EVALUATION OF A CURRENT AVIAN ENVIRONMENTAL TOXICITY GUIDELINE PRESCRIBED FOR PESTICIDE EVALUATION FOR THE PREDICTION OF THE TOXIC EFFECT OF DICLOFENAC IN VULTURES.

Dr. IZ Hassan

University of Pretoria

DECLARATION

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.

The dissertation has not previously been submitted at this University or any other academic institution of learning for consideration. It is the result of my own investigations, except where the inputs of others are acknowledged.

I, Dr Ibrahim Hassan Zubairu, declare the statements above to be correct.

Dr. Ibrahim Hassan Zubairu

Prof. Vinny Naidoo (Supervisor)

AKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor Professor Vinny Naidoo, for his painstaking guidance, patience and constructive “criticism” enabling me to put together this piece of work. I have really learnt a lot from your wealth of knowledge and experience. It’s been an honor to have worked under you sir. Thank you.

I would also like to thank my Co-supervisor Professor Neil Duncan, for conducting the necropsies and his expertise in pathology, and not to mention your time, effort and high tolerance. I won’t have to bother you with my questions while you’re on the road anymore.

I am extremely grateful to the University of Pretoria for providing this platform to advance myself and for awarding me a postgraduate research support bursary to pay for my registration fee during my second year of study.

My sincere appreciation goes to the entire staff members of the University of Pretoria Biomedical Research Centre, most especially but not limited to, Mrs. Antonette Van Wyk (“my school mother”), Dr. John Chipangura, Mrs. Ilse Janse van Rensburg, Mr. Humbelani Ratshibamda and Mr. Josias Shongwe for their assistance with managing the animal studies.

To Dr. Emmanuel O. Adawaren, thank you for guiding me through operating the HPLC machine. It would not have been this smooth without your assistance.

I am extending my gratitude to all my colleagues at the Pharmacology and Toxicology Section, members of staff of the Department of Paraclinical sciences for their support and creating an enabling working environment. I cannot but mention some few names; Ms Anette Venter, Ms Arina Ferreira, Mr. Williams Mokgojane, Mr. Rexton Ramuageli, Mrs. Madelyn de Wet and Mrs. Fransie Lothering (Of the Deputy Dean’s office Research and Postgraduate studies).

To Prof. M.M Suleiman, Dr Bala Usman, Dr S.M Tanko, Dr Abdul-Hakeem Bello, Dr Bala Abubakar Muhammed, Dr Habeeb Ismaila Ahmad, Dr Sidiama Adamu, Mr. Idris S. Evuti, Mr. Abdul-Qadir Muhammed and all other friends and colleagues, thank you for your support, frequent checks and prayers.

My heart felt gratitude to the Limans and Kolos, Dr Isa Hamza Ibrahim, Dr Rukayyah Abubakar, Dr Donald Lubembe, Mr. Alfred Omwando Mainga, Dr David Dazhia Lazarus, Dr Yusuf Ngoshe Bitrus, Dr Sam Ogundare Tolulope, Dr Onyeka Libby and Mrs. Dorcas Gado for all your support and being my family away from home. You guys made my stay in South Africa worthwhile.

Finally, to my parents; Bldr. Gimba & Hajiya Fatima H.Z. and siblings; Mr. Muhammed, Mr. Idris, Mr. Usman, Mr. Zubairu, Mrs. Fatima, Ms Fatima & Ms Fatima Zahra. I can't thank you enough for the never-ending show of love and support. I am eternally grateful.

ABSTRACT

Diclofenac was responsible for the death of millions of *Gyps* vultures (*G. bengalensis, G. indicus* and *G. tenuirostris*) in the Indian sub-region with the safety of the other members of non-steroidal anti-inflammatory drugs (NSAIDs) being questionable. This has resulted in calls to test all the available NSAIDs for their vulture toxicity potential especially as studies have shown meloxicam to be safe; and ketoprofen, carprofen, flunixin and phenylbutazone as toxic. Unfortunately, due to the cost of testing, the time taken to establish toxicity reliably and the questionable ethics of repeat toxicity testing in an endangered species, an alternate method of and model for testing is needed. For this study, we evaluated an OECD recommended method for determining the avian toxic potential of environmentally applied pesticides. We exposed young-adult Japanese quails (*Coturnix japonica*), Muscovy ducks (*Cairina moschata*) and domestic pigeons (*Columba livia*) as per model requirements to diclofenac at various doses. This was coupled to the evaluation of the plasma toxicokinetics of the mentioned drug. The aim of this study was to look at the potential of the OECD models, as a predictive tool for diclofenac's environmental toxic effect.

Intoxication was noted in Japanese quails and Muscovy ducks which appeared to be identical to the clinical signs that were previously reported in vultures viz. depression and death within 48 – 92 h of dosing; while the domestic pigeon was insensitive| not susceptible. For the birds that died, necropsy revealed signs of nephrosis with resultant urate deposits in the kidney, spleen, pericardium and liver, once again as previously seen in the vulture. The pharmacokinetic profile in the domestic pigeon showed that the drug was well absorbed and distributed with a $T_{1/2}$ generally below 6 h. The toxicokinetic profile in Japanese quails demonstrates that toxicity was related to metabolic capacity, with a $T_{1/2}$ and MRT above 6 h and 8 h respectively being associated with signs of intoxication. While the quail result is consistent with previous studies, poisoning in the Muscovy ducks was not related to metabolic constraint but elevated plasma uric acid concentration as they all demonstrated rapid metabolism [$T_{1/2}$ (1-2 h) and MRT (2-3 h)] irrespective of survival or death. This was also reflected by their almost intact micro-hepatic structure as opposed to the other species.

Interestingly, some of the Muscovy ducks recovered even though they had elevated plasma uric acid concentrations reported to kill *Gyps* vulture. To better understand this, plasma from Muscovy duck, Cape Griffon vulture (*Gyps coprotheres*) and domestic chicken (*Gallus gallus*) were subjected to a uric-acid saturation test which proved the former's higher tolerance to elevated plasma uric acid concentration. Despite evidence of intoxication from this study, the estimated oral LD_{50} was very high at 405 mg/kg and 190 mg/kg in Japanese quails and Muscovy ducks respectively. The latter was also substantially higher than the LD_{50} of 0.1 mg/kg extrapolated for *Gyps* vultures. We therefore conclude that these bird species are not suitable as surrogates for NSAID toxicity testing. More importantly the results suggest that the toxicity of diclofenac in vultures is idiosyncratic and thus completely unpredictable using current laboratory models prescribed by the OECD.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AIDS	Acquired Immune Deficiency Syndrome
BCF	Bioconcentration Factor
CNS	Central Nervous System
COX	Cyco-oxygenase enzyme
EPA	Environmental Protection Agency
HIV	Human Immunodeficiency Virus
ICH	The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IUCN	International Union for Conservation of Natural
Kd	Sorption/desorption coefficient
Koc	Sorption/desorption coefficient, normalized to organic carbon content
Kow	n-Octanol/water partitioning coefficient
NOEC	No-observed effect concentration, i.e., the test concentration at which no adverse effect occurs.
NSAID	Non-steroidal anti-inflammatory drug
OECD	Organisation for Economic Co-operation and Development
PAH	Polycyclic aromatic hydrocarbons
PEC	Predicted environmental concentration
PNEC	Predicted no effect concentration

RQ	Risk Quotient
SMS	Soil multi-species
SW	Surface water
VMP	Veterinary medical products
VICH	The International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products
WHO	World Health Organisation

1 INTRODUCTION

1.1 Introduction

Since the industrial expansion after the Second World War, the environment has been constantly exposed to a myriad of chemical substances in the form of synthetic pesticides, pharmaceuticals, industrial effluents, etc (Hoffman, 1995). These chemical substances find their way into the environment from either direct application onto fields/livestock in fields, treatment of fish in aquaculture systems and/or disposal of surplus chemicals as sewage or from industrial effluent. Similarly, the environment has also been indirectly exposed in the form of metabolites of products excreted by humans or animals following their treatment/exposure (Halling-Sørensen *et al.,* 1998). While the contamination of the environment was to a large extent ignored for many years, findings have shown that once in the environment, these pollutants are not without adverse effect. Pesticides like dichlorodiphenyltrichloroethane (DDT), an organochlorine and others belonging to the organophosphate & carbamate groups have been seen to cause devastating adverse effects to non-target species both directly and indirectly. Examples of direct effects include the killing of beneficial insect species such as the ladybird beetle (*Cycloneda sanguinea*), insidious flower bug (*Orius insidiosus*), soldier beetle *(Chauliognathus flavipes)*(Fernandes *et al.,* 2016), honeybee (*Apis species*) (Gonalons and Farina, 2015); and high mortalities in birds that fed on treated crops or contaminated prey (Fleischli *et al.,* 2004; Kohler and Triebskorn, 2013).

In a similar manner, pharmaceuticals and other medicinal substances have also been seen to cause deleterious consequences on non-target species ranging from effects on aquatic organisms such as feminisation of fish by the persistent 17 alpha ethynyl estradiol (Santos *et al.,* 2010) to the deaths of invertebrates (beetle larvae, fly larvae and other beneficial non-target invertebrates) due to their exposure to some anthelmintic compounds (Lumaret and Errouissi, 2002). Early in the twenty first century, diclofenac (a non-steroidal anti-inflammatory drug [NSAID]) was incriminated as the cause of the demise of three species of vultures (*Gyps bengalensis*, *Gyps indicus* and *Gyps tenuirostris*) in the Indian sub-region. The birds died following their secondary exposure to the drug in carcasses of ungulates that had received the drug shortly before death.

Deaths in vultures were associated with renal failure, increased plasma uric acid concentration and extensive visceral gout deposits at post mortem (Oaks *et al.,* 2004; Swan *et al.,* 2006b). India lost more than 99% of their endemic vulture species (numbering tens of millions) to diclofenac and consequently is now faced with a direct economic loss from an associated increase in feral dog and rat populations [i.e. estimated at 2.43 billion US dollars annually (Markandya *et al.,* 2008; Moleón *et al.,* 2014)] with an associated increase in incidence of human rabies and bubonic plague from the disruption of the ecosystem. The catastrophe has also had cultural and religious impact on certain populations of the people of India (Ogada *et al.,* 2012).

When looking at the toxicity of diclofenac in vultures, two important questions are raised; why was the drug so toxic and how could a registered drug end up being so toxic? When looking at the registration process of medicines, while different countries may have different processes, the basic tenets remain the same viz. laboratory animal toxicity and target animal toxicity. Little is done with regards to environmental toxicity for non-target species. For VICH (International Co-operation on Harmonisation of Technical Requirements for the Registration of Veterinary Medical Products) aligned countries, a guideline is available that attempts to answer question on environmental toxicity. Risk of environmental toxicity from veterinary medical products (VMPs) are assessed based on their physicochemical properties, effect, fate, and environmental exposure potential i.e. amount of parent drug and/or residue entering directly into the environment. Thus, most VICH recommendations are based on direct effects of the presence of such agents to the inhabitants of the environment (soil and water). In doing so, birds were precluded from the environmental toxicity testing since they do not survive directly within the soil or water. Thus, with the VICH guidelines aimed mainly at aquatic and soil toxicity, it is not surprising that toxicity was not predictable in vultures for drugs such as ketoprofen, caprofen, flunixin and phenylbutazone (Naidoo *et al.,* 2010b; Fourie *et al.,* 2015).

While there has been a general absence of focus on avian toxicity in the pharmaceutical registration process, the same does not apply for the pesticides. This is largely due to the fact that birds depend on crops and prey (i.e. invertebrates & vertebrate pests) for nourishment. Pesticides are widely used on farm lands to control various forms of pests; thus, birds are at a high risk of exposure to pesticides from contaminated prey or crops with significant consequences on the

ecosystem (Tesfahunegny and Mulualem, 2016). The two common guidelines are the Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD) guidelines. For these, the quail (*Coturnix japonica/Colinus virginianus*), mallard duck (*Anas platyrhynchos*), chicken (*Gallus gallus*) and a passerine species (House sparrow [*Passer domesticus*]) are proposed as indicator species for the entire avian clade (Kenaga, 1978), with other species such as the pigeon (*Columba livia domestica*), ring-necked pheasant (*Phasianus colchicus*), red-legged partridge (*Alectoris rufa*) (EPA, 2012) and budgerigar (*Melopsittacus undulatus*) (OECD, 2010) being less commonly recommended. These species of birds are usually exposed to the said xenobiotic either by a single-dose oral LD_{50} test, a dietary LC_{50} test or a reproduction test. It is assumed that any xenobiotic found to be safe in these species is presumably safe to the entire avian family. Noteworthy to mention is that while all birds originated from a common ancestor (Padian and Chiappe, 1998; Xu *et al.,* 2003; van Tuinen, 2016) they have become varied. Their exposure to numerous xenobiotics in their feed has appeared to cause a differentiation in their ability to metabolise different substances through mechanisms such as gene duplication and deletion over time (Thomas, 2007). Thus, they respond differently to xenobiotic insults based on such pressures (Hutchinson *et al.,* 2014; Almeida *et al.,* 2016). At present this principle appears to be valid for diclofenac toxicity, with a high LD_{50} of 9.8 mg/kg being reported in the chicken (Naidoo *et al.,* 2007) and >20 mg/kg for the Japanese quail (Hussain *et al.,* 2008) in contrast to the very low LD_{50} value in oriental white-rumped vultures (0.1 - 0.2 mg/kg) as shown by Swan *et al.,* (2006b). Similar discrepancies were observed in the LD_{50} values of some organophosphates and carbamates pesticides on mallard duck, house sparrow, Japanese quail, pigeon and chukar partridges (*Alectoris chukar*) due to apparent differences in the sensitivity of the cholinesterase enzymes (Tucker and Haegele, 1971).

For this study, I will look at the applicability of the OECD models, as a predictive tool for diclofenac's avian environmental toxic effect or whether the said toxicity was so idiosyncratic that it is a totally unpredictable toxicological event. More importantly, if the method proves to be effective in predictive drug toxicity in birds, attempts will be made to see how the VICH guidelines may be adapted to include birds in the environmental risk assessment of veterinary medicines.

1.2 Aim

To ascertain predictability of diclofenac's toxicity in Vulture if the correct battery of preclinical testing is completed

1.3 Objectives

- To determine the median lethal dose (LD_{50}) of diclofenac in the quail (*Coturnix japonica)*, Muscovy duck (*Cairina moschata*) and pigeon (*Columba livia domestica*).
- To evaluate post mortem changes and determine changes in serum uric acid concentration following exposure of birds to diclofenac.
- To determine the concentration of diclofenac in plasma samples of the birds.
- To look at the potential of establishing a risk assessment rubric for the registration of veterinary medicines, that are potentially toxic to birds.

2 LITERATURE REVIEW

2.1 Environmental effects of human activities

2.1.1 Introduction

While people and the environment have been in harmony for many years, this changed in the 1850's, when human activities became increasingly associated with environmental pollution and incidences of animal die-offs and deleterious effects (Hoffman, 1995). Industrial activities such as oil exploration, manufacture of chemicals, metal smelting, mining and drilling of drainages have all been associated with unwanted effects on the ecosystem. The situation was made worse by the discovery of synthetic organic pesticides, during the Second World War, which were used to control insects mainly on farm lands (Hoffman, 1995). These pesticides were unfortunately persistent in the environment and had a wide spectrum of biological activity. It didn't take long before undesirable effects such as sudden deaths, impaired reproduction and decline in population of certain species were noticed (Hoffman, 1995; Almeida *et al.,* 2016). Even after such discovery, it took time before necessary actions were taken. Most recently, the environmental impact of pharmaceuticals has become a burning issue with several pharmaceuticals being detected in the environment, albeit at low concentration (Jones *et al.,* 2001).

2.1.2 Industrial activities

There is no doubt that the emergence of industrialization has brought about rapid development and changed the face of the world, however, this is not without consequences. Crude exploration, manufacture of chemicals, mining etc. have all been associated with far reaching effects on the ecosystem (Kadafa, 2012). Crude oil is a naturally occurring rich source of cheap energy (when compared to renewable sources[Abbasi *et al.,* 2011]) which is made up of hydrocarbon deposit and organic material (Bojes and Pope, 2007). The drive for fossil fuel as a source of energy is a global phenomenon that has sustained exploration of oil (Hubbert, 1949). This is largely due to the fact that the world depends on energy to power machines and technologies for carrying out tasks and work. However, oil spillage/leaks, depletion of biodiversity (Kadafa, 2012), the gradual acidification of the oceans and global warming are all contentious issues (disadvantages)

associated with the use of fossil fuel as source of energy (Abbasi *et al.,* 2011). An alarming 0.7 to 1.7 million tons of crude oil is estimated to be spilled into the environment annually (Kadafa, 2012). Further to this, crude oil is an important source of polycyclic aromatic hydrocarbon, which are known to persist in the environment and to bioaccumulate and biomagnify along the food chain. They have been shown to be toxic and carcinogenic (Won *et al.,* 2013). The manufacture and use of chemicals is another area of concern. As an example, the polychlorinated biphenyls (PCBs) are a group of chlorinated hydrocarbons introduced into the market at the early part of the twentieth century (Frame *et al.,* 1996). They were used in hydraulic fluids, die-casting equipment, production of transformers and as insulating fluids for assemblage of capacitors (Bohannon, 2014). Despite their value, several of them have been associated with deleterious effects on the ecosystem and have been detected in Arctic and Antarctic regions. They bioaccumulate and biomagnify as they pass through the food chain. In birds, PCBs have been associated with developmental abnormalities, endocrine disruption, immunosuppression, and diminished reproduction (Ferni *et al.,* 2003; Lavoie and Grasman, 2007; McNabb, 2005). In mammals, increased liver tumours, cytochrome P450 induction, diminished or altered detoxification, neurological, endocrine, reproductive and immune system dysfunction have been reported (Bohannon, 2014). The compounds are also persistent, with traces of these compounds still being widely seen in environmental matrices at relevant environmental concentrations in countries where they have been banned for more than thirty years (Bhalla *et al.,* 2016; Georgieva and Stancheva, 2016).

2.1.3 Pesticides

Pesticides are poisonous substances designed to alleviate, repel, harm or cause death of pest organisms (Cox and Surgan, 2006). They are generally used on crops or on animals to protect them from the said direct damage induced by these pests or from the diseases transmitted by these pest (Berthoud *et al.,* 2011), and constitute an integral part of the agricultural industry (Margni *et al.,* 2002). It is estimated that about two million tons of pesticides are used worldwide annually (Yadav *et al.,* 2015) and unfortunately, more than 99.9% enter the environment without having contact with the supposed pest but rather affect the ecosystem (Pimentel, 1995). Contamination of the environment has mostly been associated with spray drift, volatilization, run offs and leaching causing deleterious consequences on non-target organisms living in soil and

water (Pereira *et al.,* 2009). Perhaps one very important mode of environmental exposure is through the consumption of bait or contaminated prey or carcass. In the US, studies have demonstrated the presence of pesticides in greater than 70% of common foods (fruits/vegetables), streams and in more than 50% of human adults and children tested, at low concentration (Cox and Surgan, 2006). A similar situation has been reported in France, with 91% and 59% of surface water and ground water sampled, respectively, revealing the presence of pesticides (Berthoud *et al.,* 2011). In India, Yadav *et al.,* (2015) showed that all fresh water (i.e. rivers, lakes, estuaries) were contaminated with pesticides.

The environmental impacts associated with pesticide use are numerous (Pereira *et al.,* 2009), with effects being reported in both the target and non-target aquatic (vertebrates and invertebrates) and /or terrestrial organisms (vertebrates and invertebrates). Effects such as impaired moulting / embryonic development in the *Daphni*a *magna* (Mnif *et al.,* 2011), still-birth in the little brown bat (*Myotis lucifugus*), vertebral deformities in fish, sterility/feminization of pheasant chicks (Aktar et *al.,* 2009) and anatomic alterations in the gonads of alligators are documented with resultant poor reproductive successes (Rose *et al.,*1999). In the US, 0.5% of all animal illness reported to a laboratory is due to pesticide toxicosis with an annual estimated one to two million bird die off (Pimentel, 2005). Globally, about three million human acute poisoning cases due to pesticide exposure and an associated 200,000 mortalities are reported yearly (Yadav *et al.,* 2015). Other human effects include cancers, reproductive abnormalities, reduced intellect, hormone disruption and immunosuppression (Yadav *et al.,* 2015). Some other unintentional effects have included the development of pesticide resistance (Pimentel, 1995). As a result of the impact/effect of organic pesticides (especially persistent ones), researchers and policy makers have placed much effort into understanding their fate and behaviour in the environment (Yadav *et al.,* 2015).

2.1.3.1 Mechanism associated with the most common poisoning events in humans and animals

Pesticides with anti-cholinesterase activity i.e. organophosphates and carbamate (Hoffman, 1995; Fleischli *et al.,* 2004) have been seen to cause death in humans and animals. They inhibit the cholinesterase enzymes, which are responsible for catabolizing the neurotransmitter acetylcholine. This inhibition, results in the accumulation of acetylcholine at the synaptic nerve

junctions leading to continuous nerve stimulation (Fukuto, 1990). Clinical manifestations associated with poisoning include anorexia, lacrimation, nausea, salivation, vomiting, diarrhoea, sweating, miosis, respiratory distress, muscular twitching/tremors, convulsion, coma and death (Jokanović, 2009). In birds, signs encountered include feather fluffing, incoordination, amaurosis, lacrimation, lethargy, hyper-excitability, difficult breathing, tremors and convulsion (Fleischli *et al.,* 2004). Death is usually due to asphyxiation as a result of impaired respiration (Fleischli *et al.,* 2004). Exposure is frequently via the consumption of impregnated granular products, pesticide polluted seed / water / foliage and poisoned invertebrate / vertebrate. Inhalation and dermal contact are other common exposure routes (Fleischli *et al.,* 2004; Berny, 2007).

Pesticides with anti-coagulant activity: Coumarin derivative anti-coagulant rodenticides have frequently been incriminated in causing mortalities in birds of prey following exposure. Exposure is usually by ingestion of bait (primarily) or poisoned rodents (secondarily) (Berny, 2007; Watanabe *et al.,* 2010). They induce lethal haemorrhage by inhibiting vitamin K 2,3 epoxide reductase, the enzyme responsible for catalyzing the reduction of vitamin K 2,3 epoxide to reduced vitamin K, an important process in the formation of blood clotting factors II, VII, IX and X (Watanabe *et al.,* 2010; Erickson and Urban, 2004). Clinical manifestations include severe haemorrhage with poor coagulation (Merola, 2002). Animals are usually anorexic, anaemic, weak and hypothermic with a compensatory tachycardia. Other signs include dyspnoea and haemoptysis (Mount, Kim and Kass, 2003). At post mortem, carcasses present with signs of bleeding without coagulation (Berny, 2007).

Organochlorine pesticides: These are pesticides that act by interfering with nerve impulse transmission causing a malfunction of the central nervous system (CNS) (Rose *et al.,*1999). They do this by binding to the α subunit of the sodium-ion channels on nerve cells maintaining them open and allowing the continuous influx of sodium ions causing repetitive firing of action potentials and the prevention of repolarization (Androutsopoulos *et al.,* 2013). They also inhibit the gamma aminobutyric acid receptor (Androutsopoulos *et al.,* 2013) thereby interfering with chloride-ion uptake (Rose *et al.,*1999). Clinical manifestations presented include muscular twitching that eventually leads to convulsion. Other signs of toxicosis are salivation, nausea, vomiting and abdominal pain (Rose *et al.,*1999). In birds, signs such as egg shell thinning,

reduced fertility, suppression of egg formation and acute mortalities have been reported (Fry, 1995). Death is often associated with respiratory failure due to depression of the CNS (Rose *et al.,*1999).

2.1.4 Pharmaceuticals and personal care products

The use of pharmaceuticals and personal care products has become part of the modern contemporary way of living. Large quantities of these products are used in humans and animals for several reasons. It is not surprising that these substances also find their way into the environment via numerous means, with their presence being demonstrated in various environmental compartments (Shah, 2010), from sewage, soil, surface water, ground water, sea, estuaries, atmosphere to river sediments. In animals, following the use of pharmaceuticals, excreted parent drug and/or metabolites accumulate on farm lands for some time before they eventually enter the aquatic environment through leaching and run off from the land. Others may get into the environment from aquaculture facilities. For humans, pharmaceuticals and personal care products are either excreted or washed into waste water and sewage treatment facilities. These ultimately enter the aquatic environment via effluents from sewage treatment facilities. At times, these pollutants find their way into municipal drinking water as available waste water treatment facilities do not adequately remove them (Schröder *et al.,* 2016). Although, these pollutants have not been directly linked to causing abnormalities or disease conditions in humans, their constant presence in the environment has often been associated with several unwanted effects on lower organisms.

2.1.4.1 Antibiotics

These are chemical substances synthesized by living organisms to have an effect on another living organism(s). Such chemical substance either hinder the growth and multiplication of the said organism or kills it (Aliu, 2007). They are generally classified into several groups based on their physicochemical properties and/or mode of action. They are widely and extensively used in humans and animals to treat disease conditions mainly caused directly by bacterial infections or those complicated with secondary bacterial infections. In veterinary practice, this grouping of drugs constitutes more than 70% of all medications used on animals where-as in humans they are the third most widely used drugs (Puckowski *et al.,* 2016). They occupy an important position in the prevention and treatment of diseases and in some cases, have been used as growth promoters

in livestock production (Shah, 2010). However, their constant use has resulted in some environmental consequences. They easily find their way into the environment and have been associated with toxicity to green algae and cyanobacteria. Several studies have also demonstrated the presence of different antibiotic residues in edible crops i.e. tomatoes, potatoes, carrots and sweet corn (Puckowski *et al.,* 2016) posing a threat to humans as some are known to provoke allergic reactions even at minute quantities (Kumar *et al.,* 2005; Lee *et al.,* 2001). Similarly, their constant presence in the environment has resulted in the evolvement of antibiotic resistant strains of both pathogenic and non-pathogenic bacteria (Centner, 2016). This is due to the fact that continuous presence of sub-lethal levels of these drugs leads to the development of phenotypic adaptation and/or genetic alteration by these organisms enabling them to withstand the drugs presence (Chapman, 2003; Munita and Arias, 2016). In some instances, these organisms acquire new genetic information by horizontal gene transfer from co-resident organisms to be able to express resistance (Chapman, 2003; Munita and Arias, 2016). The implications to the environment are that:

- structural composition and functions of the community of soil bacteria will be disrupted (Jechalke *et al.,* 2014).
- the ecological roles played by environmentally relevant organisms like cyanobacteria i.e. nitrogen fixation (Sharma *et al.,* 2013), carbon fixation (Sarma *et al.,* 2016), serving as important source of vitamin B_{12} and atmospheric oxygen (Mazard *et al.,* 2016) are hampered.
- disease conditions in humans and animals caused by bacteria, that normally will respond to treatment have started to become unresponsive due to antimicrobial resistance.

2.1.4.2 Antivirals

Antivirals are a class of pharmaceuticals commonly used in humans to treat viral disease conditions such as AIDS (acquired immune deficiency syndrome) cause by the Human Immunodeficiency Virus (HIV). Although not extensively explored, studies have demonstrated their presence in the environment e.g. drinking water (Peng *et al.,* 2014), raw waste water, waste water treatment plant effluents, ground water and surface water (Jain *et al.,* 2013). These studies have indicated that an increase in the use of antiviral drugs especially during severe influenza outbreaks resulted in the inhibition of bacterial growth in waste water treatment ecosystem (Jain

et al., 2013), with subsequent poorly treated waste water and undesirable effects like eutrophication, loss of aquatic life and fish mortalities (Singer *et al.,* 2011). There are also concerns that the continuous introduction of these drugs into the environment especially during viral disease outbreaks could lead to the development of resistant viral strains as has been shown by influenza viruses (Jain *et al.,* 2013). Concern has also been raised that the inability of sewage treatment plants to fully remove these drugs could result in toxicity in non-target organisms after the water and solids are released into the environment (Jain *et al.,* 2013).

2.1.4.3 Antidepressants

As indicated by their name, these are pharmaceuticals that are used to relieve major depressive disorders predominantly in humans (Kormos and Gaszner, 2013). According to World Health Organisation (WHO), depression was the leading cause of disability with approximately 350 million sufferers in 2012. Like so many pharmaceuticals, antidepressants are widely present as pollutants in the environment and make up about four percent of known pharmaceuticals in the environment (Fong and Ford, 2014). As a principle, pharmaceuticals are designed to exert or alter specific biological functions which may be conserved across species and once in the environment, they exert such an effect. These classes of drugs exert their effect by modulating neurotransmitters i.e. serotonin, norepinephrine (Feighner, 1999) and dopamine (D'Aquila *et al.,* 2000). Aquatic organisms like molluscs and crustaceans have demonstrated sensitivity to these drugs as they have these endogenous neurotransmitters (Fong and Ford, 2014). Exposed organisms have shown disorders in behaviour, reproduction, growth, metabolism, immunity, feeding, locomotion and colour physiology (Fong and Ford, 2014). In molluscs, spawning and the release of larvae in bivalves were affected with a reduced fecundity in snails. In crustaceans, spawning, growth and maturation were affected with undesirable phototactic and geotactic behaviour. They have also been shown to cause gonadal aberrations in zebra mussels (*Dreissena polymorpha*) and affect learning and memory retention in cuttlefish (*Sepia species*) (Fong and Ford, 2014).

2.1.4.4 Endocrine Disrupting Compounds

The oestrogenic hormones; oestrone, oestriol, 17 beta oestradiol and 17 alpa ethinyloestradiol among other endocrine disrupting compounds have been studied well. The latter, which is synthetic, forms an integral component of female contraceptive pills available in the market.

They are used to regulate the oestrous cycle in women (Belle *et al.,* 2002). These compounds are persistent and have been detected at environmentally relevant concentrations in effluents of waste water treatment plants being released into the environment (aquatic) (Schröder *et al.,* 2016; Wilkinson *et al.,* 2016). Vajda *et al.,* (2011) demonstrated the effects of these compounds on the ecosystem when they exposed some adult flathead minnows (*Pimephales promelas*). As a consequence, they exhibited a decrease in sperm count, an increase in plasma vitellogenin, demasculinization of the dorsal fat pads and nuptial tubercles. In the sucker fish (*Catostomus commersonii*), they affected the rate of follicular maturation and influenced the production of more female offspring in the exposed population (Woodling *et al.,* 2006). Although not proven, it is speculated that these compounds may be able to disrupt the oestrogenic hormonal pathway in exposed humans or affect development of male children (Vajda *et al.,* 2011; Fisher and Eugster, 2014; Patisaul and Adewale, 2009).

2.1.4.5 Beta Blockers

Beta blockers like metoprolol, propranolol and atenolol have generally been used in the management of cardiac arrhythmias in human and veterinary practice (Aliu, 2007). These drugs are believed to pose very little environmental risk to humans as only minute quantities have been detected. The same cannot be said for lower organisms, as Brezovšek *et al.,* (2014) demonstrated their toxic effect to algae and cyanobacteria. In another study, Huggett *et al.,* (2002) demonstrated their effect on reproduction in the amphipod crustacean (*Hyalella azteca*) and Japanese rice fish (*Oryzias latipes*). Propranolol-exposed fishes showed a decreased growth rate, alteration in sex hormones (i.e. increase in oestradiol levels in both sexes with a decreased testosterone level in males) and abnormalities in sperm with an associated decrease in motility (Huggett *et al.,* 2002). In crustaceans, the drug was associated with mortality and a decrease in production (Huggett *et al.,* 2002).

2.1.4.6 Perfluorinated Compounds

Compounds belonging to this family e.g. perfluorooctanesulfonic acid and perfluorooctanoic acid, are widely used in the manufacture of personal care products and in other industrial processes (Stahl *et al.,* 2011). They form an integral part of carpets, shoes and clothing as anti-stains and anti-grease agents (Stahl *et al.,* 2011). They are used in the manufacture of cosmetics, surfactants, hydrophobic textile coatings and have also found use in the food packaging industry.

By nature, they are stable and have been utilized in non-sticky surface coatings for cooking utensils. They persist in the environment and have the ability to bioaccumulate (Stahl *et al.,* 2011). Traces of these compounds have been detected in the liver samples of marine mammals, fish and birds. It is even more worrisome that breast milk, blood, hair, nail and urine samples from humans have been demonstrated to contain some of these compounds (Perez *et al.,* 2012; Seacat *et al.,* 2002). Although, no effect or disease condition has been conclusively linked to these compounds in humans, several effects on the lower organisms have been documented. In Zebra fish (*Danio rerio*), deformities and deaths in the F1generation, accumulation of liver droplets, inhibition of gonadal growth and stimulation of vitellogenesis has been attributed to these compounds (Du *et al.,* 2009). Neoplastic and hepatotoxic effects has been demonstrated in rats (Stahl *et al.,* 2011) and there are suggestions that metabolites from these compounds may affect thyroid hormone level, foetal development, birth weight, mitochondrial bioenergetics and cell to cell communications (Seacat *et al.,* 2002; Austin *et al.,* 2003; Hu *et al.,* 2002).

2.1.4.7 Antineoplastic drugs

Antineoplastic drugs are used in the management of cancers in human and animals (Selevan *et al.,* 1985; Zhang *et al.,* 2011). They also show some antiviral activity which is due to their mode of action (i.e. inhibition of cellular replication and growth) (Lapponi *et al.,* 2016). Unfortunately, as the human population ages, the incidence of neoplastic conditions and the need for treatment has also increased (Brezovšek *et al.,* 2014). With the drugs being cytotoxic by design (Zhang *et al.,* 2011), it will not be surprising that they exert similar effects on non-target organism(s) once in the environment. Residues have been detected in raw hospital sewage, treated hospital waste water effluents (Zounkova *et al.,* 2010), effluents of municipal sewage treatment plants(Kümmerer *et al.,* 2000) and in the aquatic environment. At present, very little information is available regarding their ecotoxicological implications at relevant concentrations in the environment (Brezovšek *et al.,* 2014) but their presence is of great concern.

2.1.4.8 Anthelmintics

They are pharmaceuticals used in the treatment of parasitic helminth infections in human and animals, with the veterinary use being much larger. Like all other drugs, some of them have been detected in the environment. For instance, studies have shown that up to 98% of macrocyclic lactones (as parent drug or active metabolites) are excreted in the faeces following treatment

(Horvat *et al.,* 2012) and because of their mode of action, they can be lethal or toxic to non-target aquatic and terrestrial organisms (Lumaret and Errouissi, 2002). The dung beetles (*Onthophagus gazella* and *Euoniticellus intermedius*) and some fly species (*Musca vetustissima* and *M. domestica*) are mostly affected with delayed larval development and an increased mortality of up to 100% in some cases (Lumaret and Errouissi, 2002). They have sub-lethal effects on growth, moulting, metamorphosis and reproduction in insects. In some cases, death of both adult and larval stages of insects have been demonstrated (McKellar, 1997). The implications are that ecological roles like formation of soil micro structure, breakdown of organic matter and release of nutrients into the soil will be affected (Diao *et al.,* 2007; Jensen and Scott-Fordsmand, 2012). More so, the continuous introduction of these pollutants will result in the development of resistance.

2.1.4.9 NSAID's

These are pharmaceuticals used to relieve pain and inflammation in humans and animals. However, some of these compounds have been associated with poisonous outcomes in the environment. Among them, diclofenac is particularly noted. In the amphipod crustacean (*Hyalella azteca*), it has been associated with oxidative DNA damage and an increase in lipid and protein oxidation (Lucero *et al.,* 2015). In rainbow trout (*Oncorhynchus mykiss*), diclofenac causes kidney damage and distortion of the gills at minute concentrations (Schwaiger *et al.,* 2004) and was also associated with acute toxicity in phytoplankton (Ferrari *et al.,* 2004). In vultures, it caused the most severe and rapid population decline ever recorded (Ogada *et al.,* 2012), following their secondary exposure to residues of the drug when they scavenged on ungulate carcasses that had received the drug shortly before death (Oaks *et al.,* 2004; Taggart *et al.,* 2009). The vultures exhibited renal failure, increased plasma uric acid concentration and extensive visceral gout deposits at post mortem (Oaks *et al.,* 2004; Swan *et al.,* 2006b). Other analgesics with proven environmental effects include ketoprofen (Naidoo *et al.,* 2010a), caprofen, flunixin and phenylbutazone (Fourie *et al.,* 2015).

2.2 World vulture population decline

2.2.1 Europe, Africa and America vulture population decline

Vultures are a group of birds generally classified into two subgroupings; the old-world vultures and the new world vultures constituting sixteen and seven species, respectively. They belong to the order Falconiformes and families Accipitridae and Cathartidae, respectively (Campbell, 2015). By nature, their heads are mostly featherless. This is to ensure that they keep clean during feeding (Kushwaha *et al.,* 2009) and also serves in thermoregulation (Ward *et al.,* 2008). They have long-hooked beaks with well-developed mandibular muscle adapted for easy feeding/scavenging. Most vultures are obligate scavengers and occupy a significant position in the ecosystem and food chain. They have characteristically large feet with weak toes (not adapted for hunting or grasping) (Kushwaha *et al.,* 2009). Their gastro-intestinal tract is well adapted for scavenging as the acidic gastric pH denatures viruses and bacteria in the food and also helps to break down ingesta faster (Houston and Cooper, 1975). They scan for food/carcasses during flight with keen eyesight (Buckley, 1996). As a defence mechanism, they vomit their acidic gastric content on an approaching "enemy" (Graham and Heatley, 2007). They do this also to achieve a lighter weight for flight. For some species, urinating on their feet is another way of regulating body temperature (Arad *et al.,* 1989). The urine however, also serves as an antibacterial and a parasiticide (Kushwaha *et al.,* 2009). Below is a table showing differences between the old-world and new-world vultures (Table 2.1).

Table 2.1: The major differences between old- world and new-world vultures

FEATURES	OLD WORLD VULTURES	NEW WORLD VULTURES
Family	Accipitridae	Cathartidae
Species	*Aegypius monachus, Gyps fulvus, Gyps bengalensis, Gyps rueppelli, Gyps indicus, Gyps tenuirostris, Gyps himalayensis, Gyps africanus, Gyps coprotheres, Gypaetus barbatus, Necrosyrtes monachus, Sarcogyps calvus, Torgos tracheliotos, Trigonoceps occipitalis, Neophron percnopterus* and *Gypohierax angolensis.*	*Coragyps atratus, Cathartes aura, Cathartes burrovianus, Cathartes melambrotus, Gymnogyps californianus, Vultur gryphus* and *Sarcoramphus papa*.
World distribution	Africa, Asia and Europe	Central and South America
Sense of smell	Very poor	Well developed (but only in a few species
Tolerance to diclofenac	Several species *(Gyps bengalensis, G. indicus, G. tenuirostris, G. coprotheres, G. fulvus and G. africanus*) are sensitive	Experimentally one species (*Cathartes aura*) was not sensitive
Urohidrosis	Not a habit	Common habit

Vultures have a wide distribution range with the old-world vultures mainly found in Africa, Asia and Europe whereas the new world vultures inhabit the central and southern part of America (Ogada *et al.,* 2012) (Figure 2.1). Historically, there are several myths associated with the vulture. In Costa Rica, vultures are believed to be responsible for putting in place the four-world support and that the middle pole of the cosmic house is held by a vulture's foot (Benson, 1996). In Egypt, they depict motherhood and represent the earliest deity. They are seen as protector of pharaoh, royalty, and Egypt (Web, 2014). In Ohio and Mississippi (U.S.A), they were regarded as flying delegates of the upper world spiritual forces and were honoured (Gehlbach, 2010). In India, Jatayu (the vulture god) is revered as a hero because he sacrificed his life to save Sita, the wife of Rama (Markandya *et al.,* 2008). Unfortunately, in the western and southern parts of Africa, vultures are hunted down as their body parts are believed to hold spiritual powers. Traditional healers use them to prepare charms and medicine (Ibrahim *et al.,* 2010; Simelane and Kerley, 1998), an act that motivates their killing and maiming by people. Ignorantly, they are often condemned, persecuted and thought to represent sloth and filth (Byrd, 2003). The father of evolution (Charles Darwin) once described them as a disgusting set of animals (Nellums, 2006). They are usually associated with death (Byrd, 2003) and evil spirits (Campbell, 2009). Perhaps the highest form of persecution is the one exhibited by poachers in which vultures are deliberately poisoned to avoid been exposed by the vultures while at their illicit act (Roxburgh

and McDougall, 2012). This contributes immensely to the decline of vulture population in Africa.

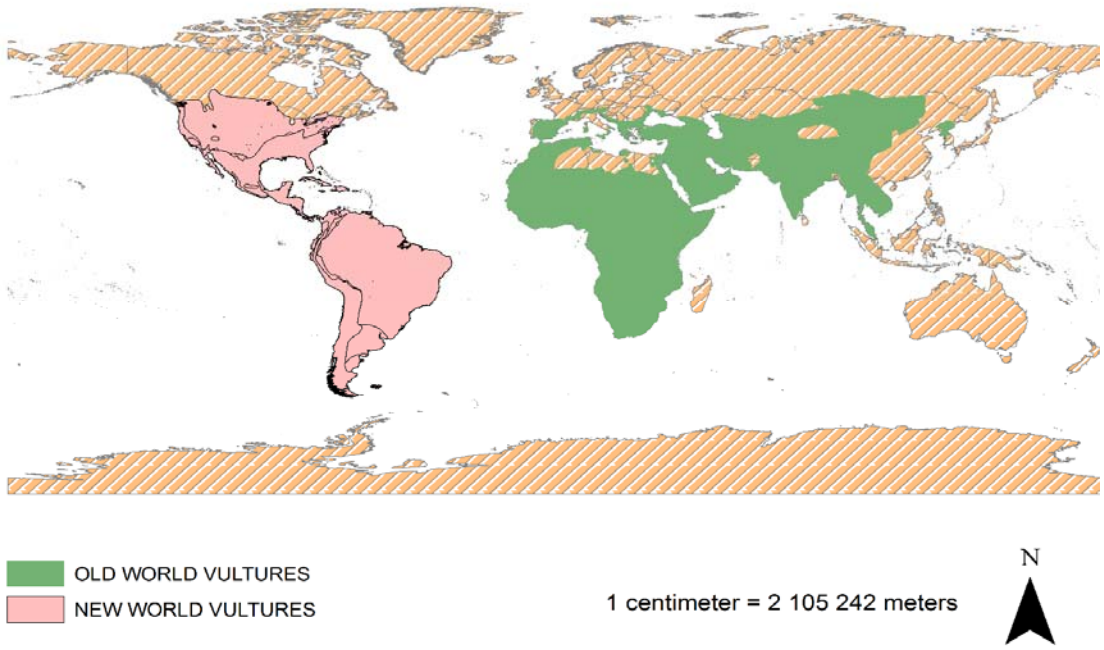


Figure 2.1: World map showing distribution of Old and New world vulture population.

From available literature, the first decline of vulture populations started in the 1860's in Europe (Bearded vultures [*Gypaetus barbatus*]) and North America (California condors [*Gymnogyps californianus*]), with both groupings nearing extinction levels within almost a century (Ogada *et al.,* 2012). The decline seen in African and Asian vulture populations are more recent with the earliest record of the Cape vulture (*Gyps coprotheres*) decline being circa 1898-1900 (Jarvis *et al.,* 1974). In the South of Asia (with exception of India), the decline of White-rumped vultures (*Gyps bengalensis*), Slender-billed vultures (*Gyps tenuirostris*) and Red-headed vultures (*Sarcogyps calvus*) started in the 1950's and it was primarily due to shortages in food supply associated with uncontrolled hunting of wild ungulates and improved husbandry practices (Pain *et al.,* 2003). In the West (Thiollay, 2006) and East of Africa (Virani *et al.,* 2011), the last four decades saw a high population decline of all vulture species in the region. The situation is not much different in North Africa where two species of vulture have already been extirpated i.e.

Lappet-faced vulture (*Torgos tracheliotos*) in Morocco, Algeria and Tunisia (Bridgeford, 2004) and Cinereous vulture (*Aegypius monachus*) in Morocco (Amezian and Khamlichi, 2016). At present numerous causes are associated with the decline of the various global vulture populations such as persecution, lead poisoning, collision, secondary poisoning (from bait), electrocution, habitat loss and decline in food supply, and most recently from the ingestion of diclofenac-tainted carcasses on the Indian subcontinent (Monadjem *et al.,* 2004)

2.2.2 India Vulture Population Decline

India is home to nine vulture species (www.iucnredlist.org). Unlike in other parts of the world, the populations in India flourished with an estimated 60 million birds being present in the 1980's to early 1990's (Shah, 2010). This was probably because of two main reasons, firstly, vultures are revered and honoured as "god" (Markandya *et al.,* 2008) thus, they were not persecuted as in Africa and secondly the year-round abundance of food. The latter was mainly due to the large population of livestock in India, estimated at about 470 million in 1992 with cattle and water buffalo making up 61.3% of the population (www.dahd.nic.in). Among persons practicing Hinduism, cattle are regarded as holy and are usually allowed to die naturally as slaughter for consumption would constitute a major religious offence (Cuthbert *et al.,* 2014). Another interesting action in India was that carcasses of dead cattle were usually left in the open for scavengers (notably vultures) to feed on. Under these conditions, it was not surprising that the vulture population ballooned to millions of birds.

However, this changed in the early 1990's and the subsequent 10-15 years, with the populations of three species of vultures in the region (Oriental-white backed vulture [*Gyps bengalensis*], Long-billed vulture [*Gyps indicus*], Slender-billed vulture [*Gyps tenuirostris*]) (Figure 2.2) undergoing a major population contraction by 99% in India, Nepal and Pakistan (Cuthbert *et al.,* 2014). At the beginning of the twenty first century, these three species were red-listed by the International Union for Conservation of Natural Resources (IUCN) as critically endangered species (IUCN, 2015). This was due to their unprecedented population decline noticed between the early 1990's to early 2000's in the Indian sub-region. According to Shah (2010), between 2000 and 2007 their population declined by 40% annually with the cumulative losses now reaching 99% (Cuthbert *et al.,* 2014). While the cause of the deaths seen on the Asian continent

was initially unknown, in 2004 Oaks *et al* showed the species to be highly sensitive to the toxic effects of diclofenac, a commonly used cattle anti-inflammatory in the region. More importantly, Oaks and collaborators were able to demonstrate that toxicity was due to secondary exposure of birds to the drug when feeding on ungulate carcasses that had received the drug shortly before death (Oaks *et al.,* 2004; Taggart *et al.,* 2009). Several researchers have since further demonstrated the toxicity of diclofenac to vultures experimentally. Although, the complete mechanism associated with its toxicity in vultures is not fully understood, zero-order pharmacokinetics (metabolic constraints) is a feature of toxicity (Hutchinson *et al.,* 2014). Death has often been linked to kidney failure, increased plasma uric acid concentration and extensive visceral gout deposit at post mortem (Oaks *et al.,* 2004; Swan *et al.,* 2006b; Naidoo *et al.,* 2009b). Other clinical manifestations include lethargy, neck drooping, increase in plasma alanine aminotransferase (ALT) activity and increased plasma potassium concentrations (Swan *et al.,* 2006b; Naidoo *et al.,* 2009b). Microscopic evaluation revealed lesions in the kidney (necrosis of the proximal convoluted tubules), liver and spleen (Swan *et al.,* 2006b; Naidoo *et al.,* 2009b).

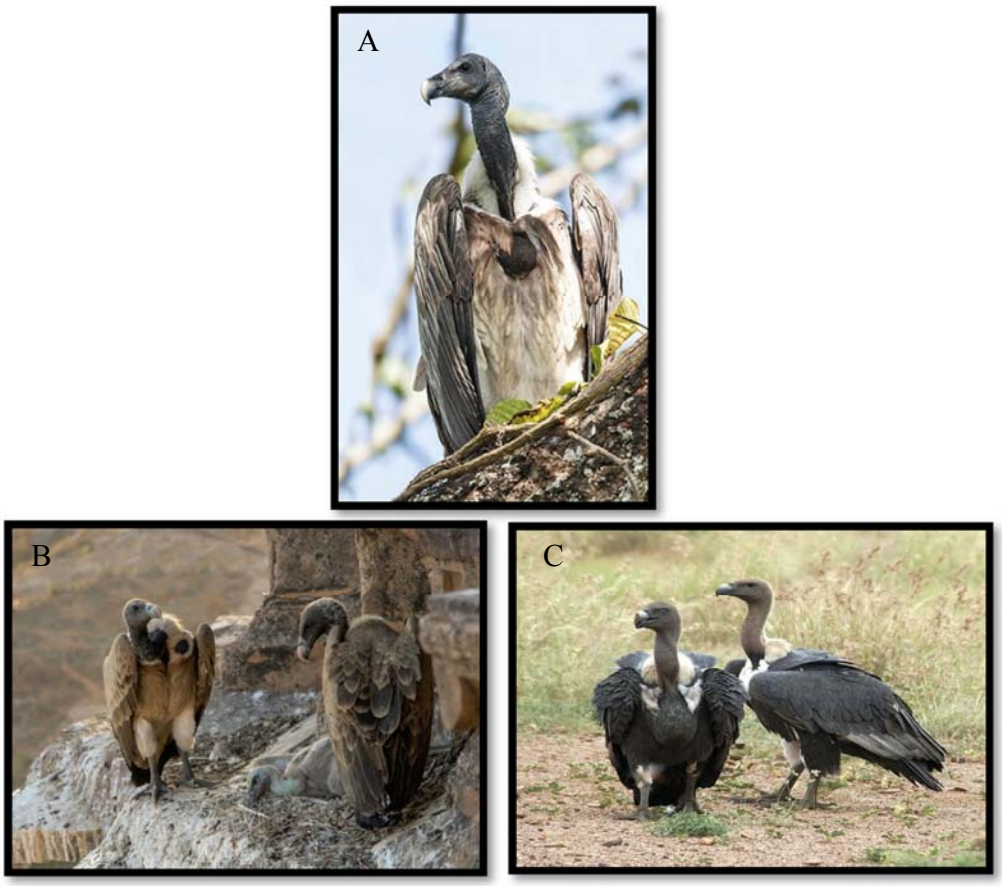


Figure 2.2: Pictures of *Gyps tenuirostris* ([A] photo credit: Pranial J Saikia), *Gyps indicus* ([B] Photo credit: Yann Forget 2012/ Wikimedia commons/ CC-BY-SA-3.0.) and *Gyps bengalensis* ([C] Jugal Tiwari 2009, discoverlife.org) in their natural environment.

2.2.3 Consequences of vulture decline

2.2.3.1 Health and wellbeing

Vultures play an important role in preventing the spread of disease to humans and animals by ridding the decaying flesh of dead animals and other organic matter which may serve as breeding ground for pathogenic bacteria (Markandya *et al.*, 2008). Due to their keen eye sight, vultures usually detect and arrive at carcasses first in large numbers. In combination with their voracious feeding, they are able to devour an entire carcass before the arrival of other (non-specialized) scavengers (Shivik, 2006). This gives them the advantage of having full meals and also helps reduce the spread of diseases like anthrax from contaminated carcasses, for which an increased number of cases have since been proposed to be associated with vulture population declines

(Mudur, 2001) as a result of people handling infected carcasses that should have been "cleaned" by the vultures.

Opportunistic scavengers like the lion (*Panthera leo*) and hyena (*Crocuta crocuta*) depend on carrion specialists like the vulture by observing their movements and behaviour. Vultures maintain the highly-structured scavenger community and regulate contact among mammalian scavengers. The absence of specialized scavengers like vultures at carcass sites, has resulted in changes in the composition of scavengers at the site (Ogada *et al.,* 2012), and potentially a related increase in number of other scavengers which may be reservoirs for disease. Two well-known examples are the increase in feral dog and rat population in India (Markandya *et al.,* 2008). In 2003, a census in the region estimated an approximate 7.25 million increase in the dog population, from the previous 21.77 million animals, in just a decade since the vulture population decline (Markandya *et al.,* 2008). This rise in feral dog population has been associated with increased incidences of dog bites and human rabies cases. Similarly, rats as primary reservoir for bubonic plague pose a high risk of disease outbreak with the increasing population of the region (Pain *et al.,* 2003). In 2012, an online media house estimated the population of rats in Mumbai to be approximately 88 million (Buncombe, 2012).

2.2.3.2 Economic concerns

A huge amount of money has to be spent to manage the increased dog population such as efforts to sterilize dog populations, dog-bites and rabies pre-and post-exposure prophylaxis (Ogada *et al.,* 2012; Markandya *et al.,* 2008). A large sum of about 2.43 billion US dollars is spent by the Indian people for these purposes annually (Moleón *et al.,* 2014). The economic impact of a reduced vulture population also trickles down to the hide and bone collectors who used to depend on vulture cleaned livestock carcasses for livelihood. The absence of vultures not only affects the availability of livestock carcasses for skin and bone collection as they are now buried or incinerated soon after death, but adds a further financial burden on the authorities who need to undertake said disposal (Markandya *et al.,* 2008; Pain *et al.,* 2003). This in turn leads to huge economic losses on the country as industries such as those for tanning, gelatine and fertilizer production depend on this source for raw materials (Markandya *et al.,* 2008). Lastly, vultures used to serve as a tourist attraction in India as tour providers included vultures in their bird watching tours thereby generating income for the country.

2.2.3.3 Cultural and religious consequences

The decline of vulture population in India has since left the Parsee community (i.e. Zoroastrian practicing members) in a dilemma. This is because among their beliefs, fire, water, air and earth are pure elements that must be preserved. To uphold their beliefs, dead bodies are left in the open at "Towers of Silence" for scavengers (notably vultures) (Subramanian, 2008). Presently, with the demise of vultures, they have relied unsuccessfully on a more expensive method (use of solar reflectors) for disposing their dead (Subramanian, 2008) and have lost a cultural heritage.

2.2.4 The solution

Fortunately, diclofenac was later banned in India, Nepal, Pakistan (Prakash *et al.,* 2012) and Bangladesh (Balmford, 2013), albeit after the damage has already been done. It was only after their population declined that the world realized the enormous role they play in the ecosystem. The world is now grappling with the consequences of their decline and trying to restore their population. Currently, in Europe, America and South Africa, the situation is better as respective governments have put in place different measures to curtail the menace e.g. banning of veterinary diclofenac in some areas, captive breeding and introduction of vulture restaurants. This is not the case for north and west Africa where urgent attention is needed (Ogada *et al.,* 2012). As part of its measures, the governments of India, Nepal, Pakistan and Bangladesh banned the use of veterinary diclofenac (Balmford, 2013) and efforts are being made to breed these birds in captivity to restore their population in the wild (Shultz *et al.,* 2004). So far, the rate of decline in India has been reduced or even stopped (Balmford, 2013) and as at 2008 a total number of 88 (in India), 11 (in Pakistan) and 18 chicks (in Nepal) had been successfully hatched in captivity (Mompati, 2012) and the numbers keep rising yearly. In 2016 alone, Nepal, Pakistan and India successfully hatched additional 1, 2 and about 60 chicks respectively for future release into the wild. Already, India has initiated their preliminary release back into the wild. In 2016, two Himalayan griffons (*Gyps himalayensis*) were released from one of the breeding centres and more are scheduled for release in 2017 (iucn-vulturenews.org)

2.3 Non-steroidal anti-inflammatory drugs (NSAIDs)

2.3.1 Introduction

NSAIDs are a group of drugs used for the management of inflammation and pain in animals. They do this by interfering with the synthesis of inflammatory mediators in the body via cyclo-oxygenase and lipo-oxygenase blockade (Azevedo *et al.,* 2013; Rai *et al.,* 2015). Inflammation is a normal protective response of the body to insult, and in such situations cytokines are released at the site of insult where they induce the release of arachidonic acid from the phospholipid membrane. Arachidonic acid is a precursor to the inflammatory mediators and once in the system, it is metabolized by either cyclo-oxygenase or lipo-oxygenase to generate mediators of inflammation i.e. prostanoids, prostaglandins, thromboxane (Papich, 2008) and leukotrienes (Funk, 2001). Three of such cyclo-oxygenase (COX) enzymes exist; COX I, responsible for protecting the gastric mucosa from gastric acid, mediating platelet aggregation and regulating blood flow to the kidney; COX II, responsible for inflammatory responses and also play important roles in protecting the duodenal mucosa and renal blood flow; COX III (also known as COX Ia), responsible for regulating pain and fever in the central nervous system (Papich, 2008; Blikslager and Jones, 2005). Most toxic episodes associated with diclofenac and other NSAIDs are due to their inhibitory effects on COX I (Vane and Botting, 1998). Undesirable effects such as renal toxicity, gastric irritation and interference with clotting mechanism are due to this blockade (Aliu, 2007). Inhibition of COX II is responsible for their anti-inflammatory and analgesic effects (Swan *et al.,* 2006b).

2.3.2 Diclofenac

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID), a potent inhibitor of the cyclo-oxygenase enzyme (Hussain *et al.,* 2008) and was first introduced in the mid 1970's (Shah, 2010). The drug is a phenyl acetic acid derivative (Aliu, 2007) with a 50% bioavailability following oral administration in humans. It undergoes first pass elimination (Willis *et al.,* 1979) and is 99% protein bound (Mestorino *et al.,* 2007). In humans, it has a plasma half-life of 1.8 h (Willis *et al.,* 1979), is metabolized in the liver, and excreted via the urine (Mestorino *et al.,* 2007). Side effects like gastric ulceraration (Silva and de Sousa, 2011; Todd and Sorkin, 1988), renal damage, hepatitis and haematological complications have all been associated with its use

(Todd and Sorkin, 1988). The drug is used in livestock for its anti-inflammatory, antipyretic and analgesic effects (Aliu, 2007). Like all other NSAIDs, it exerts it effect by inhibiting the formation of prostaglandin from arachidonic acid. In cattle, it is used to manage pain and inflammation associated with disease and trauma. Following single intramuscular administration, it was characterized by a plasma half-life of 12.2 hour with a C_{max} of 4.01 µg/ml and T_{max} of 0.5 h (Taggart *et al.,* 2007). Renal toxicity has sometimes been associated with the use of NSAID's in cattle (Desrochers and Francoz, 2014). Below is the chemical structure of diclofenac (Figure 2.3).

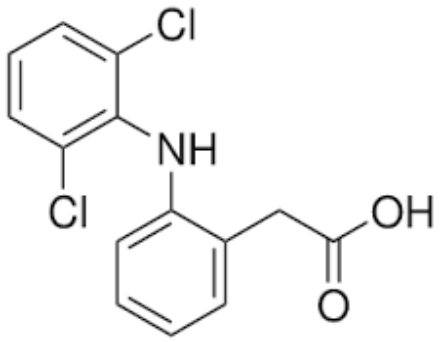


Figure 2.3: Chemical structure of diclofenac (Badwaik and Tripathi, 2012)

2.3.3 Threat from other NSAIDs

It is worthy to note that the management of pain in both mammalian and non-mammalian species through the use of pharmaceuticals is paramount in upholding animal welfare (Fenwick *et al.,* 2009). However, the safety of the drug of choice to both the target species and non-target species must be considered. Fortunately, meloxicam has been demonstrated to be safe in four species of vultures (*Gyps bengalensis*, *Gyps indicus* [Swan *et al.,* 2006a], *Gyps africanus* and *Gyps coprotheres* [Naidoo *et al.,* 2008] and a wide range of other avian species (Cuthbert *et al.,* 2007). However, there are a group of other NSAIDs that have been shown to be toxic to vultures. In 2010, Naidoo *et al.* (2010a) demonstrated the toxicity of ketoprofen in vultures and five years later carprofen, flunixin and phenylbutazone similarly showed the potential to be toxic (Fourie *et al.,* 2015). With numerous other NSAIDs available such as aceclofenac, ibuprofen, piroxicam, nimesulide, mefenamic acid, and paracetamol (Cuthbert *et al.,* 2011), it is questionable if they are safe as either the primary drug or their potential to be toxic if metabolically activated to a

toxic metabolite. This thus highlights the need for an environmental impact assessment of these veterinary pharmaceuticals in vultures and other avian species.

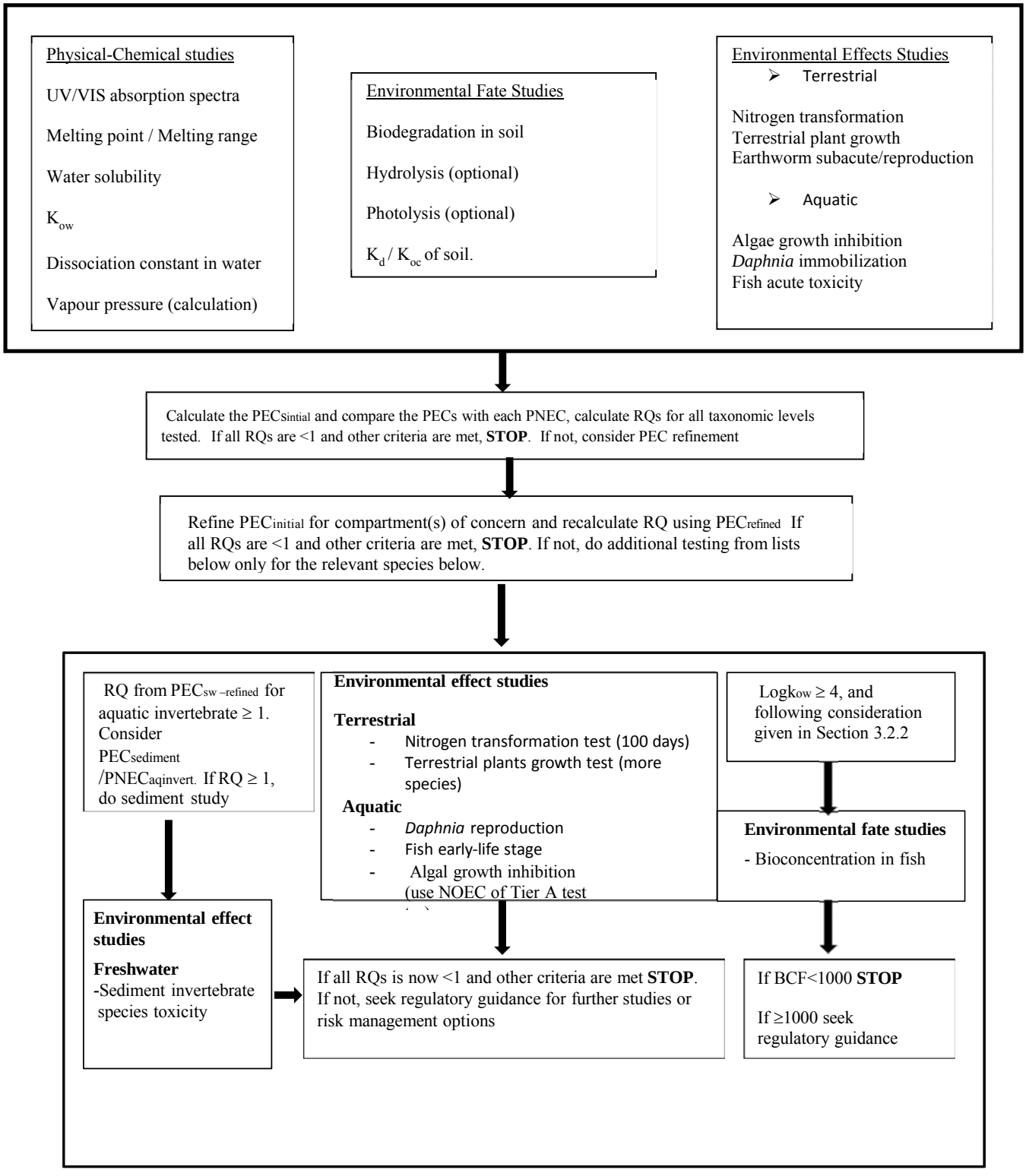
2.4 Predicting environmental effect of veterinary pharmaceuticals

While the initial toxicity of diclofenac in vultures was not predicted, the question is whether it could have been possible to predict by regulatory methods catering for the potential avian toxicity of veterinary pharmaceuticals. Currently the International Co-operation on Harmonization of Technical Requirements for Registration of Veterinary Medical Products (VICH) is a trilateral organization which was formed in 1996 with the European Union, Japan and the United State of America as signatories. The sole aim was to harmonize processes as much as possible to ease business among member countries. The organization has five main objectives, among which are to:

- Establish and implement harmonized technical requirements for the registration of veterinary medicinal products in the VICH regions, which meet high quality, safety and efficacy standards and minimize the use of test animals and costs of product development.
- Provide a basis for wider international harmonization of registration requirements.
- Monitor and maintain existing VICH guidelines, taking particular note of the ICH work program and, where necessary, update these VICH guidelines.
- Ensure efficient processes for maintaining and monitoring consistent interpretation of data requirements following the implementation of VICH guidelines.
- By means of a constructive dialogue between regulatory authorities and industry provide technical guidance enabling response to significant emerging global issues and science that impact on regulatory requirements within the VICH regions.

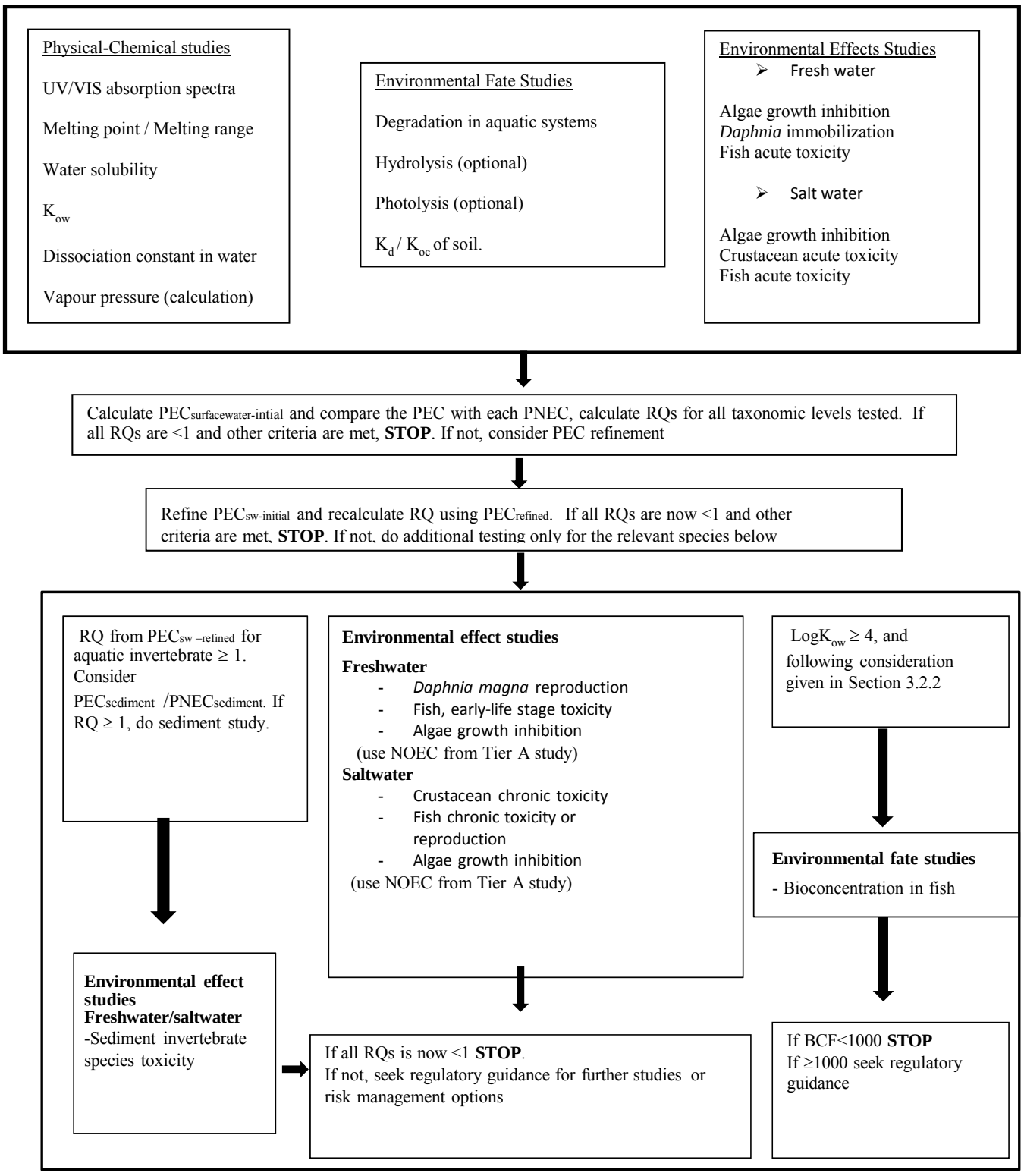
In fulfilling its objectives/mandate, the VICH provides the manner in which the potential environmental impact of veterinary medical products (VMPs) can be predicted, through the assessment of their physicochemical properties, environmental fate and effect on living organisms. An evaluation of these guidelines (VICH GL6, VICH GL38, VICH GL43 AND VICH GL46) provides an insight as to why environmental effect of diclofenac in vultures was not even considered. When assessing effect of VMPs on the environment, emphasis is placed on their effects on aquatic organisms, soil organisms and humans. No attention is given to their

effects on birds, and this is probably due to the wrong impression that birds could not be exposed secondarily to VMPs as they don't exist in soil or water (www.vichsec.org/). Their safety is only evaluated when they are considered the target species, which would explain why the toxicity of diclofenac and other NSAIDs in vultures wasn't predicted prior to the product reaching the market. Below is a very basic overview of VICH's recommendation on environmental impact assessment of VMPs in a flow chart format (Figures 2.4, 2.5 and 2.6). For more informed understanding, a detailed guide is available on the VICH website (www.vichsec.org/).



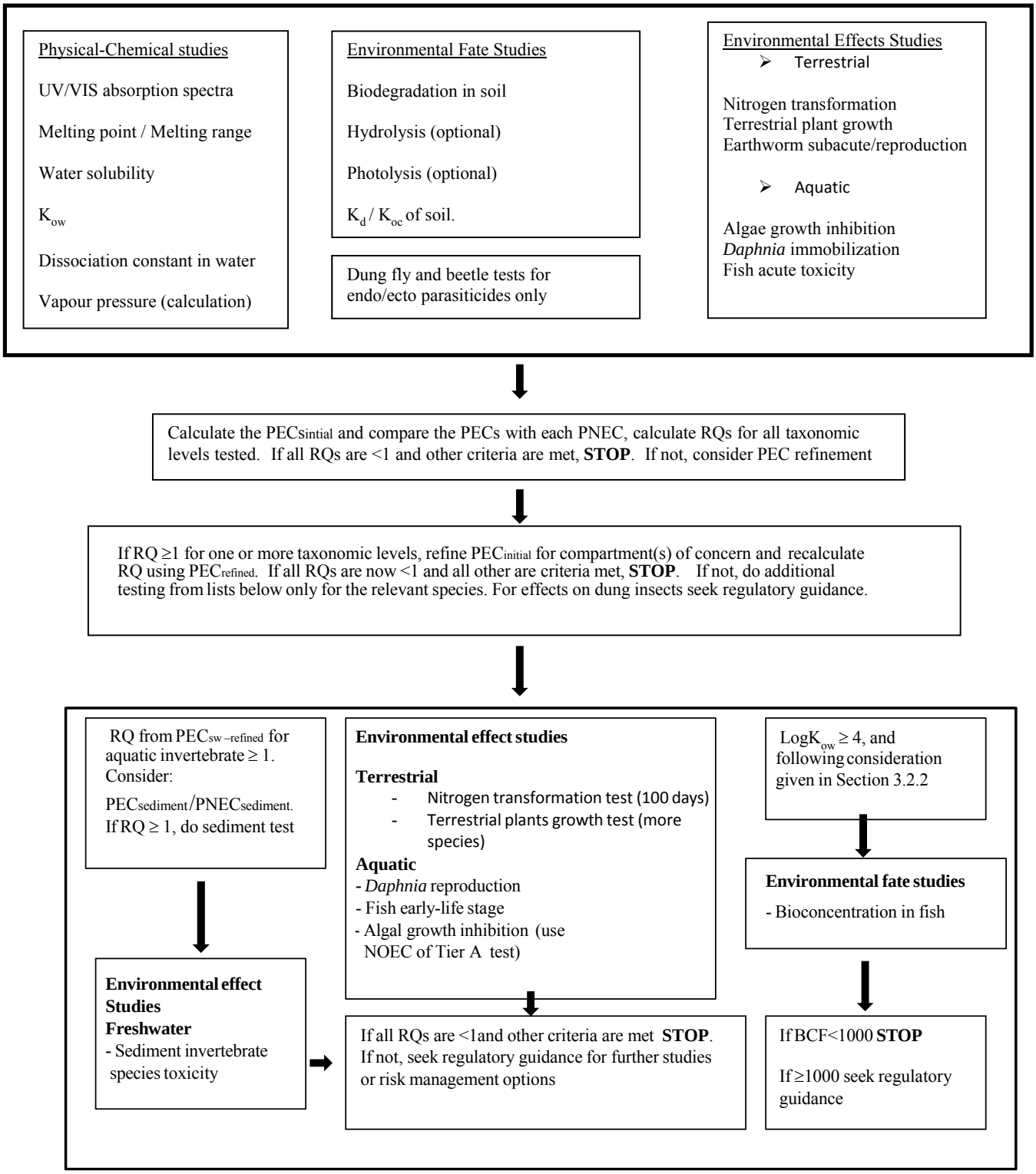
The environmental impacts (EI) of veterinary medical products (VMP) are assessed in a tiered approach. Assessments are based on physico-chemical properties, fate and effects on living organisms in the environment. A risk quotient (RQ) is calculated based on these assessments and amount of the drug entering the environment. BCF: Bioconcentration factor; K_d: Sorption/desorption coefficient; K_{oc}: Sorption/desorption coefficient, normalized to organic carbon content; K_{ow}: Water partition coefficient; NOEC: No observed effect concentration; PEC: Predicted environmental concentration; PNEC: Predicted no effect concentration; SW: Surface water. NB: If the potential for environmental exposure of a VMP is low (limited use), then it is considered less risky and it EI not assessed.

Figure 2.4: Decision tree/Flow diagram for VMPs used for intensively-reared animal



The environmental impacts (EI) of veterinary medical products (VMP) are assessed in a tiered approach. Assessments are based on physico-chemical properties, fate and effects on living organisms in the environment. A risk quotient (RQ) is calculated based on these assessments and amount of the drug entering the environment. BCF: Bioconcentration factor; K_d: Sorption/desorption coefficient; K_{oc}: Sorption/desorption coefficient, normalized to organic carbon content; K_{ow}: Water partition coefficient; NOEC: No observed effect concentration
PEC: Predicted environmental concentration; PNEC: Predicted no effect concentration; SW: Surface water. NB: If the potential for environmental exposure of a VMP is low (limited use), then it is considered less risky and it EI not assessed.

Figure 2.5: Decision tree/Flow diagram for VMPs used for aquaculture



The environmental impacts (EI) of veterinary medical products (VMP) are assessed in a tiered approach. Assessments are based on physico-chemical properties, fate and effects on living organisms in the environment. A risk quotient (RQ) is calculated based on these assessments and amount of the drug entering the environment. BCF: Bioconcentration factor; K_d: Sorption/desorption coefficient; K_{oc}: Sorption/desorption coefficient, normalized to organic carbon content; K_{ow}: Water partition coefficient; NOEC: No observed effect concentration; PEC: Predicted environmental concentration; PNEC: Predicted no effect concentration; SW: Surface water. NB: If the potential for environmental exposure of a VMP is low (limited use), then it is considered less risky and it EI not assessed.

Figure 2.6: Decision tree/Flow diagram for VMPs used for pasture animals

Presently, scientists are working towards finding an effective means of predicting the environmental effects of pharmaceuticals in birds. This is to ensure that a vulture-like tragedy does not occur again in future. In 2007 and 2011, attempts were made to validate the chicken and pied crow (*Corvus albus*) (Naidoo *et al.,* 2007; Naidoo *et al.,* 2011) as models for the environmental effect of diclofenac in vultures, albeit with mostly negative outcomes. It was thus suggested that looking at how birds metabolize xenobiotics may shed some light on the toxicosis. The cytochrome P450 (CYP 450) enzyme system, which is present in all species in various tissues, is actively involved in the biotransformation of xenobiotics in humans and animals (Hutchinson *et al.,* 2014). Ninety percent of exogenous xenobiotics are metabolized and detoxified by them (Zhu *et al.,* 2014). In animals however, huge diversification of this enzyme system exists with about thirty-seven CYP 450 families identified so far (Hutchinson *et al.,* 2014). In addition, pharmacogenomic differences may also exist within species (Naidoo *et al.,* 2010b).

Interestingly, when you look at evolution as it relates to metabolism, key factors such as feeding habit, habitat and migratory behaviour usually comes to mind. These factors play significant roles in evolution as they are tied and depend on each other (Almeida *et al.,* 2016). In birds, the enzyme system seems to be on par with their feeding habits, those with a wide range of feed types have higher hepatic biotransformation capacity towards xenobiotics when compared to those with a narrow range of feed types (Liukkonen-Anttila *et al.,* 2003; Rainio *et al.,* 2012). As an example, the yellow-legged herring gull (*Larus michahellis* [an omnivore]) has a higher hepatic detoxification capacity when compared to a fish-eating cormorant (*Phalacrocorax sp*) (Fossi *et al.,* 1995). Similarly, a kestrel (*Falco sp*), although carnivorous by nature appears to have a higher mono-oxygenase activity than the sparrow hawk (*Accipiter nisus*) because of its wider range of prey i.e. rodents, birds and insects compared to the sparrow hawk's bird meal (Liukkonen-Anttila *et al.,* 2003).

Exposure to a particular class of xenobiotic over generations seems to have enhanced the evolvement and stability of genes coding for enzyme(s) responsible for such xenobiotic's metabolism (Thomas, 2007). Thus, each species' CYP 450 enzyme system has adapted to the spectrum of xenobiotics the said species is exposed to, which may explain why different bird

species respond differently to pharmaceutical exposure. A perfect example is the safety of diclofenac in the pied crow, an omnivorous bird, in contrast to its toxicity in *Gyps* vultures, carnivores (Naidoo *et al.,* 2011). This is probably because the pied crow consumes a wide variety of feed types ranging from plants (seeds, grains, fruits) to animals (vertebrates and invertebrates). This feeding habit may have stimulated its system over time to evolve metabolic capacity (i.e. enzymes) capable of "withstanding insults" from several xenobiotics (Dean *et al.,* 2006). In the case of *Gyps* vultures, they are obligate scavengers with most of them exclusively feeding on vertebrate carcasses (Buechley and Sekercioglu, 2016), there may not have been any need for them to evolve metabolic ability (enzymes) capable of accommodating several other xenobiotics like in the case of the crow. This is exemplified by the rapid elimination of diclofenac (2.33 h) in the pied crow as opposed to that of the *Gyps* vulture (>12 h) (Naidoo *et al.,* 2011). Another factor that may explain the difference is the basal metabolic rate. Larger species of warm-blooded animals tend to have a lower basal metabolic rate compared to the smaller species, this is because they have a comparatively lower surface area to volume ratio (Singer, 2006). Small species lose more heat from their body because of the higher surface area to volume ratio, this is compensated by their higher basal metabolic rate to create a body temperature balance (Singer, 2006) which invariably speeds up metabolism of xenobiotics as seen in the case of pied crow compaired to *Gyps* vulture.

The potential for birds to have different metabolic capacity leads to an interesting question as to whether toxicity can be predicted if birds with different nutritional background can be used in a combined model to undertake said predictions. This is not an unusual approach, as a similar approach has been in place for the evaluation of environmental impact of pesticides on birds. The Organisation for Economic Co-operation and Development (OECD) and Environmental Protection Agency (EPA) have used and recommended the use of one or two of the following bird species; the quail (*Coturnix japonica*/*Colinus virginianus*), mallard duck (*Anas platyrhynchos*), feral pigeon (*Columba livia domestica*), zebra finch (*Taeniopygia guttata*), budgerigar (*Melopsittacus undulatus*), house sparrow (*Passer domesticus*) and red-wing blackbird (*Agelaius phoeniceus*) for assessment. The species of birds are usually exposed to the said xenobiotic either by a single-dose oral LD_{50} test, a dietary LD_{50} test or a reproduction test. It is assumed that any xenobiotic found to be safe in these species is presumably safe to the entire

avian family (OECD 2010; EPA 2012). These procedures are routinely carried out to evaluate the safety of a compound. While dietary LC_{50} (median lethal concentration) is the dietary concentration of a compound that causes 50% mortality in an exposed group of experimental animals (Martin and Young, 2001), the reproduction test assesses the toxic effects and/or safety of the compound on reproductive success/outcome (Ankley *et al.,* 2001).

The LD_{50} (median lethal dose) is the dose of a substance that proves to cause mortality in 50% of an exposed group of animals (the LD_{50} of diclofenac in various bird species in comparison to rats in listed in Table 2.2). Usually, experimental animals are exposed to the said compound and are observed for death or survival. A commonly used validated method for establishing LD_{50} is the probit-linear relationship model, by relating the probability of death to the log-dose of the compound. This is then used to estimate the slope and intercept by maximum likelihood estimation (Williams, 1986). The LD_{50} when derived, may be used in arriving at doses for new compounds and serves as the background for the labelling and classifying of the compound *(*Akhila *et al.,* 2007). For environmental purposes, the LD_{50s} derived from the test avian species are used to predict the overall environmental toxic potential of the tested substance, and thus can be useful in decided whether a compound can be allowed to be used in the environment. With the OECD guideline for avian environmental toxicity not specifically being applied to diclofenac and other veterinary pharmaceuticals, this guideline potentially has merit in predicting the avian environmental impact of veterinary pharmaceuticals.

Table 2.2: Median lethal doses (LD_{50}) of diclofenac in some animal species

Species	**LD_{50} (mg/kg)**	**Route**	**References**
Gyps bengalensis	0.098 – 0.225	Oral	(Swan *et al.*, 2006b)
Gyps fulvus	< 0.8	Oral	(Swan *et al.*, 2006b)
Gyps africanus	< 0.8	Oral	(Swan *et al.*, 2006b)
Gyps coprotheres	< 0.8	Intravenous	(Naidoo *et al.*,2009b)
Gallus gallus	9.8	Intramuscular	(Naidoo *et al.*, 2007)
Corvus albus	> 10	Oral	(Naidoo *et al.*, 2011)
Cathartes aura	> 25	Oral	(Rattner *et al.*, 2008)
Rattus spp	> 50	Oral	(Piao *et al.*, 2006)

2.5 Conclusion

The world population of *Gyps* vultures may still be at risk of environmental poisoning from other NSAIDs. This is more so now that the toxicity of some of these drugs have been demonstrated in vultures. With their present population status, cost and time taken to reliably establish toxicity, an alternative means of predicting the toxicity of other NSAIDs in vultures is in dire need. For this study, we investigated if the OECD guideline for single-dose acute oral toxicity study in avian could have predicted the toxic effect of diclofenac in vultures.

3 MATERIALS AND METHOD

3.1 Housing and care

The following study made use of the Japanese quail, Muscovy duck and the domestic pigeon. The project was approved by the Animal Ethics Committee of the University of Pretoria (V107/16). In all cases, the birds were housed in the experimental aviary of the Biomedical Research Centre of the University of Pretoria (UPBRC).

3.1.1 Japanese quail

Nineteen young-adult Japanese quail (*Coturnix japonica*) weighing between 286-424 g were acquired from a commercial farm in Pretoria. The birds were housed in groups and were individually wing-tagged with numbers for easy identification. The floor of the pen was covered with wood shavings to serve as bedding. The birds were allowed a one week acclimatization period and had free access to fresh commercial quail feed and municipal potable water. The birds had a daily lightening period of 14 h with room temperature and relative humidity ranging between 19.7 - 27.2 °C and 36 – 75 % respectively.

3.1.2 Muscovy duck

Nineteen young-adult Muscovy ducks (*Cairina moschata*) weighing between 1.41 - 2.46 kg were acquired from a commercial farm in Pretoria. The birds were housed in groups and were individually wing-tagged with numbers for easy identification. The birds were allowed a ten-day acclimatization period and had free access to fresh commercial flamingo feed and municipal potable water. The birds had a daily lightening period of 12 h with room temperature and relative humidity ranging between 17.9 - 26.7 °C and 44 – 89 % respectively.

3.1.3 Domestic pigeon

Nineteen young-adult domestic pigeons (*Columba livia domestica*) weighing between 371 – 516 g were acquired from a commercial farm in Pretoria. The birds were housed in groups and were individually ringed with numbers for easy identification. The birds were allowed an eight-day acclimatization period and had free access to fresh commercial quail feed and municipal potable water. The birds had a daily lightening period of 14 h with room temperature and relative

humidity ranging between 16 – 30 °C and 27 – 74 % respectively. The birds were also provided with perches (nesting boxes).

3.2 Animal treatment

All birds were fasted overnight for 15 h prior to dosing, but had free access to water. Access to feed was granted immediately after dosing. For each species, the study was divided into two discrete stages in which birds were concurrently dosed orally with diclofenac sodium or the carrier solvent without active ingredient. Propylene glycol (in Japanese quail / domestic pigeon) and sterile water (in Muscovy duck) were used as vehicles. The birds were randomly allocated to test and control groups comprising fourteen and five birds respectively. The study design made use of the prescribed method of the OECD (Figure 3.1).

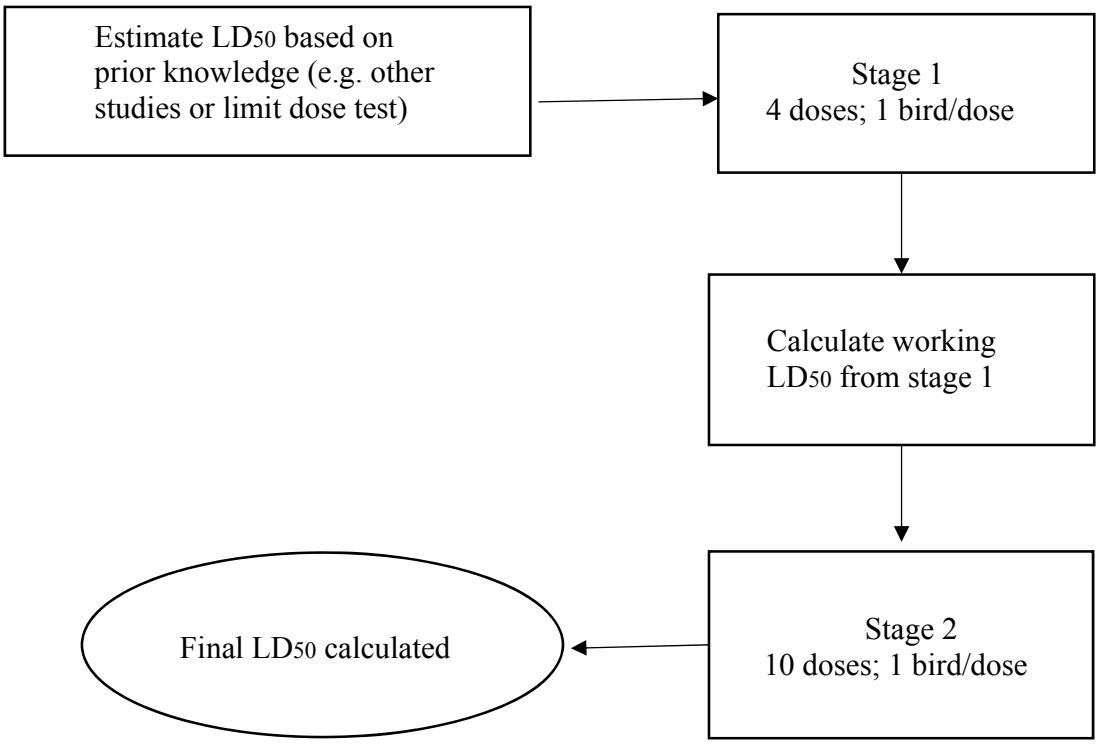


Figure 3.1: OECD sequential design procedure for LD_{50} testing of pesticide

In stage one, four birds were randomly selected from the test group and received non-replicate oral doses of diclofenac sodium (Sigma-Aldrich) dissolved in the appropriate vehicle. For the dose-range finding study, doses were calculated based on an initial guess working LD_{50} acquired

from literature (i.e. LD_{50} of diclofenac in other species) or knowledge of previous toxicity / failed limit dose test of diclofenac. Stage one doses were calculated by equally spacing the doses on a log scale around the LD_{50} estimate of diclofenac in rat (Piao *et al.,* 2006), chicken (Naidoo *et al.,* 2007) and pigeon (European medicines agency, 2014) for Japanese quail, Muscovy duck and domestic pigeon respectively, which were based on probit and log-logistic models respectively. The control groups received vehicles at same dose rate (ml/kg) used for test group. Following initiation of stage one, preliminary observations of clinical signs of poisoning and mortality were used to estimate a working LD_{50} for stage two by calculating the geometric mean of the doses that produced a transition between survival and death.

In stage two, the remaining ten birds randomly received non-replicate oral doses of diclofenac sodium calculated on the working LD_{50} estimates from stage one birds by maximum likelihood estimation (MLE). These doses were calculated using the OECD purpose written Microsoft Excel work book; SEquential DEsign Calculator (SEDEC) (Table 3.1). The final LD_{50} of diclofenac for each of the species was estimated by a probit regression model using SEDEC. This was determined by estimating the mean of the tolerance distribution in the bird population. The probit regression model is based on the principle that an individual bird will die if it receives a dose above certain value but will survive if the dose is equal to or less than this value (tolerance dose). The tolerance dose is fixed for an individual bird but vary among birds. With an assumed tolerance dose following a normal distribution, the slope (b) and intercept (a) obtained from a straight line relationship between probability of surviving and logarithm of dose can then be used to determine mean of tolerance distribution (LD_{50}) with the following equation.

$$\text{Log}(LD_{50}) = -a/b$$

Table 3.1: Doses used for the study

Species	Initial LD_{50}	Stage one doses*	Stage two working LD_{50}	Stage two doses*
Japanese quail	50 mg/kg	7, 26, 96 and 354 mg/kg	184.35 mg/kg	63.2, 80.1 ,102, 129, 164, 208, 263, 334, 424 and 538 mg/kg
Muscovy duck	9.8 mg/kg	1.39, 5.11, 18.8 and 69.3 mg/kg	133.02 mg/kg	45.6, 57.8, 73.4, 93.1, 118, 150, 190, 241, 306 and 388 mg/kg
Domestic pigeon	15.6 mg/kg	2.21, 8.13, 29.9 and 110 mg/kg	211.14 mg/kg	72.3, 91.8, 116, 148, 187, 238, 302, 383, 486 and 616 mg/kg

***Each dose listed was administered to a single bird only i.e. each bird received one single dose in a dose ascending manner**

3.3 Monitoring and sampling

Birds were monitored continuously during the first 2 h after dosing. Subsequent observation on the day of dosing occurred frequently until 9 - 12 h after dosing. Blood samples were collected from the wing vein per sampling time using needles and syringes (B|Braun) (Table 3.2). Syringes and needles were coated with heparin prior to sampling to avoid blood clotting. Samples were collected at 0, 2, 8, 24 and 48 h' post dosing or soon after death (in cases a bird died within 48 h of dosing). The volumes collected per bird are reported in Table 3.2. Collected samples were transferred into heparinized tubes and centrifuged at 1660 x g for 15 min at 25°C. Separated plasma were labelled and stored at -25°C for diclofenac and uric acid analysis. The birds were observed at least twice daily for the subsequent fourteen days. Birds were weighed on days 0 (prior to dosing), 4, 8 and 15 after dosing. Observations took note of the onset and/or signs of intoxication, remission, regurgitation, abnormal behaviour, feed consumption, changes in body weight, mortality, pathology and time to death. Final oral LD_{50} of diclofenac sodium in each species was calculated using SEDEC. All surviving birds were euthanized fifteen days after dosing using a CO_2 chamber and the carcasses were sent to the veterinary pathology section of the department for necropsy. Gross pathologic lesions seen at necropsy were recorded. For

histopathology, liver, kidney and spleen samples were collected in 10% buffered formalin, sectioned and stained using the standard H&E technique. Tissue samples (liver & kidney) were collected and preserved at -25°C for diclofenac residue analysis.

Table 3.2: Sample volume and sizes of syringes / needles used

Species	Volume of blood	Size of needle	Size of syringe
Japanese quail	0.5ml	26G	1ml
Muscovy duck	2ml	23G	3ml
Domestic pigeon	0.5ml	26G	1ml

3.4 Drug analysis

3.4.1 Plasma sample preparation

3.4.1.1 Japanese quail

Preparation of standards for calibration curve was achieved by dissolving diclofenac sodium (10 mg) in 5 ml of dosing vehicle to give a 2 mg/ml stock solution. One mililitre of the stock solution was added to 9 ml of vehicle to give a 200 µg/ml working solution. In 2 ml tubes, diclofenac sodium working standard solution (200 µg/ml) were added in volumes of 0, 0.976, 1.953, 3.906, 7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 µl to 200 µl of blank plasma to provide calibration standards of 0, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. The contents were mixed for 30 sec using a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) (El-Sayed *et al.,* 1988). The standards were then treated as par the test samples.

Plasma samples (200 µl) were transferred into 2 ml tubes and 400 µl of diethyl ether were added to each. This was then followed by a 400 µl of 0.3 M potassium dihydrogen phosphate with pH of 3.5. The tubes were capped and content mixed with a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) for 2 min. The mixtures were subsequently centrifuged at 5878 x g for 10 min at 4°C. The organic layers were separated and decanted from the solidified aqueous phase after inserting the tubes in an ice-bath (methanol/dry ice) for 3 min. Under a mild

flow of nitrogen, the organic layers were evaporated to dryness for 30 min at 50°C. Residues were preserved at -25°C until HPLC analysis. On the day of analysis, residues were dissolved in 400 µl of mobile phase and subsequently transferred into inserts that were fitted to the crimp top vials and capped (Naidoo *et al.,* 2007).

3.4.1.2 Muscovy duck and domestic pigeon

Preparation of standards for the calibration curve was achieved by dissolving diclofenac sodium (10 mg) in 5 ml of dosing vehicle to give a 2 mg/ml stock solution. One mililitre of the stock solution was added to 9 ml of vehicle to give a 200 µg/ml working solution. In 2 ml tubes, diclofenac sodium working standard solution (200 µg/ml) were added in volumes of 0, 0.976, 1.953, 3.906, 7.8125, 15.625, 31.25, 62.5, 125 and 250 µl to 200 µl of blank plasma to provide calibration standards of 0, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25 and 50 µg/ml. The contents were mixed for 30 sec using a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) (El-Sayed *et al.,* 1988). The standards were then treated as par the test samples.

Plasma samples (200 µl) were transferred into 2 ml tubes and 40 µl of phosphoric acid was added to each. This was then diluted with 200 µl deionised water. The tubes were capped and contents mixed with a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) for 30 sec. The sample mixtures were subsequently loaded onto preconditioned cartridges (1 ml 30 mg Oasis HLB cartridges from Waters, Miford, MA, USA). Cartridges were conditioned by loading 1 ml diethyl ether/methanol mixture (90:10), these were followed by 1 ml methanol and then 1ml deionised water. At every point of loading, the bed within the cartridge was maintained wet. After loading the sample mixtures, the cartridges were washed using 1 ml deionised water. Diclofenac was eluted using 1 ml diethyl ether/methanol mixture (90:10). Eluate was evaporated to dryness under a steady stream of nitrogen at 50°C for 30 min. Residues were preserved at -25°C until HPLC analysis. On the day of analysis, residues were dissolved in 250 µl of mobile phase and subsequently transferred into inserts that were fitted to crimp top vials and capped (Suenami *et al.,* 2006).

3.4.2 Liver and kidney sample preparation

For standard calibration curve preparation, liver and kidney tissue samples from a non-treated bird were weighed after defrosting. To each tissue sub-samples (0.5 g each), diclofenac sodium

working standard solution of 200 µg/ml were added in volumes of 0, 7.813, 15.625, 31.25, 62.5, 125 and 250 µl to provide calibration for blank, 1.563, 3.125, 6.25, 12.5, 25 and 50 µg. To each mixture, 2 ml acetonitrile were added and the mixed samples were treated as described for the test samples.

For each liver and kidney sample preserved at -25°C, 0.5 g of thawed tissue was weighed into a 10 ml tube and 2 ml of HPLC grade acetonitrile was added. The mixture was homogenized using a homogenizer (Pro 200, Pro Scientific Inc., Oxford, CT USA) for 1 min and subsequently vortexed for 10 min. The prepared sample was centrifuged at 4500 x g for 10 min at 4°C and the supernatant filtered using 0.22 µm disposable MS® Nylon syringe filter units. The resultant filtrates were stored at -25°C in crimp top vials until analysis using HPLC technique (Taggart *et al.,* 2007).

3.4.3 Separation and quantification of diclofenac sodium using HPLC

Samples were analysed isocratically using a Beckman System Gold HPLC. The apparatus is composed of an autosampler module 507, diode array detector (DAD) 168, programmable solvent module 126 and a 32 Karat™ software package (Beckman Instruments, Fullerton, CA, USA). Diclofenac detection was achieved using a 250 x 4.6 mm, 5 µ BDS HYPERSIL Phenyl column with a mobile phase consisting of 0.05 M sodium dihydrogen phosphate (pH 4.86-4.88): acetonitrile, 42.5:57.5. Thirty microliters of reconstituted samples were injected onto the HPLC column at a flow rate of 1 ml/min. Detection was done at a wavelength of 275 nm with a sample run time of 8 min. The retention times for diclofenac were 3.97, 4.3 and 4.7min for the pigeons, quails and ducks, respectively. The standard calibration curve showed an r^2 value above 0.99 for each run (Figure 3.3). The LOD and LOQ were 0.195 µg/ml and 0.78 µg/ml, respectively for the ducks. For the quails the LOD and LOQ were the same i.e. 0.39 µg/ml, as signal to noise ratio for the lowest standard concentration is far above 10:1. The LOD and LOQ for the pigeons were similarly the same i.e. 1.56 µg/ml. Below is a chromatograph showing diclofenac in plasma (Figure 3.2).

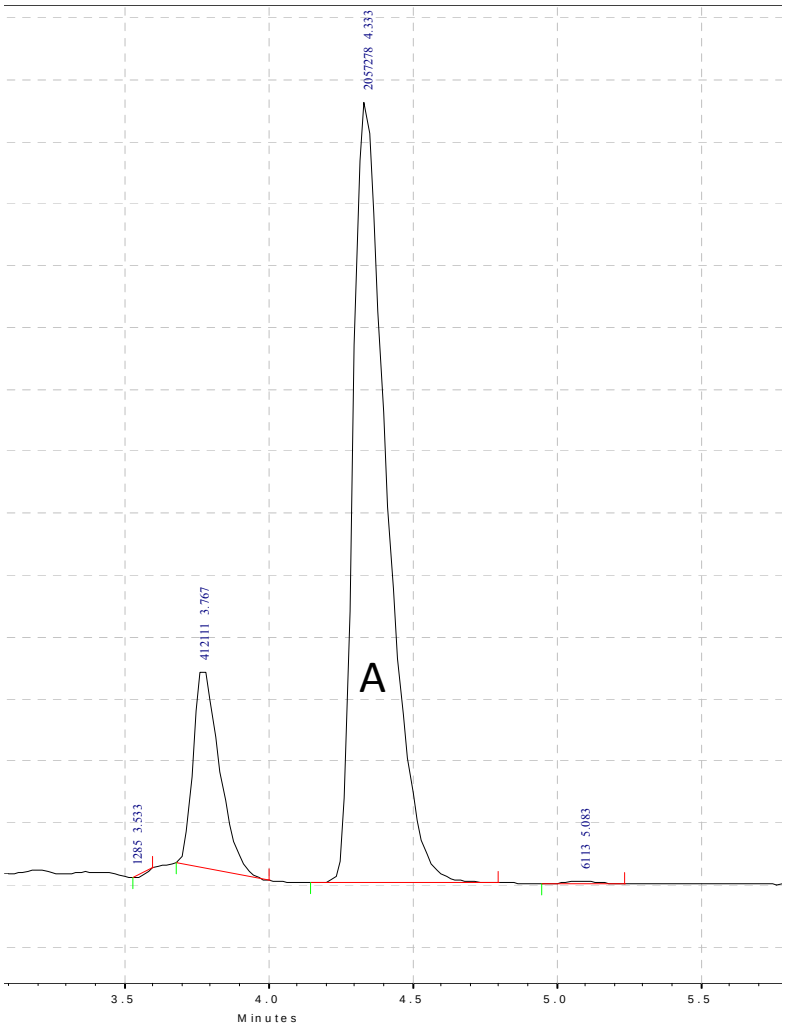


Figure 3.2: Chromatograph of diclofenac sodium (A) in plasma

3.5 Pharmacokinetic analysis

Data generated following HPLC analysis were evaluated by a non-compartmental model technique, using the Kinetica 5.0 software package developed by Thermo Scientific, with the resultant pharmacokinetic parameters i.e. $T_{1/2}$, AUC, MRT, Vd, C_{max}, CL and T_{max} of diclofenac sodium in the 3 species been generated. The maximum plasma concentration (C_{max}) and the time to maximum concentration (T_{max}) were read directly of the concentration versus time plasma profile. The area under the curve to the last quantifiable time point (AUC_{last}) was determined using the linear trapezoidal rule ($AUC_{last} = \sum_{i=1}^{n} 0{,}5\ x((C_i + C_{i+1})\ x\Delta t)$. The total area under curve extrapolated to infinity (AUC_{tot}) was calculated as $AUC_{tot} = AUC_{last} + AUC_{extra} = AUC_{last}+$

C_{Last}/λ with C_{last} being the computed last measured concentration and λ being the terminal elimination rate constant. The area under the moment curve from the time point zero to the last measured time point ($AUCM_{last}$) was calculated as $AUMC_{last} = \sum_{i=1}^{n} 0{,}5\, x(t_i\, xC_i + t_i{}^{+1}\, x\, C_{i+1})\, x\Delta t$. The half-life ($T_{1/2}$), clearance (Cl) and volume of distribution during terminal phase (V_z) and volume of distribution at steady state (V_{ss}) and the mean residence time (MRT) were determined as $T_{1/2} = \ln(2)/\lambda$; $V_z = Cl/\lambda = Dose/(AUC\ x\ \lambda)$; $V_{ss} = (Dose\ x\ MRT)/AUC$, Cl = $dose/AUC_{tot}$ and $MRT = AUMC_{tot}/AUC_{tot}$.

However, due to the detection of diclofenac at only a few sampling time point in the pigeons, pharmacokinetic evaluation for this species was undertaken using the therapeutic drug monitoring standard formulae as below.

Elimination rate constant (λ)	$\lambda = \left(In\frac{Cmax}{Cmin}\right) x\ (t2 - t1)$
Half-life ($T_{1/2}$)	$t_{1/2} = \frac{In(2)}{\lambda}$
Volume of distribution (Vd)	$vd = \frac{Dose}{Cmin} x \frac{1-e^{-n\lambda t}}{1-e^{-\lambda t}} x\ e^{-\lambda t}$
Area under curve (AUC)	$AUC = \frac{Dose}{vd\ x\ \lambda}$
Clearance rate (Cl)	$Cl = \frac{Dose}{AUC}$

3.6 Clinical chemistry analysis

Due to the paucity of samples, clinical chemistry analysis was not undertaken in the Japanese quails and domestic pigeons. For the Muscovy ducks, plasma samples were sent to the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital for evaluation. Uric acid (for all time points samples), Na^+ and K^+ (for 48-hour samples of control/test birds that succumbed) plasma levels were assessed using the Cobas Integra 400 plus analyser (Roche diagnostics, Mannheim, Germany). It is a fully automated random access, software-controlled system for photometric, fluorescence polarisation and electrolyte analysis. Uric acid concentration was determined at 552nm using the uricase enzyme colorimetric test by measuring the absorbance of the red colour intensity of quinoneimine dye formed at the end of the reaction.

Na^+ and K^+ were analysed by an Ion-selective electrode using automatically diluted plasma samples.

3.7 Comparison between Muscovy duck (C. moschata), Chicken (G. gallus) and Cape vulture (G. coprotheres)

3.7.1 Plasma electrolyte and protein analysis

Blank plasma samples from the ducks; and preserved blank plasma samples collected from chicken (V108/16) and Cape vulture (V014/17) during another study were assessed for Na^+, K^+ (using same method described above) and albumin concentration. Albumin concentration were assessed at the clinical pathology laboratory of the Onderstepoort Veterinary Academic Hospital using a fully automated electrophoresis system (Pretty, Separation Scientific, Honeydew, South Africa). The system separates proteins into distinct bands by electrophoresis on agarose gel plates. Densitometry of the patterns were eventually used to relatively quantify the protein zones.

3.7.2 Plasma uric acid saturation assay

Blank plasma samples from the ducks; and preserved blank plasma samples (at -80°C) collected from chicken (V108/16) and Cape vulture (V014/17) during another study were used for the assay. Samples were thawed at room temperature and were subsequently divided into five parts (of 1 ml each) per species. To each of the five subsets of blank sample, uric acid salt (Sigma) were added until the plasma were saturated (cloudy) and precipitated. Plasma samples were subsequently centrifuged at 2260 x g for 10 min at room temperature and cleared plasma portion (supernatant) aspirated off using pipettes. Plasma samples were then sent to the clinical pathology laboratory for uric acid evaluation using method previously described above. In this manner, the maximum concentration of uric acid that can dissolve in the plasma under the defined laboratory conditions was ascertained.

3.8 Establishing relationship between body mass and diclofenac's toxicity in some bird species

The estimates of median lethal dose of diclofenac in some bird species from previous studies were compared among the birds to establish if any relationship existed between body mass and diclofenac's toxicity. For this study, the established median lethal dose of diclofenac in *Gyps* vulture (Swan *et al.,* 2006b), chicken (Naidoo *et al.,* 2007), Muscovy duck (present study) and

Japanese quail (present study); and the average body weight per species were used for comparison on a Log scale.

4 RESULTS

4.1 Japanese-quail acute oral toxicity study

4.1.1 Clinical signs

Of all the experimental birds exposed to diclofenac, only two birds showed signs of intoxication and died. The first which died approximately 47.5 h after dosing, received a high dose of 354 mg/kg. The bird was depressed and unable to walk (i.e. sitting on the hock) 47 h 10 min after dosing, 16 min later it assumed a sternal recumbency position (head drooping) and was gasping for air. It died 6 min later. The second bird received the highest dose of the study i.e. 538 mg/kg. It vomited twice, at 2 and 8 hours after dosing during restraint for blood collection. Twenty-two h and 46 min after dosing, it was slightly depressed and separated from the rest of the group. It remained so until it was found dead at 46.5 h after dosing. All other experimental birds appeared healthy and alert except for the slight loss of body weight which was evident in both control and test animals (Table 4.1). They all recovered and gained weight after dosing and blood collection. Feed consumption increased during the study, except for days when birds were fasted or handled for weighing, dosing and/or blood collection. The resulting oral LD_{50} of diclofenac sodium in Japanese-quails was estimated at 405 mg/kg with probability and slope being 0.98 and 9.0 respectively as shown in the output result (Figures 4.1 and 8.3).

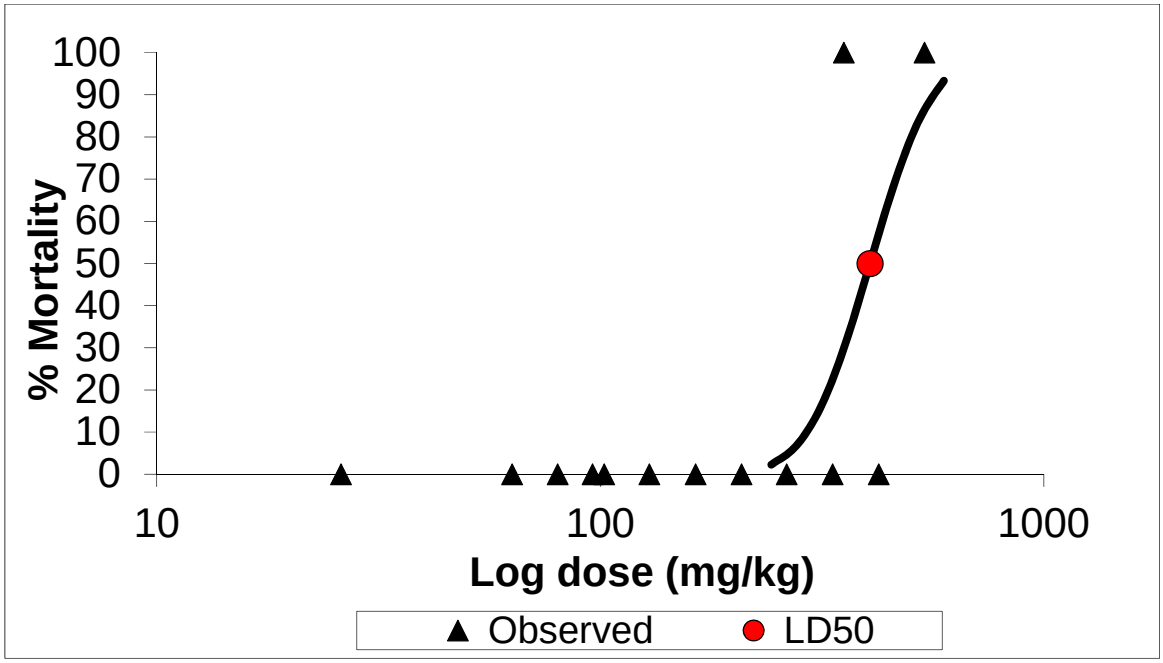


Figure 4.1: Diclofenac oral LD_{50} plot for Japanese quail

4.1.2 Pathology

The bird that received 354 mg/kg had evidence of urate crystals on the pericardium, liver and intestine (serosal surfaces of all abdominal organs). The kidneys were moderately swollen with tubules visible as white lines in the cortex. The lungs were mildly congested with marked oedema. Similar findings were noted for the bird that received the highest dose (538 mg/kg). The kidneys were pale and enlarged with severe urate deposition on all serosal surfaces (Figure 4.2). No pathologic lesions were seen on the other birds that underwent schedule euthanasia fifteen days after dosing

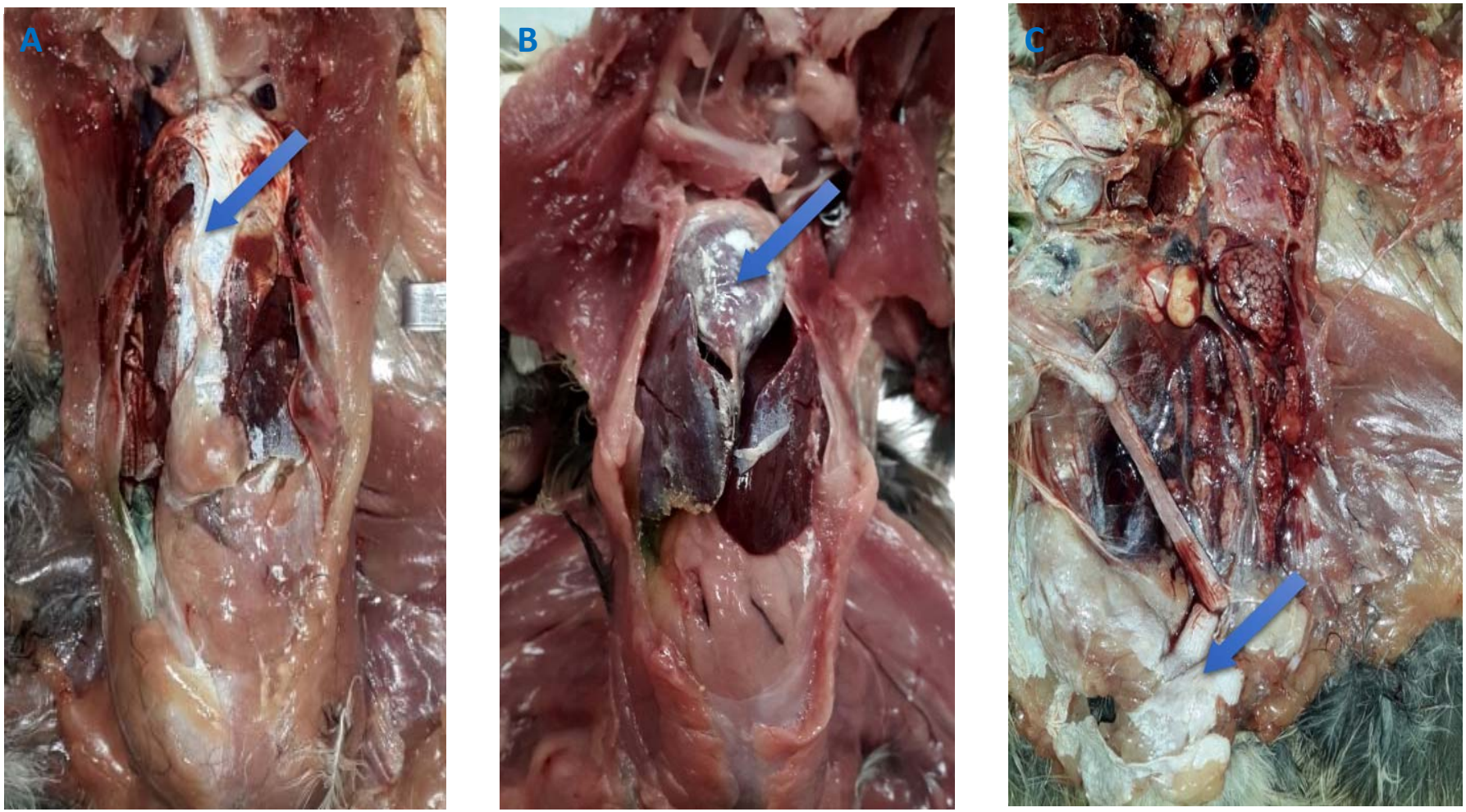


Figure 4.2: Gross pathology showing urate deposit on abdominal organs of quail; [A] Pericardium, [B] Pericardium and liver, [C] Abdominal wall (blue arrows).

Histologically, there were evidences of tophi in the kidney, spleen and liver of the two birds that died after dosing (Figure 4.3). The kidneys were particularly more affected with tubules being characterized by the presence of urate aggregates which resulted in their damage. In some instances, the aggregates had assumed a globular form. The liver was not severely affected as it had scanty presence of urate deposit within the parenchyma. The spleen also had urate aggregates deposited in it parenchyma.

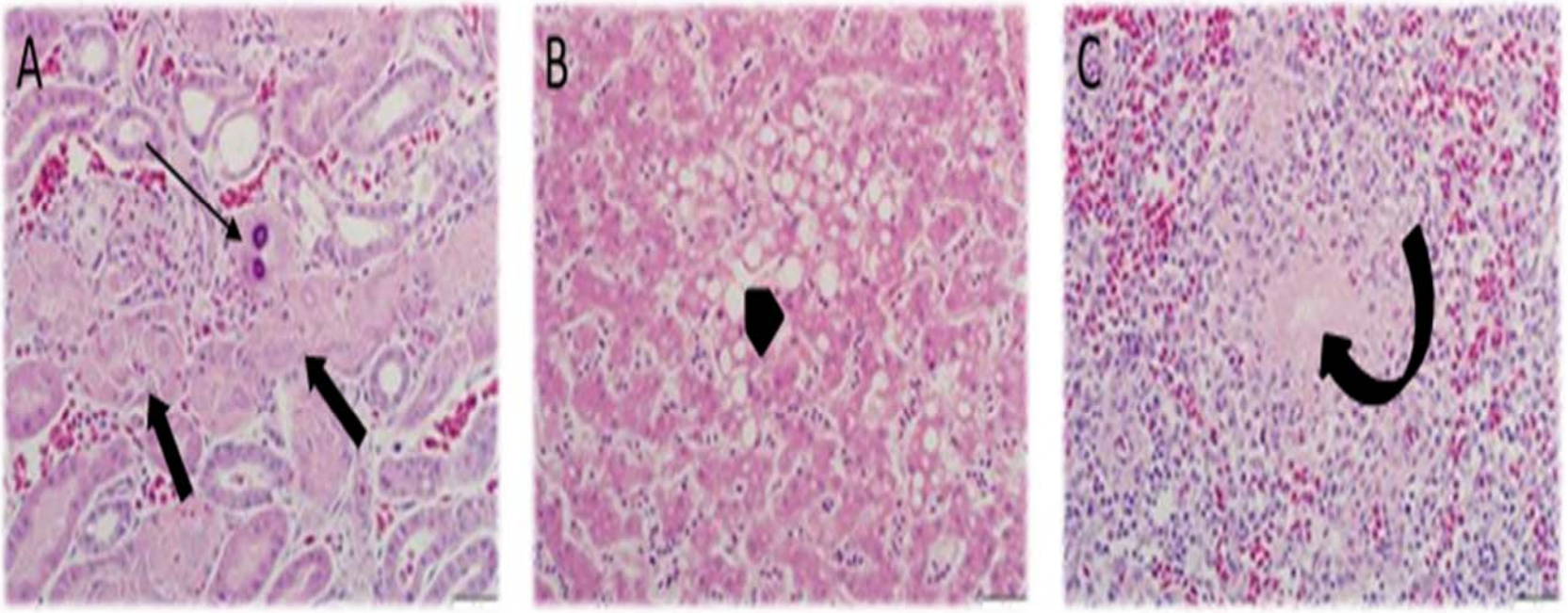


Figure 4.3: (A) Kidney HE x 40 (B) Liver HE x 40 (C) Spleen HE x 40 from a dosed quail that succumbed. Severe renal tubular damage (block arrows), renal tubule with tophi that has assumed a globular form (line arrow), urate crystals in the liver (pentagon arrow) spicule shaped tophi in the spleen (curved arrow)

Table 4.1: Japanese quail body weight measurement.

Animal ID	Dose(mg/kg)	Body weight (g)			
		Day 0	Day 4	Day 8	Day 15
9168	control	385	376	387	403
9175	control	286	291	298	297
9181	control	362	360	372	383
9184	control	309	310	318	321
9185	control	381	368	402	421
9182	7	291	289	311	305
9176	26	424	377	389	447
9187	63.2	375	363	373	373
9169	80.1	340	319	329	334
9174	96	349	334	349	366
9188	102	361	339	377	403
9170	129	373	341	355	361
9173	164	363	350	362	368
9177	208	353	340	353	356
9179	263	362	341	356	360
9186	334	350	334	357	372
9172	354	349			
9183	424	377	363	373	398
9171	538	351			

Highlighted cells, indicate the birds that died during the study

4.1.3 Pharmacokinetics

The pharmacokinetic data and diclofenac plasma profile for the birds are presented in Table 4.2 and Figure 4.4 respectively. In all cases, the plasma concentrations were at their maximum at the first sampling at 2 h. The AUC_{last} attained for each of the birds failed to uniformly increase in the same manner that the dose was increased. In general, diclofenac was well distributed after administration, and was characterised by a short half-life of elimination. The half-life of elimination was generally under 6 h, with the exception of the birds that died. These two birds showed a relatively slow elimination rate constant (Lz) with correspondent lengthy elimination half-life (above 6 h) and mean residence time (above 8 h). Liver and kidney tissues from all the birds had no detectable residues of diclofenac parent molecule following HPLC analysis.

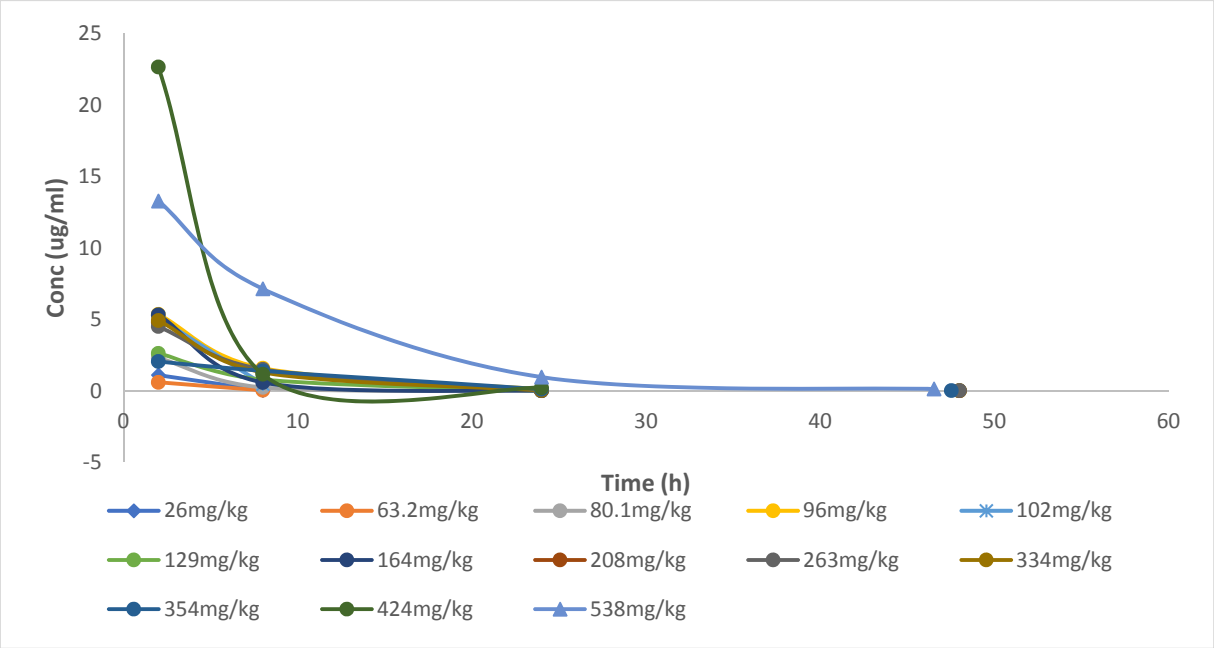


Figure 4.4: Diclofenac disposition curve following oral dosing in Japanese quails

Table 4.2: Pharmacokinetic data of diclofenac following oral dosing in Japanese quails

Animal ID	Dose	C_{max}	T_{max}	AUC_{last}	AUC_{inf}	Lz	$AUMC_{last}$	$T_{1/2}$	MRT	Clearance	V_z	V_{ss}
	mg/kg	µg/mL	h	µg/mL*h	µg/mL*h	1/h	µg/mL*	h	h	L/h*kg	L/kg	L/kg
9176	26	1.11	2	4.54	4.60	0.58	9.70	1.20	2.24	5.65	9.78	12.64
9187	63.2	0.60	2	2.53	2.62	0.45	5.79	1.54	2.56	24.12	53.58	61.73
9169	80.1	2.32	2	12.03	12.07	0.24	41.87	2.89	3.55	6.64	27.70	23.57
9174	96	5.36	2	41.60	41.67	0.13	250.50	5.27	6.10	2.30	17.50	14.06
9188	102	5.11	2	21.85	23.03	0.40	52.16	1.74	2.80	4.43	11.14	12.42
9170	129	2.63	2	19.49	19.51	0.14	97.88	5.01	5.06	6.61	47.82	33.47
9173	164	5.33	2	27.85	27.87	0.30	95.66	2.29	3.45	5.88	19.42	20.31
9177	208	4.81	2	58.26	58.29	0.14	130.89	5.01	2.27	3.57	25.79	8.10
9179	263	4.50	2	35.87	35.95	0.12	204.05	5.67	5.80	7.32	59.80	42.44
9186	334	4.93	2	34.32	34.50	0.22	161.43	3.18	4.83	9.68	44.42	46.78
9171	354	2.06	2	26.19	26.33	0.11	206.73	6.47	8.16	13.44	125.48	109.77
9183	424	22.67	2	105.20	106.08	0.19	325.86	3.68	3.31	4.00	21.22	13.25
9171	538	13.29	2	152.64	153.81	0.10	1275.64	6.88	8.73	3.50	34.74	30.55

C_{max}: Maximum plasma concentration, T_{max}: Time to maximum concentration, AUC_{last}: Area under curve to the last quantifiable time point, AUC_{inf}: Total area under curve extrapolated to infinity, Lz (λ): Terminal elimination rate constant, $AUCM_{last}$: The area under the moment curve from the time point zero to the last measured time point, $T_{1/2}$: Half-life, MRT: Mean residence time, Cl: Clearance, V_z: Volume of distribution during terminal phase, V_{ss}: Volume of distribution at steady state.
Highlighted cells indicate birds that died

4.2 Muscovy duck acute oral toxicity study

4.2.1 Clinical signs

Following their exposure to diclofenac, five birds died or were euthanized. The first bird (5854; 190 mg/kg) started limping on its left leg 2 h after dosing, shortly after blood collection. Twenty-four h later, it couldn't bear weight on its legs and by 48 h it died soon after the 48-h blood collection. The second bird (5858; 241 mg/kg) was found dead 72 h after dosing. The third bird (5855; 45.6 mg/kg) was found depressed and isolated 72 h after dosing. Its condition gradually deteriorated until it could not walk. It became comatose and died about 79 hours after dosing, before it could be euthanized for being moribund. The fourth bird (5859; 57.8 mg/kg) was noticed to be depressed and unable to bear weight about 77 h after dosing. It was euthanized about 79 h after dosing. Bird 5852 (73.4 mg/kg) was seen limping on it left limb about 77 h after dosing. It was active, alert and remained so throughout the study. The fifth bird (5861; 306 mg/kg) was euthanized for humane reasons, 98 h after dosing due to severe loss of body weight (18.5% in 4 d after dosing). In general, save for a few exception, there was a slight loss of body weight in both test and control birds (Table 4.3) with few fluctuations. Feed consumption was relatively steady throughout the study, but severely dropped on days when birds were fasted, dosed or handled for blood collection. The resulting oral LD_{50} of diclofenac sodium in Muscovy ducks was 190 mg/kg with probability and slope being 0.39 and 0.99 respectively (Figures 4.5 and 8.4).

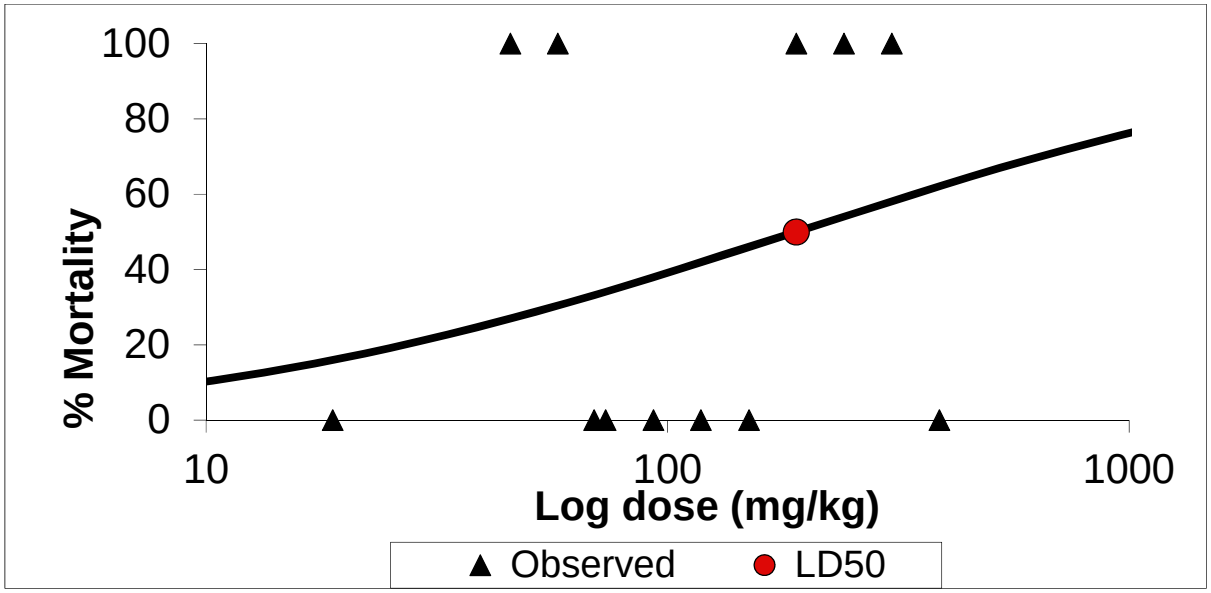


Figure 4.5: Diclofenac oral LD_{50} plot for Muscovy duck

4.2.2 Pathology

Grossly, bird 5858 (241 mg/kg) had severely swollen and pale kidneys with urate deposition on the pericardium and liver capsule (Figure 4.6). Its ovarian follicles were collapsed. For bird 5854 (190 mg/kg), the kidneys were also pale and swollen with collapsed ovarian follicles. It had a fracture of the left femur neck due to trauma with peri-articular haemorrhage being evident. For bird 5859 (57.8 mg/kg), the kidneys were equally pale and enlarged with ovarian follicles being collapsed. For bird 5855 (45.6 mg/kg), the kidneys were pale and swollen, and had urate crystals on the serosal surfaces of the liver, heart and air sacks. Urate crystals were even present in the bile. Bird 5861 (306 mg/kg) had normal kidneys but with a moderate generalised congestion. In addition, it had moderate splenomegaly and some collapsed ruptured ovarian follicles. 5851 (118 mg/kg) had a small pale liver with hard consistency. The rest of the other birds were macroscopically normal.

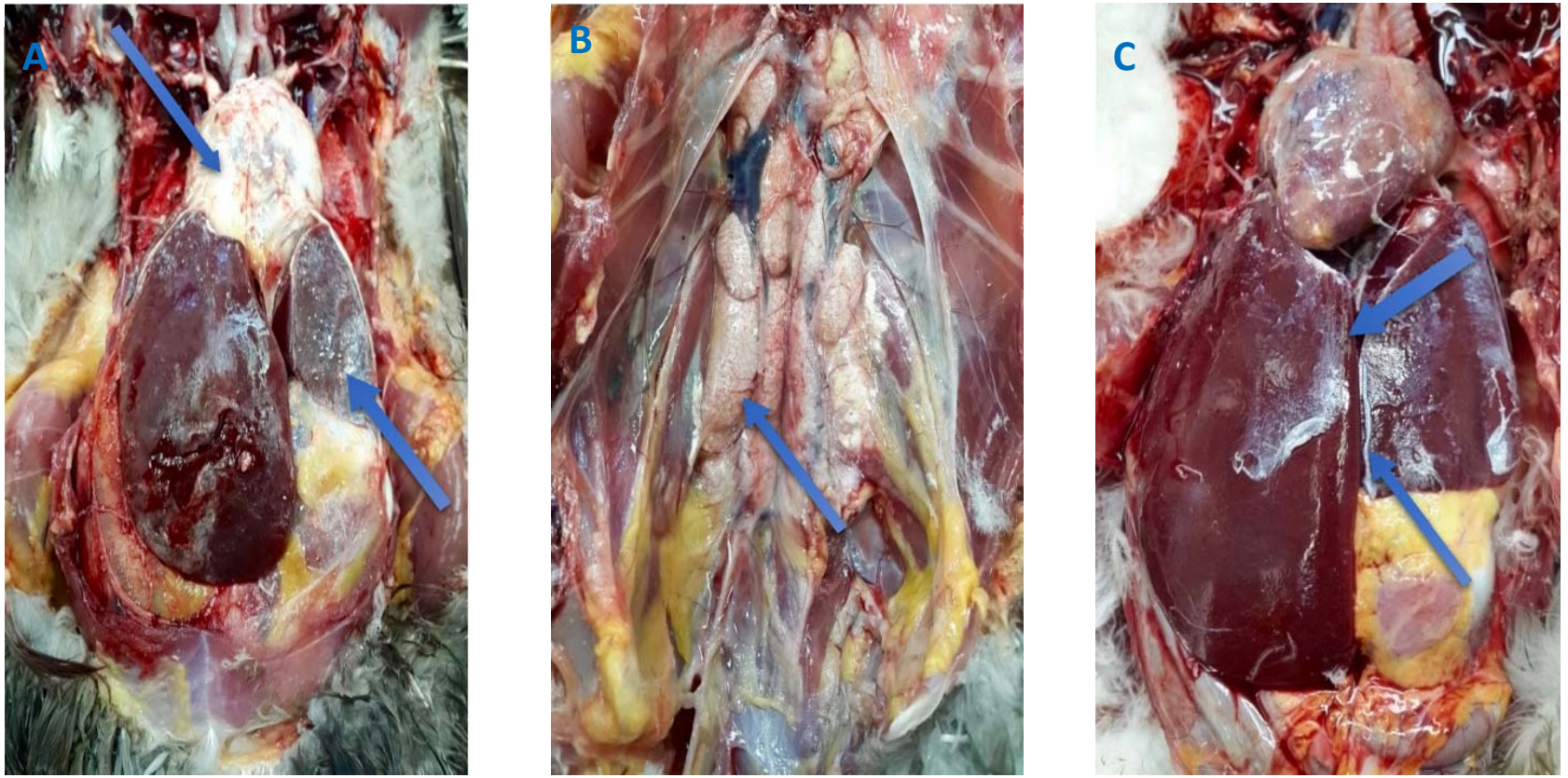


Figure 4.6: (A) Urate deposit seen on the pericardium and liver capsule; (B) Severely swollen and pale kidneys; (C) Urate crystals seen on the liver.

Numerous lesions were evident in the different birds (Figure 4.7). The lesions present on histology were:

- Bird 5858 (241 mg/kg) revealed moderate injury to the kidneys with widespread dilatation of tubules associated with loss of the normal cuboidal lining cells. The cells were replaced by a granular pink material in which cell fragments were embedded. Within some of the tubules, radiating spicules of urate crystals could be seen within this pink material filling the damaged tubules. In addition, scattered tubules showed mineralisation of individual epithelial cells and many collecting ducts contained desquamated tubular epithelium admixed with heterophils.
- Bird 5859 (57.8 mg/kg) had similar lesions as bird 5858
- Bird 5854 (190 mg/kg), the lesions on the kidneys were similar to those of bird 5858 however, the foci of radiating urate crystals within damaged tubules were much more prominent and also in addition had involvement of the adjacent interstitium. However, in contrast to bird 5858, bird 5854 had microscopic lesions in the liver. Scattered single hepatocytes were swollen, lighter in colour with identifiable small needle shaped crystals within their cytoplasm.
- Bird 5855 (45.6 mg/kg), the kidneys were massively damaged with most parts affected. There were numerous urate crystalline tophi between the damaged tubules and within the actual interstitium.
- Bird 5861 (306 mg/kg) had marked congestion of all its organs. Apart from white pulp hyperplasia in the spleen, no other morphological changes were observed.
- Bird 5851 (118 mg/kg), the liver revealed a marked replacement fibrosis together with bile duct proliferation within the liver. The replacement fibrosis and bile duct proliferation of the liver is not characteristic of diclofenac's toxicity and may have been a consequence from previous exposure to one of the hepatotoxins i.e. aflatoxin or microcystin. However, it is important to note that this duck had a plasma uric acid concentration above normal following oral gavage.
- Bird 5852 (73.4 mg/kg), the kidneys interestingly revealed urate crystals that were starting to be deposited within the cones with destruction of the lining cells associated with shrinkage and basophilia of the nuclei. Scattered collecting ducts were dilated and contain desquamated epithelial cells.

- Bird 5857 (93.1 mg/kg), the kidneys had normal proximal and distal convoluted tubules but within some of the renal cones there is an infiltrate of heterophils both into the interstitium as well as tubule lumens with an associated injury to the lining cells, which could have been due to an ascending infection.
- Bird 5866 (1.39 mg/kg), bird 5863 (5.11 mg/kg), bird 5867 (69.3 mg/kg), bird 5862 (control) and bird 5869 (control) all showed moderate congestion of the liver and kidneys and in addition had focal lymphocyte aggregate in the liver.
- Bird 5864 (control), 5868 (control) and 5870 (18.8 mg/kg) all had moderate congestion of the liver and kidneys but in addition, 5864 and 5870 had mild vacuolar change in the liver.
- Bird 5865 (control) showed marked congestion of the kidneys. It also exhibited moderate congestion with mild fatty change in the liver.
- Bird 5853 (388 mg/kg) and 5860 (150 mg/kg) appeared normal with no pathology evident.

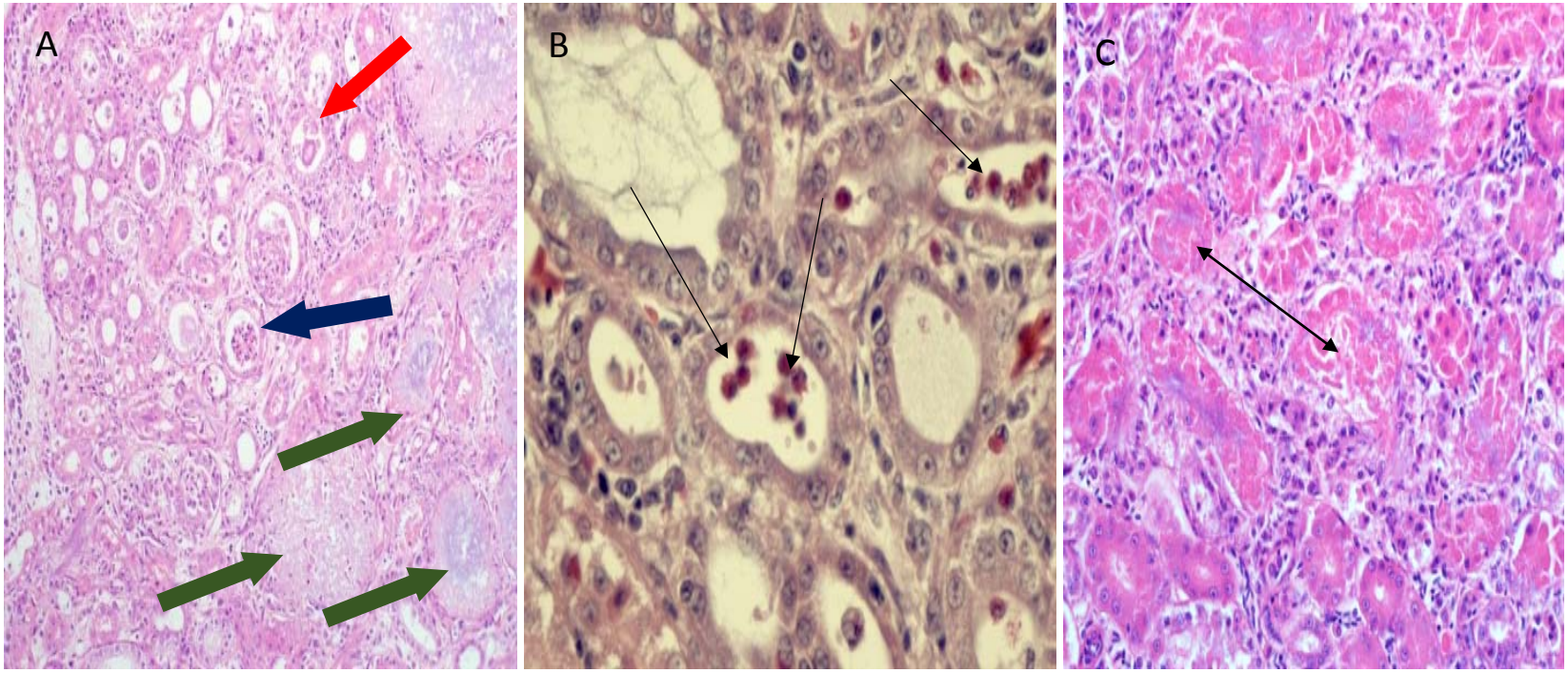


Figure 4.7: (A)Kidney HE x 20 (B) Kidney HE x 40 (C) Kidney HE x 40 from dosed ducks that succumbed. (A) Obliteration of the Bowman's capsule associated with deposit of urate crystals (green block arrows), globoid shaped urate crystal within the renal tubule (red block arrow) with cellular cast within the collecting duct (purple block arrow), (B) heterophils within the renal tubules (line arrows), (C) loss of renal architecture with swollen tubules associated with eosinophilic fragmented cytoplasm and nuclear debris (double head arrow)

Table 4.3: Body weight measurement in Muscovy duck.

Animal ID	Dosage (mg/kg)	Body weight (kg)			
		Day 0	Day 4	Day 8	Day 15
5862	control	1.78	1.82	2.12	1.7
5864	control	2.04	2	2.18	2.01
5865	control	1.97	1.85	1.93	2.02
5868	control	2.03	2.02	2.04	1.83
5869	control	1.97	1.98	2.05	1.99
5866	1.39	1.68	1.67	1.61	1.58
5863	5.11	2.31	2.48	2.72	2.77
5870	18.8	1.86	1.82	1.84	1.66
5855	45.6	1.7	1.68		
5859	57.8	1.76	1.69		
5867	69.3	1.99	1.87	1.8	1.84
5852	73.4	1.51	1.46	1.42	1.54
5857	93.1	1.85	1.88	1.87	1.93
5851	118	1.38	1.75	1.9	2.06
5860	150	1.89	1.91	1.7	1.85
5854	190	1.73			
5858	241	1.7			
5861	306	1.81	1.6		
5853	388	2.91	2.89	2.97	3.14

Highlighted cells, indicate the birds that died during the study

4.2.3 Pharmacokinetics

From the pharmacokinetic data generated (Table 4.4), there was evidence of a large variation between the birds in the metabolism of diclofenac, as evident from the half-life of elimination. These variations were consistent even when the plasma concentration were equalised to the dose administered. In all cases, the plasma concentrations were at their maximum at the first sampling at 2 h (Figure 4.8). The C_{max} attained, AUC and elimination half-life do not clearly differentiate between poisoning and survival. For all the birds dosed, the extent of drug absorption was high with a short half-life of elimination of less than 3 hours and mean residence time within 2-3 hours. Liver and kidney tissues from all the birds had no detectable residues of diclofenac parent molecule following HPLC analysis.

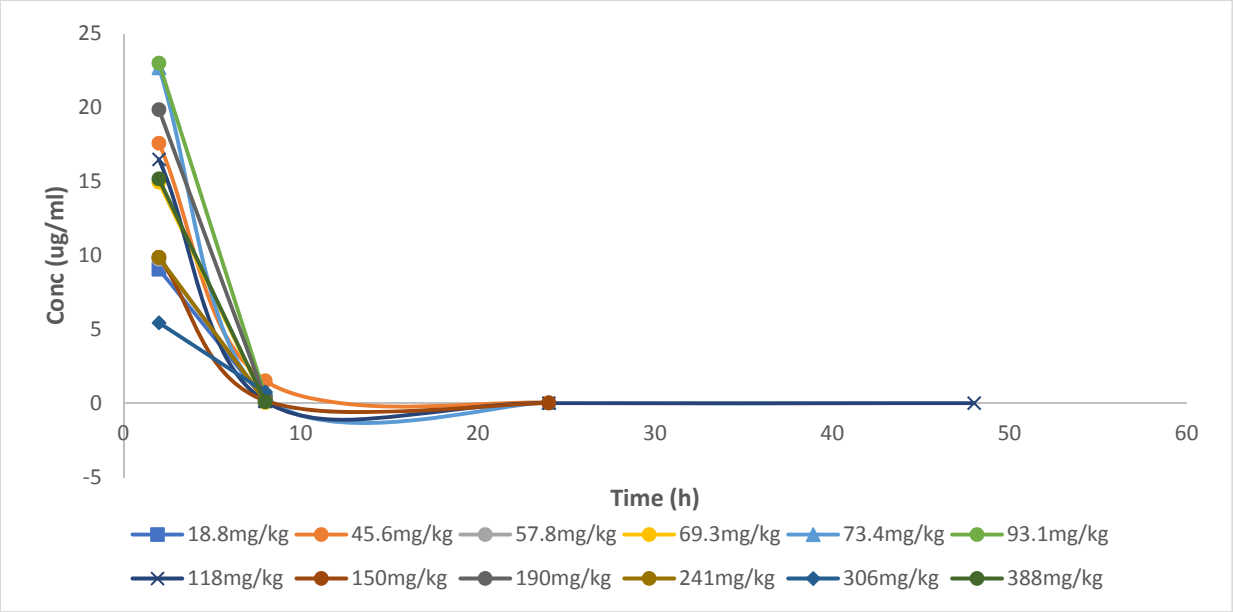


Figure 4.8: Diclofenac disposition curve following oral dosing in Muscovy ducks.

Table 4.4: Pharmacokinetic data of diclofenac following oral dosing in Muscovy duck

Animal	Dose	C_{max}	T_{max}	AUC_{last}	AUC_{tot}	L_z	$AUMC_{last}$	$T_{1/2}$	MRT	Clearance	V_z	V_{ss}
	mg/kg	µg/mL	h	µg/mL*h	µg/mL*h	1/h	µg/mL*(h)²	h	h	L/h*kg	L/kg	L/kg
5870	18.8	9.07	2	36.79	37.06	0.66	76.73	1.05	2.14	0.51	0.77	1.09
5855	45.6	17.59	2	87.53	87.69	0.26	284.26	2.7	3.29	0.52	2.03	1.71
5859	57.8	9.73	2	39.63	39.99	0.62	83.39	1.11	2.17	1.45	2.32	3.14
5867	69.3	14.95	2	59.95	59.99	0.98	120.66	0.71	2.02	1.16	1.18	2.33
5852	73.4	22.69	2	95.95	96.11	0.24	230.32	2.86	2.44	0.76	3.16	1.87
5857	93.1	23.02	2	93.52	94.28	0.64	195.83	1.08	2.15	0.99	1.54	2.13
5851	118	16.51	2	67.83	67.83	0.3	154.42	2.3	2.28	1.74	5.78	3.96
5860	150	9.87	2	41.44	41.49	0.26	97	2.71	2.37	3.62	14.11	8.57
5854	190	19.86	2	80.45	80.94	0.68	166.9	1.02	2.12	2.35	3.44	4.97
5858	241	9.87	2	39.9	40.09	0.71	82.33	0.98	2.1	6.01	8.49	12.62
5861	306	5.44	2	24.06	26.39	0.33	61.84	2.12	3.32	11.6	35.39	38.47
5853	388	15.17	2	60.99	61.11	0.84	123.74	0.82	2.04	6.35	7.56	12.97

C_{max}: Maximum plasma concentration, T_{max}: Time to maximum concentration, AUC_{last}: Area under curve to the last quantifiable time point, AUC_{tot}: Total area under curve extrapolated to infinity, Lz (λ): Terminal elimination rate constant, $AUMC_{last}$: The area under the moment curve from the time point zero to the last measured time point, $T_{1/2}$: Half-life, MRT: Mean residence time, Cl: Clearance, V_z: Volume of distribution during terminal phase, V_{ss}: Volume of distribution at steady state.
Highlighted cells: indicate the animals that died during the study

4.2.4 Clinical chemistry analysis

Table 4.5: Changes in plasma uric acid concentration in Muscovy ducks

ID	Dosage(mg/kg)	Uric Acid (mMol/l)				
		0 h	2 h	8 h	24 h	48 h
5862	Control	0.553	0.113	0.177	0.17	0.19
5864	Control	0.205	0.13	0.144	0.13	0.238
5865	Control	0.605	0.202	0.169	0.217	0.228
5868	Control	0.244	0.153	0.211	0.182	0.267
5869	Control	0.391	0.299	0.297	0.379	0.429
5866	1.39	0.291	0.192	0.158	0.11	0.15
5863	5.11	0.223	0.238	5.602	0.54	0.621
5870	18.8	0.288	0.191	0.316	3.838	0.453
5855	45.6	0.192	0.368	4.336	5.749	9.38
5859	57.8	0.23	0.29	0.774	5.133	7.85
5867	69.3	0.361	0.303	0.502	0.166	0.229
5852	73.4	0.201	0.578	4.649	6.333	4.27
5857	93.1	0.225	0.333	3.717	4.957	1.418
5851	118	0.173	0.299	0.272	0.454	1.446
5860	150	0.253	0.564	0.744	0.487	0.344
5854	190	0.164	1.018	4.48	1.072	14.36
5858	241	0.192	0.453	1.21	6.107	7.915
5861	306	0.145	0.156	0.283	0.364	0.318
5853	388	0.099	0.587	0.322	0.773	0.452

Highlighted cells: indicate the animals that died during the stud

Following plasma uric acid analysis, all birds (test and control) showed a relatively uniform concentration at 0 h of sampling before exposure to the test substance. The control birds showed uniformly steady concentration throughout the study (i.e. from 0 – 48 h of dosing). For the test birds, there was a progressive and consistent increase in plasma uric acid levels from 2 h after dosing up until 24 h of dosing. At 48 h after dosing there was a sharp decline in plasma uric acid concentrations in birds that survived, while the birds that succumbed, there was a further increase in plasma uric acid concentrations (Figure 4.9). The 48-h plasma uric acid concentration of the five test birds that died following diclofenac dosing was extremely high (7.96 ± 5.03 mMol/l) while those of the remaining test birds that survived were comparably low (1.04 ± 1.3 mMol/l). Of the test birds that survived the study, bird 5852 (73.4 mg/kg) had a relatively high plasma uric acid concentration of 4.27 mmol/l at 48 h after dosing (Table 4.5). The 48 h Na^+ and K^+ electrolyte analysis revealed relatively normal levels in birds that succumbed in comparison to the control birds which had 136.8 ± 4.63 mMol/l and 2.46 ± 0.39 mMol/l respectively. The only exception is the modest rise in K^+ concentration noticed in one of the birds that succumbed (190 mg/kg; Bird 5854) (Table 4.7).

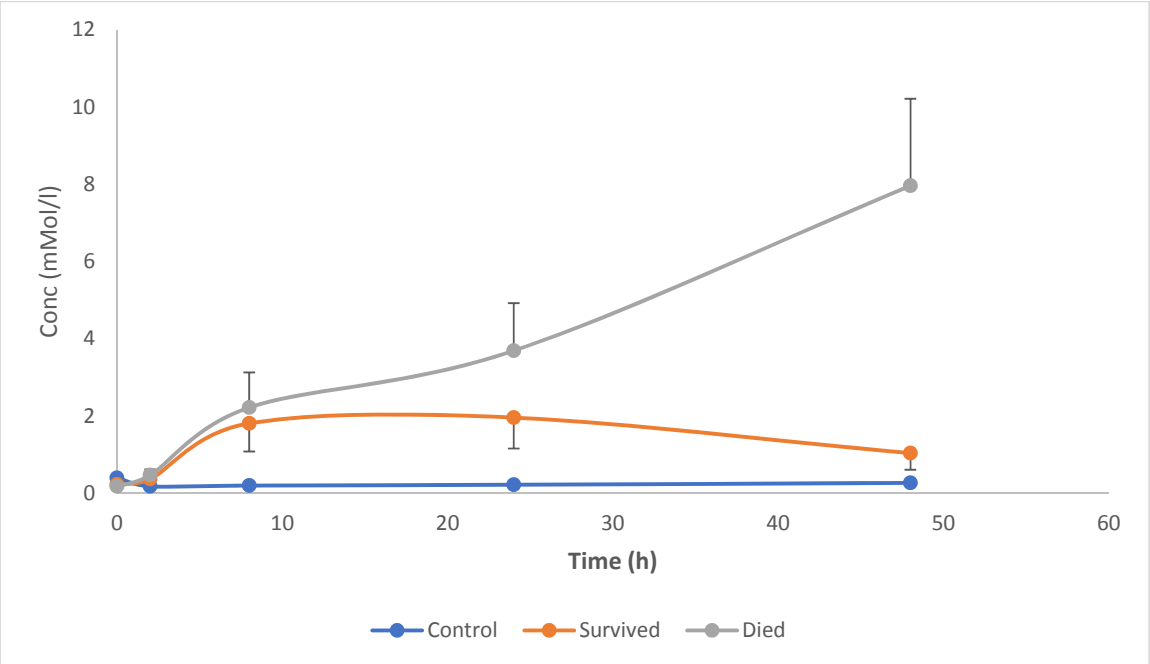


Figure 4.9: Showing changes in plasma uric acid concentration in Muscovy ducks

Table 4.6: Showing standard deviation/error of changes in plasma uric acid concentration in Muscovy ducks.

Time (h)	Control (mMol/l)			Survived (mMol/l)			Died (mMol/l)		
	Mean	S.D	S.E	Mean	S.D	S.E	Mean	S.D	S.E
0	0.4	0.18	0.08	0.23	0.08	0.03	0.18	0.03	0.01
2	0.18	0.07	0.03	0.37	0.17	0.06	0.47	0.33	0.15
8	0.2	0.06	0.03	1.81	2.19	0.73	2.22	2.03	0.91
24	0.22	0.1	0.04	1.96	2.4	0.8	3.69	2.74	1.23
48	0.27	0.09	0.04	1.04	1.3	0.43	7.96	5.03	2.25

Table 4.7: Plasma concentration of electrolytes.

Electrolyte (mMol/l)	Dose of diclofenac (mg/kg) associated with mortality					Control				
	190	45.6	241	57.8	306	1	2	3	4	5
Na^+	133.2	137.5	127.6	130.6	135.5	129.4	135.5	139.7	141.2	138.1
K^+	5.52	2.19	2.25	2.76	1.95	1.89	2.23	2.69	2.76	2.74

4.2.5 Evaluation on the potential reason why some ducks recovered

To better understand the reasons why some of the Muscovy ducks with the high uric acid at 24 h were able to recover, while all the vultures with high uric acid at the same time died, we attempted to better understand the capacity of duck plasma for uric acid. The evaluation showed that their plasma electrolytes (Na^+ / K^+) concentrations were in general lower in comparison to the chicken and Cape vulture. In contrast the total plasma protein and albumin levels were much higher in the ducks, followed by the vultures which were in turn closely followed by the chicken. The ratio of albumin concentration duck, chicken and vulture were 3.1 : 1.9 : 2. The same trend was evident for the maximum solubility of uric acid in plasma at room temperature with concentration being 8.06 ± 0.2 mMol/l in comparison to 6.98 ± 0.33 mMol/l for the vulture (Table 4.8).

Table 4.8: Clinical pathology parameters of untreated Muscovy duck, Chicken and Cape vulture in relationship to saturation capacity for uric acid

Species	Max uric acid (mMol/L)	Na (mMol/l)	K (mMol/l)	Protein (g/l)	Pre-alb (g/l)	Alb (g/l)
Duck	8.06 ± 0.2	135.98 ± 0.24	2.03 ± 0.01	41.52 ± 0.25	1.5 ± 1.79	12.34 ± 0.95
Chicken	5.83 ± 0.48	147.94 ±0.34	7.55 ± 0.03	27.06 ± 0.23		7.76 ± 1.95
Vulture	6.98 ± 0.33	147.68 ± 0.33	4.55 ± 0.01	30.7 ± 0.33	2.76 ± 2.34	8.14 ± 1.31

Alb: Albumin; Pre-alb: Pre-albumin.

4.3 Domestic pigeon acute oral toxicity study

4.3.1 Clinical signs

None of the birds succumbed following their exposure to diclofenac. However, after the oral gavage many of the birds were seen regurgitating. Within one hour the birds dosed at 72.3, 116 and 238 mg/kg regurgitated. Subsequently, the birds dosed at 302, 383, 486 and 616 mg/kg all followed suit but a little bit longer, albeit all within 2 h after dosing. Approximately 2 h after dosing one of the control birds (red) and one test bird (05985, 29.9 mg/kg) regurgitated fluid and feed respectively during their restraint for blood collection. At approximately 3 h and 5 h after dosing, bird 24983 (616 mg/kg) was seen making attempts to regurgitate. Again, at about 7 h after dosing, birds 7742 (8.13 mg/kg), BR7 (110 mg/kg), 05985 (29.9 mg/kg), 02672 (148 mg/kg), 01764 (486 mg/kg), yellow (72.3 mg/kg), 05490 (91.8 mg/kg), 1621 (116 mg/kg), 4355 (187 mg/kg), 13736 (383 mg/kg) and 24983 (616 mg/kg) all regurgitated feed during their restraint for blood collection. At approximately 24 h after dosing, birds 7745 (2.21 mg/kg), 05490 (91.8 mg/kg), and 01764 (486 mg/kg) all regurgitated feed during the course of restraint for blood collection. At about 31 h after dosing, bird 01764 (486 mg/kg) was seen attempting to regurgitate. Except for the slight loss of body weight which was observed just after dosing/series of blood collection, all birds remained active and alert throughout the study. Their body weights were steady and increasing except for the few cases where there was a slight loss of body weight in both test and control birds (Table 4.9). Feed consumption was relatively steady and increasing during the study, but dropped on days when birds were fasted, dosed or blood was collected. There were also few other cases where feed consumption dropped.

Table 4.9: Body weight measurement in domestic pigeons

Animal ID	Dose (mg/kg)	Body weight (g)			
		Day 0	Day 4	Day 8	Day 15
RED	control	395	368	401	415
5415	control	439	442	439	445
4683	control	445	441	465	474
133	control	481	466	497	497
3835	control	419	403	412	435
7745	2.21	421	417	428	474
7742	8.13	470	454	458	488
5985	29.9	497	496	508	531
YELLOW	72.3	419	415	423	414
5490	91.8	404	410	443	433
BR7	110	513	496	507	518
1621	116	515	504	515	506
2672	148	433	413	430	435
4355	187	504	486	497	497
10527	238	427	440	420	416
11952	302	394	390	411	428
13736	383	432	381	437	441
1764	486	517	495	519	542
24983	616	511	457	496	488

4.3.2 Pathology

At necropsy, no gross pathologic lesions were observed in any of the birds. Numerous non-specific microscopically changes were evident in the various birds inducted into the study; All the birds showed a moderate congestion of their kidneys except for 00133 (control) which showed a marked congestion. In addition, birds yellow (72.3 mg/kg), 05490 (91.8 mg/kg), 1621 (116 mg/kg), 02672 (148 mg/kg), 4355 (187 mg/kg), 10527 (238 mg/kg), 11952 (302 mg/kg), 13736 (383 mg/kg), 01764 (486 mg/kg), and 24983 (616 mg/kg) exhibited scattered foci of lymphocytes in their kidneys. The livers from all the birds except for 00133 (control) and 4355 (187 mg/kg) showed moderate congestion with scattered foci of lymphocytes. In addition, bird 11952 (302 mg/kg) had mild fatty change of the liver. For bird 00133, there was marked

congestion with scattered foci of lymphocytes while in the case of bird 4355, there was mild vacuolar change with scattered foci of lymphocytes.

Moderate congestion of the spleen was noticed in birds 3835 (control), 7745 (2.21 mg/kg), 7742 (8.13 mg/kg), BR7 (110 mg/kg), 5415 (control), 04683 (control), 05985 (29.9 mg/kg), red (control), 24983 (616 mg/kg), yellow (72.3 mg/kg), 11952 (302 mg/kg), and 01764 (486 mg/kg). In addition, birds 24983 (616 mg/kg), yellow (72.3 mg/kg), 11952 (302 mg/kg), and 01764 (486 mg/kg) had prominent white pulp. Marked congestion was observed in the spleen of birds 00133 (control) and 02672 (148 mg/kg) with that of the latter exhibiting prominent white pulp. The rest of the birds (4355 -187 mg/kg; 13736 - 383 mg/kg; 1621 - 116 mg/kg; 10527 - 238 mg/kg and 05490 - 91.8 mg/kg) had prominent white pulp of the spleen. There was also a marked (bird 1621) and moderate (birds 10527 & 05490) diffuse infiltrate of small lymphocytes into the red pulp.

4.3.3 Pharmacokinetics

From the pharmacokinetic data generated (Table 4.10) and diclofenac disposition curve (Figure 4.10), maximum plasma concentration was achieved at the first sampling point i.e. 2 h after dosing. Diclofenac was well absorbed and distributed following oral gavage. The half-life of elimination was generally below 6 h. In general the maximum plasma concentration reached and extent of drug absorption were directly proportional to the dosage received. None of the pigeons had detectable residues of diclofenac parent molecule in their tissues (liver and kidney) following HPLC analysis.

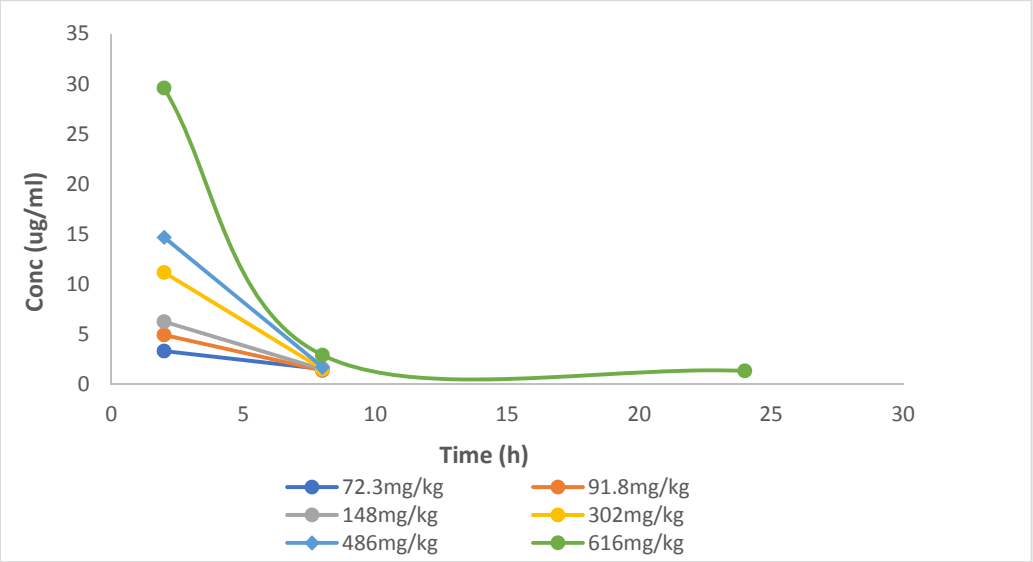


Figure 4.10: Diclofenac disposition curve following oral dosing in domestic pigeons

Table 4.10: Pharmacokinetic data of diclofenac following oral dosing in Domestic pigeon

Animal	**Dose**	**C_{max}**	**T_{max}**	**AUC_{last}**	**L_z**	**$T_{1/2}$**	**Clearance**	**Vd**
	mg/kg	µg/mL	h	µg/mL*h	1/h	h	L/h*kg	L/kg
Yellow	72.3	3.33	2	33.29	0.13	5.35	2.17	16.75
5490	91.8	4.94	2	35.67	0.21	3.28	2.57	12.18
2672	148	6.26	2	42.33	0.24	2.91	3.5	14.68
11952	302	11.17	2	65.4	0.33	2.09	4.62	13.94
1764	486	14.69	2	84.06	0.36	1.94	5.78	16.22
24983	616	29.58	2	279.1	0.14	4.94	2.21	15.73

C_{max}: Maximum plasma concentration, T_{max}: Time to maximum concentration, AUC_{last}: Area under curve to the last quantifiable time point, Lz: Terminal elimination rate constant, $T_{1/2}$: Half-life, Cl: Clearance, Vd: Volume of distribution. The birds omitted from the analysis had only one measurable time point which precluded pharmacokinetic analysis.

5 DISCUSSION

5.1 Clinical signs of toxicity

The signs presented by the Japanese quails and Muscovy ducks in this study were similar to those demonstrated by diclofenac exposed *Gyps* vultures and chicken viz. depression and acute death (Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b; Swan *et al.,* 2006b). While Japanese quails that succumbed only showed evidence of subtle depression at about 24 - 48 h and death at approximately 48 h of dosing, the Muscovy ducks exhibited a much more delayed episode of depression (approximately 48 - 72 h) that progressed to coma and death. These presentations were thus different from the usual depression evident at 24 h post dosing, which progressively worsened to coma and death at 48 h in *Gyps* vultures and chicken (Swan *et al.,* 2006b; Naidoo *et al.,* 2007). The domestic pigeons surprisingly remained healthy throughout the study.

On pathologic manifestations, again as were previously reported in chickens and *Gyps* vultures, postmortem findings in the Japanese quails and Muscovy ducks were characterized by severe visceral gout associated with renal failure (Naidoo *et al.,* 2007; Oaks et al 2004; Swan *et al.,* 2006b). The proximal renal tubules were damaged and characterized by the presence of urate aggregates. However, the lesions seen in the Japanese quails were not as severe as those demonstrated in *Gyps* vulture and chicken as there were no evidence of inflammatory cells involvement which has been previously reported in the latter species. In the case of the Muscovy ducks, there were more severe renal lesions involving the proximal tubules, interstitium, collecting duct and Bowman's capsule associated with inflammatory cells. This would support the longer interval to death seen in the Muscovy ducks in comparison to the chicken, Japanese quail and *Gyps* vulture, as the birds had sufficient time to initiate an inflammatory response. However, in sharp contrast to the former species, most of the ducks that succumbed had no hepatic or splenic lesion.

Nevertheless, the similarities in clinical manifestations and post mortem findings noted may indicate a common mechanism of toxicity as NSAID's are known to inhibit the formation of prostaglandins by cyclo-oxygenase blockade (Rai *et al.,* 2015), a very vital component in

maintaining renal blood flow (Papich, 2008). Inhibition of this important process could explain the degenerative changes (nephrosis), noted in diclofenac poisoning events, mainly manifesting in the proximal renal tubule. The proximal renal tubule is particularly vulnerable to injury from insufficient and/or lack of nutrients due to its metabolically active state. It has the reputation of being one of the most metabolic active sites in the body and thus highly susceptible to ischaemia (Constable *et al.,* 2017). The resultant kidney damage leads to the clinical signs of depression, urate crystal deposition and/or lameness as plasma uric acid concentration increases above normal due to impaired tubular secretion of uric acid (Naidoo *et al.,* 2009a). Also, with the kidneys largely responsible for maintaining plasma K^+ balance, the underlining renal impairment result in hyperkalaemia which eventually leads to death due to cardiac arrest (Naidoo *et al.,* 2007). Despite the similarities highlighted, the Muscovy duck and Japanese quail proved to be more resilient as very high oral LD_{50}'s compared to the *Gyps* vultures were determined. Also important to note, was that mortalities were not related to changes in potassium as evident in the vultures.

Surprisingly, the domestic pigeons remained active and alert throughout the study and did not show any obvious signs of intoxication except for the regurgitation noted following oral gavage. Perhaps the lack of intoxication may be due to the pigeon's inherent metabolic abilities. Despite regurgitating soon after dosing, the drug was still detected in plasma up to 24 h after dosing. Diclofenac is known to be rapidly absorbed from the gastrointestinal tract (GIT) (within an hour, in the absence of feed) following oral gavage (Naidoo *et al.,* 2007) and would have been absorbed from the GIT before the episodes of regurgitation in most of the birds. This is reflected in the high level of drug absorption and high drug plasma concentration noted in them. One may argue that the birds regurgitated the drug and the study couldn't have given a true reflection of diclofenac's toxicity in this species. However, it is important to note that only a few of the birds reacted within an hour of dosing, the remainder of the birds only exhibited signs later. Moreover, diclofenac is known to elicit gastric ulceration as a side effect (Aliu, 2007) and may explain the observations seen. In addition, the stress from handling/restraint could also trigger regurgitation as the birds were being bled at time intervals for pharmacokinetic evaluation of diclofenac.

5.2 Clinical pathology

On clinical pathology, most of the Muscovy ducks that succumbed to diclofenac's toxic effects showed a consistent rise in plasma uric acid concentration right from onset of dosing to the last bleeding time at 48 h. Although the rise is consistent with diclofenac's toxicity in chickens and *Gyps* vultures (Oaks *et al.,* 2004; Swan *et al.,* 2006b; Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b), the interesting point to note was that despite the increase in plasma uric acid concentration far above those reported to kill *Gyps* vultures and chickens, only one of the Muscovy ducks (which had the highest concentration) had shown signs of intoxication at the last blood collection time (48 h). The rest remained active and alert for another 12-24 h before expressing intoxication. This probably coincided with a further rise in plasma uric acid concentration that could no longer be handled by the body and may explain the delayed appearance of depression/lameness noted in this species as against the vultures and chickens. The finding in the duck thus brings across another interesting finding, in the temporal relationship between drug exposure and clinical signs i.e. clinical signs are clearly linked to uric acid concentrations.

Uric acid is a major by-product of protein metabolism (Lumeij, 1994) which is mainly excreted in birds by active renal tubular secretion via the para-amino hippuric acid channel (Naidoo et al 2009a). Uric acid is primarily present in the form of monosodium urate in plasma and its solubility in plasma depends on body temperature, plasma sodium ion concentration and the amount of plasma protein, most especially albumin, present. The higher these parameters are, the more soluble uric acid is in the body (Lumeij, 1994; Seaton, 1996). Once the plasma uric acid saturation point (limit of solubility) is exceeded, urate precipitates are formed and deposited (as gout) in tissues. The latter was evident in the birds that died, they showed both high plasma uric acid concentrations and tissue damage, and was similar to the effects observed in the vultures, as the increase in uric acid was soon followed by death and signs of severe gout (Swan *et al.,* 2006b; Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b).

The absence of this effect in birds that demonstrated levels of 6.3 mMol/l was a surprising finding. Based on the principle of Lumeij (1994), we take this as an indication that the birds recovered as saturation of the plasma had not yet occurred, with the result that there was no uric acid precipitation and irreversible tissue damage. This was supported by the *in vitro* study, which

demonstrated a maximum uric acid solubility of 8.06 ± 0.2 mMol/l, which as a matter of fact is not even a true reflection of the maximum limit of solubility as one can expect even greater solubility at the higher normal avian body temperature. It should also be noted that the maximum solubility in the vulture was lower at 6.98 ± 0.33 mMol/l, which may also to some extent explain their sensitivity to the effect of diclofenac. The reason for the difference may result from a lower plasma albumin and Na^+ concentration in vultures in comparison to Muscovy ducks.

The decrease seen in uric acid concentrations in some of the birds, after 24 h without death, was not something that was ever seen in exposed vultures. The decline in concentration towards normal, may therefore indicate a restoration of renal function with subsequent survival. Two potential reasons may explain this recovery. Firstly, this suggests that the drug induces the increase in plasma uric acid concentrations via a mechanistic manner. From human literature, diclofenac is known to inhibit the uric acid channels in the kidney (Khamdang *et al.,* 2002), which may suggest that the drug functions in a similar manner in birds. If this is the case, the degree of inhibition of the channels may be more pronounced in the vulture, which may explain their higher sensitivity. However, since some of the birds that recovered did still show signs of pathology, it could also be indicative that the drug only induced partial renal damage in the said birds, and that the recovery was from the functional renal reserves which can be as high as 70% or more in some bird species (Lierz, 2003).

While the above changes in uric acid could not be evaluated in the Japanese quail due to a paucity in sample volumes (the 200 µl collected being subjected to toxico-kinetic analysis), the presence of extensive urate deposit (gout) in the carcasses of Japanese quails and Muscovy ducks that succumbed translates to uric acid accumulation within the plasma (i.e. plasma saturation) (Echols, 2006; Merriman and Dalbeth, 2011), a common characteristic of diclofenac poisoning in *Gyps* vultures (Oaks *et al.,* 2004; Shultz *et al.,* 2004; Swan *et al.,* 2006b; Naidoo *et al.,* 2009b) and chicken (Naidoo *et al.,* 2007).

5.3 Pharmacokinetics

For a comparison of the pharmacokinetic parameters between doses and species, the applicable parameters were equilibrated to 1 mg/kg. The pharmacokinetic data as reflected in table 5.1

indicates that the AUC in the pied crow (Naidoo *et al.,* 2011), Japanese quail, Muscovy duck and domestic pigeon are relatively lower compared to what was obtained in the chicken and *Gyps* vulture (Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b). This would indicate that the extent of drug absorption and thus extent of exposure to the said drug is much higher in the chicken and *Gyps* vulture compared to the former species. Further to this, it also tends to suggest that there is some degree of pre-systemic elimination following dosing. Interestingly, the observed volume of distribution of diclofenac in the chicken and *Gyps* vulture (Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b) were quite negligible compared to estimates in the pied crow (Naidoo *et al.,* 2011), Japanese quail, Muscovy duck and pigeon which may indicate that the absorbed drug is either rapidly excreted or that the drug is overall poorly absorbed in the latter species. At this point, the reason for the poor volume of distribution in the vulture remains unexplained. However, the poor clearance of the drugs is probably contributory. The three bird species used in this study demonstrated a much more rapid clearance (at least 108-fold higher) and a shorter biological half-life of elimination (at least 2-fold lower) compared to *Gyps* vultures (Naidoo *et al.,* 2009b) which tends to suggest an inferior metabolic capacity or elimination in the latter species. In general, the pied crow demonstrated the fastest clearance rate with a biological half-life of elimination fairly similar to those of Japanese quail, pigeon and Muscovy duck (Naidoo *et al.,* 2011). From the above information and available toxicity data (Table 2.2), diclofenac's toxicity is clearly linked to the extent of exposure to the drug and inability of the body to eliminate the drug in a timely manner. The higher the extent of exposure and time taken to metabolize the drug by a species, the more toxic it becomes, which was the case between *Gyps* vulture and bird species used for this study.

Table 5.1: Average equalized pharmacokinetic parameters in bird species following exposure to diclofenac

Species	C_{max}	AUC_{last}	$T_{1/2}$	$AUMC_{last}$	Cl	MRT	Vd	References
	µg/mL	µg/mL*h	h	µg/mL*(h)²	L/h*kg	h	L/kg	
Quail	0.03	0.19	3.41	0.74	7.29	3.82	30.74	Present study
Quail	0.02	0.18	6.68	1.48	8.47	8.45	80.12	Present study
Duck	0.21	0.88	1.65	1.91	2.16	2.21	4.88	Present study
Duck	0.14	0.65	1.58	1.82	4.38	2.6	10.33	Present study
Pigeon	0.04	0.33	3.42		3.48		14.92	Present study
Chicken	2.64	5.41	0.89		0.19		0.24	Naidoo *et al.*, 2007
Chicken		1.26	14.34		0.65			Naidoo *et al.*, 2007
AWBV		77.44	16.78	1357.2	0.02	26.1	0.3	Naidoo *et al.*, 2007
CGV		100.35	12.24	1020.25	0.01	15.11	0.18	Naidoo *et al.*, 2009b
Turkey vulture		5.65	6.29		0.26			Rattner *et al.*, 2008
Turkey vulture		1.73	6.43		0.79			Rattner *et al.*, 2008
Pied crow	0.01	0.05	2.33	0.3	17.36		58.35	Naidoo *et al.*, 2011

C_{max}: Maximum plasma concentration, AUC_{last}: Area under curve to the last quantifiable time point, $T_{1/2}$: Half-life, $AUMC_{last}$: The area under the moment curve from the time point zero to the last measured time point, Cl: Clearance, MRT: Mean residence time,Vd: Volume of distribution. AWBV: African White Backed Vulture; CGV: Cape Griffon Vulture. Highlighted cells indicate birds that died

The data (Table 5.2), also showed an increasing tendency towards a superior metabolic ability/capacity among the bird species with every decrease in average body weight. The same was true for body clearance rates which also increased with every decrease in average body weight. Correspondingly, the time taken to metabolize the drug (denoted by $T_{1/2}$) decreases with decreasing average body weight. This conforms with the allometric theory of larger species of animals having a relatively slower metabolic rate when compared to smaller species (Munshi-South and Wilkinson, 2010). However, in an unexpected manner, the Muscovy ducks (large species) was an exception to this rule as they demonstrated a shorter half-life of elimination compared to the Japanese quails/pigeons (small species) despite the latter's higher volume of distribution and more rapid clearance rate.

Table 5.2: Comparing pharmacokinetic parameters in relation to species average body weight.

Specie	Average body weight (kg)	CL (L/h*kg)	$T_{1/2}$ (h)	Reference
Japanese quail	0.3	7.47 ± 5.80	3.91 ± 1.93	Present study
Domestic pigeon	0.4	3.48 ± 1.47	3.42 ± 1.43	Present study
Muscovy duck	1.8	3.09 ± 3.35	1.62 ± 0.84	Present study
Leghorn chicken	1.8	0.65	14.34	Naidoo *et al*., 2007
African White Backed Vulture	4.5	0.02	16.78	Naidoo *et al*., 2009b

Cl: Clearance rate, $T_{1/2}$: Half-life

5.4 Toxicity

The established oral median lethal dose of diclofenac in the three bird species from this study were much higher than those reported in *Gyps* vultures and the chicken. While the Japanese quail and Muscovy duck had an estimated oral LD_{50} of 405 mg/kg and 190 mg/kg respectively, the pigeon like the pied crow and turkey vulture, were resistant to diclofenac's toxic effects (Naidoo *et al.,* 2011; Rattner *et al.,* 2008). These variations could be due to the species peculiar metabolic abilities and/or susceptibility, which tends to suggest that diclofenac's toxicity may not be predictable from one avian species to another. A comparison of diclofenac's LD_{50} across these species of bird that demonstrated toxicity showed no direct relationship between toxicity and body mass i.e. *Gyps* vultures (4.5kg) 0.1 mg/kg < chicken (1.8kg) 10 mg/kg < Muscovy duck (1.8kg) 190 mg/kg < Japanese quail (0.3kg) 405 mg/kg (Swan *et al.,* 2006b; Naidoo *et al.,* 2007; Present study). The unexpected result was the high LD_{50} in the Muscovy ducks, which had an LD_{50} that was almost 20-fold higher than the chicken, which is of a similar weight to the duck. It is not exactly clear why this was the case. More so, the intoxication in the ducks did not appear to be from any metabolic constraints as the birds that died did not demonstrate major differences in metabolism of the drug i.e. the zero-order kinetics and change in half-life seen between surviving and dead vultures exposed to ketoprofen was not evident. In contrast, it should be noted that a relationship was present between the half-life, elimination and toxicity in the quail in this study in the birds that died compared to the birds that survived (longer half-life in the birds that died). This would thus suggest, as mentioned above, that toxicity may be more mechanistic in effect such as decrease in blood supply to the kidney or altered function or uric acid changes

in the kidney with metabolic capacity dictating the overall susceptibility as opposed to being the driver of toxicity.

6 CONCLUSION

The outcome from this study suggests that the bird species used have very high metabolic capacity with wide variations; and with the observed lethal doses in them being far much greater than what has been established in *Gyps* vultures, it is safe to conclude that the use of these bird species as models would not have predicted diclofenac's environmental effects on *Gyps* vultures. However, the similarities in the clinical manifestations and pathology suggest a common mechanism of intoxication which thus strengthens the possibility of using these birds as models to better understand the pathophysiology behind diclofenac-induced intoxication in vultures.

7 REFERENCES

Abbasi, T., Premalatha, M. and Abbasi, S.A. (2011) 'The return to renewables: Will it help in global warming control?', *Renewable and Sustainable Energy Reviews,* 15(1), pp. 891-894. doi: http://dx.doi.org/10.1016/j.rser.2010.09.048.

Akhila, J.S., Shyamjith, D. and Alwar, M. (2007) 'Acute toxicity studies and determination of median lethal dose', *Current Science,* 93(7), pp. 917-920.

Aktar, W., Sengupta, D. and Chowdhury, A. (2009) 'Impact of pesticides use in agriculture: their benefits and hazards', *Interdisciplinary Toxicology,* 2(1), pp. 1-12.

Aliu, Y.O. (2007) 'Veterinary Pharmacology', First edition, Tamaza Publishing Company Limited, Zaria-Nigeria

Almeida, D., Maldonado, E., Khan, I., Silva, L., Gilbert, M.T., Zhang, G., Jarvis, E.D., O'Brien, S.J., Johnson, W.E. and Antunes, A. (2016) 'Whole-Genome Identification, Phylogeny, and Evolution of the Cytochrome P450 Family 2 (CYP2) Subfamilies in Birds', *Genome Biology and Evolution,* 8(4), pp. 1115-1131. doi: 10.1093/gbe/evw041 [doi].

Amezian, M. and Khamlichi, R.E. (2016) 'Significant population of Egyptian Vulture Neophron percnopterus found in Morocco', *Ostrich,* 87(1), pp. 73-76.

Androutsopoulos, V.P., Hernandez, A.F., Liesivuori, J. and Tsatsakis, A.M. (2013) 'A mechanistic overview of health associated effects of low levels of organochlorine and organophosphorous pesticides', *Toxicology*, 307, pp 89-94.

Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J. and Makynen, E.A. (2001) 'Description and evaluation of a short-term reproduction test with the fathead minnow (Pimephales promelas)', *Environmental Toxicology and Chemistry,* 20(6), pp. 1276-1290.

Arad, Z., Midtgård, U. and Bernstein, M.H. (1989) 'Thermoregulation in Turkey Vultures. Vascular anatomy, arteriovenous heat exchange, and behavior', *Condor,* 91(3), pp. 505-514.

Austin, M.E., Kasturi, B.S., Barber, M., Kannan, K., MohanKumar, P.S. and MohanKumar, S.M. (2003) 'Neuroendocrine effects of perfluorooctane sulfonate in rats', *Environmental Health Perspectives,* 111(12), pp. 1485-1489.

Azevedo, M.S., De La Côrte, F.D., Brass, K.E., Dalmora, S.L., Machado, F.T., Pompermayer, E., Dau, S.L. and Santa'Ana, L.A. (2013) 'Bioavailability and Tolerability of Topical and Oral Diclofenac Sodium Administration in Healthy Ponies', *Journal of Equine Veterinary Science,* 33(1), pp. 22-26. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.jevs.2012.04.013.

Badwaik, H. and Tripathi, D.K. (2012) 'In vitro evaluation of commercially available enteric coated tablet containing diclofenac sodium', *Inflammation,* 3(2), pp. 875-881.

Balmford, A. (2013) 'Ecology. Pollution, politics, and vultures', *Science (New York, N.Y.),* 339(6120), pp. 653-654. doi: 10.1126/science.1234193 [doi].

Belle, D.J., Callaghan, J.T., Gorski, J.C., Maya, J.F., Mousa, O., Wrighton, S.A. and Hall, S.D. (2002) 'The effects of an oral contraceptive containing ethinyloestradiol and norgestrel on CYP3A activity', *British Journal of Clinical Pharmacology*, *53*(1), pp.67-74.

Benson, E.P. (1996) 'The vulture: The sky and the earth', *Palenque Round Tables,* 10, pp. 309-319.

Berny, P. (2007) 'Pesticides and the intoxication of wild animals', *Journal of Veterinary Pharmacology and Therapeutics,* 30(2), pp. 93-100.

Berthoud, A., Maupu, P., Huet, C. and Poupart, A. (2011) 'Assessing freshwater ecotoxicity of agricultural products in life cycle assessment (LCA): a case study of wheat using French agricultural practices databases and USEtox model', *The International Journal of Life Cycle Assessment,* 16(8), pp. 841-847.

Bhalla, R., Tehrani, R. and Van Aken, B. (2016) 'Toxicity of hydroxylated polychlorinated biphenyls (HO-PCBs) using the bioluminescent assay Microtox®', *Ecotoxicology,* 25(7), pp. 1438-1444.

Blikslager, A. and Jones, S. (2005) 'NSAIDs', *Journal of Equine Veterinary Science,* 25(3), pp. 98-102. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.jevs.2005.02.004.

Bohannon, M.E. (2014) 'Effects of PCB exposure on hepatic gene expression and enzyme activity in avian species', Doctoral dissertation, University of Maryland, College Park.

Bojes, H.K. and Pope, P.G. (2007) 'Characterization of EPA's 16 priority pollutant polycyclic aromatic hydrocarbons (PAHs) in tank bottom solids and associated contaminated soils at oil exploration and production sites in Texas', *Regulatory Toxicology and Pharmacology,* 47(3), pp. 288-295. doi: http://dx.doi.org/10.1016/j.yrtph.2006.11.007.

Brezovšek, P., Eleršek, T. and Filipič, M. (2014) 'Toxicities of four anti-neoplastic drugs and their binary mixtures tested on the green alga Pseudokirchneriella subcapitata and the cyanobacterium Synechococcus leopoliensis', *Water Research,* 52, pp. 168-177.

Bridgeford, P. (2004) 'Lappet-faced Vulture Torgos tracheliotos', *The Vultures of Southern Africa–Quo Vadis?,* , pp. 28.

Buckley, N.J. (1996) 'Food finding and the influence of information, local enhancement, and communal roosting on foraging success of North American vultures', *The Auk*, pp.473-488.

Bucombe A. (2012) 'The verminators: On the frontline of Mumbai's battle with 88 million rats', *Independent.* www.independent.co.uk. 18th June 2017.

Buechley, E.R. and Sekercioglu, C.H. (2016) 'Vultures', *Current Biology,* 26(13), pp. R560-R561.

Byrd, B. (2003) 'Of Love and Loathing: The Role of the Vulture in Three Cultures'.

Campbell, M. (2009) 'Factors for the presence of avian scavengers in Accra and Kumasi, Ghana', *Area,* 41(3), pp. 341-349.

Campbell, M.O. (2015) *Vultures: Their Evolution, Ecology and Conservation.* CRC Press.

Centner, T.J. (2016) 'Efforts to slacken antibiotic resistance: Labeling meat products from animals raised without antibiotics in the United States', *Science of the Total Environment,* 563, pp. 1088-1094. doi: 10.1016/j.scitotenv.2016.05.082.

Chapman, J.S. (2003) 'Disinfectant resistance mechanisms, cross-resistance, and co-resistance', *International Biodeterioration & Biodegradation,* 51(4), pp. 271-276.

Constable, P.D., Hinchcliff, K.W., Done, S.H., Grünberg, W., Colwell, D.D., Connelly, S., Dirikolu, L., Gasser, R., Hovda, L., Ikede, B., Larsen, J., Witola, W. and Woolums, A.R. (2017) ' Diseases of the Urinary System. In: Constable, P.D., Hinchcliff, K.W., Done, S.H., Grünberg, W.(Eds.), Veterinary Medicine' Eleventh edition, *W.B. Saunders,* pp. 1095-1154.

Cox, C. and Surgan, M. (2006) 'Unidentified inert ingredients in pesticides: implications for human and environmental health', *Environmental Health Perspectives,* 114(12), pp. 1803-1806.

Cuthbert, R.J., Dave, R., Chakraborty, S.S., Kumar, S., Prakash, S., Ranade, S.P. and Prakash, V. (2011) 'Assessing the ongoing threat from veterinary non-steroidal anti-inflammatory drugs to Critically Endangered Gyps vultures in India', *Oryx,* 45(3), pp. 420-426.

Cuthbert, R., Parry-Jones, J., Green, R.E. and Pain, D.J. (2007) 'NSAIDs and scavenging birds: potential impacts beyond Asia's critically endangered vultures', *Biology Letters,* 3(1), pp. 91-94.

Cuthbert, R.J., Taggart, M.A., Prakash, V., Chakraborty, S.S., Deori, P., Galligan, T., Kulkarni, M., Ranade, S., Saini, M., Sharma, A.K., Shringarpure, R. and Green, R.E. (2014) 'Avian scavengers and the threat from veterinary pharmaceuticals', *Philosophical Transactions of the Royal Society of London.Series B, Biological Sciences,* 369(1656), pp. 10.1098/rstb.2013.0574. doi: 10.1098/rstb.2013.0574 [doi].

D'Aquila, P.S., Collu, M., Gessa, G.L. and Serra, G. (2000) 'The role of dopamine in the mechanism of action of antidepressant drugs', *European Journal of Pharmacology,* 405(1), pp. 365-373.

Dean, W., Milton, S. and Anderson, M. (2006) 'Use of road kills and roadside vegetation by Pied and Cape Crows in semi-arid South Africa', *Ostrich-Journal of African Ornithology,* 77(1-2), pp. 102-104.

Desrochers, A. and Francoz, D. (2014) 'Clinical management of septic arthritis in cattle', *Veterinary Clinics of North America: Food Animal Practice,* 30(1), pp. 177-203.

Diao, X., Jensen, J. and Hansen, A.D. (2007) 'Toxicity of the anthelmintic abamectin to four species of soil invertebrates', *Environmental Pollution,* 148(2), pp. 514-519.

Du, Y., Shi, X., Liu, C., Yu, K. and Zhou, B. (2009) 'Chronic effects of water-borne PFOS exposure on growth, survival and hepatotoxicity in zebrafish: a partial life-cycle test', *Chemosphere,* 74(5), pp. 723-729.

Echols, M. (2006) 'Evaluating and treating the kidneys', *Clinical Avian Medicine,* 2, pp. 451-492.

El-Sayed, Y.M., Abdel-Hameed, M.E., Suleiman, M.S. and Najib, N.M. (1988) 'A rapid and sensitive high-performance liquid chromatographic method for the determination of diclofenac sodium in serum and its use in pharmacokinetic studies', *Journal of Pharmacy and Pharmacology,* 40(10), pp. 727-729.

Erickson, W.A. and Urban, D.J. (2004) *Potential risks of nine rodenticides to birds and nontarget mammals: a comparative approach.* US Environmental Protection Agency, Office of Prevention, Pesticides and Toxic Substances Washington, DC.

European Medicines Agency Report (2014) ' On the risk to vultures and other necrophagus bird populations in the European Union in connection with the use of veterinary medicinal products containing the substance diclofenac, EMA/CVMP/721170/2014.

Feighner, J.P. (1999) *Mechanism of action of antidepressant medications.* In Assessing Antidepressant Efficacy: A reexamination., Jan, 1988, Phoenix, AZ, US. Physicians Postgraduate Press.

Fenwick, N., Griffin, G. and Gauthier, C. (2009) 'The welfare of animals used in science: How the “Three Rs” ethic guides improvements', *The Canadian Veterinary Journal,* 50(5), pp. 523.

Fernandes, M.E.S., Alves, F.M., Pereira, R.C., Aquino, L.A., Fernandes, F.L. and Zanuncio, J.C. (2016) 'Lethal and sublethal effects of seven insecticides on three beneficial insects in laboratory assays and field trials.', *Chemosphere,* 156, pp. 45-55. doi: 10.1016/j.chemosphere.2016.04.115 ER.

Fernie, K., Bortolotti, G. and Smits, J. (2003) 'Reproductive abnormalities, teratogenicity, and developmental problems in American kestrels (Falco sparverius) exposed to polychlorinated biphenyls', *Journal of Toxicology and Environmental Health, Part A, 66*(22), pp.2089-2103.

Ferrari, B., Mons, R., Vollat, B., Fraysse, B., Paxēaus, N., Giudice, R.L., Pollio, A. and Garric, J. (2004) 'Environmental risk assessment of six human pharmaceuticals: are the current environmental risk assessment procedures sufficient for the protection of the aquatic environment?', *Environmental Toxicology and Chemistry,* 23(5), pp. 1344-1354.

Fisher, M.M. and Eugster, E.A. (2014) 'What is in our environment that effects puberty?', *Reproductive Toxicology,* 44, pp. 7-14.

Fleischli, M.A., Franson, J., Thomas, N., Finley, D. and Riley Jr, W. (2004) 'Avian mortality events in the United States caused by anticholinesterase pesticides: a retrospective summary of National Wildlife Health Center records from 1980 to 2000', *Archives of Environmental Contamination and Toxicology,* 46(4), pp. 542-550.

Fong, P.P. and Ford, A.T. (2014) 'The biological effects of antidepressants on the molluscs and crustaceans: a review', *Aquatic Toxicology,* 151, pp. 4-13.

Fossi, M.C., Massi, A., Lari, L., Marsili, L., Focardi, S., Leonzio, C. and Renzoni, A. (1995) 'Interspecies differences in mixed function oxidase activity in birds: Relationship between feeding habits, detoxication activities and organochlorine accumulation', *Environmental Pollution,* 90(1), pp. 15-24. doi: http://dx.doi.org/10.1016/0269-7491(94)00098-X.

Fourie, T., Cromarty, D., Duncan, N., Wolter, K. and Naidoo, V. (2015) 'The safety and pharmacokinetics of carprofen, flunixin and phenylbutazone in the Cape Vulture (Gyps coprotheres) following oral exposure', *PloS One,* 10(10), pp. e0141419.

Fukuto, T.R. (1990) 'Mechanism of action of organophosphorus and carbamate insecticides'. *Environmental Health Perspectives*, *87*, p.245.

Frame, G.M., Wagner, R.E., Carnahan, J.C., Brown, J.F., May, R.J., Smullen, L.A. and Bedard, D.L. (1996) 'Comprehensive, quantitative, congener-specific analyses of eight Aroclors and complete PCB congener assignments on DB-1 capillary GC columns', *Chemosphere*, *33*(4), pp.603-623.

Fry, D.M. (1995) 'Reproductive effects in birds exposed to pesticides and industrial chemicals', *Environmental Health Perspectives,* 103 Suppl 7, pp. 165-171.

Funk, C.D. (2001) 'Prostaglandins and leukotrienes: advances in eicosanoid biology', *Science (New York, N.Y.),* 294(5548), pp. 1871-1875. doi: 10.1126/science.294.5548.1871 [doi].

Gehlbach, D. (2010) 'Bird Head Effigy Pipes Were Important Prehistoric Talismans', .

Georgieva, S. and Stancheva, M. (2016) 'Indicator polychlorinated biphenyl residues in muscle tissue of fish from Black Sea coast of Bulgaria.', *Agricultural Science and Technology,* 8(2), pp. 149-152.

Gonalons, C.M. and Farina, W.M. (2015) 'Effects of sublethal doses of Imidacloprid on young adult honeybee behaviour.', *PLoS One,* 10(10), pp. e0140814. doi: 10.1371/journal.pone.0140814 ER.

Government of Indian. (1997) '16th Indian livestock census summary report', 1A, New Delhi - India. Retrieved from www.dahd.nic.in 18th June 2017.

Graham, J.E. and Heatley, J.J. (2007) 'Emergency care of raptors', *Veterinary Clinics of North America: Exotic Animal Practice*, *10*(2), pp.395-418.

Halling-Sørensen, B., Nielsen, S.N., Lanzky, P., Ingerslev, F., Lützhøft, H.H. and Jørgensen, S. (1998) 'Occurrence, fate and effects of pharmaceutical substances in the environment-A review', *Chemosphere,* 36(2), pp. 357-393.

Hoffman, D.J. (1995) 'Wildlife toxicity testing', *Handbook of Ecotoxicology,* , pp. 356-391.

Horvat, A.J., Babić, S., Pavlović, D., Ašperger, D., Pelko, S., Kaštelan-Macan, M., Petrović, M. and Mance, A. (2012) 'Analysis, occurrence and fate of anthelmintics and their transformation products in the environment', *TrAC Trends in Analytical Chemistry,* 31, pp. 61-84.

Houston, D.C. and 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), pp.306-313.

Hu, W., Jones, P.D., Upham, B.L., Trosko, J.E., Lau, C. and Giesy, J.P. (2002) 'Inhibition of gap junctional intercellular communication by perfluorinated compounds in rat liver and dolphin kidney epithelial cell lines in vitro and Sprague-Dawley rats in vivo', *Toxicological Sciences : An Official Journal of the Society of Toxicology,* 68(2), pp. 429-436.

Hubbert, M.K. (1949) 'Energy from fossil fuels', *Science*, *109*(2823), pp.103-109.

Huggett, D., Brooks, B., Peterson, B., Foran, C. and Schlenk, D. (2002) 'Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms', *Archives of Environmental Contamination and Toxicology,* 43(2), pp. 229-235.

Hussain, I., Khan, M.Z., Khan, A., Javed, I. and Saleemi, M.K. (2008) 'Toxicological effects of diclofenac in four avian species', *Avian Pathology,* 37(3), pp. 315-321.

Hutchinson, T.H., Madden, J.C., Naidoo, V. and 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 of London.Series B, Biological Sciences,* 369(1656), pp. 10.1098/rstb.2013.0583. doi: 10.1098/rstb.2013.0583 [doi].

Ibrahim, J.A., Muazzam, I., Jegede, I.A. and Kunle, O.F. (2010) 'Medicinal plants and animals sold by the Yan-Shimfidas of Sabo Wuse in Niger State, Nigeria', *African Journal of Pharmacy and Pharmacology*, *4*(6), pp.386-394.

IUCN (2015) 'The IUCN red list of threatened species 2015'. Retrieved from www.iucnredlist.org 18th June 2017

IUCN (2016) 'Vulture updates-October 2106- Around the world of vultures & VSG activities', *Vulture News,* 71, pp. 65-70. Retrieved from iucn-vulturenews.org 18th June 2017

Jain, S., Kumar, P., Vyas, R.K., Pandit, P. and Dalai, A.K. (2013) 'Occurrence and removal of antiviral drugs in environment: a review', *Water, Air, & Soil Pollution,* 224(2), pp. 1410.

Jarvis, M., Siegfried, W. and Currie, M. (1974) 'Conservation of the Cape vulture in the Cape Province', *Journal of South African Wildlife Management Association,* 4(1), pp. 29-34.

Jechalke, S., Heuer, H., Siemens, J., Amelung, W. and Smalla, K. (2014) 'Fate and effects of veterinary antibiotics in soil ', *Trends in Microbiology,* 22(9), pp. 536-545.

Jensen, J. and Scott-Fordsmand, J.J. (2012) 'Ecotoxicity of the veterinary pharmaceutical ivermectin tested in a soil multi-species (SMS) system', *Environmental Pollution,* 171, pp. 133-139.

Jokanović, M. (2009) 'Medical treatment of acute poisoning with organophosphorus and carbamate pesticides', *Toxicology Letters,* 190(2), pp. 107-115. doi: http://dx.doi.org/10.1016/j.toxlet.2009.07.025.

Jones, O., Voulvoulis, N. and Lester, J. (2001) 'Human pharmaceuticals in the aquatic environment a review', *Environmental Technology,* 22(12), pp. 1383-1394.

Kadafa, A.A. (2012) 'Oil exploration and spillage in the Niger Delta of Nigeria', *Civil and Environmental Research,* 2(3), pp. 38-51.

Kenaga, E.E. (1978) 'Test organisms and methods useful for early assessment of acute toxicity of chemicals', *Environmental Science & Technology,* 12(12), pp. 1322-1329.

Khamdang, S., Takeda, M., Noshiro, R., Narikawa, S., Enomoto, A., Anzai, N., Piyachaturawat, P. and Endou, H. (2002) 'Interactions of human organic anion transporters and human organic cation transporters with nonsteroidal anti-inflammatory drugs', *Journal of Pharmacology and Experimental Therapeutics*, *303*(2), pp.534-539.

Kohler, H.R. and Triebskorn, R. (2013) 'Wildlife ecotoxicology of pesticides: can we track effects to the population level and beyond?', *Science (New York, N.Y.),* 341(6147), pp. 759-765. doi: 10.1126/science.1237591 [doi].

Kormos, V. and Gaszner, B. (2013) 'Role of neuropeptides in anxiety, stress, and depression: from animals to humans', *Neuropeptides,* 47(6), pp. 401-419.

Kumar, K., Gupta, S., Baidoo, S., Chander, Y. and Rosen, C. (2005) 'Antibiotic uptake by plants from soil fertilized with animal manure', *Journal of Environmental Quality,* 34(6), pp. 2082-2085.

Kümmerer, K., Al-Ahmad, A., Bertram, B. and Wießler, M. (2000) 'Biodegradability of antineoplastic compounds in screening tests: influence of glucosidation and of stereochemistry', *Chemosphere,* 40(7), pp. 767-773.

Kushwaha, S., Kanaujia, A. and Basu, D. (2009) 'Nature's scavengers: Vultures', *Research in Environment and Life Science,* 2, pp. 185-192.

Lapponi, M.J., Rivero, C.W., Zinni, M.A., Britos, C.N. and Trelles, J.A. (2016) 'New developments in nucleoside analogues biosynthesis: A review ', *Journal of Molecular Catalysis B: Enzymatic,* 133, pp. 218-233.

Lavoie, E.T. and Grasman, K.A. (2007) 'Effects of in ovo exposure to PCBs 126 and 77 on mortality, deformities and post-hatch immune function in chickens', *Journal of Toxicology and Environmental Health, Part A*, *70*(6), pp.547-558.

Lee, M., Lee, H. and Ryu, P. (2001) 'Public health risks: Chemical and antibiotic residues-review', *Asian-Australasian Journal of Animal Sciences,* 14(3), pp. 402-413.

Lierz, M. (2003) 'Avian renal disease: pathogenesis, diagnosis, and therapy', *Veterinary Clinics of North America: Exotic Animal Practice*, *6*(1), pp.29-55.

Liukkonen-Anttila, T., Honkanen, H., Peltokangas, P., Pelkonen, O. and Hohtola, E. (2003) 'Cytochrome P450 enzyme activity in five herbivorous, non-passerine bird species', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 134(1), pp. 69-77. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/S1532-0456(02)00211-9.

Lucero, G.A., Marcela, G., Sandra, G., Manuel, G.L. and Celene, R. (2015) 'Naproxen-enriched artificial sediment induces oxidative stress and genotoxicity in Hyalella azteca', *Water, Air, & Soil Pollution,* 226(6), pp. 1-10.

Lumaret, J. and Errouissi, F. (2002) 'Use of anthelmintics in herbivores and evaluation of risks for the non target fauna of pastures', *Veterinary Research,* 33(5), pp. 547-562.

Lumeij, J.T. (1994) 'Nephrology', In: Ritichie, B.W., Harrison, G.J., Harrison, L.R.(Eds.), Avian Medicine: Principles and Application', *Wingers, Florida,* pp. 538-555.

Margni, M., Rossier, D., Crettaz, P. and Jolliet, O. (2002) 'Life cycle impact assessment of pesticides on human health and ecosystems', *Agriculture, Ecosystems & Environment,* 93(1), pp. 379-392.

Markandya, A., Taylor, T., Longo, A., Murty, M., Murty, S. and 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), pp. 194-204.

Martin, T.M. and Young, D.M. (2001) 'Prediction of the acute toxicity (96-h LC50) of organic compounds to the fathead minnow (Pimephales promelas) using a group contribution method', *Chemical Research in Toxicology,* 14(10), pp. 1378-1385.

Mazard, S., Penesyan, A., Ostrowski, M., Paulsen, I.T. and Egan, S. (2016) 'Tiny microbes with a big impact: the role of cyanobacteria and their metabolites in shaping our future', *Marine Drugs,* 14(5), pp. 97.

McKellar, Q. (1997) 'Ecotoxicology and residues of anthelmintic compounds', *Veterinary Parasitology,* 72(3-4), pp. 413-435.

McNabb, F.M. (2005) 'Biomarkers for the assessment of avian thyroid disruption by chemical contaminants', *Avian and Poultry Biology Reviews*, *16*(1), pp.3-10.

Merola, V. (2002) 'Anticoagulant rodenticides: deadly for pests, dangerous for pets', *Veterinary Medicine-bonner Springs then Edwardsville-,* 97(10), pp. 716-727.

Merriman, T.R. and Dalbeth, N. (2011) 'The genetic basis of hyperuricaemia and gout', *Joint Bone Spine,* 78(1), pp. 35-40.

Mestorino, N., Hernandez, E.M., Marchetti, L. and Errecalde, J. (2007) 'Pharmacokinetics and tissue residues of an oxytetracycline/diclofenac combination in cattle', *Revue Scientifique et Technique-Office International des Epizooties,* 26(3), pp. 679.

Mnif, W., Hassine, A.I.H., Bouaziz, A., Bartegi, A., Thomas, O. and Roig, B. (2011) 'Effect of endocrine disruptor pesticides: a review', *International Journal of Environmental Research and Public Health,* 8(6), pp. 2265-2303.

Moleón, M., Sánchez-Zapata, J.A., Margalida, A., Carrete, M., Owen-Smith, N. and Donázar, J.A. (2014) 'Humans and scavengers: the evolution of interactions and ecosystem services', *Bioscience,* 64(5), pp. 394-403.

Mompati, K.F. (2012) *The insensitivity of the crow (Corvusalbus) to diclofenac toxicity,* Master dissertation, University of Pretoria, South Africa.

Monadjem, A., Anderson, M., Piper, S. and Boshoff, A. (2004) *The vultures of southern Africa-quo vadis.* In Proceedings of workshop on vulture research and conservation in southern Africa, Birds of Prey Working Group, Endangered Wildlife Group, Johannesburg.

Mount, M.E., Kim, B.U. and Kass, P.H. (2003) 'Use of a test for proteins induced by vitamin K absence or antagonism in diagnosis of anticoagulant poisoning in dogs: 325 cases (1987-1997)', *Journal of the American Veterinary Medical Association,* 222(2), pp. 194-198.

Mudur, G. (2001) 'Human anthrax in India may be linked to vulture decline', *BMJ: British Medical Journal,* 322(7282), pp. 320.

Munita, J.M. and Arias, C.A. (2016) 'Mechanisms of Antibiotic Resistance', *Microbiology Spectrum,* 4(2), pp. 10.1128/microbiolspec.VMBF-0016-2015. doi: 10.1128/microbiolspec.VMBF-0016-2015 [doi].

Munshi-South, J. and Wilkinson, G.S. (2010) 'Bats and birds: exceptional longevity despite high metabolic rates', *Ageing Research Reviews,* 9(1), pp. 12-19.

Naidoo, V., Duncan, N., Bekker, L. and 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), pp. 260-266.

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

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

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

Naidoo, V., Mompati, K.F., Duncan, N. and Taggart, M.A. (2011) 'The pied crow (Corvus albus) is insensitive to diclofenac at concentrations present in carrion', *Journal of Wildlife Diseases,* 47(4), pp. 936-944.

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

Naidoo, V., Wolter, K., Cromarty, D., Diekmann, M., Duncan, N., Meharg, A.A., Taggart, M.A., Venter, L. and Cuthbert, R. (2010b) 'Toxicity of non-steroidal anti-inflammatory drugs to Gyps vultures: a new threat from ketoprofen', *Biology Letters,* 6(3), pp. 339-341. doi: 10.1098/rsbl.2009.0818 [doi].

Nellums, E.K. (2006) *The Expansion of Black Vultures, Coragyps atratus, into Southwestern Ohio,* Doctoral dissertation , Miami University.

Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad, H., Ahmed, S., Chaudhry, M.J.I. and Arshad, M. (2004) 'Diclofenac residues as the cause of vulture population decline in Pakistan', *Nature,* 427(6975), pp. 630-633.

OECD, 2010. Test No. 223: Avian Acute Oral Toxicity Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. DOI: http://dx.doi.org/10.1787/9789264090897-en

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

Padian, K. and Chiappe, L.M. (1998) 'The origin and early evolution of birds', *Biological Reviews,* 73(1), pp. 1-42.

Pain, D.J., Cunningham, A., Donald, P., Duckworth, J., Houston, D., Katzner, T., Parry-Jones, J., Poole, C., Prakash, V. and Round, P. (2003) 'Causes and effects of temporospatial declines of Gyps vultures in Asia', *Conservation Biology,* 17(3), pp. 661-671.

Papich, M.G. (2008) 'An Update on Nonsteroidal Anti-Inflammatory Drugs (NSAIDs) in Small Animals', *Veterinary Clinics of North America: Small Animal Practice,* 38(6), pp. 1243-1266. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.cvsm.2008.09.002.

Patisaul, H.B. and Adewale, H.B. (2009) 'Long-term effects of environmental endocrine disruptors on reproductive physiology and behavior', *Frontiers in Behavioral Neuroscience,* 3, pp. 10. doi: 10.3389/neuro.08.010.2009 [doi].

Peng, X., Wang, C., Zhang, K., Wang, Z., Huang, Q., Yu, Y. and Ou, W. (2014) 'Profile and behavior of antiviral drugs in aquatic environments of the Pearl River Delta, China', *Science of the Total Environment,* 466, pp. 755-761.

Pereira, J.L., Antunes, S.C., Castro, B.B., Marques, C.R., Gonçalves, A.M., Gonçalves, F. and Pereira, R. (2009) 'Toxicity evaluation of three pesticides on non-target aquatic and soil organisms: commercial formulation versus active ingredient', *Ecotoxicology,* 18(4), pp. 455-463.

Perez, F., Llorca, M., Farré, M. and Barceló, D. (2012) 'Automated analysis of perfluorinated compounds in human hair and urine samples by turbulent flow chromatography coupled to tandem mass spectrometry', *Analytical and Bioanalytical Chemistry,* 402(7), pp. 2369-2378.

Piao, H., Kamiya, N., Watanabe, J., Yokoyama, H., Hirata, A., Fujii, T., Shimizu, I., Ito, S. and Goto, M. (2006) 'Oral delivery of diclofenac sodium using a novel solid-in-oil suspension', *International Journal of Pharmaceutics,* 313(1), pp. 159-162.

Pimentel, D. (1995) 'Amounts of pesticides reaching target pests: environmental impacts and ethics', *Journal of Agricultural and Environmental Ethics,* 8(1), pp. 17-29.

Pimentel, D. (2005) 'Environmental and economic costs of the application of pesticides primarily in the United States.', *Environment, Development and Sustainability,* 7(2), pp. 229-252. doi: 10.1007/s10668-005-7314-2 ER.

Prakash, V., Bishwakarma, M.C., Chaudhary, A., Cuthbert, R., Dave, R., Kulkarni, M., Kumar, S., Paudel, K., Ranade, S. and Shringarpure, R. (2012) 'The population decline of Gyps vultures in India and Nepal has slowed since veterinary use of diclofenac was banned', *PLoS One,* 7(11), pp. e49118.

Puckowski, A., Mioduszewska, K., Lukaszewicz, P., Borecka, M., Caban, M., Maszkowska, J. and Stepnowski, P. (2016) 'Bioaccumulation and analytics of pharmaceutical residues in the environment: A review', *Journal of Pharmaceutical and Biomedical Analysis,* 127, pp. 232-255. doi: 10.1016/j.jpba.2016.02.049.

Rai, N., Sarkar, M. and Raha, S. (2015) 'Piroxicam, a traditional non-steroidal anti-inflammatory drug (NSAID) causes apoptosis by ROS mediated Akt activation', *Pharmacological Reports,* 67(6), pp. 1215-1223. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.pharep.2015.05.012.

Rainio, M.J., Kanerva, M., Wahlberg, N., Nikinmaa, M. and Eeva, T. (2012) 'Variation of basal EROD activities in ten passerine bird species–relationships with diet and migration status', *PloS One,* 7(3), pp. e33926.

Rattner, B.A., Whitehead, M.A., Gasper, G., Meteyer, C.U., Link, W.A., Taggart, M.A., Meharg, A.A., Pattee, O.H. and 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), pp. 2341-2345.

Rose, R.L., Hodgson, E. and Roe, R.M. (1999) 'Chapter 28 - Pesticides', In: Marquardt, H., Schäfer, S.G., McClellan, R. and Welsch, F. (eds.) *Toxicology* San Diego: Academic Press, pp. 663-697.

Roxburgh, L. and McDougall, R. (2012) 'Vulture poisoning incidents and the status of vultures in Zambia and Malawi', *Vulture News,* 62(1), pp. 33-39.

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

Sarma, M.K., Kaushik, S. and Goswami, P. (2016) 'Cyanobacteria: A metabolic power house for harvesting solar energy to produce bio-electricity and biofuels', *Biomass and Bioenergy,* 90, pp. 187-201.

Schröder, P., Helmreich, B., Škrbić, B., Carballa, M., Papa, M., Pastore, C., Emre, Z., Oehmen, A., Langenhoff, A. and Molinos, M. (2016) 'Status of hormones and painkillers in wastewater effluents across several European states—considerations for the EU watch list concerning estradiols and diclofenac', *Environmental Science and Pollution Research,* 23(13) pp. 12835-12866.

Schwaiger, J., Ferling, H., Mallow, U., Wintermayr, H. and Negele, R. (2004) 'Toxic effects of the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout', *Aquatic Toxicology,* 68(2), pp. 141-150.

Seacat, A.M., Thomford, P.J., Hansen, K.J., Olsen, G.W., Case, M.T. and Butenhoff, J.L. (2002) 'Subchronic toxicity studies on perfluorooctanesulfonate potassium salt in cynomolgus monkeys', *Toxicological Sciences : An Official Journal of the Society of Toxicology,* 68(1), pp. 249-264.

Seaton, K. (1996) 'Gout, uric acid, albumin, and aging', *Journal of the National Medical Association*, *88*(8), p.473.

Selevan, S.G., Lindbohm, M., Hornung, R.W. and Hemminki, K. (1985) 'A study of occupational exposure to antineoplastic drugs and fetal loss in nurses', *New England Journal of Medicine,* 313(19), pp. 1173-1178.

Shah, S. (2010) 'As pharmaceutical use soars, drugs taint water and wildlife', *Yale Environment,* 360, pp. 15.

Sharma, N.K., Rai, A.K. and Stal, L.J. (2013) *Cyanobacteria: an economic perspective.* John Wiley & Sons.

Shivik, J.A. (2006) 'Are vultures birds, and do snakes have venom, because of macro-and microscavenger conflict?', *AIBS Bulletin,* 56(10), pp. 819-823.

Shultz, S, Pain, D.J., Cunningham, A.A., Green, R.E., Parry-Jones, J. and Prakash, V. (2004) 'Current conservation priorities for the Asian Gyps vulture crisis'. In: Monadjem, A., Anderson, M.D., Piper, S.E. & Boshoff, A.F. (Eds). Vultures in The Vultures of Southern Africa – Quo Vadis?. Proceedings of a workshop on vulture research and conservation in southern Africa. Birds of Prey Working Group, Johannesburg.

Silva, M.I.G. and de Sousa, Francisca Cléa Florenço (2011) 'Gastric ulcer etiology', *Peptic Ulcer Disease,* 1.

Simelane, T.S. and Kerley, G.I.H. (1998) 'Conservation implications of the use of vertebrates by Xhosa traditional healers in South Africa', *South African Journal of Wildlife Research-24-month delayed open access*, *28*(4), pp.121-126.

Singer, A.C., Colizza, V., Schmitt, H., Andrews, J., Balcan, D., Huang, W.E., Keller, V.D., Vespignani, A. and Williams, R.J. (2011) 'Assessing the ecotoxicologic hazards of a pandemic influenza medical response', *Environmental Health Perspectives,* 119(8), pp. 1084.

Singer, D. (2006) 'Size relationship of metabolic rate: Oxygen availability as the "missing link" between structure and function?', *Thermochimica Acta,* 446(1), pp. 20-28.

Stahl, T., Mattern, D. and Brunn, H. (2011) 'Toxicology of perfluorinated compounds', *Environmental Sciences Europe,* 23(1), pp. 1.

Subramanian, M. (2008) 'Towering silence: for millennia Zoroastrians have used vultures to dispose of their dead. What will happen when the birds disappear?', *Science & Spirit,* 19(3), pp. 34-39.

Suenami, K., Lim, L.W., Takeuchi, T., Sasajima, Y., Sato, K., Takekoshi, Y. and Kanno, S. (2006) 'Rapid and simultaneous determination of nonsteroidal anti-inflammatory drugs in human plasma by LC–MS with solid-phase extraction', *Analytical and Bioanalytical Chemistry,* 384(7-8), pp. 1501-1505.

Swan, G., Naidoo, V., Cuthbert, R., Green, R.E., Pain, D.J., Devendra Swarup, Vibhu Prakash, Taggart, M., Bekker, L., Devojit Das, Diekmann, J., Diekmann, M., Killian, E., Meharg, A., Patra, R.C., Mohini Saini and Wolter, K. (2006a) 'Removing the threat of diclofenac to critically endangered Asian vultures.', *PLoS Biology,* 4(3), pp. e66. doi: 10.1371/journal.pbio.0040066 ER.

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. and Wolter, K. (2006b) 'Toxicity of diclofenac to Gyps vultures.', *Biology Letters,* 2(2), pp. 279-282. doi: 10.1098/rsbl.2005.0425 ER.

Taggart, M., Cuthbert, R., Das, D., Sashikumar, C., Pain, D., Green, R., Feltrer, Y., Shultz, S., Cunningham, A. and Meharg, A. (2007) 'Diclofenac disposition in Indian cow and goat with reference to Gyps vulture population declines', *Environmental Pollution,* 147(1), pp. 60-65.

Taggart, M.A., Senacha, K.R., Green, R.E., Cuthbert, R., Jhala, Y.V., Meharg, A.A., Mateo, R. and Pain, D.J. (2009) 'Analysis of nine NSAIDs in ungulate tissues available to critically endangered vultures in India', *Environmental Science & Technology,* 43(12), pp. 4561-4566.

Thiollay, J. (2006) 'The decline of raptors in West Africa: long-term assessment and the role of protected areas', *Ibis,* 148(2), pp. 240-254.

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

Todd, P.A. and Sorkin, E.M. (1988) 'Diclofenac sodium', *Drugs,* 35(3), pp. 244-285.

Tucker, R.K. and Haegele, M. (1971) 'Comparative acute oral toxicity of pesticides to six species of birds', *Toxicology and Applied Pharmacology,* 20(1), pp. 57-65.

U S EPA, 2012. Ecological Effects Test Guidelines, OCSPP 850.2100: Avian Acute Oral Toxicity Test.

Vajda, A.M., Barber, L.B., Gray, J.L., Lopez, E.M., Bolden, A.M., Schoenfuss, H.L. and Norris, D.O. (2011) 'Demasculinization of male fish by wastewater treatment plant effluent', *Aquatic Toxicology,* 103(3), pp. 213-221.

van Tuinen, M. (2016) 'Birds, Diversification of', In : Kliman, R.M. (Ed.) *Encyclopedia of Evolutionary Biology* Oxford: Academic Press, pp. 241-245. https://doi-org.uplib.idm.oclc.org/10.1016/B978-0-12-800049-6.00281-X

Vane, J.R. and Botting, R.M. (1998) 'Mechanism of Action of Nonsteroidal Anti-inflammatory Drugs', *The American Journal of Medicine,* 104(3, Supplement 1), pp. 2S-8S. doi: http://dx.doi.org/10.1016/S0002-9343(97)00203-9.

Virani, M.Z., Kendall, C., Njoroge, P. and Thomsett, S. (2011) 'Major declines in the abundance of vultures and other scavenging raptors in and around the Masai Mara ecosystem, Kenya ', *Biological Conservation,* 144(2), pp. 746-752.

VICH. (2000) 'Environmental impact assessment for veterinary medicinal products - Phase 1 (VICH GL6)'. Retrieved from www.vichsec.org/ 3rd April 2017

VICH. (2004) 'Environmental impact assessment for veterinary medicinal products - Phase 2 (VICH GL38)'. Retrieved from www.vichsec.org/ 3rd April 2017

VICH. (2008) 'Target animal safety for pharmaceuticals (VICH GL43)'. Retrieved from www.vichsec.org/ 3rd April 2017

VICH. (2011) 'Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Metabolism study to determine the quantity and identify the nature of the residues (VICH GL46)'. Retrieved from www.vichsec.org/ 3rd April 2017

Ward, J., McCafferty, D.J., Houston, D.C. and Ruxton, G.D. (2008) 'Why do vultures have bald heads? The role of postural adjustment and bare skin areas in thermoregulation', *Journal of Thermal Biology,* 33(3), pp. 168-173. doi: http://dx.doi.org/10.1016/j.jtherbio.2008.01.002.

Watanabe, K.P., Saengtienchai, A., Tanaka, K.D., Ikenaka, Y. and Ishizuka, M. (2010) 'Comparison of warfarin sensitivity between rat and bird species', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 152(1), pp. 114-119.

Web, A. (2014) 'Egyptian Vultures', .

Tesfahunegny, W. and Mulualem, G. (2016) 'Impact of pesticides on birds from DDT to current fatality: a literature review.', *Journal of Zoology Studies,* 3(2), pp. 44-55.

Wilkinson, J.L., Hooda, P.S., Barker, J., Barton, S. and Swinden, J. (2016) 'Ecotoxic pharmaceuticals, personal care products, and other emerging contaminants: A review of environmental, receptor-mediated, developmental, and epigenetic toxicity with discussion of proposed toxicity to humans', *Critical Reviews in Environmental Science and Technology,* 46(4), pp. 336-381.

Williams, D. (1986) 'Interval estimation of the median lethal dose', *Biometrics,* 42(3), pp. 641-645.

Willis, J., Kendall, M., Flinn, R., Thornhill, D. and Welling, P. (1979) 'The pharmacokinetics of diclofenac sodium following intravenous and oral administration', *European Journal of Clinical Pharmacology,* 16(6), pp. 405-410.

Won, E., Rhee, J., Shin, K., Jung, J., Shim, W.J., Lee, Y. and Lee, J. (2013) 'Expression of three novel cytochrome P450 (CYP) and antioxidative genes from the polychaete, Perinereis nuntia exposed to water accommodated fraction (WAF) of Iranian crude oil and Benzo[α]pyrene', *Marine Environmental Research,* 90, pp. 75-84. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.marenvres.2013.05.014.

Woodling, J.D., Lopez, E.M., Maldonado, T.A., Norris, D.O. and Vajda, A.M. (2006) 'Intersex and other reproductive disruption of fish in wastewater effluent dominated Colorado streams', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology,* 144(1), pp. 10-15.

Xu, X., Zhou, Z., Wang, X., Kuang, X., Zhang, F. and Du, X. (2003) 'Four-winged dinosaurs from China', *Nature,* 421(6921), pp. 335-340.

Yadav, I.C., Devi, N.L., Syed, J.H., Cheng, Z., Li, J., Zhang, G. and Jones, K.C. (2015) 'Current status of persistent organic pesticides residues in air, water, and soil, and their possible effect on neighboring countries: A comprehensive review of India', *Science of The Total Environment,* 511, pp. 123-137. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.scitotenv.2014.12.041.

Zhang, Y., Wang, B., Wang, Y., Qiao, W. and Shao, P. (2011) 'A novel hypurgia for cancer chemotherapy: Programmable release of antineoplastics and cytothesis agents from core–shell micro-/nano-particles', *Medical Hypotheses,* 76(2), pp. 201-203.

Zhu, W., Zhang, Z., Wang, X., Xu, S., Li, M. and Li, S. (2014) 'Effects of avermectin on microsomal cytochrome P450 enzymes in the liver and kidneys of pigeons', *Environmental Toxicology and Pharmacology,* 38(2), pp. 562-569. doi: http://dx.doi.org.uplib.idm.oclc.org/10.1016/j.etap.2014.08.011.

Zounkova, R., Kovalova, L., Blaha, L. and Dott, W. (2010) 'Ecotoxicity and genotoxicity assessment of cytotoxic antineoplastic drugs and their metabolites', *Chemosphere,* 81(2), pp. 253-260. doi: http://dx.doi.org/10.1016/j.chemosphere.2010.06.029.

8 ADDENDUM

8.1 Validation technique

Preparation of standards for calibration curve was achieved by dissolving diclofenac sodium (10 mg) in 5 ml of dosing vehicle to give a 2 mg/ml stock solution. 1ml of the stock solution was added to 9 ml of vehicle to give a 200 µg/ml working solution. In 2 ml tubes, diclofenac sodium working standard solution (200 µg/ml) were added in volumes of 0, 0.976, 1.953, 3.906, 7.8125, 15.625, 31.25, 62.5, 125, 250 and 500 µl to 200 µl of blank plasma to provide calibration standards of 0, 0.195, 0.391, 0.781, 1.563, 3.125, 6.25, 12.5, 25, 50 and 100 µg/ml. The contents were mixed for 30 sec using a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) (El-Sayed *et al.,* 1988). The standards were then treated as par the test samples. For the validation, prepared samples were analysed in duplicate and the average of chromatogram (peak) area taken (Table 8.1). Calibration curve was drawn by plotting equivalent concentration over mean chromatogram (peak) area. The limit of detection (LOD) and limit of quantification (LOQ) were determined by estimating the signal (peak area of diclofenac) to noise (average area of two peaks, one from either side of diclofenac's) ratio. A signal to noise ratio of 3:1 and 10:1 were used for the LOD and LOQ respectively.

Table 8.1: Showing measured peak area with corresponding diclofenac concentration in plasma

Conc (µg/ml)	Vol (µl)	Equivalence (µg)	Area 1	R. Time 1	Area 2	R. Time 2	Mean Area
200	1.953125	0.390625	53691	4.383	148185	4.35	100938
200	3.90625	0.78125	92080	4.35	239747	4.333	165913.5
200	7.8125	1.5625	206791	4.35	204238	4.367	205514.5
200	15.625	3.125	463216	4.35	463693	4.35	463454.5
200	31.25	6.25	809098	4.35	811594	4.35	810346
200	62.5	12.5	2054089	4.35	2049789	4.317	2051939
200	125	25	4099781	4.317	3787790	4.317	3943785.5
200	250	50	8693464	4.267	8681817	4.267	8687640.5
200	500	100	15021180	4.233	14939334	4.233	14980257

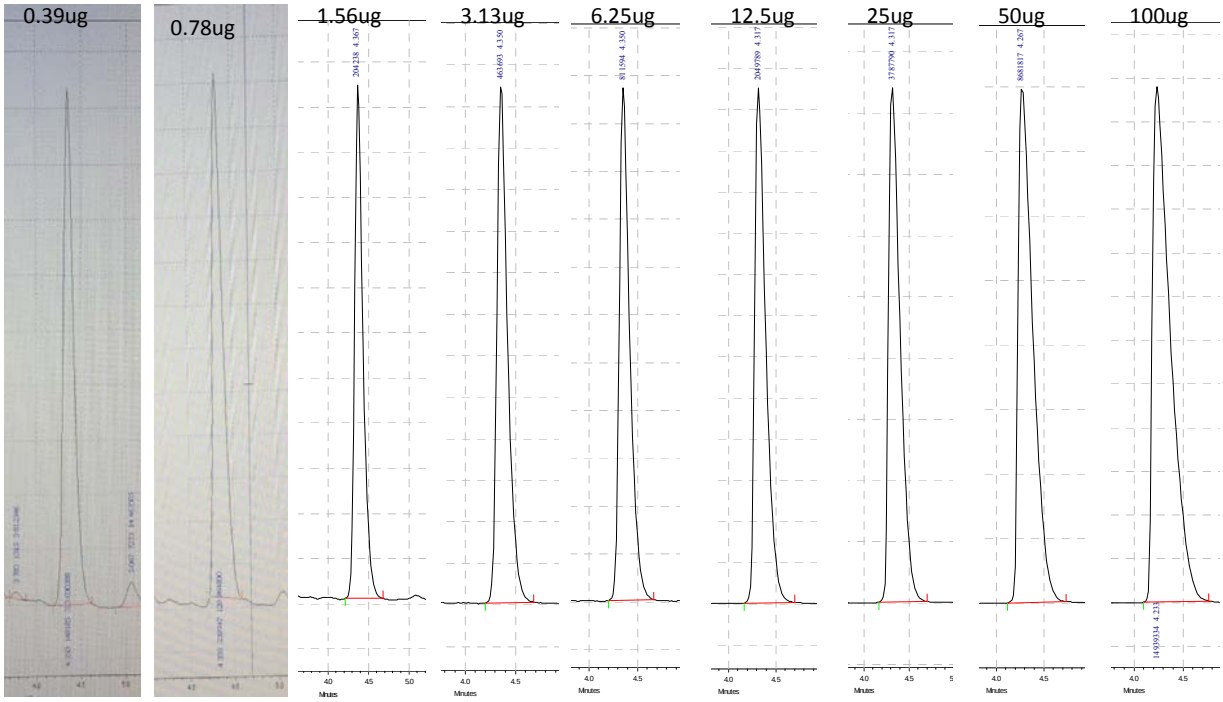


Figure 8.1: Chromatograms of different diclofenac concentration in plasma used for calibration

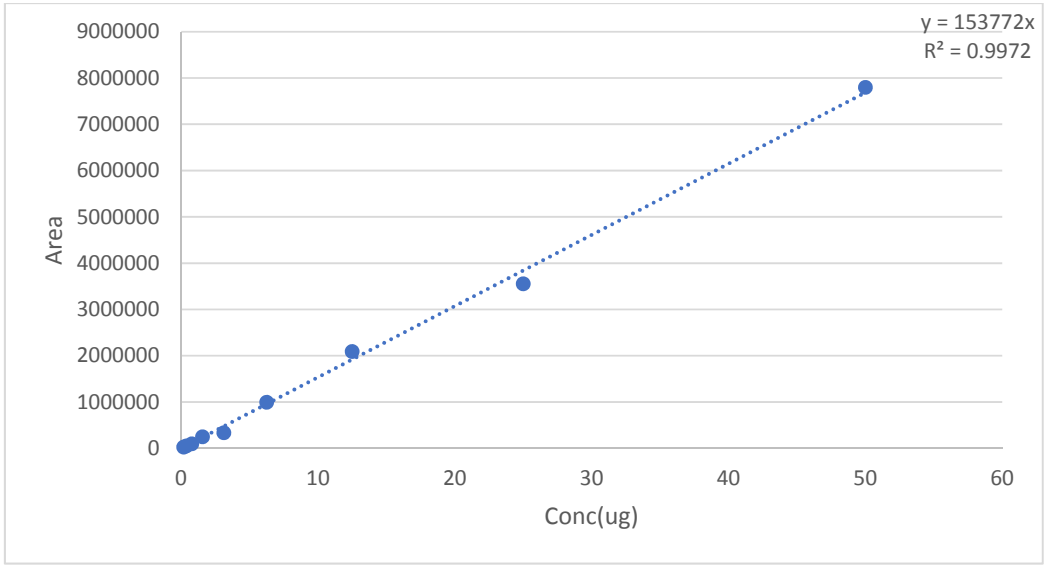


Figure 8.2: Standard calibration curve for diclofenac

SEDEC V1.3 Sequential Design Calculator

Study Identification

Project Number =	Master-10
Test Substance =	Diclofenac
Dose Units =	mg formulation/kg
Test Species =	Coturnix (Japanese Quail)
Study Type =	Dose-Response: LD50 Only
Limit Dose =	NA
Initial LD50 Guess =	50
Step Size =	1.269429701
Date =	3-Nov-16
Initials =	u14437296
Study Status Code =	24
Min % Dose Sep =	1
Stage 3 Type =	B

(Combine doses differing by < Min% for analysis)

Doses / Responses

Stage 1			Stage 2			Stage 3			Stage 4		
Dose	N Tested	N Respondi	Dose	N Tested	N Respondi	Dose	N Tested	N Respondi	Dose	N Tested	N Respondi
7.07	1	0	63.2	1	0	252	2				
26	1	0	80.1	1	0	319	2				
96	1	0	102	1	0	405	2				
354	1	1	129	1	0	515	2				
			164	1	0	653	2				
			208	1	0						
			263	1	0						
			334	1	0						
			424	1	0						
			538	1	1						

Analysis

Probit Analysis Results

Iterations	Chi-square	Probability	G	N
8	4.174481822	0.980078007	2.235324089	14

Slope =	8.995478846		
95% Confidence Limits=	-4.453661816	and	22.44461951
LD50 =	405.4150008		
95% Confidence Limits=	0	and	+ Infinity
n of Reversals =	1	n of Partials =	0

Figure 8.3: Japanese quail acute oral toxicity output result

SEDEC V1.3 Sequential Design Calculator

Study Identification

Project Number =	v107/16
Test Substance =	Diclofenac
Dose Units =	mg formulation/kg
Test Species =	Muscovy duck
Study Type =	Dose-Response: LD50 Only
Limit Dose =	NA
Initial LD50 Guess =	9.8
Date =	15-Mar-17
Initials =	u14437296
Study Status Code =	23
Min % Dose Sep =	1
Stage 3 Type =	A

Step Size = 118.0320636

(Combine doses differing by < Min% for analysis)

Doses / Responses

Stage 1			Stage 2			Stage 3			Stage 4		
Dose	N Tested	N Respondin	Dose	N Tested	N Respondin	Dose	N Tested	N Respondin	Dose	N Tested	N Respondin
1.39	1	0	45.6	1	1	17.5	5				
5.11	1	0	57.8	1	1	2063	5				
18.8	1	0	73.4	1	0						
69.3	1	0	93.1	1	0						
			118	1	0						
			150	1	0						
			190	1	1						
			241	1	1						
			306	1	1						
			388	1	0						

Analysis

Probit Analysis Results

Iterations	Chi-square	Probability	G	N
5	12.67591108	0.393030185	2.342181534	14

Slope =	0.99287661			
95% Confidence Limits=		-0.526640365	and	2.512393585
LD50 =	189.9223689			
95% Confidence Limits=		0	and	+ Infinity
n of Reversals =	2	n of Partials =	0	

Figure 8.4: Muscovy duck acute oral toxicity output result