., Ranade, S., Shringarpure, R.N., Galligan, T.H. & Green, R.E. (2016) Continuing mortality of vultures in India associated with illegal veterinary use of diclofenac and a potential threat from nimesulide. *Oryx*, **50**(1), 104-112.

Daly, A.K., Aithal, G.P., Leathart, J.B.S., Swainsbury, R.A., Dang, T.S. & Day, C.P. (2007) Genetic Susceptibility to Diclofenac-Induced Hepatotoxicity: Contribution of UGT2B7, CYP2C8, and ABCC2 Genotypes. *Gastroenterology*, **132**(1), 272-281.

Dewse, C.D. & Potter, C.G. (1975) Inhibitory effect of phenylbutazone and oxyphenbutazone on DNA synthesis in normal human bone marrow cells in vitro. *The Journal of pharmacy and pharmacology*, **27**(7), 523-526.

Dudas, P.L., Pelis, R.M., Braun, E.J. & Renfro, J.L. (2005) Transepithelial urate transport by avian renal proximal tubule epithelium in primary culture. *The Journal of experimental biology*, **208**(Pt 22), 4305-4315.

Eguchi, K., Nishibe, Y., Baba, T. & Ohno, K. (1996) Quantitation of cytochrome P450 enzymes (CYP1A1/2, 2B11, 2C21 and 3A12) in dog liver microsomes by enzyme-linked immunosorbent assay. *Xenobiotica; the fate of foreign compounds in biological systems*, **26**(7), 755-763.

Eleni, C., Neri, B., Giannetti, L., Grifoni, G., Meoli, R., Stravino, F., Friedrich, K.G., Scholl, F., Di Cerbo, P. & Battisti, A. (2019) Death of captive-bred vultures caused by flunixin poisoning in Italy. *Environmental Toxicology and Pharmacology*, **68**, 91-93.

Elwood, P.C. (2001) Aspirin: past, present and future. *Clinical Medicine (London, England)*, **1**(2), 132-137.

EPA (2012). *Ecological Effects Test Guidelines OCSPP 850.2100: Avian Acute Oral Toxicity Test*. In Ecological Effects Test Guidelines OCSPP 850.2100: Avian Acute Oral Toxicity Test.

EU (2013). *Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy Text with EEA relevance*. In Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 amending Directives 2000/60/EC and 2008/105/EC as regards priority substances in the field of water policy Text with EEA relevance.

Fatemeh, J.-K., Zahra, L. & Hossein, A.-K. (2018). *Medicinal plants: Past history and future perspective*. In Medicinal plants: Past history and future perspective pp. 1-7.

Fink-Gremmels, J. (2008) Implications of hepatic cytochrome P450-related biotransformation processes in veterinary sciences. *European Journal of Pharmacology*, **585**(2-3), 502-509.

Fourie, T., Cromarty, D., Duncan, N., Wolter, K., Naidoo, V. & Lierz, M.E. (2015). *The Safety and Pharmacokinetics of Carprofen, Flunixin and Phenylbutazone in the Cape Vulture (Gyps coprotheres) following Oral Exposure*. In The Safety and Pharmacokinetics of

Carprofen, Flunixin and Phenylbutazone in the Cape Vulture (Gyps coprotheres) following Oral Exposure.

Galligan, T., Bhusal, K., Paudel, K., Chapagain, D., Joshi, A., P. Chaudhary, I., Chaudhary, A., Baral, H., J. Cuthbert, R. & Green, R. (2019). Partial recovery of Critically Endangered Gyps vulture populations in Nepal.

Galligan, T.H., Bhusal, K.P., Paudel, K., Chapagain, D., Joshi, A.B., Chaudhary, I.P., Chaudhary, A., Baral, H.S., Cuthbert, R.J. & Green, R.E. (2019) Partial recovery of Critically Endangered Gyps vulture populations in Nepal. *Bird Conservation International*, 1-16.

Galligan, T.H., Taggart, M.A., Cuthbert, R.J., Svobodova, D., Chipangura, J., Alderson, D., Prakash, V.M. & Naidoo, V. (2016) Metabolism of aceclofenac in cattle to vulture-killing diclofenac. *Conservation Biology*, **30**(5), 1122-1127.

Gilbert, M., Watson, R.T., Ahmed, S., Asim, M. & Johnson, J.A. (2007) Vulture restaurants and their role in reducing diclofenac exposure in Asian vultures. *Bird Conservation International*, **17**(1), 63-78.

Gonzalez, F.J. & Nebert, D.W. (1990) Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. *Trends in genetics : TIG*, **6**(6), 182-186.

Guo, J., Sinclair, C.J., Selby, K. & Boxall, A.B.A. (2016) Toxicological and ecotoxicological risk-based prioritization of pharmaceuticals in the natural environment. *Environmental Toxicology and Chemistry*, **35**(6), 1550-1559.

Hasegawa, T., Nakanishi, S., Minami, K., Higashino, H., Kataoka, M., Shitara, Y. & Yamashita, S. (2017) Increase in the systemic exposure of primary metabolites of Midazolam in rat arising from CYP inhibition or hepatic dysfunction. *Drug Metabolism and Pharmacokinetics*, **32**(1), 69-76.

Hassan, I.Z., Duncan, N., Adawaren, E.O. & Naidoo, V. (2018) Could the environmental toxicity of diclofenac in vultures been predictable if preclinical testing methodology were applied? *Environmental Toxicology and Pharmacology*, **64**, 181-186.

He, B.S., Wang, J., Liu, J. & Hu, X.M. (2017) Eco-pharmacovigilance of non-steroidal anti-inflammatory drugs: Necessity and opportunities. *Chemosphere*, **181**, 178-189.

Henriques, M., Granadeiro, J.P., Monteiro, H., Nuno, A., Lecoq, M., Cardoso, P., Regalla, A. & Catry, P. (2018) Not in wilderness: African vulture strongholds remain in areas with high human density. *PloS One*, **13**(1), e0190594.

Hill, J.E., DeVault, T.L., Beasley, J.C., Rhodes, O.E. & Belant, J.L. (2018) Effects of vulture exclusion on carrion consumption by facultative scavengers. *Ecology and Evolution*, **8**(5), 2518-2526.

Houston, D.C. & Cooper, J.E. (1975) The digestive tract of the whiteback griffon vulture and its role in disease transmission among wild ungulates. *Journal of wildlife diseases*, **11**(3), 306-313.

Hunter, R.P., Mahmood, I. & Martinez, M.N. (2008) Prediction of xenobiotic clearance in avian species using mammalian or avian data: how accurate is the prediction? *Journal of Veterinary Pharmacology and Therapeutics*, **31**(3), 281-284.

Hussain, I., Khan, M.Z., Khan, A., Javed, I. & Saleemi, M.K. (2008) Toxicological effects of diclofenac in four avian species. *Avian pathology : journal of the W.V.P.A*, **37**(3), 315-321.

Hutchinson, T.H., Madden, J.C., Naidoo, V. & Walker, C.H. (2014) Comparative metabolism as a key driver of wildlife species sensitivity to human and veterinary pharmaceuticals. *Philosophical Transactions of the Royal Society B-Biological Sciences*, **369**(1656).

Johnson, J.A., Gilbert, M., Virani, M.Z., Asim, M. & Mindell, D.P. (2008) Temporal genetic analysis of the critically endangered oriental white-backed vulture in Pakistan. *Biological Conservation*, **141**(9), 2403-2409.

Joseph, C.K., Michael, J.M., Karyn, D.H., Dorothy, E.F. & Badar, S. (2006). *Hepatic CYP Isoforms and Drug-Metabolizing Enzyme Activities in Broiler Chicks*. In Hepatic CYP Isoforms and Drug-Metabolizing Enzyme Activities in Broiler Chicks. Asian Network for Scientific Information.

Kam, P.C.A. & So, A. (2009) COX-3: Uncertainties and controversies. *Current Anaesthesia and Critical Care*, **20**(1), 50-53.

Kawalek, J., Myers, M., Howard, K., Farrell, D. & Shaikh, B. (2006) Hepatic CYP Isoforms and Drug-Metabolizing Enzyme Activities in Broiler Chicks. *International Journal of Poultry Science*, **5**.

Kinney, C.A., Furlong, E.T., Werner, S.L. & Cahill, J.D. (2006) Presence and distribution of wastewater-derived pharmaceuticals in soil irrigated with reclaimed water. *Environmental Toxicology and Chemistry*, **25**(2), 317-326.

Koenig, R. (2006) Vulture Research Soars as the Scavengers' Numbers Decline. *Science*, **312**(5780), 1591-1592.

Köhler, H.-R. & Triebskorn, R. (2013) Wildlife Ecotoxicology of Pesticides: Can We Track Effects to the Population Level and Beyond? *Science*, **341**(6147), 759-765.

Konstandi, M., Johnson, E.O. & Lang, M.A. (2014) Consequences of psychophysiological stress on cytochrome P450-catalyzed drug metabolism. *Neuroscience and Biobehavioral Reviews*, **45**, 149-167.

Leemann, T., Transon, C. & Dayer, P. (1993) Cytochrome P450TB (CYP2C): a major monooxygenase catalyzing diclofenac 4'-hydroxylation in human liver. *Life sciences*, **52**(1), 29-34.

Lierz, M. (2003) Avian renal disease: pathogenesis, diagnosis, and therapy. *The veterinary clinics of North America. Exotic animal practice*, **6**(1), 29-55.

Mahapatro, G.K. & Arunkumar, K. (2014) The case for banning diclofenac and the vanishing vultures. *Biodiversity*, **15**(4), 265-268.

Marcelo, N.M. & John, L.W. (2006). *COX-Inhibiting Nitric Oxide Donors (CINODs): Potential Benefits on Cardiovascular and Renal Function*. In COX-Inhibiting Nitric Oxide Donors (CINODs): Potential Benefits on Cardiovascular and Renal Function pp. 155-164.

Marchant, A., Bastnet, P.B. & Friederich, G. (2007) Vulture snippets from around the world : Lappet-faced Vulture in East Griqualand, KwaZulu-Natal, South Africa; Diclofenac remains a threat to Nepal's vultures; Vultures breeding in palm trees. *Vulture News*, **38**(57), 70-72.

Margalida, A., Sánchez-Zapata, J.A., Blanco, G., Hiraldo, F. & Donázar, J.A. (2014) Diclofenac Approval as a Threat to Spanish Vultures. *Conservation Biology*, **28**(3), 631-632.

Markandya, A., Taylor, T., Longo, A., Murty, M.N., Murty, S. & Dhavala, K. (2008) Counting the cost of vulture decline—An appraisal of the human health and other benefits of vultures in India. *Ecological Economics*, **67**(2), 194-204.

Martignoni, M., Groothuis, G. & De Kanter, R. (2007) Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology*, **2**, 875-894.

Meteyer, C.U., Rideout, B.A., Gilbert, M., Shivaprasad, H.L. & Oaks, J.L. (2005) Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-backed vultures (Gyps Bengalensis). *Journal of Wildlife Diseases*, **41**(4), 707-716.

Naidoo, V. (2013). *Diclofenac in Gyps vultures : a molecular mechanism of toxicity*. In Diclofenac in Gyps vultures : a molecular mechanism of toxicity.

Naidoo, V., Duncan, N., Bekker, L. & Swan, G. (2007) Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in Gyps vultures. *Environmental Toxicology and Pharmacology*, **24**(3), 260-266.

Naidoo, V., Mompati, K.F., Duncan, N. & Taggart, M.A. (2011) The Pied Crow (Corvus albus) is insensitive to diclofenac at concentrations present in carrion. *Journal of wildlife diseases*, **47**(4), 936-944.

Naidoo, V. & Swan, G.E. (2009) Diclofenac toxicity in Gyps vulture is associated with decreased uric acid excretion and not renal portal vasoconstriction. *Comparative Biochemistry and Physiology, Part C*, **149**(3), 269-274.

Naidoo, V., Taggart, M.A., Duncan, N., Wolter, K., Chipangura, J., Green, R.E. & Galligan, T.H. (2018) The use of toxicokinetics and exposure studies to show that carprofen in cattle tissue could lead to secondary toxicity and death in wild vultures. *Chemosphere*, **190**, 80-89.

Naidoo, V., Venter, L., Wolter, K., Taggart, M. & Cuthbert, R. (2010) The toxicokinetics of ketoprofen in Gyps coprotheres: toxicity due to zero-order metabolism. *Archives of Toxicology*, **84**(10), 761-766.

Naidoo, V., Wolter, K., Cromarty, A.D., Bartels, P., Bekker, L., McGaw, L., Taggart, M.A., Cuthbert, R. & Swan, G.E. (2008) The pharmacokinetics of meloxicam in vultures. *Journal of Veterinary Pharmacology and Therapeutics*, **31**(2), 128-134.

Naidoo, V., Wolter, K., Cromarty, D., Diekmann, M., Duncan, N., Meharg, A.A., Taggart, M.A., Venter, L. & Cuthbert, R. (2010) Toxicity of non-steroidal anti-inflammatory drugs to Gyps vultures: a new threat from ketoprofen. *Biology letters*, **6**(3), 339-341.

Naidoo, V., Wolter, K., Cuthbert, R. & Duncan, N. (2009) Veterinary diclofenac threatens Africa's endangered vulture species. *Regulatory Toxicology and Pharmacology*, **53**(3), 205-208.

National Research Council . Subcommittee on, E. & Continuous Exposure Guidance Levels for Selected Submarine, C. (2009). *Emergency and continuous exposure guidance levels for selected submarine contaminants. Volume 3*. In Emergency and continuous exposure guidance levels for selected submarine contaminants. Volume 3. National Academies Press, Washington, D.C.

Nelson, D.R., Goldstone, J.V. & Stegeman, J.J. (2013) The cytochrome P450 genesis locus: the origin and evolution of animal cytochrome P450s. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, **368**(1612), 20120474.

Ng, L.E., Halliwell, B. & Wong, K.P. (2008) Nephrotoxic cell death by diclofenac and meloxicam. *Biochemical and Biophysical Research Communications*, **369**(3), 873-877.

Ng, L.E., Vincent, A.S., Halliwell, B. & Wong, K.P. (2006) Action of diclofenac on kidney mitochondria and cells. *Biochemical and Biophysical Research Communications*, **348**(2), 494-500.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M., Mahmood, S., Ali, A. & Khan, A.A. (2004) Diclofenac residues as the cause of vulture population decline in Pakistan. *Nature*, **427**(6975), 630-633.

Oberle, R.L., Das, H., Wong, S.L., Chan, K.K.H. & Sawchuk, R.J. (1994) Pharmacokinetics and Metabolism of Diclofenac Sodium in Yucatan Miniature Pigs. *Pharmaceutical Research : An Official Journal of the American Association of Pharmaceutical Scientists*, **11**(5), 698-703.

OECD (1984). Test No. 205: Avian Dietary Toxicity Test.

OECD (1984). Test No. 206: Avian Reproduction Test.

OECD (2016). Test No. 223: Avian Acute Oral Toxicity Test.

Ogada, D.L., Keesing, F. & Virani, M.Z. (2012) Dropping dead: causes and consequences of vulture population declines worldwide. *Annals of the New York Academy of Sciences*, **1249**(1), 57-71.

Olson, E.J., Morales, S.C., McVey, A.S. & Hayden, D.W. (2005) Putative metronidazole neurotoxicosis in a cat. *Veterinary Pathology*, **42**(5), 665-669.

Pelkonen, O., Maenpaa, J., Taavitsainen, P., Rautio, A. & Raunio, H. (1998) Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica*, **28**(12).

Peredo, H.A. (2003) Opposite effects of endogenous nitric oxide and prostaglandin F2a in the rat mesenteric bed. *AUTONOMIC AND AUTACOID PHARMACOLOGY*, **23**(3), 167-172.

Pino, M.R., Muñiz, S., Val, J. & Navarro, E. (2016) Phytotoxicity of 15 common pharmaceuticals on the germination of Lactuca sativa and photosynthesis of Chlamydomonas reinhardtii. *Environmental Science and Pollution Research*, **23**(22), 22530-22541.

Prakash, V., Green, R.E., Pain, D.J., Ranade, S.P., Saravanan, S., Prakash, N., Venkitachalam, R., Cuthbert, R., Rahmani, A.R. & Cunningham, A.A. (2007) RECENT CHANGES IN POPULATIONS OF RESIDENT GYPS VULTURES IN INDIA. *The Journal of the Bombay Natural History Society.*, **104**(2), 127-133.

Prakash, V., Pain, D.J., Cunningham, A.A., Donald, P.F., Prakash, N., Verma, A., Gargi, R., Sivakumar, S. & Rahmani, A.R. (2003) Catastrophic collapse of Indian white-backed Gyps

bengalensis and long-billed Gyps indicus vulture populations. *Biological Conservation*, **109**(3), 381-390.

Preissner, S.C., Hoffmann, M.F., Preissner, R., Dunkel, M., Gewiess, A., Preissner, S. & Nie, D.E. (2013). *Polymorphic Cytochrome P450 Enzymes (CYPs) and Their Role in Personalized Therapy*. In Polymorphic Cytochrome P450 Enzymes (CYPs) and Their Role in Personalized Therapy.

Rattner, B.A. (2009) History of wildlife toxicology. *Ecotoxicology (London, England)*, **18**(7), 773-783.

Rattner, B.A., Whitehead, M.A., Gasper, G., Meteyer, C.U., Link, W.A., Taggart, M.A., Meharg, A.A., Pattee, O.H. & Pain, D.J. (2008) Apparent tolerance of turkey vultures (Cathartes aura) to the non-steroidal anti-inflammatory drug diclofenac. *Environmental Toxicology and Chemistry*, **27**(11), 2341-2345.

Ribas, J.o.L.C., Sherry, J.P., Zampronio, A.R., Silva de Assis, H.C. & Simmons, D.B.D. (2017) Inhibition of immune responses and related proteins in Rhamdia quelen exposed to diclofenac. *Environmental Toxicology and Chemistry*, **36**(8), 2092-2107.

Richards, N.L. & Scott, K.S. (2011) Qualitative detection of the NSAIDs diclofenac and ibuprofen in the hair of Eurasian otters (Lutra lutra) occupying UK waterways with GC-MS. *European Journal of Wildlife Research*, **57**(5), 1107-1114.

Rochette, F., Engelen, M. & Vanden Bossche, H. (2003) Antifungal agents of use in animal health--practical applications. *Journal of Veterinary Pharmacology and Therapeutics*, **26**(1), 31-53.

Ronis, M. & Walker, C.H. (1989) The microsomal monooxygenases of birds. *Rev. Biochem. Toxicol.*, **10**, 301-384.

Santos, L.H., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C. & Montenegro, M.C. (2010) Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *Journal of Hazardous Materials*, **175**(1-3), 45-95.

Sarda, S., Page, C., Pickup, K., Schulz-Utermoehl, T. & Wilson, I. (2012) Diclofenac metabolism in the mouse: Novel in vivo metabolites identified by high performance liquid chromatography coupled to linear ion trap mass spectrometry. *Xenobiotica*, **42**(2), 179-194.

Schäbitz, W.R., Berger, C., Knauth, M., Meinck, H.M. & Steiner, T. (2001) Hypoxic brain damage after intramuscular self-injection of diclofenac for acute back pain. *European Journal of Anaesthesiology*, **18**(11), 763-765.

Schmidt, M., Sørensen, H.T. & Pedersen, L. (2018) Diclofenac use and cardiovascular risks: series of nationwide cohort studies. *BMJ (Clinical research ed.)*, **362**, k3426.

Shafi, M., Hussain, K., Garg, U.K. & Baba, O.K. (2015) Haemato-biochemical and immunological study on NSAIDs induced acute toxicity in broiler chicken. *Journal of Animal Research*, **5**(2), 297-302.

Shang, S., Jiang, J. & Deng, Y. (2013) Chicken cytochrome P450 1A5 is the key enzyme for metabolizing T-2 toxin to 3'OH-T-2. *International Journal of Molecular Sciences*, **14**(6), 10809-10818.

Sharma, A.K., Saini, M., Singh, S.D., Prakash, V., Das, A., Dasan, R.B., Pandey, S., Bohara, D., Galligan, T.H., Green, R.E., Knopp, D. & Cuthbert, R.J. (2014) Diclofenac is toxic to the Steppe Eagle Aquila nipalensis: widening the diversity of raptors threatened by NSAID misuse in South Asia. *Bird Conservation International*, **24**(3), 282-286.

Sharma, P. (2012) ACECLOFENAC AS A POTENTIAL THREAT TO CRITICALLY ENDANGERED VULTURES IN INDIA: A REVIEW. *Journal of Raptor Research*, **46**(3), 314-318.

Shen, S., Hargus, S.J., Martin, B.M. & Pohl, L.R. (1997) Cytochrome P4502C11 is a target of diclofenac covalent binding in rats. *Chemical Research in Toxicology*, **10**(4), 420-423.

Shen, S., Marchick, M.R., Davis, M.R., Doss, G.A. & Pohl, L.R. (1999) Metabolic activation of diclofenac by human cytochrome P450 3A4: role of 5-hydroxydiclofenac. *Chemical Research in Toxicology*, **12**(2), 214-222.

Shou, M., Norcross, R., Sandig, G., Lu, P., Li, Y., Lin, Y., Mei, Q., Rodrigues, A.D. & Rushmore, T.H. (2003) Substrate specificity and kinetic properties of seven heterologously

expressed dog cytochromes p450. *Drug metabolism and disposition: the biological fate of chemicals*, **31**(9), 1161-1169.

Shultz, S., Baral, H.S., Charman, S., Cunningham, A.A., Das, D., Ghalsasi, G.R., Goudar, M.S., Green, R.E., Jones, A., Nighot, P., Pain, D.J. & Prakash, V. (2004) Diclofenac poisoning is widespread in declining vulture populations across the Indian subcontinent. *Royal Society of London. Proceedings. Biological Sciences*, **271**(6), S458-s460.

Small, R.E. (1989) Diclofenac sodium. *Clinical Pharmacy*, **8**(8), 545-558.

Stierlin, H., Faigle, J.W., Sallmann, A., Küng, W., Richter, W.J., Kriemler, H.P., Alt, K.O. & Winkler, T. (1979) Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I. Isolation and identification of principal metabolites. *Xenobiotica; the fate of foreign compounds in biological systems*, **9**(10), 601-610.

Swan, G., Naidoo, V., Cuthbert, R., Green, R.E., Pain, D.J., Swarup, D., Prakash, V., Taggart, M., Bekker, L., Das, D., Diekmann, J., Diekmann, M., Killian, E., Meharg, A., Patra, R.C., Saini, M. & Wolter, K. (2006) Removing the threat of diclofenac to critically endangered Asian vultures. *PLoS biology*, **4**(3), e66.

Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A., Duncan, N., Meharg, A.A., Oaks, J.L., Parry-Jones, J., Shultz, S., Taggart, M.A., Verdoorn, G. & Wolter, K. (2006) Toxicity of diclofenac to Gyps vultures. *Biology letters*, **2**(2), 279-282.

Szotáková, B., Baliharová, V., Lamka, J., Nožinová, E., Wsól, V., Velı, x, k, J., Machala, M., Neča, J., Souček, P., Šusová, S. & Skálová, L. (2004) Comparison of in vitro activities of biotransformation enzymes in pig, cattle, goat and sheep. *Research in Veterinary Science*, **76**(1), 43-51.

Takanohashi, T., Kubo, S., Nakayama, A., Mihara, R. & Hayashi, M. (2010) Inhibition of human liver microsomal CYP by nateglinide. *Journal of Pharmacy and Pharmacology*, **62**(5), 592-597.

Thomas, J.H. (2007) Rapid birth-death evolution specific to xenobiotic cytochrome P450 genes in vertebrates. *PLoS Genetics*, **3**(5), e67.

Toutain, P.L. & Bousquet-Mélou, A. (2004) Plasma clearance. *Journal of Veterinary Pharmacology and Therapeutics*, **27**(6), 415-425.

Trepanier, L.A. (2006) Cytochrome P450 and its role in veterinary drug interactions. *The Veterinary clinics of North America. Small animal practice*, **36**(5), 975-985.

Vannini, A., Paoli, L., Vichi, M., Bačkor, M., Bačkorová, M. & Loppi, S. (2018) Toxicity of Diclofenac in the Fern Azolla filiculoides and the Lichen Xanthoria parietina. *Bulletin of Environmental Contamination and Toxicology*, **100**(3), 430-437.

VICH (1998). *Validation of Analytical Method: Methodology*. In Validation of Analytical Method: Methodology.

VICH (2000). *Environmental Impact Assessment (EIAs) for veterinary medicinal products (VMPs) - Phase 1*. In Environmental Impact Assessment (EIAs) for veterinary medicinal products (VMPs) - Phase 1.

VICH (2004). *Environmental Impact Assessment (EIAs) for Veterinary Medicinal Products (VMPs) - Phase II*. In Environmental Impact Assessment (EIAs) for Veterinary Medicinal Products (VMPs) - Phase II.

Vié, J.-C., Hilton-Taylor, C., Stuart, S.N., Union, I.U.-.-T.W.C. & Commission, I.S.S. (2009). Wildlife in a changing world : an analysis of the 2008 IUCN red list of threatened species. IUCN ;
Lynx Edicions, Gland, Switzerland
Barcelona, Spain.

Wang, J., Zhao, S.-q., Zhang, M.-y. & He, B.-s. (2018) Targeted eco-pharmacovigilance for ketoprofen in the environment: Need, strategy and challenge. *Chemosphere*, **194**(Suppl. 1), 450-462.

Wasfi, I.A., Hussain, M.M., Elghazali, M., Alkatheeri, N.A. & Abdel Hadi, A.A. (2003) The disposition of diclofenac in camels after intravenous administration. *The Veterinary Journal*, **166**(3), 277-283.

Watanabe, K.P., Kawai, Y.K., Ikenaka, Y., Kawata, M., Ikushiro, S., Sakaki, T. & Ishizuka, M. (2013) Avian cytochrome P450 (CYP) 1-3 family genes: isoforms, evolutionary relationships, and mRNA expression in chicken liver. *PloS One*, **8**(9), e75689.

Watson, R. (2003) Eureka! We found the cause of the Asian vulture population crash. *The Peregrine Fund*(34), 6-7.

Wilson, H.R. & Miles, R.D. (1988) Plasma uric acid of broiler breeder and Leghorn male chickens: effect of feeding time. *Poultry science*, **67**(2), 345-347.

Wishart, D.S., Knox, C., Guo, A.C., Cheng, D., Shrivastava, S., Tzur, D., Gautam, B. & Hassanali, M. (2008) DrugBank: a knowledgebase for drugs, drug actions and drug targets. *Nucleic Acids Res*, **36**(Database issue), D901-906.

Woodward, K.N. (2005) Veterinary pharmacovigilance. Part 3. Adverse effects of veterinary medicinal products in animals and on the environment. *Journal of Veterinary Pharmacology and Therapeutics*, **28**(2), 171-184.

Zhai, X.-j., Shi, F., Chen, F. & Lu, Y.-n. (2013) Capsaicin pretreatment increased the bioavailability of cyclosporin in rats: Involvement of P-glycoprotein and CYP 3A inhibition. *Food and Chemical Toxicology*, **62**, 323-328.

# 8 Addendum

## 8.1 Maximum Likelihood Estimate Method for Determining Diclofenac Doses

The calculations of *ldose, hdose* and the *step* for Stage 2 of the OECD study design were as follows:

(1) The lowest and highest treatment doses were calculated using the following equations, where the working $LD_{50}$ was known; 9.8mg/kg BW (Naidoo et al., 2007):

*ldose* = dose1 = 0.3425 x working $LD_{50}$

*hdose* = dose10 = 2.919 x working $LD_{50}$.

(2) The *step* interval was calculated using the following equation: $step = (hdose/ ldose)^{1/9}$.

(3) The eight intermediate doses were calculated as follows: $dose_i = ldose \times step^{i-1}$, for i = 2 to 9.

(4) Each of the ten birds was randomly assigned to one of the calculated doses.

## 8.2 Drug Analysis Validation Report

For validation of the diclofenac and 4'- and 5'-hydroxydiclofenac (referred to as the metabolite combination) HPLC analysis, samples were prepared as discussed in section 3.6.2.1 (Naidoo et al., 2007) above. The average chromatogram (peak) and retention time values were obtained. The method was validated according to the VICH GL2 Validation of Analytic Procedures (VICH, 1998).

Bird samples were run over 2 sessions, therefore the calibration curve was repeated for diclofenac prior to the second run. The metabolite peaks could not be separated, and as individual plasma concentrations could therefore not be calculated, the calibration curve for the metabolite combination was run only once to validate specificity and linearity.

### *8.2.1 Specificity*

Diclofenac and the metabolite combination calibration dilutions demonstrated a clear and consistent peak at a mean retention time of 4.51 and 3.45 min, respectively (figure 8.1 and 8.2). An overlay graph of diclofenac peaks produced during the first calibration run, shows good retention time consistency (figure 8.3). Analysis of blank plasma samples showed no interfering peaks (figure 8.4). Fluconazole, administered as a CYP2C9 inhibitor for 3 days prior to diclofenac treatment, did not elute using the method discussed in 3.6.2.1 above.

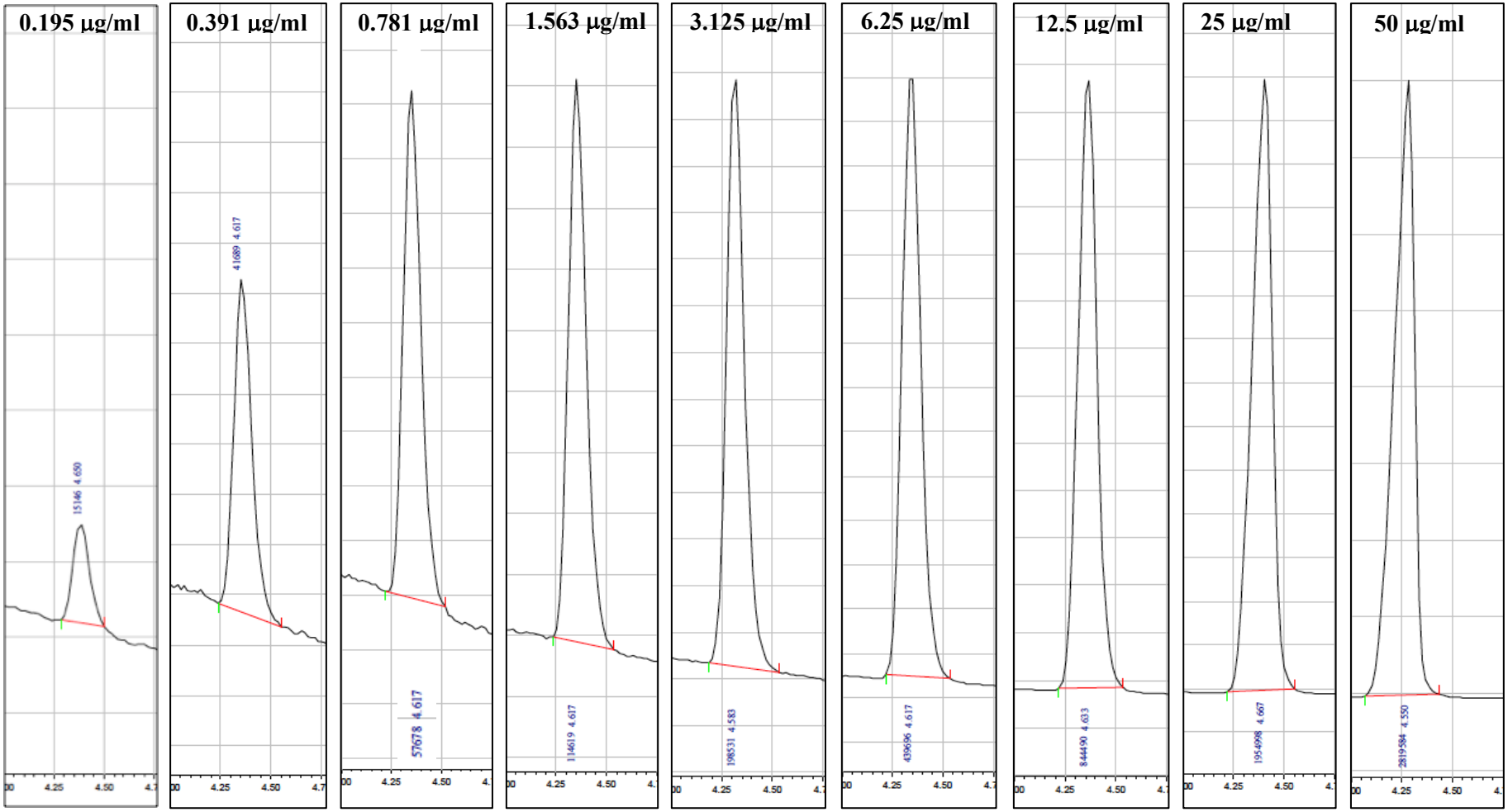


**Figure 8.1 Calibration curve peaks for diclofenac in chicken plasma, over a range of calibration standards.**

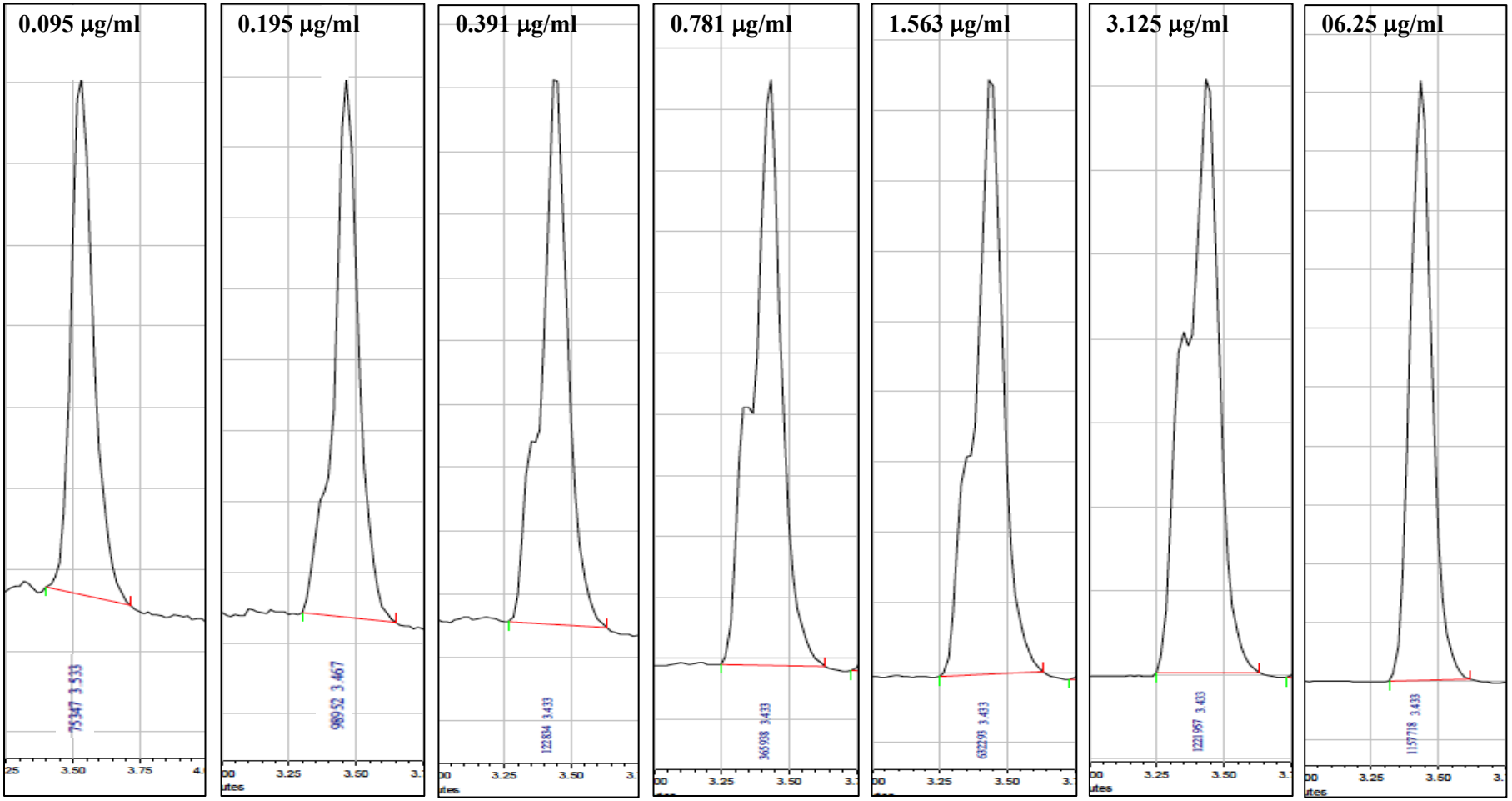


**Figure 8.2 Calibration curve peaks for combination 4'- and 5'-hydroxydiclofenac in chicken plasma, over a range of calibration standards.**

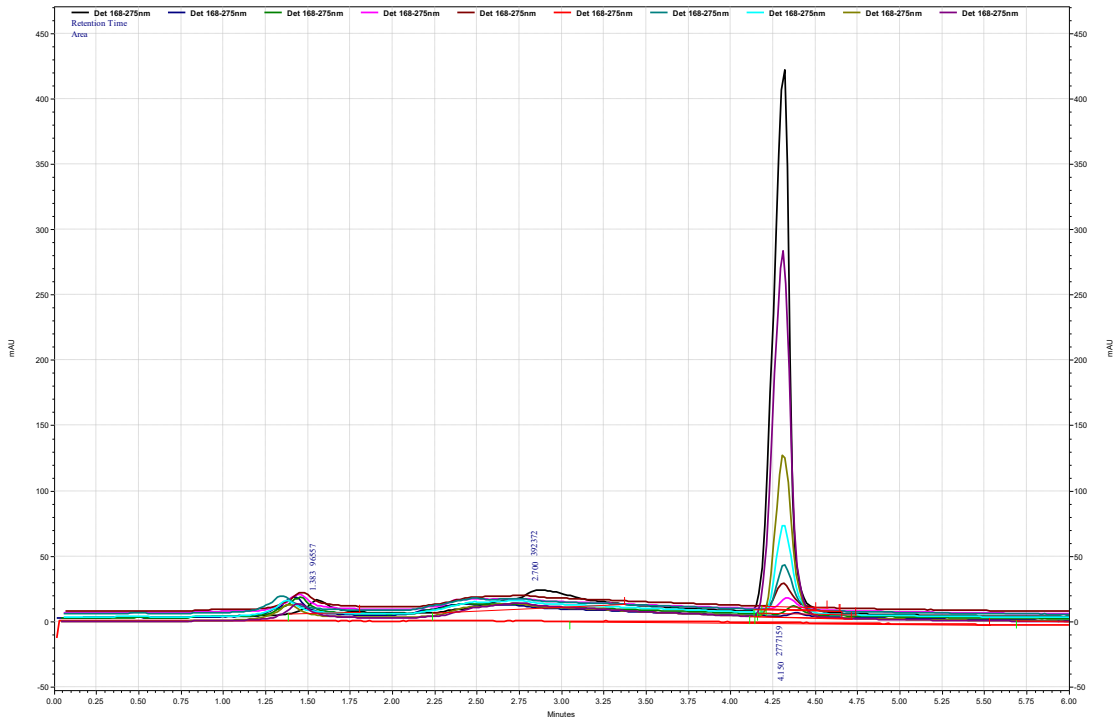


**Figure 8.3 Overlay of all peaks produced during first calibration run for diclofenac standards.**

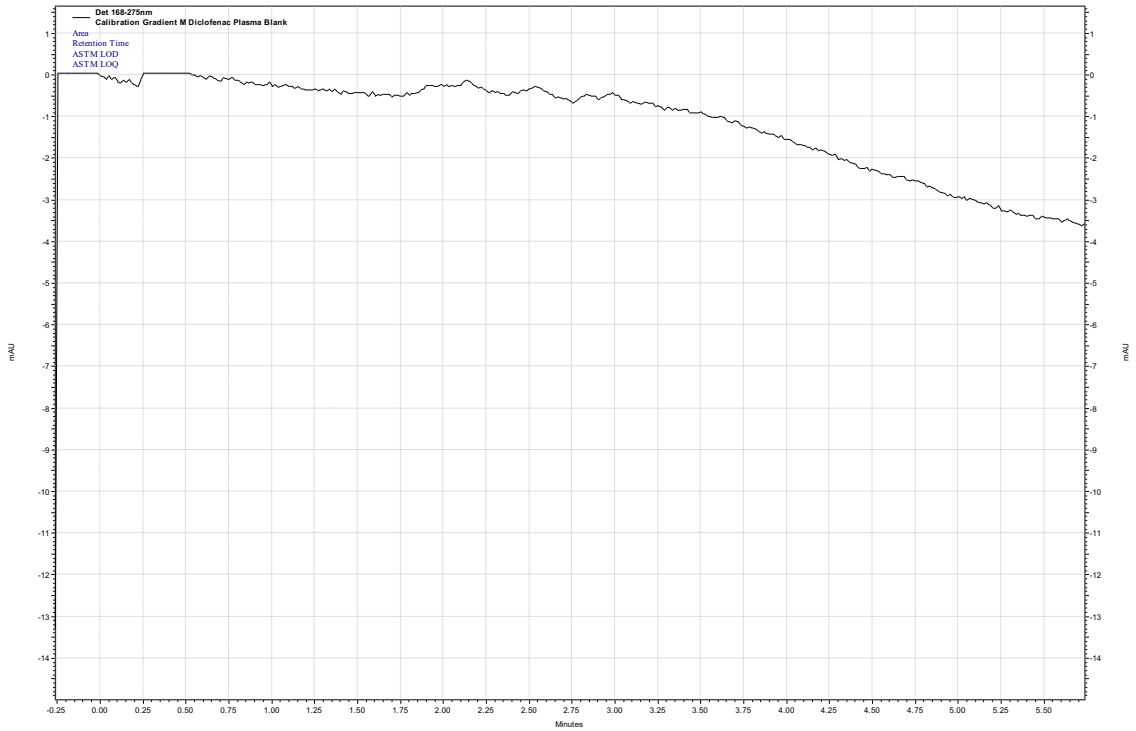


**Figure 8.4 Blank chicken plasma.**

### *8.2.2 Linearity*

A total of 8 and 7 calibration concentrations were used to obtain a calibration curve for diclofenac and the metabolite combination, respectively. The samples were run in triplicate for the first and second run of diclofenac and singularly for the metabolite combination (table 8.1, 8.2 and 8.3).

The resultant calibration curves for the mean area values are shown in figure 8.5, 8.6 and 8.7. The correlation coefficient, y-intercept and slope of the regression line are shown in table 8.4.

**Table 8.1 Diclofenac in chicken plasma calibration curve, first run.**

| **Conc** (µg/ml) | **Vol** (µl) | **Equivalence** (µg) | **Area 1** mAu | **R. Time 1** (min) | **Area 2** (m Au) | **R. Time 2** (min) | **Area 3** (m Au) | **R. Time 3** (min) | **Mean Area** (m Au) |
|---|---|---|---|---|---|---|---|---|---|
| **200** | 0.967 | 0.195 | 15146 | 4.650 | 15441 | 4.367 | 18121 | 4.367 | 16236.00 |
| **200** | 1.953 | 0.391 | 41689 | 4.617 | 40602 | 4.367 | 40531 | 4.333 | 40941.67 |
| **200** | 3.906 | 0.781 | 57678 | 4.617 | 57798 | 4.317 | 57965 | 4.283 | 57813.67 |
| **200** | 7.812 | 1.563 | 114619 | 4.617 | 116494 | 4.283 | 115534 | 4.250 | 115549.00 |
| **200** | 15.625 | 3.125 | 198531 | 4.583 | 217800 | 4.283 | 224694 | 4.267 | 213675.00 |
| **200** | 31.25 | 6.25 | 439696 | 4.617 | 441376 | 4.283 | 450846 | 4.267 | 443972.67 |
| **200** | 62.5 | 12.5 | 844490 | 4.633 | 842582 | 4.283 | 853212 | 4.267 | 846761.33 |
| **200** | 125 | 25 | 1954998 | 4.667 | 2003800 | 4.283 | 1997431 | 4.267 | 1985409.76 |

R.Time; Retention Time

**Table 8.2 Diclofenac in chicken plasma calibration curve, second run.**

| **Conc** (µg/ml) | **Vol** (µl) | **Equivalence** (µg) | **Area 1** mAu | **R. Time 1** (min) | **Area 2** (m Au) | **R. Time 2** (min) | **Area 3** (m Au) | **R. Time 3** (min) | **Mean Area** (m Au) |
|---|---|---|---|---|---|---|---|---|---|
| **200** | 0.967 | 0.195 | 52288 | 4.500 | 42181 | 4.783 | 41164 | 4.400 | 45211.00 |
| **200** | 1.953 | 0.391 | 45730 | 4.817 | 40927 | 4.783 | 37877 | 4.367 | 41511.33 |
| **200** | 3.906 | 0.781 | 70451 | 4.817 | 65123 | 4.800 | 65511 | 4.367 | 67028.33 |
| **200** | 7.812 | 1.563 | 148220 | 4.783 | 134210 | 4.833 | 136419 | 4.383 | 139616.33 |
| **200** | 15.625 | 3.125 | 331159 | 4.783 | 305221 | 4.833 | 316419 | 4.100 | 331159.00 |
| **200** | 31.25 | 6.25 | 433718 | 4.783 | 422350 | 4.817 | 419702 | 4.367 | 425256.67 |
| **200** | 62.5 | 12.5 | 1032862 | 4.767 | 992247 | 4.817 | 974226 | 4.017 | 1032862.00 |
| **200** | 125 | 25 | 2014531 | 4.850 | 1963637 | 4.867 | 1967155 | 4.450 | 1981774.30 |

R.Time; Retention Time

**Table 8.3 Metabolite (4'- and 5'-hydroxydiclofenac) combination in chicken plasma calibration curve.**

| **Conc** (µg/ml) | **Vol** (µl) | **Equivalence** (µg) | **Area 1** (m Au) | **R. Time 1** (min) |
|---|---|---|---|---|
| **50** | 1.953 | 0.095 | 75347 | 3.533 |
| **50** | 3.906 | 0.195 | 98952 | 3.467 |
| **50** | 7.812 | 0.391 | 122834 | 3.430 |
| **50** | 15.625 | 0.781 | 365938 | 3.433 |
| **50** | 31.25 | 1.563 | 632293 | 3.433 |
| **50** | 62.5 | 3.125 | 1221957 | 3.433 |

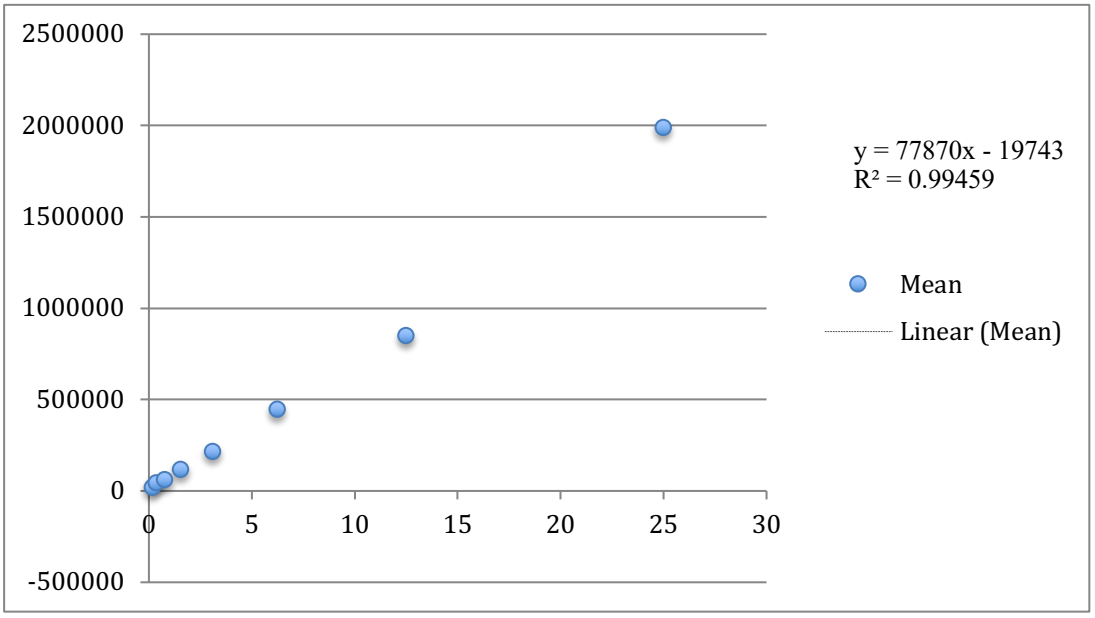


**Figure 8.5 Calibration curve for diclofenac in chicken plasma across 8 concentrations, first run.**

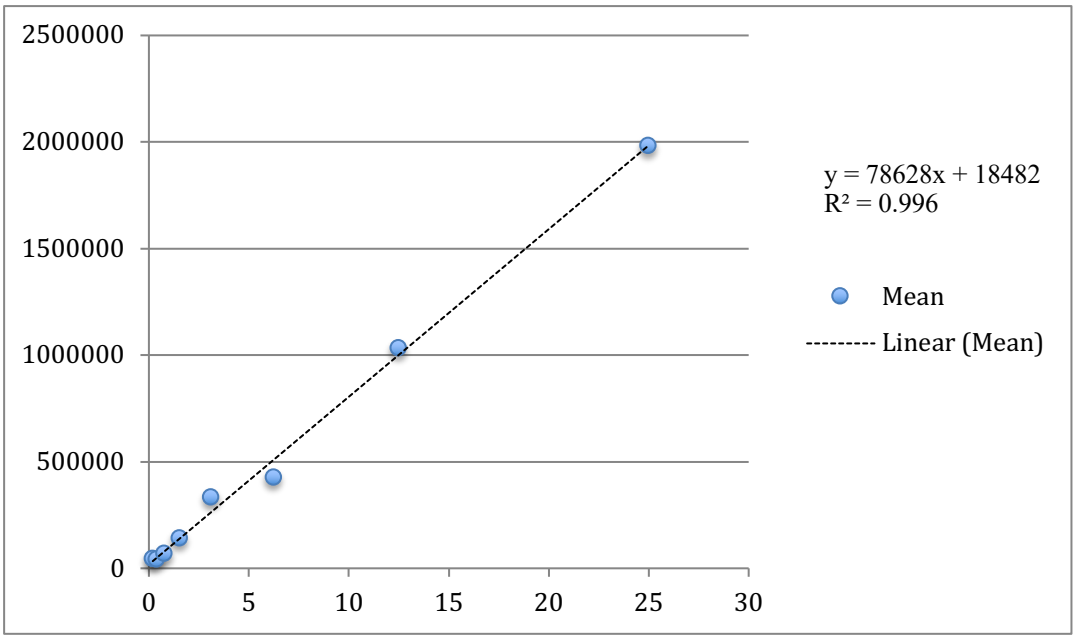


**Figure 8.6 Calibration curve for diclofenac in chicken plasma across 8 concentrations, second run.**

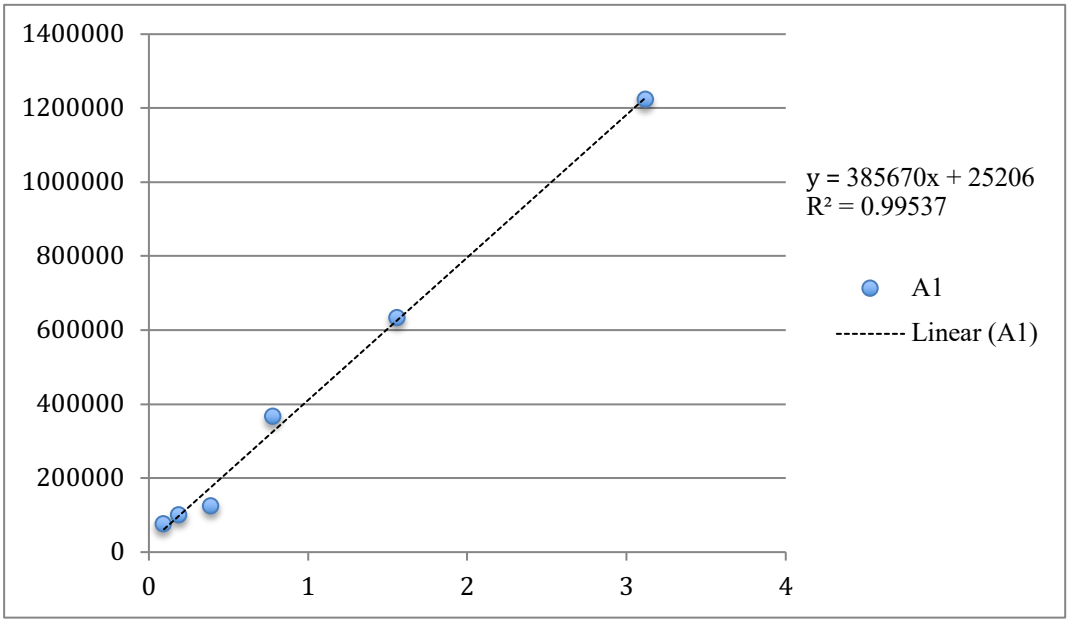


**Figure 8.7 Calibration curve for the metabolite combination, 4'- and 5'-hydroxydiclofenac, in chicken plasma across 8 concentrations.**

**Table 8.4 Linearity assessment parameters for diclofenac and metabolite combination in chicken plasma.**

| | **Diclofenac, first run** | **Diclofenac, second run** | **Metabolite combination** |
|---|---|---|---|
| **Correlation Coefficient ($R^2$)** | 0.995 | 0.996 | 0.995 |
| **y-intercept (m Au)** | -19743 | 18482 | 25206 |
| **Slope** | 77870 | 78628 | 385670 |

### *8.2.3 Accuracy*

Accuracy was determined over the 8 concentrations run in triplicate for diclofenac. Accuracy was determined by comparing the mean response to an analyte of known concentration. Accuracy was deemed acceptable for concentrations between 0.781 and 25 µg (table 8.5).

**Table 8.5 Diclofenac in chicken plasma accuracy assessments, first and second run.**

| **Mean response, first run** (m Au) | **Mean response, second run** (m Au) | **Theoretical concentrat-ion, first run** (µg) | **Theoretical concentrat-ion, second run** (µg) | **Expected concentrat-ion** (µg) | **Mean % recovery** |
|---|---|---|---|---|---|
| 16236 | 45211 | 0.46 | 0.34 | **0.195** | 206% |
| 40941 | 41511 | 0.78 | 0.29 | **0.391** | 137% |
| 57814 | 67028 | 1.00 | 0.62 | **0.781** | 103% |
| 115549 | 139616 | 1.74 | 1.54 | **1.563** | 105% |
| 213675 | 331159 | 3.00 | 3.98 | **3.125** | 112% |
| 443973 | 425257 | 5.95 | 5.17 | **6.25** | 89% |
| 846761 | 1032862 | 11.13 | 12.90 | **12.5** | 96% |
| 1985410 | 1981774 | 25.75 | 24.97 | **25** | 101% |
| 2789635 | 2548017 | 36.08 | 32.17 | **50** | 68% |

* Red values are those falling outside accuracy range of 90 – 110%.

### *8.2.4 Precision*

Only intermediate precision was assessed for diclofenac for the HPLC method used. Different operators conducted the first and second calibration runs on different days. The 95% confidence interval was used, and precision was deemed acceptable for the intended purpose of the analytical method (table 8.6 and 8.7).

**Table 8.6 Intermediate precision of peak response for diclofenac in chicken plasma.**

| **Equival-ence concent-ration** (µg) | **Mean peak response, both runs** (m Au) | **Standard deviation** (m Au) | **Relative Standard Deviation** (%) | **Lower confidence interval** (µg) | **Upper confidence interval** (µg) |
|---|---|---|---|---|---|
| **0.196** | 30723.50 | 16372.80 | 53.29 | 13538.51 | 47908.49 |
| **0.396** | 41226.00 | 2556.48 | 6.20 | 38542.70 | 43909.30 |
| **0.782** | 62421.00 | 5386.17 | 8.63 | 56767.65 | 68074.35 |
| **1.562** | 127582.67 | 14029.17 | 11.00 | 112857.57 | 142307.77 |
| **3.125** | 265637.33 | 58149.39 | 21.89 | 204603.36 | 326671.31 |
| **6.25** | 434614.67 | 11904.67 | 2.74 | 422119.45 | 447109.88 |
| **12.5** | 923269.83 | 86011.29 | 9.32 | 832991.83 | 1013547.83 |
| **25** | 1983592.00 | 24670.21 | 1.24 | 1957697.99 | 2009486.01 |
| **50** | 2668825.67 | 139625.01 | 5.23 | 2522274.37 | 2815376.97 |

**Table 8.7 Intermediate precision of retention time for diclofenac in chicken plasma.**

| **Equival-ence concent-ration** (µg) | **Mean retention time, both runs** (min) | **Standard deviation** (min) | **Relative standard deviation** (%) | **Lower confidence interval** (min) | **Upper confidence interval** (min) |
|---|---|---|---|---|---|
| **0.196** | 4.511 | 0.17 | 3.80 | 4.331 | 4.691 |
| **0.396** | 4.547 | 0.22 | 4.86 | 4.315 | 4.779 |
| **0.782** | 4.534 | 0.24 | 5.36 | 4.278 | 4.789 |
| **1.562** | 4.525 | 0.25 | 5.63 | 4.258 | 4.792 |
| **3.125** | 4.475 | 0.30 | 6.74 | 4.158 | 4.792 |
| **6.25** | 4.522 | 0.25 | 5.51 | 4.261 | 4.784 |
| **12.5** | 4.464 | 0.32 | 7.20 | 4.127 | 4.801 |
| **25** | 4.564 | 0.27 | 5.91 | 4.281 | 4.847 |
| **50** | 4.506 | 0.32 | 7.18 | 4.166 | 4.845 |

### *8.2.5 Range*

The HPLC method employed provides acceptable linearity, accuracy and precision over the concentration range 0.718 – 25 µg for diclofenac and acceptable linearity over the concentration range 0.095 – 3.125 µg for the metabolite combination.

### *8.2.6 Limit of Detection (LOD) and Limit of Quantification (LOQ)*

The LOD and LOQ were determined by estimating the signal (peak area of diclofenac or metabolite combination) to noise (average area of two highest peaks, one from either side of the diclofenac or metabolite combination peak) ratio. A signal to noise ratio of 3:1 and 10:1

were used for the LOD and LOQ respectively. The values are listed in table 8.8 and were identical for the first and second diclofenac calibration runs.

**Table 8.8 LOD and LOD for diclofenac and the metabolite combination in plasma.**

| | **Diclofenac, first run** (µg) | **Diclofenac, second run** (µg) | **Metabolite combination** (µg) |
|---|---|---|---|
| **LOD** | 0.196 | 0.196 | 0.095 |
| **LOQ** | 0.396 | 0.396 | 0.095 |

## 8.3 Chi Square Analysis to Assess Relationship between Weight and Mortality.

**Case Processing Summary**

| | Cases | | | | | |
|---|---|---|---|---|---|---|
| | Valid | | Missing | | Total | |
| | N | Percent | N | Percent | N | Percent |
| WtAbAV * Mortality | 20 | 100.0% | 0 | 0.0% | 20 | 100.0% |

**WtAbAV * Mortality Crosstabulation**

| | | | Mortality | | |
|---|---|---|---|---|---|
| | | | Alive | Dead | Total |
| WtAbAV | Above | Count | 5 | 3 | 8 |
| | | Expected Count | 4.4 | 3.6 | 8.0 |
| | | % within WtAbAV | 62.5% | 37.5% | 100.0% |
| | | % within Mortality | 45.5% | 33.3% | 40.0% |
| | | % of Total | 25.0% | 15.0% | 40.0% |
| | Below | Count | 6 | 6 | 12 |
| | | Expected Count | 6.6 | 5.4 | 12.0 |
| | | % within WtAbAV | 50.0% | 50.0% | 100.0% |
| | | % within Mortality | 54.5% | 66.7% | 60.0% |
| | | % of Total | 30.0% | 30.0% | 60.0% |
| Total | | Count | 11 | 9 | 20 |
| | | Expected Count | 11.0 | 9.0 | 20.0 |
| | | % within WtAbAV | 55.0% | 45.0% | 100.0% |
| | | % within Mortality | 100.0% | 100.0% | 100.0% |
| | | % of Total | 55.0% | 45.0% | 100.0% |

**Chi-Square Tests**

| | Value | df | Asymptotic Significance (2-sided) | Exact Sig. (2-sided) | Exact Sig. (1-sided) |
|---|---|---|---|---|---|
| Pearson Chi-Square | .303[a] | 1 | .582 | | |
| Continuity Correction[b] | .008 | 1 | .927 | | |
| Likelihood Ratio | .305 | 1 | .581 | | |
| Fisher's Exact Test | | | | .670 | .465 |
| N of Valid Cases | 20 | | | | |

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 3.60.

b. Computed only for a 2x2 table

**Figure 8.8 SPSS output for Chi Square analysis to assess the relationship between bird weight and mortality.**

## 8.4 Uric Acid Analysis

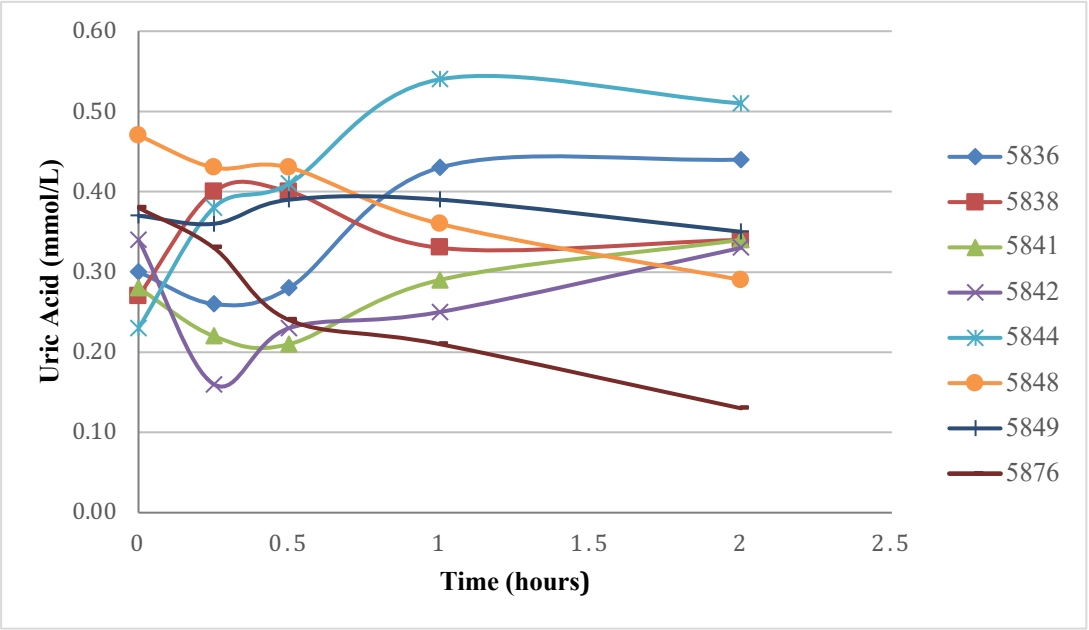


Figure 8.9 Uric acid concentration over time for test group birds.

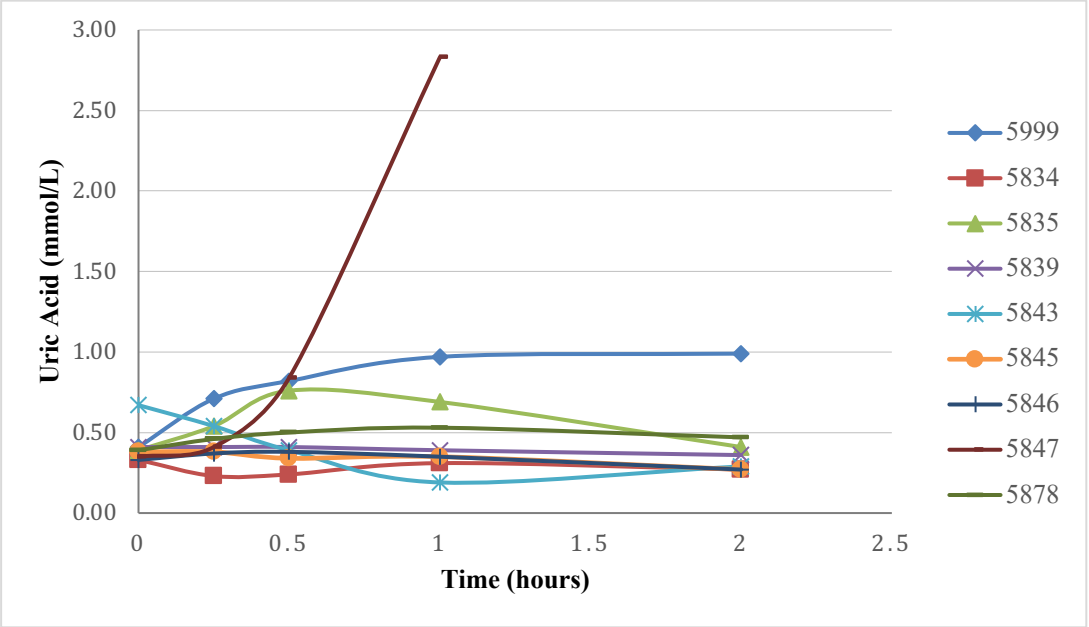


Figure 8.10 Uric Acid concentration over time for negative control group birds.

## T-Test

### Group Statistics

| | Group | N | Mean | Std. Deviation | Std. Error Mean |
|---|---|---|---|---|---|
| AUC | Test | 8 | 20.0631 | 4.50280 | 1.59198 |
| | Control | 9 | 53.5267 | 54.51453 | 18.17151 |

### Independent Samples Test

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| AUC | Equal variances assumed | 8.496 | .011 | -1.725 | 15 | .105 | -33.46354 | 19.40271 | -74.81943 | 7.89235 |
| | Equal variances not assumed | | | -1.835 | 8.123 | .103 | -33.46354 | 18.24111 | -75.41725 | 8.49017 |

**Figure 8.11 SPSS output for Independent samples T-Test for comparing means of uric acid AUC between test and negative control groups.**

## 8.5 Pharmacokinetic Diclofenac Plasma Concentration-Time Curves and Statistical Analysis

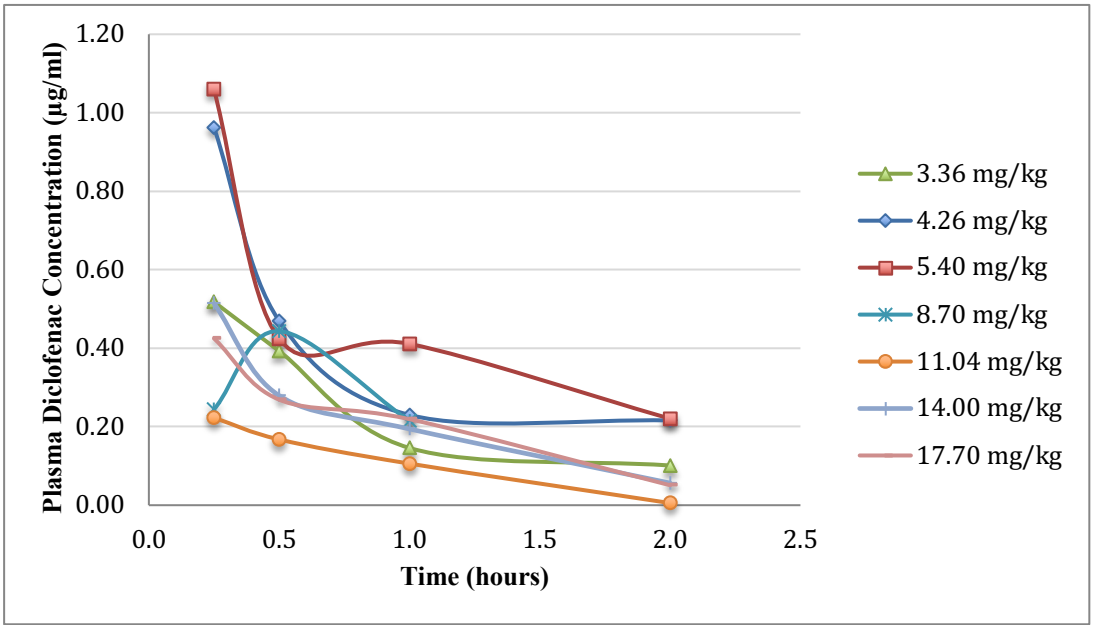


Figure 8.12 Diclofenac plasma concentration time profiles for the test group.

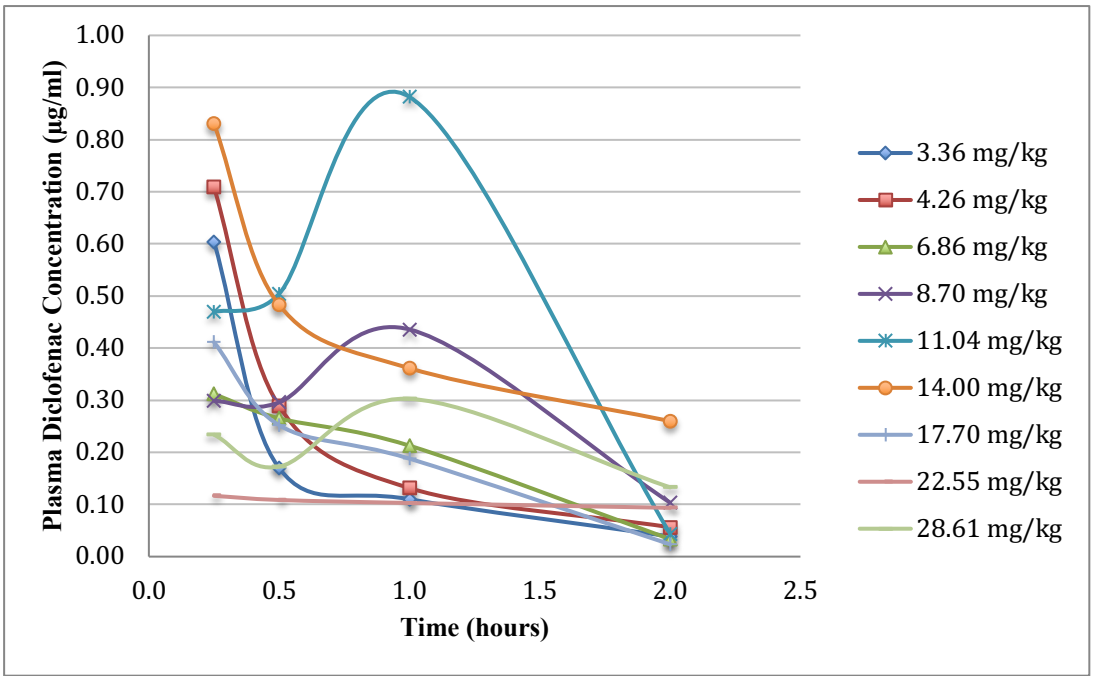


Figure 8.13 Diclofenac plasma concentration time profiles for the negative control group.

**Descriptives**

AUClast

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 7 | .4657 | .22802 | .08619 | .2548 | .6766 | .20 | .84 |
| 2 | 9 | .4322 | .17634 | .05878 | .2967 | .5678 | .19 | .79 |
| Total | 16 | .4469 | .19410 | .04853 | .3434 | .5503 | .19 | .84 |

**Test of Homogeneity of Variances**

| | | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| AUClast | Based on Mean | .671 | 1 | 14 | .426 |
| | Based on Median | .321 | 1 | 14 | .580 |
| | Based on Median and with adjusted df | .321 | 1 | 13.189 | .581 |
| | Based on trimmed mean | .625 | 1 | 14 | .442 |

**ANOVA**

AUClast

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .004 | 1 | .004 | .110 | .745 |
| Within Groups | .561 | 14 | .040 | | |
| Total | .565 | 15 | | | |

**Figure 8.14 SPSS output for one-way ANOVA for comparing $AUC_{last}$ between test and negative control groups.**

**Descriptives**

Cmax

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 7 | .5614 | .32957 | .12456 | .2566 | .8662 | .22 | 1.06 |
| 2 | 9 | .4633 | .22572 | .07524 | .2898 | .6368 | .12 | .83 |
| Total | 16 | .5063 | .27045 | .06761 | .3621 | .6504 | .12 | 1.06 |

**Test of Homogeneity of Variances**

| | | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| Cmax | Based on Mean | .948 | 1 | 14 | .347 |
| | Based on Median | .430 | 1 | 14 | .522 |
| | Based on Median and with adjusted df | .430 | 1 | 11.463 | .525 |
| | Based on trimmed mean | .813 | 1 | 14 | .382 |

**ANOVA**

Cmax

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | .038 | 1 | .038 | .501 | .491 |
| Within Groups | 1.059 | 14 | .076 | | |
| Total | 1.097 | 15 | | | |

**Figure 8.15 Figure 8.15. SPSS output for one-way ANOVA for comparing $C_{max}$ between test and negative control groups.**

**Descriptives**

Thalf

| | N | Mean | Std. Deviation | Std. Error | 95% Confidence Interval for Mean | | Minimum | Maximum |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower Bound | Upper Bound | | |
| 1 | 7 | .9486 | .57022 | .21552 | .4212 | 1.4759 | .32 | 1.94 |
| 2 | 9 | 1.7167 | 2.12432 | .70811 | .0838 | 3.3496 | .43 | 7.01 |
| Total | 16 | 1.3806 | 1.64065 | .41016 | .5064 | 2.2549 | .32 | 7.01 |

**Test of Homogeneity of Variances**

| | | Levene Statistic | df1 | df2 | Sig. |
|---|---|---|---|---|---|
| Thalf | Based on Mean | 2.744 | 1 | 14 | .120 |
| | Based on Median | .942 | 1 | 14 | .348 |
| | Based on Median and with adjusted df | .942 | 1 | 8.544 | .359 |
| | Based on trimmed mean | 2.122 | 1 | 14 | .167 |

**ANOVA**

Thalf

| | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Between Groups | 2.323 | 1 | 2.323 | .855 | .371 |
| Within Groups | 38.053 | 14 | 2.718 | | |
| Total | 40.376 | 15 | | | |

**Figure 8.16 SPSS Output for one-way ANOVA for comparing $T_{1/2}$ between test and negative control groups.**

## 8.6 Metabolite Statistical Analysis

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| DicMetRatioP1 | Equal variances assumed | 1.039 | .324 | .110 | 15 | .914 | 9.06653 | 82.79687 | -167.41081 | 185.54387 |
| | Equal variances not assumed | | | .105 | 10.037 | .918 | 9.06653 | 85.97456 | -182.40215 | 200.53520 |

**Figure 8.17 Independent samples T-Test for comparing dose corrected diclofenac: metabolite peak $AUC_{last}$ values, Peak 1.**

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | Sig. (2-tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| DicMetRatioP2 | Equal variances assumed | .157 | .698 | -.600 | 15 | .558 | -132.91528 | 221.62024 | -605.28764 | 339.45709 |
| | Equal variances not assumed | | | -.598 | 14.567 | .559 | -132.91528 | 222.27328 | -607.90990 | 342.07934 |

**Figure 8.18 Independent samples T-Test for comparing dose corrected diclofenac: metabolite peak $AUC_{last}$ values, Peak 2.**

**Independent Samples Test**

| | | Levene's Test for Equality of Variances | | t-test for Equality of Means | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Double-click to activate | | | 95% Confidence Interval of the Difference | |
| | | F | Sig. | t | df | tailed) | Mean Difference | Std. Error Difference | Lower | Upper |
| DicMetRatioP3 | Equal variances assumed | 6.558 | .022 | .878 | 15 | .394 | 98.41194 | 112.02380 | -140.36113 | 337.18502 |
| | Equal variances not assumed | | | .848 | 10.311 | .416 | 98.41194 | 116.09653 | -159.21424 | 356.03813 |

**Figure 8.19 Independent samples T-Test for comparing dose corrected diclofenac: metabolite peak $AUC_{last}$ values, Peak 3.**

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|
| Step 1[a] | Dose | -25.389 | 11917.488 | .000 | 1 | .998 | .000 | .000 | . |
| | AUCMetDCP2 | -.146 | 148.638 | .000 | 1 | .999 | .864 | .000 | 2.868E+126 |
| | AUCMetDCP3 | -1.069 | 536.858 | .000 | 1 | .998 | .343 | .000 | . |
| | AUCMetDCP4 | -.025 | 218.159 | .000 | 1 | 1.000 | .975 | .000 | 4.857E+185 |
| | Constant | 556.637 | 272236.914 | .000 | 1 | .998 | 5.549E+241 | | |

a. Variable(s) entered on step 1: Dose, AUCMetDCP2, AUCMetDCP3, AUCMetDCP4.

Double-click to activate

**Figure 8.20 Binary logistic regression to assess relationship and significance thereof between dose corrected metabolite $AUC_{last}$ peaks (National Research Council . Subcommittee on & Continuous Exposure Guidance Levels for Selected Submarine) and mortality for the test group birds.**

**Variables in the Equation**

| | | B | S.E. | Wald | df | Sig. | Exp(B) | 95% C.I.for EXP(B) Lower | Upper |
|---|---|---|---|---|---|---|---|---|---|
| Step 1[a] | Dose | 3.663 | 1924.928 | .000 | 1 | .998 | 38.972 | .000 | . |
| | AUCMetDCP2 | -.053 | Double-click to activate | .000 | 1 | 1.000 | .948 | .000 | 1.109E+155 |
| | AUCMetDCP3 | -.689 | | .000 | 1 | .999 | .502 | .000 | . |
| | AUCMetDCP4 | .622 | 172.210 | .000 | 1 | .997 | 1.862 | .000 | 7.173E+146 |
| | Constant | -110.948 | 47393.753 | .000 | 1 | .998 | .000 | | |

a. Variable(s) entered on step 1: Dose, AUCMetDCP2, AUCMetDCP3, AUCMetDCP4.

**Figure 8.21 Binary logistic regression to assess relationship and significance thereof between dose corrected metabolite $AUC_{last}$ peaks (National Research Council . Subcommittee on & Continuous Exposure Guidance Levels for Selected Submarine) and mortality for the negative control group birds.**

## 8.7 SEDEC Calculator $LD_{50}$ Outputs

**SEDEC V1.3 Sequential Design Calculator**

**Study Identification**

| | |
|---|---|
| Project Number = | V078-18 |
| Test Substance = | Diclofenac |
| Dose Units = | mg formulation/kg |
| Test Species = | Pheasant |
| Study Type = | Dose-Response: LD50 Only |
| Limit Dose = | NA |
| Initial LD50 Guess = | 10 |
| Date = | 09-Oct-19 |
| Initials = | SLL |
| Study Status Code = | 23 |
| Min % Dose Sep = | 1 |
| Stage 3 Type = | A |

Step Size = 3.6246416

(Combine doses differing by < Min% for analysis)

**Doses / Responses**

| Stage 1 | | | Stage 2 | | | Stage 3 | | | Stage 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose | N Tested | N Responding | Dose | N Tested | N Responding | Dose | N Tested | N Responding | Dose | N Tested | N Responding |
| 1,41 | 1 | 0 | 3,43 | 1 | 0 | 6,26 | 5 | | | | |
| 5,21 | 1 | 0 | 4,35 | 1 | 0 | 22,7 | 5 | | | | |
| 19,2 | 1 | 1 | 5,52 | 1 | 0 | | | | | | |
| 70,7 | 1 | 1 | 7,00 | 1 | 1 | | | | | | |
| | | | 8,88 | 1 | 0 | | | | | | |
| | | | 11,3 | 1 | 0 | | | | | | |
| | | | 14,3 | 1 | 1 | | | | | | |
| | | | 18,1 | 1 | 0 | | | | | | |
| | | | 23,0 | 1 | 1 | | | | | | |
| | | | 29,2 | 1 | 1 | | | | | | |

**Analysis**

Probit Analysis Results

| Iterations | Chi-square | Probability | G | N |
|---|---|---|---|---|
| 5 | 9.653303091 | 0.561815595 | 0.836397661 | 14 |

| | | | |
|---|---|---|---|
| Slope = | 3.704862156 | | |
| 95% Confidence Limits= | 0.316588657 | and | 7.093135655 |
| LD50 = | 11.91827154 | | |
| 95% Confidence Limits= | 3.870084213 | and | 61.17224713 |
| n of Reversals = | 2 | n of Partials = | 0 |

**Figure 8.22 SEDEC output for test group.**

## SEDEC V1.3 Sequential Design Calculator

### Study Identification

| | |
|---|---|
| Project Number = | VP78-18_2 |
| Test Substance = | Diclofenac |
| Dose Units = | mg formulation/kg |
| Test Species = | Pheasant |
| Study Type = | Dose-Response: LD50 Only |
| Limit Dose = | NA |
| Initial LD50 Guess = | 10 |
| Date = | 09-Oct-19 |
| Initials = | SLL |
| Study Status Code = | 24 |
| Min % Dose Sep = | 1 |
| Stage 3 Type = | B |

Step Size = 1.2694297

(Combine doses differing by < Min% for analysis)

### Doses / Responses

| Stage 1 | | | Stage 2 | | | Stage 3 | | | Stage 4 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Dose | N Tested | N Responding | Dose | N Tested | N Responding | Dose | N Tested | N Responding | Dose | N Tested | N Responding |
| 1,41 | 1 | 0 | 3,43 | 1 | 0 | 7,18 | 2 | | | | |
| 5,21 | 1 | 0 | 4,35 | 1 | 0 | 9,12 | 2 | | | | |
| 19,2 | 1 | 1 | 5,52 | 1 | 1 | 11,6 | 2 | | | | |
| 70,7 | 1 | 1 | 7,00 | 1 | 0 | 14,7 | 2 | | | | |
| | | | 8,88 | 1 | 0 | 18,7 | 2 | | | | |
| | | | 11,3 | 1 | 0 | | | | | | |
| | | | 14,3 | 1 | 0 | | | | | | |
| | | | 18,1 | 1 | 1 | | | | | | |
| | | | 23,0 | 1 | 1 | | | | | | |
| | | | 29,2 | 1 | 1 | | | | | | |

### Analysis

| Probit Analysis Results | | | | |
|---|---|---|---|---|
| Iterations | Chi-square | Probability | G | N |
| 6 | 7.229654264 | 0.780192224 | 0.855914697 | 14 |

| | | | |
|---|---|---|---|
| Slope = | 3.326620254 | | |
| 95% Confidence Limits= | 0.248975638 | and | 6.404264871 |

| | | | |
|---|---|---|---|
| LD50 = | 11.57858016 | | |
| 95% Confidence Limits= | 3.312382788 | and | 78.61821541 |

| | | | |
|---|---|---|---|
| n of Reversals = | 1 | n of Partials = | 1 |

**Figure 8.23 SEDEC output for negative control group.**