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The safety of carprofen, flunixin and phenylbutazone in the Cape Vulture (*Gyps coprotheres*) – a pilot study.

A dissertation submitted in partial fulfilment of the requirements for the degree of

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In

Laboratory Animal Science

By

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I: DECLARATION

I declare that this dissertation, which I hereby submit for the degree Master in Veterinary Medicine in Laboratory Animal Science at the University of Pretoria, except where acknowledgements indicate such, and, with advice from my supervisor, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

Signed:

Date:

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II: DEDICATION

I dedicate this to my late grandfather, Jim Robinson who planted the seed of veterinary science in my young mind and my late grandmother, Joy Robinson. I miss you Gran.

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III: ACKNOWLEDGEMENTS

I would like to express my gratitude to the following persons / institutes, without whom this project would have been impossible:

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My friend and Saviour Jesus Christ: Your love astounds me. It is only by Your Grace that this work was completed. Your grace is sufficient, it always has been and it always will be. Praise Your Name!

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VII: ABBREVIATIONS

Abbreviation	Description
AA	Arachidonic acid
ALB	Albumin
ALT	Alanine transferase
AUC	Area under the curve
WBV	White-backed Vulture
Bov	Bovine
Ca^{2+}	Ionised calcium
Can	Canine
CV	Cape Vulture
Cl	Total body clearance
COX	Cycloxygenase
Eq	Equine
Fel	Feline
IM	Intra-muscular
IS	Internal Standard
IV	Intravenous
K	Potassium
KZN	Kwazulu Natal
LC/MS-MS	Liquid chromatography mass spectrometry mass spectrometry
LL	Lower Limit
LOX	Lipoxygenase
MRT	Mean residence time
Na^{2+}	Ionised Sodium
NAD^{+}	Oxidised form of nicotinamide adenine dinucleotide
NADH	Reduced form of nicotinamide adenine dinucleotide
NSAID	Non-steroidal anti-inflammatory drug
Por	Porcine
PBZ	Phenylbutazone
PG	Prostaglandin
PO	Per Os

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ROS	Reactive oxygen species
RTE	Renal tubular epithelium
T½	Elimination half life
UA	Uric Acid
UL	Upper Limit
UP	University of Pretoria
Vz	Volume of distribution
WD	Withdrawal Period/Time

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VIII: ABSTRACT

The safety of a single oral dose of carprofen (11.5 mg/kg), flunixin (1 mg/kg) and phenylbutazone (1.7 mg/kg) was evaluated in the Cape Vulture (*Gyps coprotheres*) by means of a four-way parallel study, using two birds per treatment. Clinical observations, clinical pathology and necropsy examinations were determining factors. Clinical signs of lethargy and depression were noted in one of the carprofen (CRP), two of the flunixin (FXN) and one of the phenylbutazone (PBZ) treated birds. Serum alanine transferase (ALT), albumin, sodium, calcium, potassium and uric acid (UA) concentrations were monitored up to 48 hours post dosing. Mild reversible inhibition of UA excretion was evident in all three groups, although UA remained within population reference intervals. All treatment groups had a drug concentration responsive ALT increase. No pathological lesions where noted on histopathology.

Oral absorption of CRP, FXN and PBZ was characterised by a maximum plasma concentration of 1051.8 ± 620.7 ng/ml, 335.9 ± 36.3 ng/ml and 11150 ± 2474.9 ng/ml obtained in 4 ± 4.3, 0.45 ± 0.02 and 5.3 ± 5.2 hours respectively and a half-life of elimination of 13.3 ±5, 1.8±1 and 18.7 ±11.4 hours respectively. The volume of distribution was 13.62 ± 9.91 L/kg (CRP); 3.29± 0.75 L/kg (FXN) and 0.13 ± 0.03 L/kg (PBZ). Area under the curve until the last time point was 21.72± 20.10; 0.78± 0.28 and 263.35 ±68.69 µg/mL*h for CRP, FXN and PBZ respectively. Glucuronidation was identified in FXN and CRP treated birds. The long half-life of PBZ and CRP creates concern that accumulative toxicity may occur. Both FXN and PBZ are potentially hepatotoxic indicating that clinical use or the presence thereof in the food-chain should be avoided. CRP may be of clinical benefit in the vulture, but only as a single treatment. This drug should also be safe in the case of possible contamination of the food chain, as it is unlikely that vultures will be exposed to the drug often enough for it to be cumulative

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1 INTRODUCTION

When considering the plains of Africa, images of the majestic big five usually come to mind. Very rarely, does one consider the vulture, probably due to their often portrayal in literature or satire as bad omens and the villains of the animal kingdom. The latter is unfortunate as these magnificent animals are an integral part of the ecosystem due to their very efficient method of cleaning a carcass of all its flesh in less than an hour (Houston & Cooper, 1975). The loss of this valuable keystone species would be catastrophic to any ecosystem. Unfortunately, at present this is becoming reality in South Africa, with seven of the nine species found in southern Africa being listed as endangered, vulnerable or critically endangered (Anonymous, 2012). A general decline in vulture populations has also been noted in various Africa countries over the past three decades (Ogada et al., 2012). These declines have been attributed to poisonings (intentional persecution and accidental) (Anderson, 2000); loss of habitat mostly due to changes in the utilisation of land and resultant reduction in available food (Anderson, 2000); electrocutions and collisions with electricity pylons (Ledger & Annegarn, 1981); harvesting of these birds for traditional medicinal purposes (Mander et al., 2007) and drowning in farm reservoirs (Anderson et al., 1999). Additional to such pressures, a vulture's reproductive cycle is not adapted to rapid population renewal. Typical to old world vultures, a pair will lay one egg per year, with breeding only starting after 5-6 years of age.

Various mitigating measures have been implemented to minimise these impacts on the vulture population. However, with the actual cause of decline being uncertain, it is impossible to mitigate against every scenario. An example of such a threat, which has occurred in recent years, was the tragic demise of large populations of Gyps vultures in Asia due to the unfortunate entry of the residue of one specific drug into the vultures' food chain (Prakash et al., 2003; Oaks et al., 2004). The drug in question, diclofenac a non-steroidal anti-inflammatory drug (NSAID), was used extensively in large food producing animals as a measure to alleviate inflammation and pain. However, with cattle being treated with the drug till death, due to religious beliefs, a significant amount of lethal concentrations of diclofenac ended up within the vulture food chain.

With the wide scale seemingly safe use of diclofenac in both human and veterinary medicine, (having fairly typical side effects that are rarely fatal), it was extremely alarming that

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diclofenac was so lethal in vultures. Most importantly, with numerous other NSAIDS being available globally for veterinary use, the safety of these "other" drugs has been questioned - especially with recent information demonstrating the unpredictable nature of said toxicity in CV e.g. recent studies have shown ketoprofen to have a similar toxic profile to diclofenac (Naidoo et al., 2010), while meloxicam was found to be safe (Naidoo et al., 2008). As a result it is believed that the only method to demonstrate toxicity would be to test all possible drugs for their effect in vivo. The following study looks at establishing the overt acute toxicity of flunixin, phenylbutazone (PBZ) and carprofen in a pilot study in the Cape Vulture using pharmacokinetic modelling.

1.1 Hypothesis

Carprofen (11.5 mg/kg), flunixin (1 mg/kg) and phenylbutazone (1.7 mg/kg) is fatal to Cape Vultures following single acute oral exposure.

1.2 Study Aim

The study aims to determine the acute oral toxicity of three veterinary NSAIDs in South Africa in the Cape Vulture.

1.3 Objectives

1. To ascertain the overt toxicity of carprofen, flunixin and phenylbutazone using clinical, clinical pathological and post-mortem evaluations.
2. To ascertain the concurrent pharmacokinetic profile of carprofen, flunixin and phenylbutazone.

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2 LITERATURE REVIEW

2.1 The Cape Vulture

Seven of South Africa's vulture species are listed in the Red Data Book as being endangered or vulnerable. Of these, the Cape Vulture (CV) (*Gyps coprotheres*) from the falconiformes family and classified as an Old World accipitird vulture is a species of high concern (Piper, 2004). The reasons for concern are their limited geographic distribution to southern Africa as well as a small population size of 3000 breeding pairs (estimated in 2004) out of a total population of 10 000 individuals (Anonymous, 2012). Endemic to Southern Africa, breeding pairs are centred around two core populations, one in Eastern South Africa and Lesotho namely the Drakensberg mountains and the other the central part of northern Southern Africa and Botswana. At present the species most northern border is Namibia, where it is believed to be extinct. The range of these birds has declined dramatically over the last century, with only a few remaining birds in the Western Cape regions (Mundy et al., 1992).

Figure 2-1: An Adult Cape Vulture (Photograph K Wolter (www.vulPro.com) used with permission)

The CV (Figure 2-1) is the largest of the South African vultures with wingspans up to 2.5m (Piper, 2005). These obligate scavengers are high fliers, reaching high altitudes from which they are able to sight carcasses through the use of their keen eyesight, with minimal energy expenditure (Mundy et al., 1992). Typical of the old world vultures, CV's are long necked birds, with large curved beaks adapted to tearing flesh from carcasses at such a rate, their crop is filled in as little as 5 minutes (Piper, 2005) with up to 1kg of flesh (Mundy, 1991).

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The CV is easily confused with the White-backed Vulture (WBV) (*Gyps africanus*) vulture, a smaller vulture within the same family. While the two have a large overlapping range, the WBV is easy to distinguish due to it being smaller with a white portion visible on the back during flight, a darker underbelly, darker neck skin and a propensity to nest in tree tops. In contrast, the CV adult's creamy- white plumage of the body contrasts sharply with the dark brown inner secondary feathers and outer black primary feathers (Mundy et al., 1992; Piper, 2005).

The CV's are seasonal breeders, with nest building activity commencing in March/April with an egg being laid around April/May. The egg has an incubation period of 56 days with fledglings taking their first flight at approximately 4 months of age although they are sustained by the parents until the 6 or 7th month. The birds are diurnal, roosting two hours before sunset (Piper, 2005) and gregarious in nature. Nest sites are occupied by many nests and, although not territorial, they have been seen to deter unpaired birds from the nesting sites.

2.2 Threats to the survival of CV

2.2.1 Historical

Although the exact reason for the historic population decline of the CV remains unknown, numerous plausible theories have been put forth. In the early part of the twentieth century, the move towards commercial agriculture resulted in the re-location of the large predators to preserves, with a resultant decline of wild carcasses in the birds' diet and replacement with natural livestock mortalities (Robertson & Boshoff, 1986). While their energy and protein dietary needs were met by natural domestic cattle mortalities, the lack of bone fragments from predator kills and scavenging (mostly the fragmentation of long bones brought about by spotted hyena and lions) resulted in calcium deficiencies and high chick mortalities (Richardson et al., 1986). A further complication arose in the latter part of the twentieth century, due to improvements in farming/hygiene practices in combination with better veterinary care, resulting in a decrease in the available cattle carcasses, with subsequent starvation amongst the birds. The latter was a major cause of population decline, until supplementary feeding stations were started in the early 1980's (Mundy et al., 1992).

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2.2.2 Current

Unfortunately, despite heavy feeding supplementations, various threats to the survival of Cape Vultures still exist (Anderson, 2000). Although each threat in itself may not account for the vast declines in the population, the toll / mortalities of each of the numerous threats is additive, especially when combined with the poor reproductive performance of the species.

2.2.2.1 *Loss of habitat / declining food sources*

It has also been speculated that the decline in vulture populations in Africa is related to the effects of bush encroachment following overgrazing of pastoral land, which leads to increased shrub density (Bamford et al., 2009). Not only does the vegetation density play a role in the ability of the birds to locate carcasses from high altitudes, but the vegetation surrounding a carcass directly influences whether or not the birds will land. It has been noted that birds are reluctant to land at carcasses when the tops of the vegetation surrounding the site creates an angle to the ground of greater than 6° (WBV) and 4° (CV), most likely due to the inability of the bird to regain flight from such an angle (Bamford et al., 2009).

2.2.2.2 *Poisoning / persecution*

Incidental poisonings occur when poison laced carcasses, placed out in the lands to kill farm predators such as jackal (specifically in sheep farming areas), result in the accidental death within vulture populations. While it has been impossible to quantify the numbers of birds, in the last year it is suspected that 400 birds have been accidentally poisoned (K Wolter, Per Comm 2013). Carcasses which are shot with lead containing bullets pose a risk to raptors, and there have been incidences of toxicity documented (Mateo et al., 1997). Contaminants of carcasses (e.g. NSAIDs, antibiotics, barbiturates, animals dying from plant intoxications) laid out at feeding sites have become a concern for conservationists (NSAIDs are further discussed in section 2.6.5). Pesticides placed on livestock to prevent blowfly strike, ticks etc. are another potential source for introducing organophosphate type pesticides into the vultures' food chain.

The intentional poisoning of vultures is also on the increase. An example of such a threat was the death of 183 vultures (species unlisted) in one poisoning incident by poachers (SAPA, 2012). It is believed that this was an attempt by poachers to evade detection, as vultures circling

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above poached carcasses could alert rangers to nefarious activity much sooner than accidentally finding a poached carcass.

2.2.2.3 *Electrocution / Collision with towers*

The electrocution of CV's on power lines was highlighted in the 1970's (Ledger & Annegarn, 1981). In 1972, Markus noted that 148 vultures were killed as a result of electrocutions in a 27 month period between January 1970 and March 1972 (Markus, 1972). The Electricity Supply Commission (ESKOM) reported the deaths of 169 CVs, that appeared to have died from electrocutions as the retrieved carcasses had singed feathers or were found surrounded by burnt patches of grass (Ledger & Annegarn, 1981). A study in 2011, using retrospective power line mortalities in CV for a period greater than 50 years, estimated power line mortalities could result in the extinction of the species in as little as 20 to 35 years based on current population numbers (Boshoff et al., 2011).

Various factors have been found to influence the risk of electrocution (Lehman et al., 2007). The main ones are believed to be the bird's 2.6 m wingspan which is greater than the distances between tower frames and conductors allowing the electricity to ground; landscapes with higher prey densities and lower perch densities increase the electrocution risk, as the only place to perch is the power lines; and the gregarious nature of the bird leads to groups of vultures utilising towers simultaneously, decreasing available space on the structure with the chance of multiple deaths increasing. Further risks are the behaviour of birds under wet conditions, whereby their wings are extended to dry out, which as stated above, increases the risk of grounding/earthing (Lehman et al., 2007).

2.2.2.4 *Use in traditional medicines*

The use of plants and animal parts, with or without communicating with ancestral spirits, form an important component of traditional medicine in Africa. Traditional medicine is considered by a large portion of the population as a first line of choice for healthcare, success in life and the warding off of misfortune. One of the concerns with indigenous medicine practices is the non-sustainable manner by which flora and/or fauna is harvested (Mander et al., 2007). The vulture is one of the species used in traditional medicine especially to grant the traditional healers clairvoyance (Mander et al., 2007). Due to the secretive nature of harvesting, it is

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difficult to obtain figures for the entire county specific to the CV. It was, however, estimated that 160 vulture carcasses are traded a year in eastern South Africa alone for traditional medicine purposes (Mander et al., 2007).

2.2.2.5 *Farm reservoir drowning*

In dry areas (particularly north western southern Africa) and during the dry seasons, steep sided circular farm reservoirs used as an artificial water source by farmers for domestic livestock, are the only available water sources for vultures. The specific design of these reservoirs poses an adverse risk to the survival of vultures, as birds either fall in or fails to get out when they enter to bathe, as the sides are too steep and high for the waterlogged birds to climb out (Anderson et al., 1999). During 1970 – 1999, 120 CV's in a total of 21 incidents of drowning were reported, of which 12 incidents were mass drowning events. Mass drowning in itself is unique to the vultures, most likely related to the gregarious nature of the birds (Anderson et al., 1999).

2.2.3 Factors preventing the rapid growth of the population

Typical of long-lived old world vultures, the CV has a low reproductive rate and long maturation periods. This can be demonstrated by reviewing the biology of CV's:

- Breeding maturity is reached relatively late, after 5 years of age (Piper et al., 1981),
- The CV usually produces only one egg per season with an incubation period of 56 days (Pickford et al., 1989), nestlings are wholly dependent on the parents for 140 days (Piper et al., 1981). Added to this is the poor survival of hatchlings to maturity which further hinders a population increase. An estimated 35 – 45 % average annual survival for all age classes has been reported (Piper et al., 1981). It can therefore take a breeding pair 15 years to produce 2 offspring which survive to adulthood (Mundy, 1991). It is suspected that the birds pair for life (Piper, 2005), with the result that the death of one mate, further restricts the colony's breeding potential.
- The CV's selectively build nests on sandstone and quartzite formations which curtails the breeding sites available to the species (Pickford et al., 1989).

On their own, while none of these factors would be restrictive to population growth, in combination with the numerous pressures placed on the population numbers it is understandable that CVs do not have the capacity to sustain their numbers.

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2.3 Mitigation measures

2.3.1 Food availability

Declines in the chick survival, noted in the 1980s, were suspected to have resulted from calcium deficiency in the chicks. Metabolic bone disease (osteodystrophy) was diagnosed in chicks with paper thin wings that bent or broke easily. When palpating the underside of the wing, breaks and deformities were found in chicks (Richardson et al., 1986; Robertson & Boshoff, 1986). This was linked to the decrease in long bone fragments available to the vulture parents. As a mitigation tool, supplementary feeding sites with cracked / fragment bones being placed out (vulture restaurants) were initiated. The introduction of these sites was believed to be of tremendous assistance to the birds as the chick survival rate increased from 42.3% to 68.9% in the first year (Piper et al., 1999). More importantly, the deaths in the first year CVs was lowest amongst the age classes (Piper et al., 1981). These deaths were mostly due to causes other than food shortages such as shootings, collisions with overhead wires and being caught by dogs.

2.3.2 Poisoning

One of the mainstays of conservation has been education. A tremendous amount of work has been done raising awareness of the plight of these birds, including such initiatives as International Vulture Day held in September every year. Such initiatives endeavour to debunk the myths surrounding these birds, in an attempt to reduce accidental and deliberate poisonings.

2.3.3 Electrocutions

ESKOM has researched pylon mitigation factors and funded conservation efforts in terms of rehabilitating injured birds. Mitigating measures, with variable success, include insulation of the conductors, moving the tower cross arms and providing alternate perches in the vicinity (Lehman et al., 2007).

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2.3.4 Drowning

Drowning in farm reservoirs has been prevented in 300 reservoirs, which have been fitted with floaters, wooden logs or ladders or covered with nets. Landowner education has been critical to this success (Anderson et al., 1999).

2.3.5 Release of captive bred CVs

Organisations such as the Vulture Programme (VulPro) (www.vulpro.com) are dedicated to the conservation of vultures. Injured birds, such as those electrocuted on pylons or poisoned, are treated and, when possible, released. On occasion the birds recover and are unfit to be released. From this population of animals, captive breeding has been successfully initiated. Captive bred chicks are raised by wild bred vulture parents (in captivity) and are released into areas where the CVs are naturally found (K Wolter, 2012 Pers Comm).

Figure 2-2: A breeding pair of Cape Vultures at the VulPro centre, assisting the hatching of a captive bred chick destined for release (Photo W.Neser, sourced from www.vulpro.com, used with permission)

2.3.6 Traditional medicine

A reward programme was attempted in Blouberg CV population, where any person bringing in a living CV that had been captured was given a monetary reward. Although 39 birds were brought in, in a year period (2011-2012), it is unsure how many of these birds were deliberately

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caught to obtain a monetary reward. Many of the birds sustained capture injuries that prevented rehabilitation for release (Cape Vulture Task Force Report, 2012).

2.4 Vulture restaurants: Friend or foe

Despite the great success of vulture restaurants in alleviating osteodystrophy in vulture chicks, these feeding stations have their inherent dangers which need to be carefully considered.

- Position: Placement of the restaurant is important to mitigate against injuries. With electrocution on power lines being of concern (Ledger & Annegarn, 1981), restaurants should not be placed in the vicinity of power lines. Further consideration needs to also be given to placement near reservoirs in which the birds could drown (Anderson et al., 1999; Anderson, 2000). Relative positions to other dangers needs to also be considered as wind farm turbines in Spain between 2006 and 2009, resulted in the deaths of 221 Griffon vultures (de Lucas et al., 2012).

- Poisoning: While the provision of food at the restaurant was always based on the principle of safe food, this cannot in most cases be verified or in some cases the toxicity of veterinary drugs therein may not be known. The latter was highlighted in India, where residues of the veterinary drug diclofenac in the carcasses placed at open carcass dumps, frequented by vultures, resulted in the toxicity and subsequent death of a large number of the birds (Oaks et al., 2004). As a result the three species endemic to South Asia, *Gyps bengalensis, Gyps indicus* and *Gyps tenuirostris*, now face impending extinction across the Indian subcontinent (IUCN listing of critically endangered) (Prakash et al., 2003). More importantly statistical analysis, indicated that as little as one in 130 to one in 760 carcasses needed to contain lethal levels of diclofenac for this catastrophic population decline to occur (Green et al., 2004).

2.5 Mitigation of vulture restaurants

In order to circumvent the dangers of vulture restaurants, yet maintain the benefit of supplementary feeding for the birds, education regarding the establishment and maintenance of these sites is important. The Endangered Wildlife Trust has done this by publishing a booklet (The Sasol guide to the establishment and maintenance of supplementary feeding sites for

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vultures) that is available online (www.ewt.org.za). The booklet highlights important factors such as position and physical characteristics of the site, the erection of observation hides that do not disturb the birds and guidance on the provision of safe carcasses.

Unfortunately little focus is given to the NSAIDs, mainly because the toxic profiles of this class of chemically distinct molecules remains largely unknown. At present South Africa makes use of a number of NSAIDs in use in cattle and horses (see section 2.7). Of the two, cattle carcasses are the most common source of food to the vultures and pose a potential risk of NSAID poisoning. Until more information is available, feeding site operators are advised to not provide carcasses of animals treated with antibiotics, barbiturates, organophosphate dips and NSAIDs other than meloxicam nor animals shot with lead bullets (Anonymous, 2011).

2.6 NSAID's

2.6.1 History

The NSAID drugs originate from the 17th century discovery that willow bark treated fever and inflammation (Burke et al., 2006). The extract from the tree was discovered to be salicin which led to the mass production of aspirin in the 19th century (Rao & Knaus, 2008). However, the mechanism by which this drug worked was only elucidated in 1960's and 1970's (by Sir John Vane), with the result that the development of NSAID drugs came to the fore front (Rao & Knaus, 2008). Since then, many novel NSAID's have been developed and tested.

2.6.2 Mechanism of action

The mechanism of action of these drugs relates to the reduction of inflammation through inhibition in the synthesis of a subgroup of the inflammatory eicosanoids (Burke et al., 2006; Smyth et al., 2006). In the presence of stimuli such as fever, infection or trauma intracellular or extracellular (e.g. snake venom) phospholipase A_2 is stimulated, acting on the phospholipid layer of the cell membrane to release arachidonic acid (AA). Two main enzymatic pathways, lipo-oxygenase (LOX) and cyclo-oxygenase (COX) pathways, utilise AA as a substrate in a cascade of reactions resulting in the formation of the eicosanoid (Figure 2-3) which plays a role in the inflammatory process, mechanical and chemical stimulation of the pain receptors and/or hyperalgesia processes (Burke et al., 2006; Smyth et al., 2006; Rao & Knaus, 2008). One such cascade begins when the COX enzyme cleaves the AA, under the influence of

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hydroperoxidase, to prostaglandin G_2 (PGG_2) and subsequently to prostaglandin H_2 (PGH_2). A range of tissue specific synthases thereafter utilise PGH_2 to produce pro-inflammatory mediators. To date three isoforms of the COX enzyme have been described namely COX1, COX2 and COX3 (Burke et al., 2006; Rao & Knaus, 2008). COX1 is a constitutive enzyme responsible for the production of, amongst others, gastro-protective prostaglandins. COX2 is an inducible enzyme and thought to mediate a large portion of the inflammatory response whereas the clinical significance of COX3, also known as COX1a, has yet to be elucidated although there is some speculation that it may be one of the targets of paracetamol (Clark, 2006).

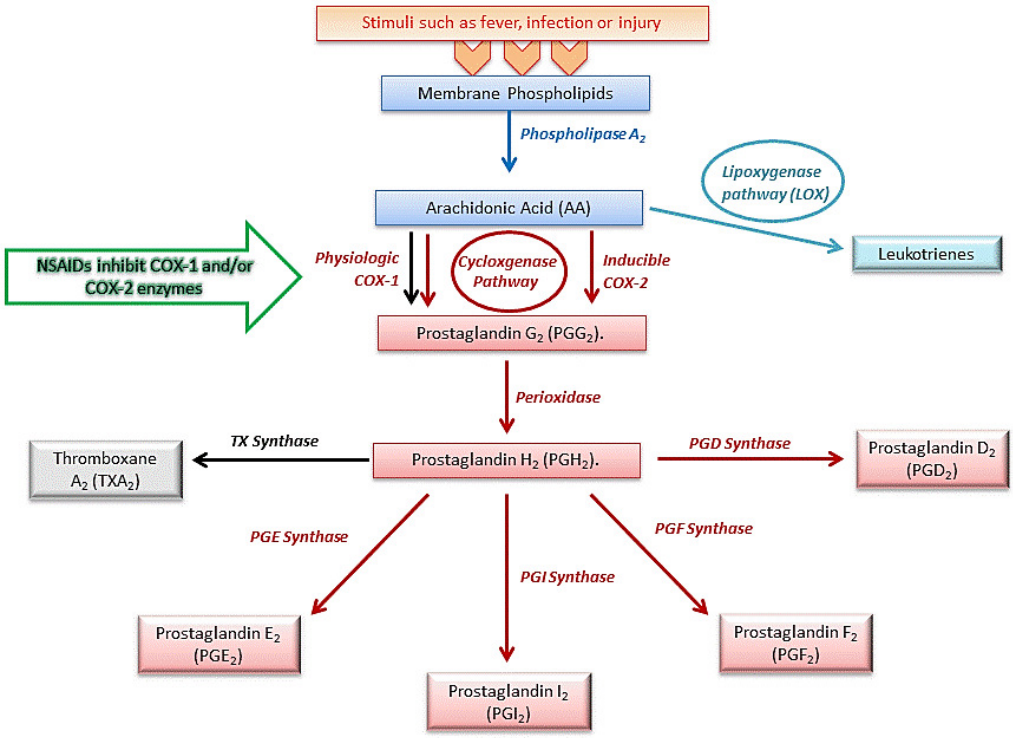

Figure 2-3: Schematic illustration of the COX enzyme mediated cascade responsible for the production of pro-inflammatory mediators and other cytokines from membrane phospholipids. The red shaded areas are inhibited by the inhibition of COX enzymes via NSAID's. (COX- Cycloxgenase, Tx synthase– Thromboxane synthase, PGE synthase – prostaglandin E synthase, PGI Synthase – Prostaglandin I synthase, PGF synthase – Prostaglandin F synthase, PGD synthase – prostaglandin D synthase.) (Figure adapted from Rao & Knaus, 2008; Smyth et al., 2006 & Burke et al., 2006)

The second cascade involves the LOX enzymes, which metabolise AA to unstable intermediates, hydroperoxy eicosatetraenoic acids (HPETEs), which are rapidly converted to corresponding hydroxyl fatty acid (HETE) (Smyth et al., 2006). Five types of cell-specific LOX enzymes have been identified in humans (5-LOX, 12(S)-LOX, 15-LOX-1, 12(R)-LOX and 15-LOX-2), each promoting the production of leukotrienes and/or lipoxins. Leukotrienes are known to play an important role in the development and persistence of the inflammatory

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response such as bronchospasms in asthmatic patients (Smyth et al., 2006). The traditional NSAID class of drugs works by predominantly inhibiting the COX enzymes, thereby inhibiting the production of pro-inflammatory mediators (Figure 2-3). While less common it is also possible that LOX pathway could also be simultaneously inhibited (Burke et al., 2006).

2.6.3 Classification

Traditionally chemical structures (Table 2-1) were used to classify NSAID's. The carboxylic acid derivatives include acetic acids, propionic acids, anthranilic acids, aminonicotinic acids and acetylsalicylic acids. The enolic acids include the pyrazolones and oxicams (Baert & De Backer, 2003).

Table 2-1: Chemical classification of NSAID drugs

Chemical Classification	Drug Names
Acetic acid derivatives	Indomethacin, diclofenac
Arylpropionic acid derivatives	Ketoprofen, carprofen
Enolic acid derviatives	Oxicams (Meloxicam)
Nicotinic acid derivatives	Flunixin
Pyrazolones	Phenylbutazone (PBZ)
N-phenylanthranilic acid	Mefenamic acid, meclofenamate

With the elucidation of the mechanisms by which the drugs function, the NSAIDs are nowadays more commonly classified on their ability to inhibit the COX1 and COX2 enzymes and the ratio thereof (Table 2-2). Drugs with a ratio of less than 1 are preferred as they have a more favourable safety profiles in terms of gastrointestinal effects (Hanson & Maddison, 2008). Table 1-2 provides an example of such classification in humans. NSAIDs are used extensively in human medicine for the alleviation of arthritic pain and other chronic conditions, requiring daily use for lengthy periods of time (Burke et al., 2006). Long term use, high doses and advanced age have been associated with higher risk of gastrointestinal (GI) adverse events, these GI events can be severe and even life threatening (Simon, 1995). GI events have been attributed to the inhibition of COX1 and the development of COX2 selective (or COX1 sparing) drugs were found to minimise these affects. As a result, the classification of NSAIDs according to COX selectivity became important for therapeutic use.

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Table 2-2: Classification of NSAID drugs according to their COX selectivity in people (Rao & Knaus, 2008)

Group	Description of COX selectivity	Examples of drugs
Group 1	Inhibit both COX-1 & COX-2 completely	Aspirin, Ibuprofen, Diclofenac, Indomethacin, Piroxicam
Group 2	Inhibit COX-2 with a 5 – 50 fold selectivity	Celecoxib, meloxicam, etodolac, nimesulide
Group 3	Inhibit COX-2 with > 50 fold selectivity	Refocoxib
Group 4	Weak inhibitors of both COX enzymes	Salicylate, 5-aminosalicylic acid, sulfasalazine

2.6.4 Adverse effects of the NSAIDs

The common adverse effects of NSAID's are gastro intestinal signs such as vomiting, abdominal pain, nausea, gastrointestinal erosions / ulcers / haemorrhage / perforation and diarrhoea, which are all linked to the inhibition of constitutive COX1 enzymes (Galati et al., 2002; Burke et al., 2006). Other manifestations of the adverse effects of NSAID therapy include changes in renal function due to loss of functional renal mass, platelet dysfunction and prolonged gestation. While these side effects can occur following short term use, these side effects are more pronounced following the long term clinical use of these agents especially for chronic ailments such as rheumatoid arthritis in people (Burke et al., 2006).

Renal effects of NSAIDs are unimportant in clinical healthy patients, however in compromised patients the lack of prostaglandin-inhibition results in reabsorption of chlorine and retention of salt and water (via antidiuretic hormone) exacerbating hypertension, oedema and renal function in these already compromised patients (Burke et al., 2006). Additionally there is a decrease in renal blood flow, glomerular filtration and suppressed renin secretion. Other minor side effects are idiosyncratic hepatic toxicity, documented in humans, horses and dogs (Simon, 1995; Hanson & Maddison, 2008).

To overcome the limitations of the predominantly COX-1 inhibiting NSAIDS, drugs which preferentially inhibit COX2 enzymes where developed, with the intention that COX1 enzymes would remain functional and gastrointestinal adverse effects thereby minimised (Clark, 2006).

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Although COX2 selective NSAIDs are not free from side effects, gastrointestinal effects are considerably minimised. COX2 selective NSAIDs are not in themselves safe as they have been associated with an increased risk in cardiovascular events such as stroke, postulated to be a result of increased blood pressure (Simon, 1995; Modi et al., 2012).

2.6.5 NSAID and the CV

The CV is as susceptible to diclofenac as their Asian counterparts (Swan et al., 2006a; Naidoo et al., 2009). Clinical signs associated with toxicity were noted within 12 – 24 hours and included lethargy, neck drooping and death associated with renal failure and an increase in serum uric acid concentrations (Swan et al., 2006b; Naidoo et al., 2009). Clinical pathological changes included an increase in alanine amino transferase (ALT) activities and uric acid (UA) concentrations (Naidoo et al., 2009). Post mortem changes were visceral gout (formation of uric acid crystals in the tissues) on the air sacs, heart and liver. Tophi were present in the lung, spleen, kidney and liver, with resultant hepatic injury and renal necrosis, on histopathology. Similar toxicity with identical clinical, clinical pathology and necropsy signs were also seen with ketoprofen (Naidoo et al., 2010). In contrast to diclofenac and ketoprofen, meloxicam was safe in vultures (Swan et al., 2006b; Naidoo et al., 2008) with no signs of pathology evident on any of the evaluated parameters.

In order to elucidate the mechanism of toxicity, both pharmacokinetic analysis and cell culture models have been used (Naidoo et al., 2009; Naidoo & Swan, 2009; Naidoo et al., 2010). From these studies it appears that toxicity was linked to the production of reactive oxygen species (ROS) and interference with uric acid excretion, in direct relation to the length of time the renal tissue was exposed to the drug. In vivo using Cape Vultures this correlated with the half-life of the drugs as both diclofenac and ketoprofen were characterised by long half-lives (12.24 ±0.99 hr. and 7.38 hr.) while meloxicam was characterised by a short half-life (0.32 hr.).

In surveys of veterinary treatments of scavenging birds with NSAIDs diclofenac, carprofen (13% of cases), flunixin (30% of cases), ibuprofen and PBZ were associated with renal disease, gout and mortalities (Cunningham, 1991; Cuthbert et al., 2007)

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2.7 NSAID in South Africa

The risk of NSAID introduction into the vulture food chain is highest from food producing animals (Cattle, sheep, goats, pigs) and horses due to the practice of operational vulture restaurants in South Africa. In order to assess the risk of NSAIDs entering the food chain for South African vultures, NSAIDs registered for use in the country were listed (Table 2-3) and chosen through an elimination process. Ketoprofen and meloxicam, having already been investigated, were not included in the study. Firocoxib, being costly and registered solely for companion animals was eliminated, while vedaprofen although a possible contaminant of horse carcasses was not frequently used and as such is no longer marketed in South Africa (Carrington et al., 2013).

This therefore left carprofen, flunixin and PBZ. While carprofen has only been recently registered for use in horses and cattle, both PBZ and flunixin are extensively used in equines and production animals respectively.

Table 2-3 NSAIDs registered for use in South Africa at the time of the study (Carrington, 2009)

Active NSAID	Trade Names	Formulation[#]	Species Registered	Meat WD
Carprofen	Rimadyl and Norocarp	Injectable and tablets	Can	None listed
Ketoprofen	Ketofen 1% Ketofen 10%	Injectable and tablets	Can and Fel Bov, Eq, Por	None listed 4 d (Bov), 4 d (P)
Flunixin	Cronyxin Finadyne Hexasol HB injection Pyroflam NS	Injectable	Bov and Eq Bov, Eq, P and Can Bov Eq and Bov	7 d 5 d (Bov),28 d (P) 21 d 10 d
Firocoxib	Previcox Tablets	Tablets	Can	None listed
Meloxicam	Metacam and Petcam	Oral suspension	Can	None listed

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Table 2-3 cont. NSAIDs registered for use in South Africa at the time of the study (Carrington, 2009)

Active NSAID	Trade Names	Formulation[#]	Species Registered	Meat WD
PBZ	Equipalazone	Powder (oral)	Equine	None listed
	Fenylbutazone 20%	Injectable	Bov, Eq, Por and Can	3 d
	Phenylarthrite	Injectable	Bov**, Eq, Por and Can	None listed*
Key: WD – withdrawal ; Bov – Bovine; Can – Canine; Eq – Equine; Fel – Feline; Por – Porcine; d – days; *not registered for use in food producing animals; [#] All registered for daily use at most unless indicated; **Registered for 48 hourly dosing in bovines				

2.7.1 Carprofen

Carprofen is a moderately potent inhibitor of phospholipase A2 and reversible inhibitor of COX enzymes, with selectivity for COX-2 *in-vitro* using dog blood (Hanson & Maddison, 2008). Its registered indications include the alleviation of post-operative pain and inflammation, osteoarthritic and musculoskeletal conditions in dogs (Carrington et al., 2013). Use in farm animals in other countries has been documented and, recently, it has been registered in South Africa for equine and bovine use as a conventional formulation (Carrington et al., 2013). Toxicity associated with the use of this drug has been tentatively documented in raptor species although the mechanism of such toxicity has not been clarified (Cuthbert et al., 2007).

2.7.2 Flunixin

Flunixin an aminonicotinic acid is indicated for the alleviation of acute inflammation and pain due to bovine respiratory diseases and acute mastitis, in equines for musculoskeletal pain and visceral pain associated with colic (Carrington et al., 2013), it is also use for the treatment of endotoxic shock in various species and in some countries is registered for use in dogs for osteoarthritic and musculoskeletal conditions. Significant renal dysfunction has been documented in dogs with the use of this drug (Hanson & Maddison, 2008). Toxicity of flunixin in avian species was demonstrated when administered at 0, 1.25, 2.5, 5 and 10 mg/kg body IM daily for four days to broilers with clinical signs analogous to diclofenac toxicity in vultures, namely stiff gait, anorexia, depression, lethargy and coma (Ramzan et al., 2012). Mortality rates in 2.5, 5 and 10 mg/kg groups were 20, 40 and 60% respectively within 48 – 72 hours of administration. Pathological lesions of visceral gout were confirmed by histopathology of urate

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tophi in the tissues. Concurrent elevation of serum uric acid and creatinine concentrations was associated with the drug's toxicity. Therapeutic use of the drug in scavenger birds has also been subjectively associated with mortalities with renal disease and gout (Cuthbert et al., 2007).

2.7.3 Phenylbutazone

One of the first NSAIDs in canine medicine, this pyrazolone NSAID is effective for osteoarthritic conditions in dogs, and equines. Postoperative use is limited to equines. At the time of this study, phenylbutazone was still registered for use in South Africa. From May 2014, the use of this NSAID has been declared prohibited for both human and veterinary medicine due to its propensity to cause irreversible agranulocytosis (Burke et al., 2006) and aplastic anaemia in people, even at concentrations present in food of animal origin. At 50 mg/kg and 100 mg/kg administered intramuscularly twice a day to broilers, PBZ was found to be toxic even though uric acid (UA) concentrations were not elevated nor was there any post-mortem lesions in the kidney up to 9 days post treatment. The study concluded that the drug was hepatotoxic in broilers with elevations in serum activities of AST, ALT and ALP as well as post mortem lesions in the liver (Awan et al., 2003).

2.8 Conclusion

Cape Vultures are an ecologically vital species that are declining in number due to numerous threats. Conservation of the vulture is a challenge due to the large number of threats, varied nature of these threats and large home ranges extending outside of a single country's border. Being endemic to southern Africa, and with the vast majority of the breeding population situated within South Africa, there is a need for local research to conserve the CV. One of the tools used with much success are supplementary feeding sites or so-called "vulture restaurants". However, in recent years the benefit of these sites was brought into question following the discovery that diclofenac, a NSAID veterinary drug utilised in cattle and horses, was lethal to vultures. With the general class of NSAIDs, being extensively used for the alleviation of pain and inflammation in veterinary medicine, it is imperative to establish the relative toxicities of the NSAIDs to ensure carcasses placed out at vulture feeding sites are safer. This study will add to this database of information, by evaluating the acute single dose toxicity of carprofen, flunixin and phenylbutazone in CV's in a pilot study.

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3 MATERIALS AND METHODS

3.1 Animals

Eight female non-releasable captive Cape Vultures (*Gyps coprotheres*) kept at the Rhino and Lion non-profit organisation's Vulture Programme Centre in Rietfontein were used for this project. Five of the birds were immature (1-3 year old), one bird a fledgling, one bird between 2 and 4 years old and the eighth bird between 3 and 5 years old. The birds were pair housed in aviaries of 5 x 3 m in size, with soil floors, perches and diamond mesh sides under natural environmental conditions. Each aviary contained a wooden dog house, large enough for a giant breed dog, for the birds to use for protection from inclement weather. The birds had last eaten two days before dosing and had free access to potable water during the study. Ethical considerations for the study were approved by the Animal Use and Care Committee of the University of Pretoria (Protocol Number: V006-10).The Vulture Programme centre activities were under approval of Gauteng Nature Conservation and North-West Nature Conservation.

3.2 Study design

The study was based on previously published study designs used by Swan et al, 2006a for the *Gyps africanus* and Naidoo et al., 2009 for *G. coprotheres,* evaluating the safety of diclofenac. The study schedule is presented in Table 3-1.

Table 3-1: Study schedule of events

Date	Day of Study	Event
03/05/2010	-1	Birds weighed and assigned to groups.
04/05/2010	0	Birds dosed, PK study started and birds monitored for toxicity
05/05/2010	1	PK Study and monitoring for toxicity
06/05/2010	2	PK Study and study termination
09/2010 and 11/2011		Pharmacokinetic analysis performed
18/01/2012		Clinical Pathology analysis performed
02/11/2012		Repeat of the PBZ analysis Metabolite analysis performed
02/2013		Metabolite identification attempted

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3.3 Treatment

Animals were randomly assigned to a treatment group on the day before dosing. The study contained 4 treatment groups namely carprofen (drug 1), flunixin (drug 2), PBZ (drug 3) and a water control (drug 4). The drug details are listed in Table 3-2.

Table 3-2: Treatment information: Drug details

Product Name	Manufacturer	Expiry Date	Batch	Active	Strength (mg/ml)	Dilution
Fenylbutazone (injectable)	Virbac	05/11	LL0812 01/98	Phenyl-butazone	200	1:4
Finadyne	Scherring Plough	08/2012	9462	Flunixin	50	1:1
Rimadyl	Pfizer	07-2012	P722/028 SA	Carprofen	50	None

Doses were determined using the method described by (Swan et al., 2006b). Namely the maximum concentration of the drug in the tissue of either cattle, horses or pigs (Table 3-3), was used to calculate the estimated dose an adult Cape Vulture of approximately 8 kg would ingest at one meal should the animal eat a meal of 2 kg. The latter was based on Komen's work, who found that an adult Cape Vulture required approximately 0.52 kg of meat per day for a 4 day period (Komen, 1992). In order to account for inaccuracy or higher population tolerances, the estimated intake doses were doubled. To allow for clinical use of the product, the current clinical indicated dose was also considered.

With regards to the clinical use of these NSAIDs in vultures, only carprofen has been considered a therapeutic agent, with a dose of 10 mg/kg (Anonymous, 2005). As a result, the birds were treated with carprofen, flunixin and PBZ at 10, 1 and 1.7 mg/kg respectively.

For treatment, the birds were manually caught within the aviary, the beak opened and the required volume placed into the mouth. Birds were restrained until swallowing was confirmed.

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Table 3-3: Maximum concentration of carprofen, PBZ and flunixin in tissues from either cattle, horses or pigs according to EMEA reference values

Drug	Concentration in meat (mg/kg)	Species	Tissue	Estimated intake (mg/kg)[a]	Double the estimated dose
Carprofen[b]	4.62	Horse	Kidney	1.16	3.32
PBZ (and oxyphenbutazone an active metabolite)[d]	3.4	Horse	Kidney	0.85	1.70
Flunixin[c]	1.95	Cattle	Liver	0.49	0.98

[a] Based on 2kg meal in an 8 kg bird; [b] The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit, (January 1999) Carprofen summary report; [c] The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit, (August 1999) Flunixin summary report. [d] The European Agency for the Evaluation of Medicinal Products, Veterinary Medicines Evaluation Unit. (October 1997) Phenylbutazone summary report.

3.4 Clinical observations

The observations for any signs of toxicity were recorded on day 0 at 15h30 (6.5 h post dosing); 17h05 (8 h post dosing) and 21h00 (12 h post dosing) and on day 1 at 06h00 (21 h post dosing), 08h00 (23 h post dosing), 12h30 (27.5 h post dosing) and 19h00 (34 h post dosing). Additional to these times, the birds were observed for signs of toxicity each time blood was collected. Signs of toxicity included any sign of malaise or disease, such as lethargy, drooping neck, sitting on hocks, dehydration etc. Dehydration was estimated by looking at the tenting of a skin fold on the neck.

3.5 Blood collection

Blood was collected from the tarsal vein (when necessary the wing vein or jugular vein was used) immediately before drug administration and at 0, 5 and 30 min; 1, 1.5, 2, 3, 5, 7, 9, 12, 24, 32 and 48 hours after treatment with a 21G needle and 5 ml syringe. One to five millilitres of blood was collected per bird, half of which was placed into either an EDTA or anti-coagulant free evacuated tube (Vacutainer, Becton Dickinson, South Africa). The serum samples were left to clot for 30 minutes before centrifuging (Capricorn Laboratory Equipment),

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while the EDTA samples were centrifuged immediately at 450 x g for 20 minutes, all at ambient temperature. All samples were centrifuged within 2 hours of collection.

The supernatant was pipetted off with a glass pipette and rubber bulb, into labelled cryo tubes (NUNC, South Africa). The samples were placed in a -20°C freezer on site until transported on ice to the -20°C freezer at the Department of Pharmacology, Faculty of Veterinary Sciences, University of Pretoria (UP). The serum samples were stored for 2 years in a freezer after which time clinical pathology analysis was performed on the samples. The plasma samples were sent on ice to the Department of Pharmacology, UP for LC/MS-MS analysis.

3.6 Clinical pathology methods

Plasma samples were analysed by the Department of Companion Animal Studies, Clinical Pathology Laboratory, University of Pretoria in January 2012, using the Cobas Integra 400 (Roche Diagnostics) for activities of alanine transferase, concentrations of albumin, calcium, potassium, sodium and uric acid while uric acid was also measured with a patient-side electronic monitor. The ranges of the individual tests and the references are tabulated in Table 3-4.

Table 3-4: Details of the clinical chemistry tests utilised, as described in the Roche Diagnostics methods manual for the Cobas Integra 400

Parameter	Test ID	Methods Manual Reference	Test Range
ALT	0-495	2008-07 V5 EN	2 – 700 U/L
ALB	0-592	2007-11 V4 EN ALB2	2 – 60 g/L
Ca	0-012	2006-11 V7 EN	0.1 – 5.0 mmol/L
K	ISE Direct	2008-02 V4 EN	0.2 – 30 mmol/L
Na	ISE Direct	2008-02 V4 EN	20 – 250 mmol/L
Uric Acid (UA)	0-615	2008-01 V4 EN UA2	11.9 – 15000 umol/L

The clinical pathology parameters were graphed over time for each bird with the normal values, derived in the Cape Vulture using 24 birds published by (Naidoo et al., 2009), drawn as bold linear lines at the upper limit (Max) and lower limit (Min). The exact methods used per analyte are also presented below. It is important to note that the two year storage of the samples before analysis is of concern. The impact on the study results is discussed in section 5.1.2. In some

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instances, a blood sample was unable to be obtained or the amount of serum drawn off from the sample was an insufficient volume for analysis, NS (no sample) was used to denote this. A comparison between the handheld UA meter and the clinical pathology results was also performed, with corresponding time data points being compared.

3.6.1 Albumin (ALB)

The Cobas Integra Albumin Gen 2 test works on the basis of a colorimetric assay described by Doumas, Watson and Briggs (Doumas et al., 1971), namely that at a pH of 4.1 albumin becomes sufficiently cationic in character than it binds to an anionic dye (bromcresol green or BCG) to form a blue green complex. The intensity of the blue green colour is measured by monitoring the increase in absorbance at 583 nm and is directly proportional to the concentration of albumin.

$$ALB + BCG \xrightarrow{pH\ 4.1} ALB\text{-}BCG\ Complex$$

3.6.2 Alanine transferase (ALT)

ALT is tested according to the method described by the International Federation of Clinical Chemistry (IFCC) but without pyridoxal-5'phosphate (Schiele et al., 1992). The test utilises the principle that the catalytic activity of ALT is directly proportional to the rate at which nicotinamide adenine dinucleotide (NADH) is oxidized in the following set of reactions:

$$L\text{-}alanine + 2\text{-}oxoglutarate \xrightarrow{ALT} pyruvate$$

$$Pyruvate + NADH \xrightarrow{LDH} L\text{-}lactate + NAD^{+}$$

Oxidation of NADH is measured by a decrease in absorbance at 340 nm.

3.6.3 Ionised calcium (Ca^{2+})

Utilising the method according to Schwarzenbach (Schwarzenbach, 1955) calcium concentration is directly proportional to the colour intensity of the violet complex formed when calcium ion reacts with o-cresolphthaleincomplexane (O-CPC), measured by an increased absorbance at 552 nm.

$$Ca^{2+} + O\text{-}CPC \xrightarrow{alkaline\ pH} calcium - O - CPC - complex$$

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3.6.4 Potassium (K) and sodium (Na)

Potassium and sodium were measured by ion selective electrodes (ISE) in the Cobas Integra using the methods according to Shono et al., 1982 for potassium and Pioda et al., 1969 for sodium. The potential which developed across the membrane is automatically converted to the ion concentration by using the Nernst equation for the specific ion (Pioda et al., 1969; Shono et al., 1982).

$$E = \mathrm{Eo} + \mathrm{RT} / \mathrm{nF} \cdot \ln(\mathrm{f} - \mathrm{Ct}) / (\mathrm{f} - \mathrm{Ci})$$

Where E =electrode EMF; $E_{o=}$ standard EM;, R= constant; T= temperature; n= charge of the ion; F= faraday's constant; f=activity coefficient; Ct= ion concentration in test solution and Ci= ion concentration in internal filling solution.

3.6.5 Uric acid (UA)

The Cobas Integra UA ver 2 test (UA2) is an enzymatic colourimetric test based on the method described by Prætorius and Poulsen in 1953 with modifications by Siedel. Uricase cleaves uric acid to form allantoin and hydrogen peroxide which is further oxidised to quinone-diimine dye. Uric acid concentration is directly proportional to the colour intensity of the quinone-diimine dye which is measured by an increase in absorbance at 552 nm.

$$UA + 2H_20 + O_2 \xrightarrow{uricase} allantoin + CO_2 + H_2O_2$$

$$2\,H_2O_2 + H^+ + TOOS + 4\text{-}aminophenazone \xrightarrow{peroxidase} quinone\text{-}diimine\ dye + 4H_2O$$

3.6.6 Uric acid (handheld meter)

Uric acid (UA) was also determined using the UASure ® Blood Uric Acid Meter (Apex Biotechnology Corporation) at 8, 9, 12, 24 and 32 hours post dosing. The meter was calibrated, as per manufacturer's instructions, with the code card packaged with the test strips. The UASure ® blood uric acid test strip, a biosensor-based electrode, was inserted into the slot on the handheld meter and a drop of whole blood from the bird touched onto the tip of the strip, filling the reaction zone of the strip. The meter reads uric acid between 3.0 mg/dL and 20 mg/dL, values outside of this range are indicated by "Lo" or "Hi" on the display screen. The results obtained from this meter were compared to the Cobas integra results by means of a scatter plot and Pearson correlation to determine if this meter could be a useful tool for

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"bedside" UA measurements in further vulture studies. The method by which the meter measures uric acid was not made available by the manufacturer.

3.7 Post mortem examination

The animals underwent euthanasia after the final observation point (48 hours post treatment). Sodium pentobarbitone (Euthapent, Bayer) was administered intravenously into the tarsal vein at 1 ml/kg. The carcasses from birds 1 – 6 where sent to the Department of Pathology, Faculty of Veterinary Sciences, University of Pretoria, for post mortem examination. Samples were collected from parenchymatous organs in 10% buffered formalin and routinely processed for histopathology examination with H and E staining.

3.8 Quantification of drug in the plasma

The pharmacokinetics data was determined using a liquid chromatography tandem mass spectrometry (LC/MS-MS) under the supervision of Dr Duncan Cromarty at the Department of Pharmacology, Faculty of Health Sciences, University of Pretoria.

3.8.1 Equipment

- Applied Biosystems/MDS Sciex 4000 Q Trap mass spectrometer with a "Turbo V" ion spray source (electrospray ionisation source ESI)
- Agilent 1100 series High Pressure Liquid Chromatograph system with a temperature controlled autosampler and six port switching valve
- Diode Array detector (Prominence) SPD-M20A*
- RF-10AXL Shimadzu Fluorescence detector*
- Shimadzu Prominence liquid chromatography LC-20AT
- Analyst 1.5.2 Software

* This equipment was utilised to collect additional confirmatory data, this data was not utilised in the final analysis as the mass spectrometer information was sufficient.

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3.8.2 Method: carprofen and flunixin analysis

Plasma samples were thawed and 200 µL aliquots were diluted 1:1 with water and 25 µL meloxicam (2 ug / mL) added as an internal standard, centrifuged at 850g's for 5 minutes to remove the insolubles and the supernatant placed into the autosampler vials. From the temperature controlled (set point of 12°C) auto-sampler, a volume of 10 µL was injected and transferred to a capture column [BDS 10 x 4.6 mm C18] extracting the analytes from the sample while allowing salts and protein to pass through to the waste. After washing with 0.01% formic acid in water at a reduced flow rate for 3.2 minutes the valve switched allowing elution onto a Hypersil C18 DB analytical column [50x4.6 mm]. Elution off the capture column to the main analytical column was achieved with and then resolved with a mobile phase consisting of A: 0.1% formic acid in water at a pH 3.1; B: 0.1% Formic acid in MeCN. Gradient elution was performed using the following gradient (total time indicated): Step 1: 0.8 min, 100% A; Step 2: 0.9 min, 100% A; Step 3: 3 min, 100% A; Step 4: 3.8 min, 5% A and 95% B; Step 5: 5.2 min, 5% A and 95% B; Step 6: 5.25 min, 100% A; Step 7:7 min, 100% A. The eluted sample was passed through a diode array detector before introduction into the mass spectrometer. Mass spectrometer was set on a negative polarity mode, MRM scan type and a unit resolution for both Q1 and Q3.The ionisation source voltage was -4000V, extraction potential -10 V and collision cell extraction potential -10 V.

Parameters were optimised individually for each analyte as determined during instrument tuning with at least two mass transitions being monitored for quantitation and confirmation of analyte identity. These mass transitions and related instrument settings are summarised in Table 3-5.

Table 3-5: Tuning parameters of the mass spectrometer for each analyte

Analyte	Q1 mass (Da)	Q3 mass (Da)	Dwell (msec)	DP	CE
Carprofen 1	272.300	228.000	80.00	-40.00	-32.00
Carprofen 2	272.300	190.100	80.00	-40.00	-55.00
Flunixin 1	295.400	251.200	80.00	-35.00	-28.00
Flunixin2	295.400	209.300	80.00	-35.00	-35.00
Meloxicam 1	350.400	146.200	80.00	-35.00	-30.00
Meloxicam 2	350.400	286.400	80.00	-35.00	-20.00
PBZ 1	307.400	279.500	80.00	-35.00	-28.00
PBZ 2	307.400	131.000	80.00	-35.00	-40.00

KEY: DP – Declustering Potential; CE – Collision Energy; Da – Dalton; msec - millisecond

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3.8.2.1 *Calibration of the method: Carprofen and Flunixin*

Method development and calibration validation was performed using chicken plasma due to the unavailability of large volumes of vulture plasma. Freshly drawn chicken plasma was spiked with the different analytes at seven different concentrations covering the expected concentration ranges to be found in the vulture plasma samples.

A fixed concentration of an internal standard (meloxicam) was included in all vulture samples. The meloxicam stock solution was initially made up in methanol at 2 mg/ml and sequentially diluted 1/10 in methanol then 1/100 in methanol:water (1:1) to give a 2 µg/ml solution. A volume of 25 µL of this internal standard stock solution was added to 225 µL plasma to give a concentration of 200 ng/ml per sample. The internal standard CPS values were graphed in Microsoft Excel (2010), analyte values were considered to be valid when the internal standards were within 2 standard deviations of the mean for each drug.

Stock solution of standards of each analyte (carprofen, flunixin and PBZ) were made up to 10 µg/ml in methanol. From these stock solutions the different concentrations of standards was made up in either 10% methanol in water or into chicken plasma. Three concentration series of 31.3, 62.5, 125, 250, 500, 1000 and 2000 ng/ml of each standard were made and analysed separately (run 1 – 3). This was done by directly spiking the diluent at 1/40, 1/20, 1/10 and 1/5 for the highest concentrations. The lower concentrations for the calibration curve were made by making a further 1/20 dilution of the 10 µg/ml stock in methanol and then making a ¼, 1/8 and a 1/16 dilution into the diluent for the lower concentrations. Quality control standards were made from a separate weighing of the dry standards and made to a final concentration of 100 and 1250 ng/ml.

A response factor for each analyte was determined in Microsoft Office Excel from the calibration curve by plotting a regression line on the average peak versus concentration graph for each analyte. The slope of the line was used as the response factor. The conversion of the peak area counts to concentrations was performed in Microsoft Excel 2010 using the response factor. The value of the zero hour sample for each bird was subtracted from each subsequent time point's concentration value to account for background noise. These corrected concentrations were used for kinetic analysis. Signal to noise ratios of the calibration curve

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were obtained using the Analyst software. Accuracy and precision of the curves was calculated in Microsoft Excel 2010, using the formulas and methods as described in the ICH harmonised tripartite guideline on the validation of analytical procedures (VICH, 1998) text and methodology Q2(R1) (ICH, 2005).

3.8.3 Method: PBZ analysis

The PBZ analysis performed using the method described in 3.8.2 above, produced questionable and variable results. The analysis was therefore repeated using a manual extraction method of the analyte.

The plasma samples were thawed, 100 µL aliquots were diluted 1:1 with 100 µL methanol containing ketoprofen (0.5 ml ketoprofen 2.7 mg/ml was diluted in 24.5 ml of methanol) and sonicated for 10 minutes in the ultrasonic bath. A further 100 µL of methanol/ketoprofen was added into the sample and sonicated for 10 minutes in the ultrasonic bath. The sample was centrifuged for 10 minutes at 14800 rpm (Beckman Coulter Microfuge 16 centrifuge) to obtain a pellet of protein precipitates in the sample. The 100 µL of the supernatant was drawn off and pipetted into labelled autosampler vials. From the temperature controlled (set point of 12°C) auto-sampler a volume of 10 µL, was injected onto a Hypersil C18 DB analytical column [50x4.6 mm]. The mobile phase consisted of A: 0.1% Formic acid in water adjusted to pH 6.1 with ammonium hydroxide; B: 5mM Ammonium formate in 27% water: acetonitrile at a pH of 6.1. Gradient elution was performed using the following gradient (total time indicated): Step 1: 0.75 min, 75% A and 25% B; Step 2: 4 min, 5% A and 95% B; Step 3:5 min, 5% A and 95% B; Step 4: 5.5.min, 75% A and 25% B; Step 5: 7 min, 75% A and 25% B. The sample was passed through a diode array detector and into the mass spectrometer. Mass spectrometer was set to a negative polarity mode, MRM scan type and a unit resolution for both Q1 and Q3.The ionisation source voltage was -4000 V, extraction potential -10 V and collision cell extraction potential -10 V. The diode array with UV lamp on and visible lamp off was set in spectral scanning mode, from 210 to 400 nm with a 2 nm step width.

3.8.3.1 *Calibration of the method (PBZ)*

Method development and calibration validation was performed in chicken plasma and solvent. Freshly drawn chicken plasma or solvent was spiked with the PBZ (10.2 mg/ml) at seven

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different concentrations (0.41, 1.02, 2.04, 4.08, 10.2, 13.6 and 20.4 µg/ml) covering the expected concentrations to be found in the vulture plasma samples.

Ketoprofen was used as an internal standard (IS), 0.5 ml ketoprofen (2.7 mg/ml) in 24.5 ml MeOH was used. 100 µL PBZ 10.2 mg/ml was aliquotted into 5000 µL of water: methanol/ketoprofen (50:50) to create a solution of 204 µg/ml PBZ. A volume of 50 µL of this solution was diluted in 450 µL of plasma for a 1:10 (20.4 µg/ml); 20 µL in 280 µL plasma for the 1:15 (13.6 µg/ml). 100 µL of the 20.4 µg/ml was added to 100 µL of plasma for the 10.2 µg/ml solution; 50 µL into 200 µL plasma for the 4.08 µg/ml concentration and 20 µL into 180 µL of plasma for the 2.04 µg/ml concentration. A volume of 100 µL of the 2.04 µg/ml solution was added to 100 µL of plasma to create 1.02 µg/ml and 50 µL was added to 200 µL of plasma for the final solution of 0.41 µg/ml.

The calibration was run three times for the solvent and plasma and plotted in Analyst software. The conversion of peak areas to concentrations was performed using the response factor determined from the calibration curve. The Analyst software plotted the ratio of the PBZ to IS, using the regression line slope of this plot as a response factor to convert peak analyte areas to analyte concentrations. The precision, accuracy and signal to noise ratio was determined in Analyst.

3.8.4 Method: Metabolite identification

Plasma samples for bird 1-4 were thawed and 50 µL diluted with 50 µL of methanol. The samples were sonicated for 10 minutes and thereafter centrifuged for 10 minutes. The supernatant was pipetted into labelled autosampler vials. The samples prepared for bird 5 and 6 as per 3.8.3.1 above were used for the metabolite studies. Samples were chosen to reflect the elimination phase of the plasma concentration time curve.

A search for metabolites was performed using Light sight version 2.3 from ABSciex on the MRM Q1 data obtained during the pharmacological analysis of the samples. The following molecular weights were used: PBZ ($C_{19}H_{20}N_2O_2$) 308,37 Da; flunixin ($C_{14}H_{11}F_3N_2O_2$) 296,24 Da and carprofen ($C_{15}H_{12}ClNO_2$) 273,71 Da. A zero hour sample from bird 5 was used as the control sample. The program was set to detect both phase I and II metabolites.

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The graphs obtained were manually assessed using the criteria listed below (D. Cromarty, Per. comm., 2013)

1) If the peak corresponded to the parent molecule's retention time, as was the case with decarboxylation and hydrogenation for carprofen, this was deemed an artefact of ionisation in source of the MSMS and excluded.
2) If the peak occurred simultaneously with a peak found in the untreated control sample, it was excluded.
3) Decarboxylation and demethylation with retention times similar to the parent molecule were excluded as it is expected with these metabolites to have retention times different from the precursor molecule.
4) Dehydrogenation occurring long after the parent molecule was excluded, as dehydrogenation of the parent molecule would result in metabolite retention times close to the precursor.
5) Sulfonation with long retention times was excluded. Due to the increased polarity of sulfonated metabolites retention times are expected to be earlier that the precursor.
6) Methylation with short retention times was excluded. Due to the decrease in polarity of these metabolites the retention time is expected to be increased.
7) Peaks occurring on total ion chromatographs with a lot of noise at the same intensity of the peaks were excluded.
8) Peaks with an AUC that by visual inspection appeared to be very small compared to the noise where considered less likely to be relevant.
9) Loss of water occurring at retention times of more than 5 minutes after the precursor's retention time was considered to be an artefact.

3.9 Hysteresis plots

Hysteresis plots were graphed in Microsoft excel 2010, by utilising a scatter plot with the plasma concentration of the drug on the x-axis and the corresponding time point's clinical pathology parameter serum concentration plotted on the y-axis. The population reference intervals were included for comparative purposes. Previously collected data for diclofenac, ketoprofen and meloxicam, were also analysed for comparative purposes.

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3.10 Pharmacokinetic and statistical analysis

All calculations were performed in Microsoft office Excel (2010), using the standard formulae. All graphs were plotted in Microsoft Excel 2010. The pharmacokinetic curve fitting was performed in Kinetica 5.2 (ThermoElectron Corporation) using non-compartmental and compartmental modelling. Compartmental results were used for the curve fitting plots. The terminal phase was utilised to determine the elimination half-life (T½) and elimination rate constant (λ). The area under the plasma concentration versus time curve (AUC) was obtained using the linear trapezoidal rule, up until the last measurable concentration (C_{last}), with extrapolation to infinity (AUC_{inf}) using the elimination rate constant (C_{last}/λ). Total body clearance (Cl), volume of distribution (Vz) and the mean residence time (MRT) were calculated using standard formulae. The compartmental modelling was performed according to the macro extravascular model available in Kinetica.

The results from the two uric acid meters was analysed to determine correlation. The two readings were plotted in Microsoft Office Excel (2010) using a scatter plot for visual inspection. A Pearson correlation coefficient was thereafter calculated in Excel (2010). A p-value <0.05 was interpreted as a statistically significant finding. An r value of >0.9 was considered a strong correlation.

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4 RESULTS

4.1 Treatment

The dose that each bird received was based on the previous day's body weight and calculated in Excel (Office 2010, Microsoft). The final doses and volumes administered per bird are presented in Table 4-1.

Table 4-1: Dose determination and allocation of bird to treatment group

Bird Number	Treatment	Weight (kg)	Dose	Total Dose (mg)	Conc. (mg/ml)	Dose (ml)	Dose Administered (ml)
1	Rimadyl	7.2	11.5	82.8	50	1.66	1.65
2	Rimadyl	9.0	11.5	103.5	50	2.07	2.10
3	Flunixin	7.0	1.0	7.0	25	0.28	0.28
4	Flunixin	9.0	1.0	9.0	25	0.36	0.36
5	Fenylbutazone (injectable)	7.2	1.7	12.2	25	0.49	0.49
6	Fenylbutazone (injectable)	8.0	1.7	13.6	25	0.54	0.55
7	Water	8.0	0.0	0.0	0	0.00	1.00
8	Water	9.2	0.0	0.0	0	0.00	1.00

4.2 Clinical observations

The significant findings are presented in Table 4-2. While no unscheduled mortalities were recorded following treatment, short term depression was noted in one of the carprofen and both PBZ treated birds approximately 6 and 1 hour after dosing respectively. Depression was characterised as a bird being less active than a pen mate or the controls, sitting still for long periods of time or not coming out of the refuge in the pen. Clinical signs of lethargy and depression were noted in the 1/2 carprofen treated birds, 2/2 flunixin treated birds, 1/2 PBZ treated birds and 0/2 control birds during the monitoring period. Forty-eight hours post treatment the depression noted in the flunixin treated birds had resolved.

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Table 4-2: Significant findings noted during observations of the birds for signs of disease, malaise or toxicity

Bird	Drug	Significant Findings
Bird 1	Car	None
Bird 2	Car	Day 0: Slightly depressed at 15h30 only
Bird 3	Flx	Day 1: at 19h00 mild depression
Bird 4	Flx	Day 0: at 15:30 the bird was not as active and alert and sitting on its hocks Day 1: at 19h00 mild depression
Bird 5	PBZ	Day 1: at 6h00 bird was less active
Bird 6	PBZ	Day 0: One hour after dosing the bird was depressed. Seven hours after dosing 5% dehydration was present, the bird remained depressed for the rest of the day. Day 1: dehydration was noted until 12h30
Bird 7	Control	Five hours post treatment: the wing stump sustained an injury characterised by oozing blood however the wound was considered to be minor, resulting from self-mutilation behaviour.
Bird 8	Control	None

KEY: Car – Carprofen; Flx – Flunixin; PBZ - Phenylbutazone; Control – Control

4.3 Clinical pathology

None of the clinical chemistry results fell outside the reference range of measurement supplied in the Roche manual for the Cobas integra 400. Table 4-3 summarises all the clinical pathology results for all 4 treatment groups.

4.3.1 Population changes

The results from this study fell within the population reference interval except for the time points listed below (Figure 4-1).

- **Albumin** concentrations were marginally lower than the population reference interval (9.46 – 17.31 g/l)
    - Control: Bird 7 between 2 to 12h and Bird 8 at 9h.

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- **ALT** serum activities exceeded the population upper reference interval (31.2 – 60.1 u/l):
  - Carprofen: Bird 1 from 7 h and Bird 2 between 9 and 24 h;
  - Flunixin: Bird 4 from 7 h;
  - PBZ: Bird 5 from 48 h and bird 6 from 32 h;
  - Control: Bird 8 at 9, 12 and 24 h.

- **ALT** serum activity was below the population reference intervals in many of the birds:
  - Carprofen: Bird 2 (up to 3 h);
  - Flunixin: Bird 3 (1-1.5 h);
  - PBZ: Bird 5 (up to 2 h); Bird 6 at 0.5 h;
  - Control: Bird 7 (up to 7 h and at 48 h); Bird 8 (up to 2 h).

- **Ionised calcium** concentrations were all above the population reference interval (0.44 – 1.35 mmol/l) except
  - Carprofen: Bird 2 (12 to 24 h);
  - Flunixin: Bird 4 (12h);
  - PBZ: Bird 5 (12 h);
  - Control: Bird 7 (7h) and Bird 8 (7 to 9 h).

- **Potassium** concentrations were above the population reference interval (1.49 – 7.15 mmol/l) for
  - Carprofen: Bird 1, 2 at 9 h;
  - PBZ: Bird 6 at 5h;
  - Control: Bird 8 at 9h.

- **Sodium** concentrations were below the population reference interval (136.36 – 149.45 mmol/l) for
  - Carprofen: Bird 1 at 9h;
  - PBZ: Bird 5 at 12h and Bird 6 at 1h;
  - Control: Bird 8 at 7 to 9 and 24 to 32 h.

- **Sodium** concentrations exceeded the upper population reference interval in
  - Carprofen: Bird 1 up to 2 h and Bird 2 up to 1 h and again at 48h;

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- Flunixin: Bird 4 until 7 h;
- PBZ: Bird 5 at 9 h and 48 h, Bird 6 up to 2 h;
- Control: Bird 7 up to 3 h; Bird 8 at 1 h.

- **UA** concentrations were above the population upper reference interval (0.15 – 0.65 mmol/l) at the following points:
  - Carprofen: bird 1 at 0.5, 1 and 2h;
  - Flunixin: bird 4 at 1.5 – 7 h;
  - **C**ontrol: bird 8 was marginally below the lower population reference interval from 9 h and remained low until the last sample at 48 h.

Additional to the population reference interval comparisons, various trends in the ALT serum activities and uric acid time concentration graphs could be identified.

- A time related treatment response for ALT serum activities was present for birds 1 and 2 (carprofen), with a gradual increase peaking at 12 and 24 h before declining. The control bird 8 showed a similar response for ALT, however to a lower magnitude, which also declined to normal reference intervals by 48 h. Bird 4 (flunixin) had a time related treatment response peaking at 32 h before declining. Bird 5 and 6's (PBZ) graphs indicate a steadily increasing ALT serum activity, over the time period which failed to peak by the last sampling point, with the data after 32 h being above the population upper limit.

- After an initial small peak in UA concentration, marginally above the upper population interval for bird 1, Bird 1 and 2 (carprofen)'s UA concentrations gradually declined until the 48 h point. A similar trend was evident for the control birds (7 and 8) albeit at lower concentrations. A response of increasing plasma concentration to increasing UA was present in bird 4 (flunixin) with the peak concentration being almost double baseline concentration at 5 h. The UA concentrations in this bird declined to a level within the reference interval by 7 h, and were not very different concentrations to that of the control birds by the 48 h. The UA trend for birds 3, 5, 6 and 7 remained within the reference interval for all time points, while bird 8 had concentrations that were below the population reference interval from 9 h.

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4.3.2 Average concentrations

In addition to evaluation of graphs for individuals, average profiles per drug were also evaluated Figure 4-2 and Table 4-4.

- **Albumin:** Despite all the treated groups having higher concentrations at all-time points in comparison to the control group, they remained within the population reference intervals.

- **ALT** activities increased 4.6 fold at 12 h for the carprofen treated birds, prior to returning to the control bird concentrations by 48 h. Flunixin treated birds had ALT serum activities outside of population upper reference interval from 9 h, and remained above the population upper interval by a magnitude of 6.2 fold at 32 h. PBZ treated birds' ALT activity remained within the population reference interval until 32 h, where an approximate 5 times increase from zero hour was seen. Nonetheless this increased ALT level was only marginally above the population upper reference interval.

- **Ionised calcium** concentration in treated groups was marginally higher than the control, albeit with no trend being present between the treatment groups and control groups. Almost all the time points were above the population upper reference interval.

- **Potassium** concentrations were similar in the control and treatment groups, with the exception of the 9 hour sample for the PBZ and flunixin groups which did not show a peak above the population upper reference interval as seen in the control group.

- **Sodium:** The carprofen and control group were comparable until 12 h, after which the control group was below the population lower reference interval. At 48 h, the control group was lower than all 3 treatment groups although only one group (carprofen) was above the population upper reference interval.

- **UA** concentration in all the treated groups was higher than the control group from 3 to 32 h, however this increase did not exceed the upper limit of the population reference interval for carprofen or PBZ. The flunixin treated group did experience a transient hyperureamia at the 5 h point, but this transient increase had returned to the population reference interval by the 7 h time point.

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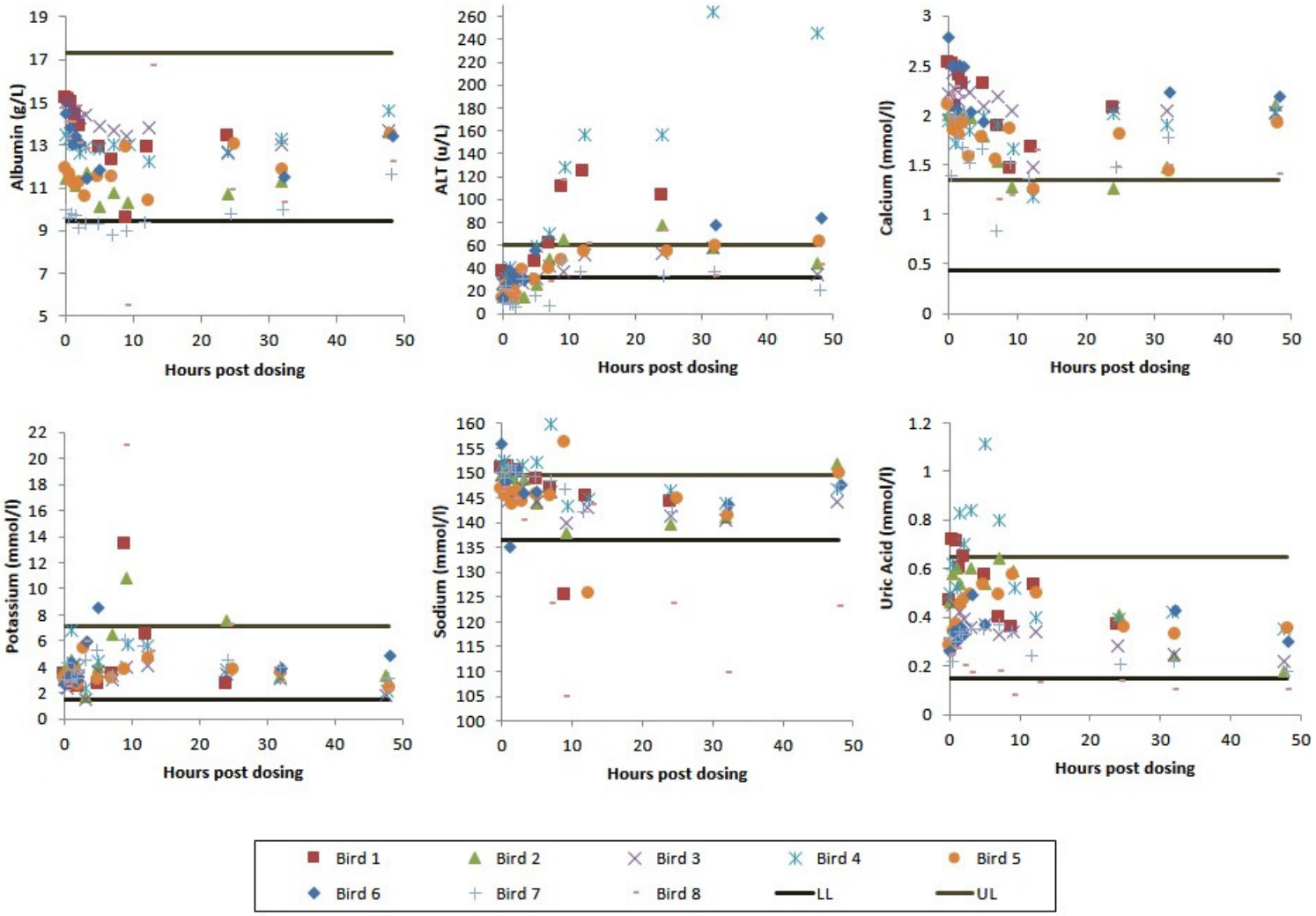

Figure 4-1: A scatter plot of a specific clinical pathology parameter (Albumin, ALT, ionised calcium, Sodium, Potassium and Uric Acid) by concentration, plotted over time per treatment group (n=2 per group). Bird 1 & 2 – Carprofen; Bird 3 & 4 – Flunixin; Bird 5 & 6 – PBZ, with the upper limit and lower limit for the species represented by the horizontal lines.

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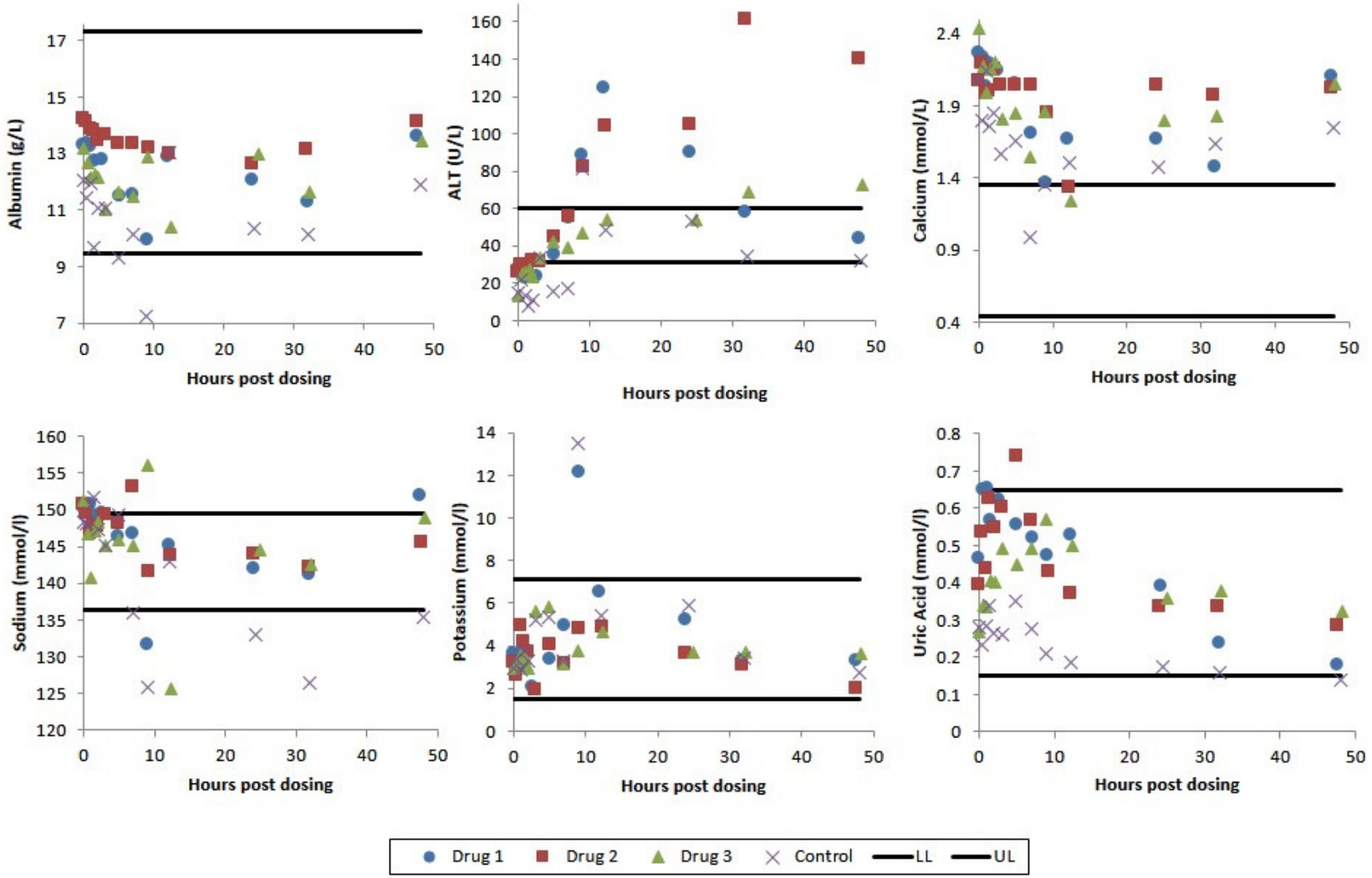

Figure 4-2: Average of a specific clinical pathology parameter (Albumin, ALT, Ionised calcium, Sodium, Potassium and Uric Acid) by concentration, plotted over time per treatment group (n=2 per group). Drug 1 – Carprofen; Drug 2 – Flunixin; Drug 3 – PBZ, with the upper limit and lower limit for the species represented by the horizontal lines.

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Table 4-3: Clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each bird with the actual time of sample collection indicated in hours.

Drug 1 – Carprofen	Time Point	0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
Bird 1	Actual Time (h)	0.0	0.6	1.0	1.5	2.1		5.0	7.0	9.0	12.1	24.0		
	ALB (g/l)	15.2	15.1	15.0	14.4	13.9	NS	12.9	12.3	9.6	12.9	13.4	NS	NS
	ALT (U/l)	36.0	34.0	29.0	32.0	33.0	NS	45.0	61.0	111.0	124.0	103.0	NS	NS
	Ca^{2+} (mmol/l)	2.53	2.52	2.09	2.40	2.32	NS	2.31	1.88	1.46	1.67	2.07	NS	NS
	Na (mmol/l)	151.0	149.7	151.2	149.0	150.4	NS	148.7	147.0	125.5	145.2	144.3	NS	NS
	K (mmol/l)	3.47	2.96	2.72	2.78	2.47	NS	2.67	3.43	13.44	6.49	2.74	NS	NS
	UA (mmol/l)	0.47	0.72	0.71	0.60	0.65	NS	0.57	0.40	0.36	0.53	0.37	NS	NS
Bird 2	Actual Time (h)	0.00	0.52	1.00	1.50		3.15	5.08	7.08	9.13		24.07	31.92	47.65
	ALB (g/l)	11.4	11.6	11.5	11.1	NS	11.7	10.1	10.8	10.3	NS	10.7	11.3	13.6
	ALT (U/l)	18.0	15.0	17.0	13.0	NS	14.0	25.0	48.0	65.0	NS	77.0	58.0	44.0
	Ca^{2+} (mmol/l)	2.00	1.95	1.97	2.00	NS	1.97	1.79	1.53	1.27	NS	1.26	1.47	2.10
	Na (mmol/l)	149.7	151.4	150.0	149.0	NS	148.6	143.9	146.3	137.8	NS	139.7	141.1	151.8
	K (mmol/l)	3.80	3.42	4.48	3.29	NS	1.68	4.00	6.46	10.86	NS	7.61	3.27	3.30
	UA (mmol/l)	0.46	0.58	0.60	0.54	NS	0.60	0.54	0.64	0.59	NS	0.41	0.24	0.18

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. NS – No sample.
Reference values: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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Table 4-3 (cont.): Clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each bird with the actual time of sample collection indicated in hours.

		Time Point	0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
Drug 2 - Flunixin	Bird 3	Actual Time (h)	0.0	0.5	1.0	1.5	2.1	3.1	5.0	7.1	9.1	12.3	24.0	31.9	47.7
		ALB (g/l)	15.0	15.0	14.6	14.6	14.3	14.4	13.9	13.7	13.4	13.8	12.6	13.0	13.7
		ALT (U/l)	27.0	30.0	20.0	24.0	30.0	27.0	30.0	41.0	36.0	52.0	53.0	58.0	34.0
		Ca^{2+} (mmol/l)	2.21	2.43	2.25	2.14	2.29	2.23	2.09	2.18	2.04	1.48	2.07	2.05	2.03
		Na (mmol/l)	149.6	146.8	144.4	145.4	144.7	147.0	144.2	146.1	140.0	142.9	141.3	140.4	144.3
		K (mmol/l)	3.06	2.36	3.06	4.16	3.38	1.50	3.66	3.07	3.94	4.13	3.46	3.09	1.80
		UA (mmol/l)	0.29	0.45	0.35	0.42	0.39	0.36	0.37	0.33	0.34	0.34	0.28	0.25	0.22
	Bird 4	Actual Time (h)	0.0	0.4	1.0	1.5	2.1	3.1	5.0	7.1	9.4	12.3	24.1	31.8	47.7
		ALB (g/l)	13.5	13.3	13.1	13.0	12.6	12.9	12.8	13.0	13.0	12.2	12.7	13.3	14.6
		ALT (U/l)	25.0	30.0	40.0	29.0	34.0	36.0	59.0	70.0	128.0	156.0	157.0	264.0	246.0
		Ca^{2+} (mmol/l)	1.94	1.96	1.71	1.86	2.02	1.85	1.99	1.90	1.66	1.18	2.02	1.90	2.02
		Na (mmol/l)	151.7	152.3	148.8	150.2	150.5	151.7	152.1	159.9	143.2	144.6	146.5	143.8	146.7
		K (mmol/l)	3.38	2.91	6.83	4.25	3.97	2.38	4.42	3.29	5.70	5.58	3.78	3.14	2.14
		UA (mmol/l)	0.50	0.62	0.52	0.83	0.70	0.84	1.11	0.80	0.52	0.40	0.39	0.42	0.35

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. NS – No sample.
Reference values: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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Table 4-3 (cont.): Clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each bird with the actual time of sample collection indicated in hours.

Drug 3 - Phenylbutazone		Time Point	0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
	Bird 5	**Actual Time (h)**	**0.0**	**0.7**	**1.0**	**1.6**	**2.1**	**3.0**	**5.0**	**7.0**	**9.0**	**12.4**	**25.0**	**32.2**	**48.1**
		ALB (g/l)	11.9	11.6	11.3	11.1	11.2	10.6	11.5	11.5	12.9	10.4	13.0	11.8	13.5
		ALT (U/l)	13.0	24.0	14.0	22.0	16.0	38.0	29.0	39.0	47.0	54.0	54.0	59.0	62.0
		Ca^{2+} (mmol/l)	2.10	1.84	1.93	1.80	1.92	1.58	1.77	1.55	1.86	1.24	1.80	1.43	1.92
		Na (mmol/l)	146.8	145.3	146.3	143.5	146.1	144.3	145.5	145.2	156.1	125.6	144.6	141.4	150.0
		K (mmol/l)	3.14	3.32	2.79	3.91	2.60	5.39	3.07	3.12	3.77	4.68	3.72	3.53	2.38
		UA (mmol/l)	0.28	0.34	0.37	0.45	0.47	0.49	0.53	0.49	0.57	0.50	0.36	0.33	0.35
	Bird 6	**Actual Time (h)**	**0.0**	**0.6**	**1.1**	**1.6**	**2.1**	**3.3**	**5.0**					**32.2**	**48.3**
		ALB (g/l)	14.5	13.8	13.0	13.4	13.1	11.4	11.8	NS	NS	NS	NS	11.5	13.4
		ALT (U/l)	13.0	26.0	38.0	32.0	31.0	29.0	55.0	NS	NS	NS	NS	78.0	83.0
		Ca^{2+} (mmol/l)	2.78	2.50	2.06	2.50	2.49	2.03	1.93	NS	NS	NS	NS	2.23	2.19
		Na (mmol/l)	155.9	148.2	135.2	150.6	150.6	145.8	146.2	NS	NS	NS	NS	143.5	147.7
		K (mmol/l)	2.72	3.20	4.28	2.90	3.24	5.91	8.57	NS	NS	NS	NS	3.83	4.85
		UA (mmol/l)	0.26	0.34	0.30	0.36	0.33	0.49	0.37	NS	NS	NS	NS	0.43	0.30

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. NS – No sample.
Reference values: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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Table 4-3 (cont.): Clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each bird with the actual time of sample collection indicated in hours.

			0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
Control	Bird 7	Actual Time (h)	0.0	0.4	0.9	1.5	2.0	3.1	4.9	7.0	9.0	11.7	24.3	32.0	48.0
		ALB (g/l)	10.0	9.6	9.8	9.7	9.1	9.3	9.3	8.8	9.0	9.4	9.8	10.0	11.6
		ALT (U/l)	9.0	24.0	8.0	8.0	6.0	31.0	16.0	7.0	46.0	36.0	33.0	36.0	21.0
		Ca^{2+} (mmol/l)	2.02	1.39	1.99	1.76	1.67	1.52	1.66	0.84	1.51	1.36	1.47	1.78	2.09
		Na (mmol/l)	150.2	149.1	151.0	151.6	150.1	149.7	149.2	148.4	146.8	142.1	142.3	143.2	147.5
		K (mmol/l)	3.03	4.27	2.97	3.75	3.09	4.50	5.34	3.34	6.04	5.63	4.52	3.98	3.15
		UA (mmol/l)	0.32	0.22	0.30	0.34	0.33	0.35	0.35	0.37	0.34	0.24	0.21	0.22	0.18
	Bird 8	Actual Time (h)	0.0	0.4	1.0		2.0	3.1		7.0	9.0	12.8	24.3	32.0	48.0
		ALB (g/l)	14.1	13.3	14.1	NS	13.1	12.8	NS	11.5	5.5	16.7	10.9	10.3	12.2
		ALT (U/l)	21.0	20.0	19.0	NS	16.0	36.0	NS	28.0	117.0	61.0	73.0	33.0	43.0
		Ca^{2+} (mmol/l)	2.14	2.20	2.29	NS	2.02	1.61	NS	1.14	1.19	1.65	1.47	1.49	1.40
		Na (mmol/l)	146.2	147.0	150.2	NS	144.6	140.6	NS	123.6	104.8	143.6	123.6	109.7	123.2
		K (mmol/l)	2.84	2.45	2.83	NS	3.50	5.91	NS	3.22	20.97	5.16	7.23	2.80	2.31
		UA (mmol/l)	0.24	0.25	0.27	NS	0.20	0.17	NS	0.18	0.08	0.13	0.14	0.10	0.10

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. NS – No sample.
Reference values: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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Table 4-4: Average clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each treatment group with the average actual time of sample collection indicated in hours.

	Time Point	0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
Carprofen	Actual Time (h)	0.0	0.5	1.0	1.5	2.1*	3.2*	5.0	7.1	9.1	12.1*	24.0	31.9*	47.7*
	ALB (g/l)	13.3	13.4	13.3	12.8	13.9	11.7	11.5	11.6	10.0	12.9	13.4	11.3	13.6
	ALT (U/l)	27.0	24.5	23.0	22.5	33.0	14.0	35.0	54.5	88.0	124	103.0	58.0	44.0
	Ca^{2+} (mmol/l)	2.3	2.2	2.0	2.2	2.3	2.0	2.1	1.7	1.4	1.7	2.1	1.5	2.1
	Na (mmol/l)	150.4	150.6	150.6	149.0	150.4	148.6	146.3	146.7	131.7	145.2	144.3	141.1	151.8
	K (mmol/l)	3.64	3.19	3.60	3.04	2.47	1.68	3.34	4.95	12.15	6.49	2.74	3.27	3.30
	UA (mmol/l)	0.47	0.65	0.66	0.57	0.65	0.60	0.56	0.52	0.48	0.53	0.37	0.24	0.18
Flunixin	**Actual Time (h)**	**0.0**	**0.5**	**1.0**	**1.5**	**2.1**	**3.1**	**5.0**	**7.1**	**9.2**	**12.3**	**24.1**	**31.9**	**47.7**
	ALB (g/l)	14.3	14.2	13.9	13.8	13.5	13.7	13.4	13.4	13.2	13.0	12.7	13.2	14.2
	ALT (U/l)	26.0	30.0	30.0	26.5	32.0	31.5	44.5	55.5	82.0	104.0	105.0	161.0	140.0
	Ca^{2+} (mmol/l)	2.1	2.2	2.0	2.0	2.2	2.0	2.0	2.0	1.9	1.3	2.0	2.0	2.0
	Na (mmol/l)	150.7	149.6	146.6	147.8	147.6	149.4	148.2	153.0	141.6	143.8	143.9	142.1	145.5
	K (mmol/l)	3.22	2.64	4.95	4.21	3.68	1.94	4.04	3.18	4.82	4.86	3.62	3.12	1.97
	UA (mmol/l)	0.40	0.54	0.44	0.63	0.55	0.60	0.74	0.57	0.43	0.37	0.34	0.34	0.29

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. * - indicates only 1 sample was available for use. **Reference values**: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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Table 4-4 (cont.): Average clinical pathology parameters (ALB, ALT, Ca^{2+}, Na, K and UA) for each treatment group with the average actual time of sample collection indicated in hours.

	Time Point	0 h	0.5 h	1 h	1.5 h	2 h	3 h	5 h	7 h	9 h	12 h	24 h	32 h	48 h
PBZ	**Actual Time (h)**	**0.0**	**0.6**	**1.1**	**1.6**	**2.1**	**3.1**	**5.0**	**7.0***	**9.0***	**12.4***	**25.0***	**32.2**	**48.2**
	ALB (g/l)	13.2	12.7	12.2	12.3	12.2	11.0	11.7	11.5	12.9	10.4	13.0	11.7	13.5
	ALT (U/l)	13.0	25.0	26.0	27.0	23.5	33.5	42.0	39.0	47.0	54.0	54.0	68.5	72.5
	Ca^{2+} (mmol/l)	2.4	2.2	2.0	2.2	2.2	1.8	1.9	1.6	1.9	1.2	1.8	1.8	2.1
	Na (mmol/l)	151.4	146.8	140.8	147.1	148.4	145.1	145.9	145.2	156.1	125.6	144.6	142.5	148.9
	K (mmol/l)	2.93	3.26	3.54	3.41	2.92	5.65	5.82	3.12	3.77	4.68	3.72	3.68	3.62
	UA (mmol/l)	0.27	0.34	0.34	0.41	0.40	0.49	0.45	0.49	0.57	0.50	0.36	0.38	0.33
Controls	**Actual Time (h)**	**0.0**	**0.4**	**1.0**	**1.5***	**2.0**	**3.1**	**4.9***	**7.0**	**9.0**	**12.2**	**24.3**	**32.0**	**48.0**
	ALB (g/l)	12.1	11.5	12.0	9.7	11.1	11.1	9.3	10.2	7.3	13.1	10.4	10.2	11.9
	ALT (U/l)	15.0	22.0	13.5	8.0	11.0	33.5	16.0	17.5	81.5	48.5	53.0	34.5	32.0
	Ca^{2+} (mmol/l)	2.1	1.8	2.1	1.8	1.8	1.6	1.7	1.0	1.4	1.5	1.5	1.6	1.7
	Na (mmol/l)	148.2	148.1	150.6	151.6	147.4	145.2	149.2	136.0	125.8	142.9	133.0	126.5	135.4
	K (mmol/l)	2.94	3.36	2.90	3.75	3.30	5.21	5.34	3.28	13.51	5.40	5.88	3.39	2.73
	UA (mmol/l)	0.28	0.24	0.29	0.34	0.27	0.26	0.35	0.28	0.21	0.19	0.18	0.16	0.14

Key: Red shaded cells indicate values above and blue shaded cells indicate values below the population reference interval. * - indicates only 1 sample was available for use. **Reference values**: **ALB** 9.46 – 17.31 g/l; **ALT** 31.2 – 60.1 u/l; **Ca^{2+}** 0.44 – 1.35 mmol/l; **K** 1.49 – 7.15 mmol/l; **Na** 136.36 – 149.45 mmol/l; **UA** 0.15 – 0.65 mmol/l

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4.3.3 UA handheld meter comparison

The uric acid concentration readings obtained via the benchtop analyser (Roche Cobas Integra 400) were compared to the UA sure ® handheld meter readings, Table 4-5 provides the direct data used for this comparison.

Table 4-5: Comparison of the UA readings obtained via the Cobas Integra analyser and the handheld UASure ® meter (meter output of mg/dL converted to mmol/L)

	Cobas Integra UA (mmol/l)	UASure ® UA (mmol/L)	UASure ® UA (mg/dL)
	0.08	0	0
	0.1	0	0
	0.14	0.50	8.4
	0.18	0	0
	0.21	0	0
	0.22	0	0
	0.24	0	0
	0.25	0	0
	0.28	0	0
	0.33	0	0
	0.34	0.73	12.2
	0.34	0.21	3.6
	0.35	0.41	6.9
	0.36	0.32	5.3
	0.37	0.20	3.4
	0.39	0.28	4.7
	0.41	0.32	5.4
	0.42	0.21	3.6
	0.43	0.37	6.2
	0.52	0.54	9.1
	0.57	0.23	3.8
	0.59	0.94	15.8
	0.64	0.54	9
Mean	**0.34**	**0.41**	**4.23**
SD	**0.15**	**0.22**	**4.46**

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A statistically significant but weak correlation [A pearson r value of 0.6372 with a calculated p-value of 0.001] was found on non-log transformed data between the handheld meter UA reading and the clinical pathology bench top analyser readings (Figure 4-3).

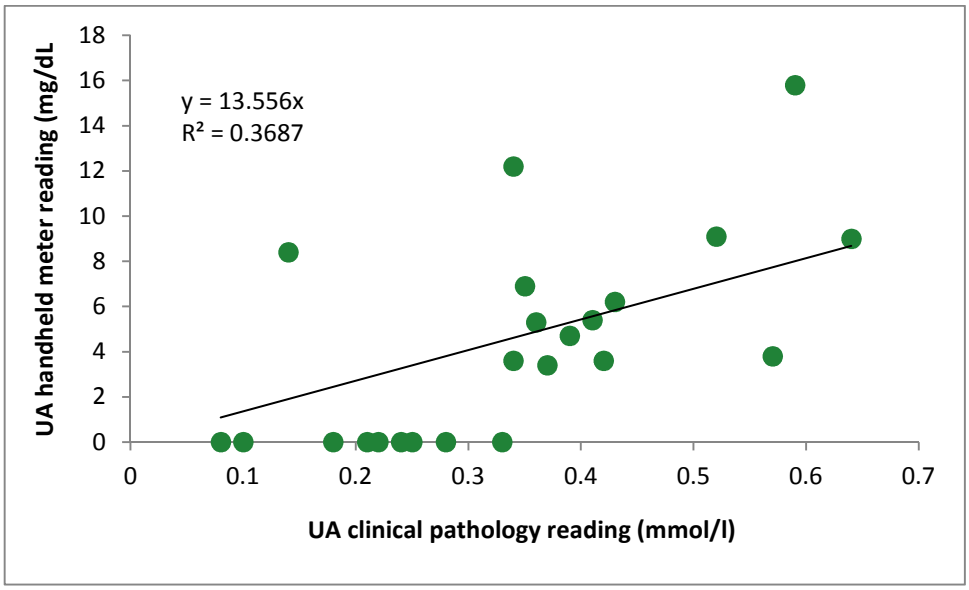

Figure 4-3: Uric acid (UA) readings from the clinical pathology laboratory (x-axis) and handheld meter (y-axis) with a linear regression line set at 0 Y-intercept.

4.4 Post mortem examination

All necropsies were unremarkable, with none of the treated birds showing any signs of urate crystals on the serosal surfaces or tophi in the parenchymatous organs. This was confirmed on histopathological evaluation, as the only sign evident were scattered renal tubules showing some signs of pyknosis and karryorhexis with no granular or cellular casts.

4.5 Pharmacokinetic drug analysis

4.5.1 Calibration of the method

4.5.1.1 *Carprofen*

The method proved to be accurate and precise above 125 ng/ml (Table 4-6), with a linear response between concentration and peak area described by a regression equation of y = 1405.6x + 32482 with a regression coefficient of 0.9979 over the concentration range of 31.3 –

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2000 ng/ml. The signal to noise ratio at the lowest concentration (31.3 ng/ml) of the curve was 28.9 indicating the possibility to detect concentrations lower than the lowest concentration assessed during the method development, however the accuracy fails validation at these low concentrations.

Table 4-6: Accuracy and precision of the carprofen calibration curve

Concentration (ng/ml)	Average concentration (ng/ml)	SD Concentration (ng/ml)	Accuracy (%)	Precision (% CV)
31.3	50.47	13.11	161	26
62.5	81.85	14.62	131	18
125	138.23	7.74	111	6
250	251.11	12.62	100	5
500	472.31	24.71	94	5
1000	939.59	36.37	94	4
2000	2035.28	19.55	102	1

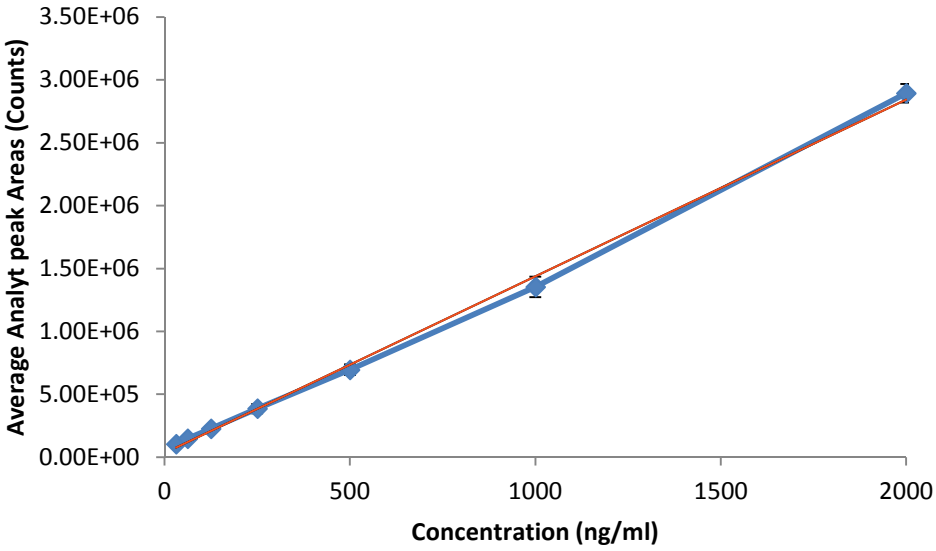

Figure 4-4: Calibration curve for carprofen, blue line indicating the average of 3 runs and error bars (±1 SD)

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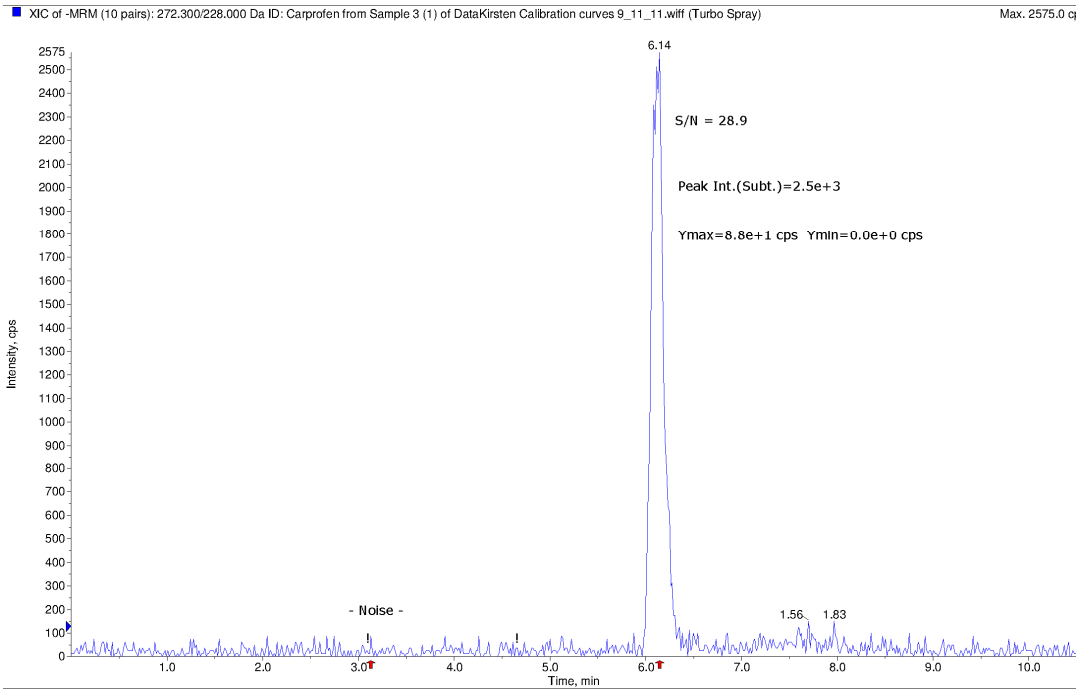

Figure 4-5: Chromatogram of carprofen sample at 31.3 ng/ml for the calibration curve, demonstrating the signal to noise ratio calculation as performed in Analyst software.

4.5.1.2 *Flunixin*

The method proved to be accurate and precise between 62.5 and 500 ng/ml (Table 4-7) with a linear relationship between concentration and peak area described by a regression equation of y = 5627.8x + 443552 with a regression coefficient of 0.9987 over the concentration range of 31.3 – 500 ng/ml. The signal to noise ratio at the lowest concentration (31.3 ng/ml) of the curve was 25.8.

Table 4-7: Accuracy and precision of the flunixin calibration curve

Analyte Concentration (ng/mL)	Mean concentration (ng/ml)	SD concentration (ng/ml)	Accuracy (%)	Precision (% CV)
31.3	24.00	3.04	77	13
62.5	60.94	5.50	97	9
125	130.86	4.11	105	3
250	257.64	17.23	103	7
500	495.37	7.33	99	1

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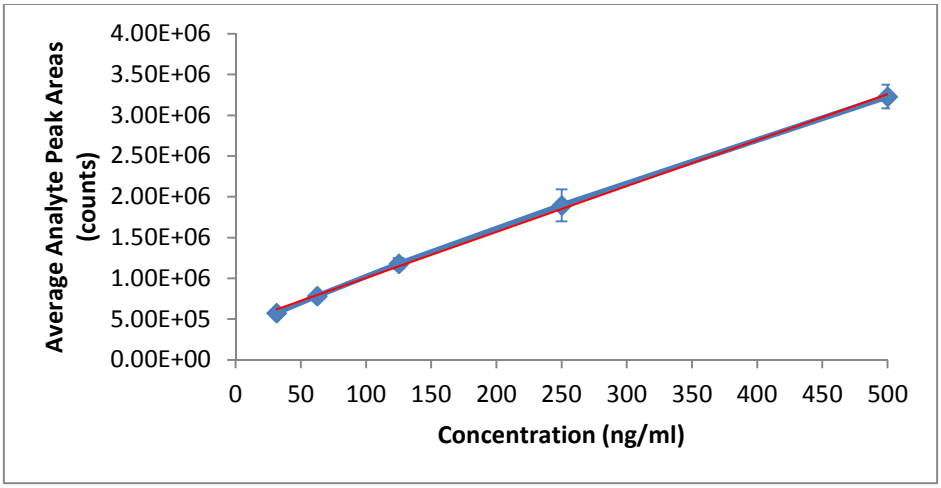

Figure 4-6: The calibration curve for flunixin, the blue line indicating an average of 3runs and error bars (±1 SD)

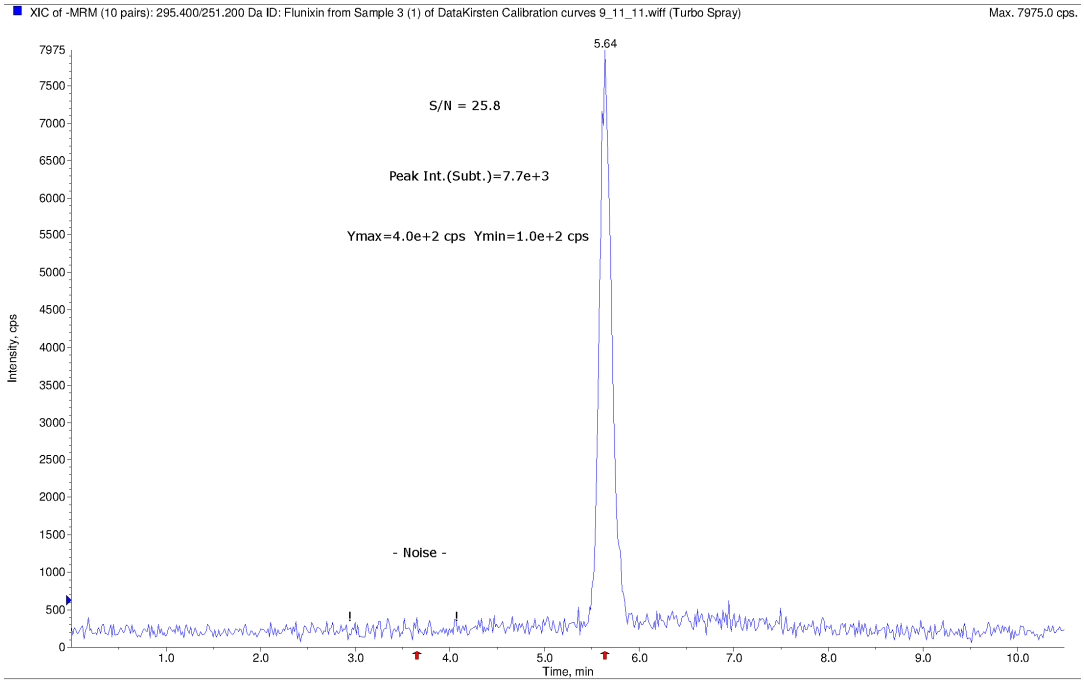

Figure 4-7: Chromatogram of flunixin sample at 31.3 ng/ml for the calibration curve, demonstrating the signal to noise ratio calculation as performed in Analyst software.

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4.5.1.3 *Phenylbutazone*

The method as described in section 3.8.2 for carprofen and flunixin was attempted for the PBZ analysis. Although the calibration curve was linear up to 1000 ng/ml (R^2=0.9814), the data obtained in vulture plasma was so variable that the online extraction method was deemed to have failed, and thereafter requiring a manual extraction method as described section 3.8.3.

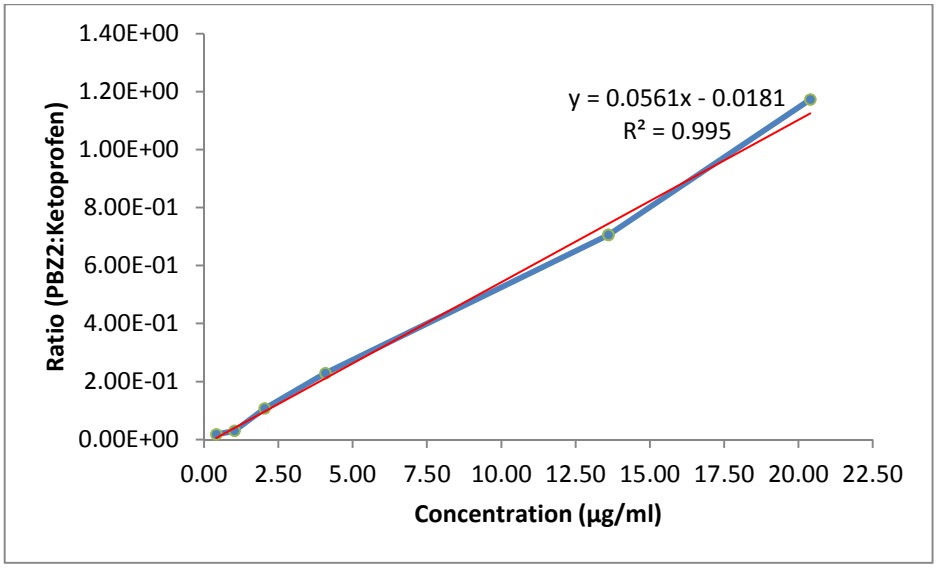

Figure 4-8: The calibration curve for PBZ, the blue line indicating an average of 3runs

Table 4-8: Accuracy and precision of the PBZ calibration curve (as calculated by Analyst software)

Concentration (µg/ml)	Number Of Values Used	Mean concentration (µg/ml)	SD (µg/ml)	Precision (%CV)	Accuracy (%)
0.41	3 of 3	0.54	0.21	38.80	131.19
1.02	3 of 3	0.91	0.36	39.59	89.08
2.04	3 of 3	2.16	0.37	17.04	105.89
4.08	3 of 3	4.03	0.12	2.90	98.83
10.2	2 of 2	8.94	0.92	10.27	87.69
13.6	3 of 3	13.43	0.46	3.41	98.74
20.4	3 of 3	20.92	0.41	1.98	102.57

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The method proved to be accurate and precise above 2.04 µg/ml (Table 4-8) with a linear relationship between concentration and peak area described by a regression equation of y = 0.0561x -0.0181 with a regression coefficient of 0.995 over the concentration range of 0.41 – 20.4 µg/ml. The signal to noise ratio at the lowest concentration of the curve was 144.9 (Figure 4-9).

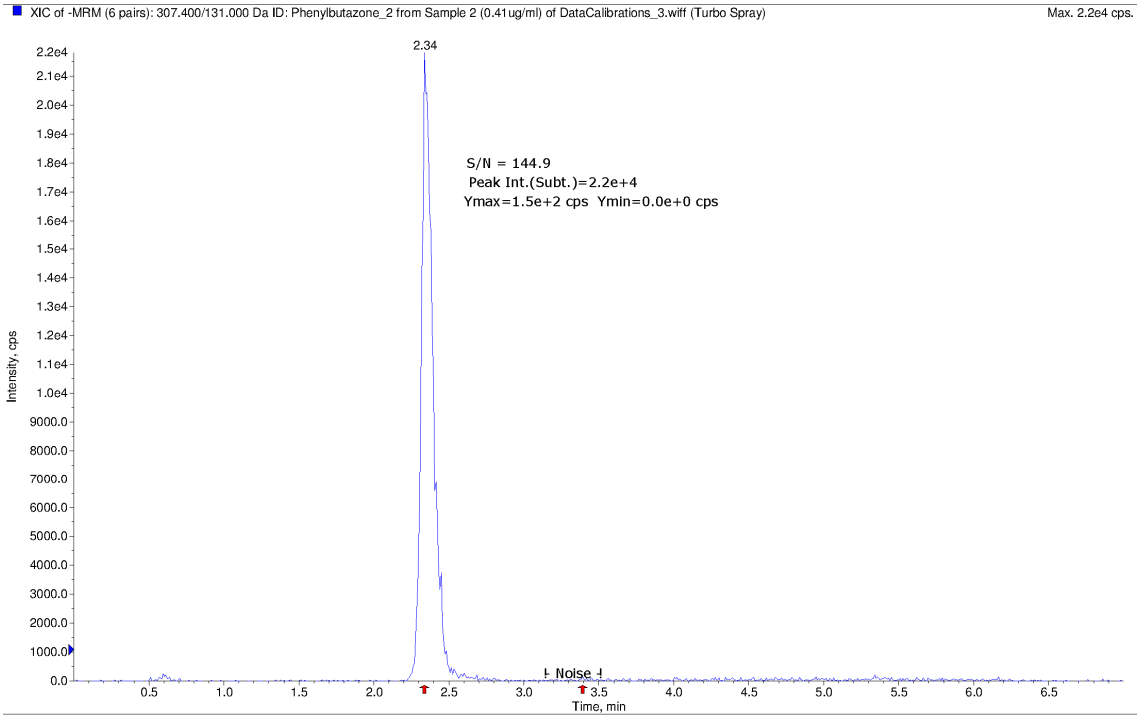

Figure 4-9: Chromatogram of PBZ sample at 0.41 µg/ml for the calibration curve, demonstrating the signal to noise ratio calculation as performed in Analyst software.

4.5.2 Internal standard analysis

The internal standard peaks 1 and 2 for each time point were graphed in excel and visually evaluated (Figure 4-10).

The %CV of the internal standard peak 1 and peak 2 were 12.2% and 12.3% respectively for the flunixin and carprofen analysis. All the IS values were within two standard deviations except for Bird 1 at 0 h time point and Bird 4 at the 30 minute time point. The online extraction of the NSAID was therefore considered to be adequate. The PBZ analysis utilised ketoprofen as

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an internal standard and the internal standard as a ratio to the analyte was utilised to determine the response factor

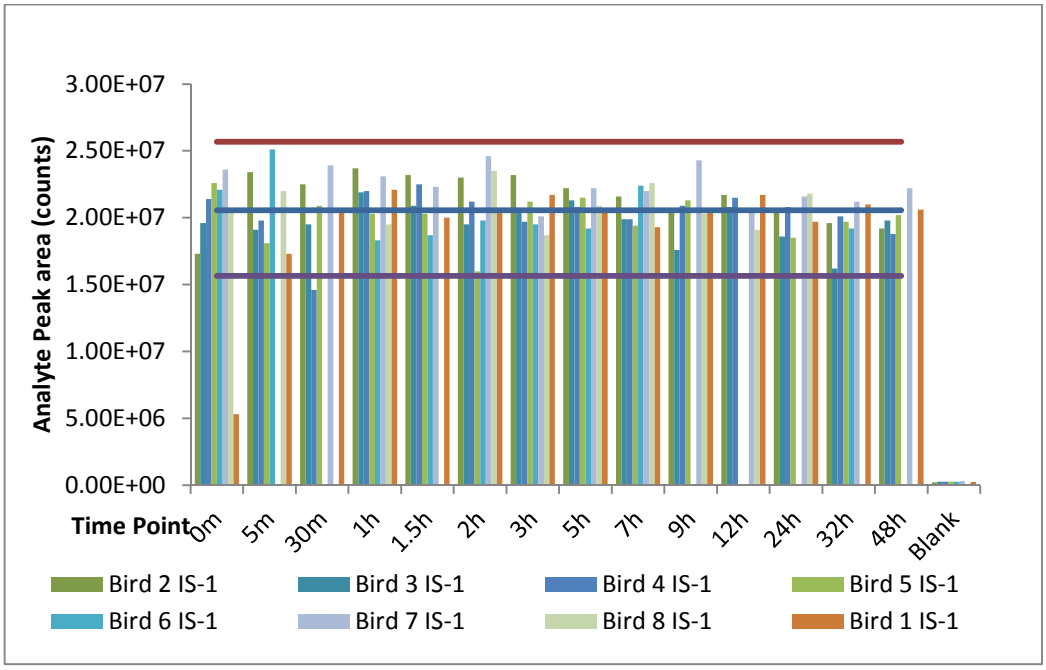

Figure 4-10: Internal standard of the carprofen and flunixin treatment groups indicating the extraction of the standard

4.5.3 Drug plasma concentration results

The drug plasma concentration (ng/ml) was graphed in Microsoft Excel (2010) as a scatter plot over the actual time (h) for each bird. The pharmacokinetic curve fitted plots, graphed in Kinetica software, using the compartmental analysis are presented (Figure 4-11 a and b, Figure 4-12 a and b and Figure 4-13 a and b).

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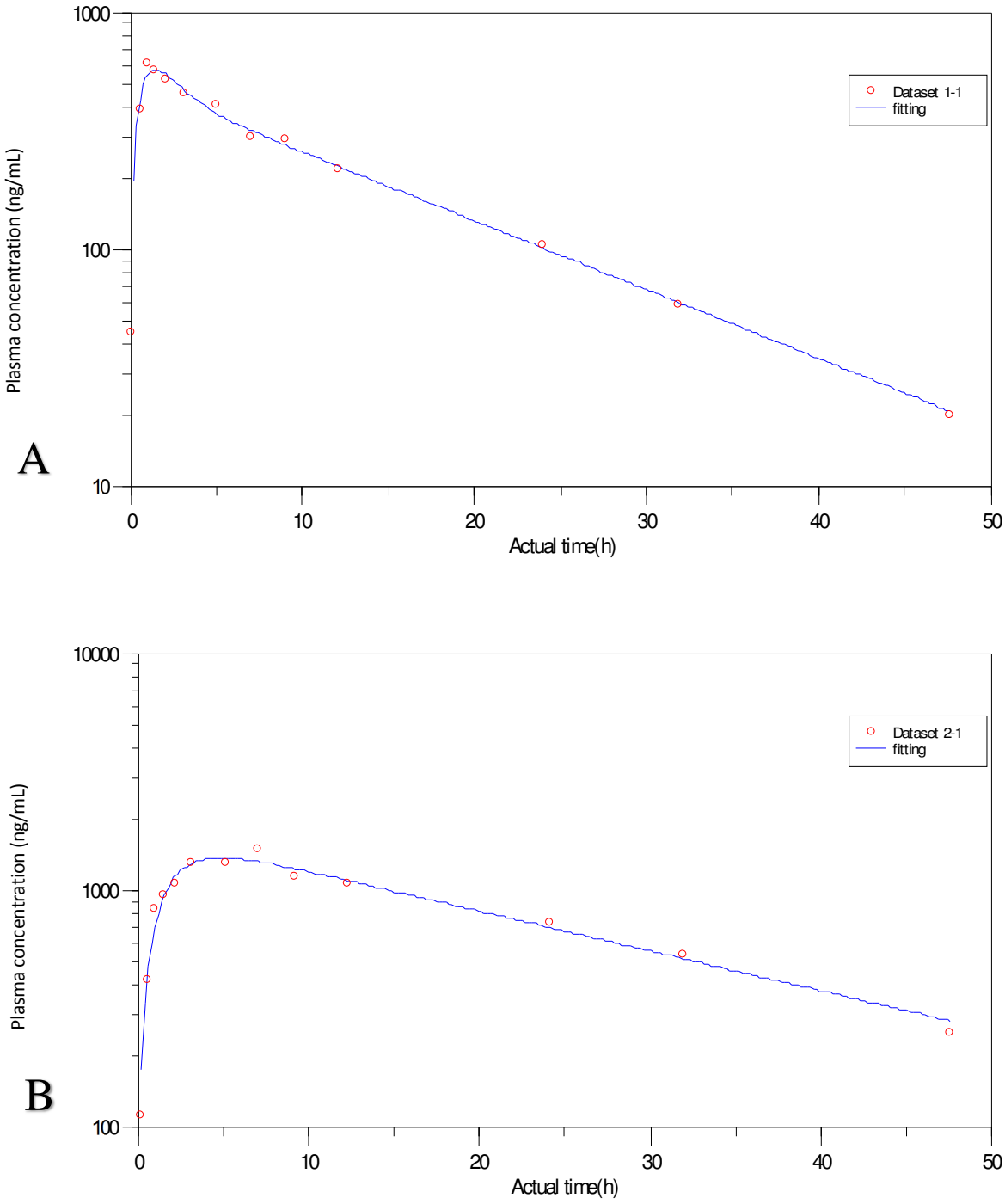

Figure 4-11: Plasma concentration (ng/ml) over time after dosing (h) plot with the fitted PK curve in blue for (A) Bird 1 and (B) bird 2 (dosed with carprofen at 11.5 mg/kg PO)

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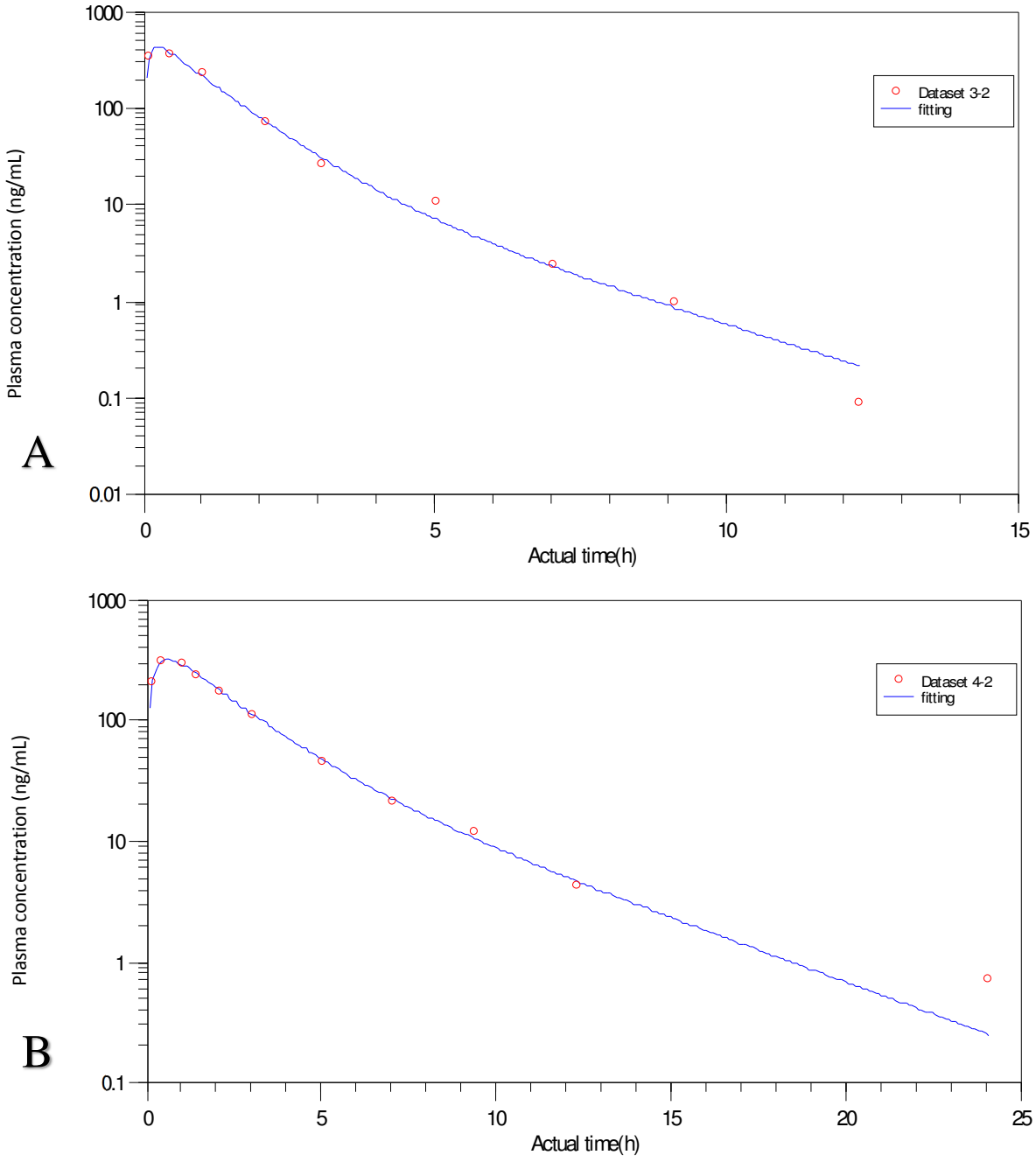

Figure 4-12: Plasma concentration (ng/ml) over time after dosing (h) plot with the fitted PK curve in blue for (A) bird 3 and (B) bird 4 (dosed with flunixin at 1 mg/kg PO)

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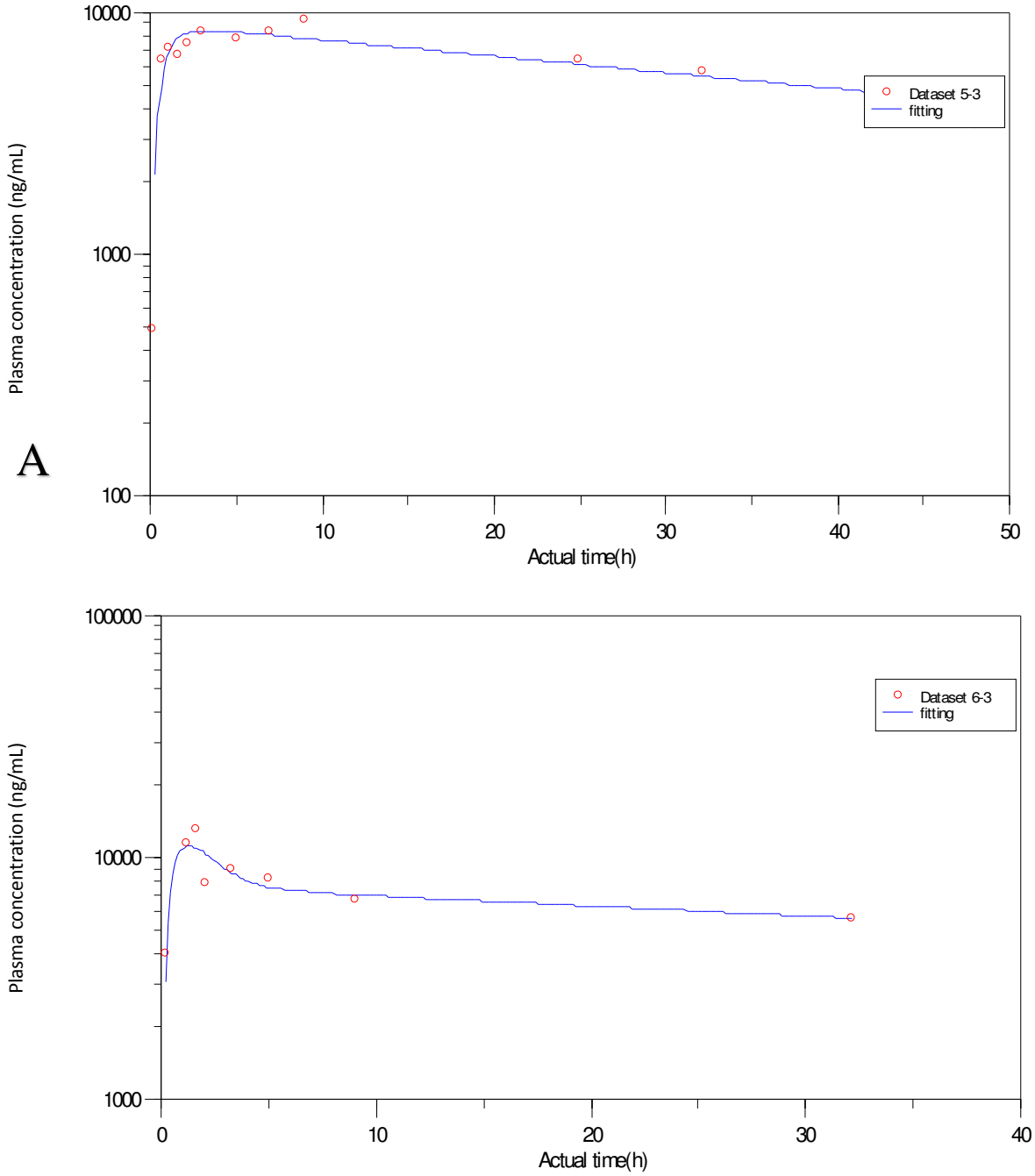

Figure 4-13: Plasma concentration (ng/ml) over time after dosing (h) plot with the fitted PK curve in blue for (A) bird 5 and (B) bird 6 (dosed with PBZ at 1.7 mg/kg PO)

4.5.4 Pharmacokinetic analysis

The pharmacokinetic parameters obtained for non-compartmental modelling are provided in Table 4-9 to Table 4-11. Oral absorption of carprofen, flunixin and PBZ was characterised by a maximum concentration of 1051.8 ± 620.7 ng/ml, 335.9 ± 36.3 ng/ml and 11150 ± 2474.9

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ng/ml obtained in 4 ± 4.3, 0.45 ± 0.02 and 5.3 ± 5.2 hours respectively and an elimination half-life of 13.3 ±5, 1.8±1 and 18.7 ±11.4 hours respectively. Volume of distribution was 13.62 ± 9.91 L/kg; 3.29± 0.75 L/kg and 0.13 ± 0.03 L/kg for carprofen, flunixin and PBZ respectively. The %AUC extra was 3.43%; 15.01%; 0.03%, 0.02%, 30.4% and 9.18% for Birds 1 to 6 respectively. Except for Bird 5 having a %AUC extra above 20%, the curves are considered to be adequately captured. Area under the curve until the last time point was 21.72± 20.1; 0.78± 0.28 and 263.35 ±68.69 µg/mL*h for carprofen, flunixin and PBZ respectively.

The curves best fitted a one compartmental model when visually appraised. The absorption constant (Ka) determined via compartmental modelling, was calculated to be 0.86 ±0.36 (carprofen); 7.02± 4.62 (flunixin) and 1.27 ±0.24 (PBZ) yielded a half-life of absorption of 0.88 ±0.37 h (carprofen), 0.13 ± 0.08h (flunixin) and 0.56 ± 0.10h (PBZ) respectively. The alpha half-life could not be calculated for carprofen and PBZ as only one bird in each group had reportable alpha half-lives. The alpha half-life for flunixin was 1.39 ±1.6 h. The beta half-life for each drug was 14.16 ±5.37 h (carprofen); 1.74 ±0.62 h (flunixin) and 22.6 ±31.15 h (phenylbutazone).

Table 4-9: Non-compartmental and compartmental results for carprofen

Carprofen (11.5 mg/kg)						
	Parameter	**Unit**	**Bird 1**	**Bird 2**	**Average**	**SD**
Non-compartmental results	**Cmax**	**ng/mL**	612.87	1490.68	1051.78	620.70
	Tmax	**h**	1.00	7.08	4.04	4.30
	AUClast	**µg/mL*h**	7.51	35.93	21.72	20.10
	AUCextra	**µg/mL*h**	0.27	6.35	3.31	4.30
	AUCtot	**µg/mL*h**	7.78	42.28	25.03	24.40
	%AUCextra		3.427	3.43	15.01	9.22
	Lz	**1/h**	0.07	0.04	0.06	0.02
	AUMClast	**µg/mL*(h)²**	92.44	634.37	363.40	383.21
	AUMCextra	**µg/mL*(h)²**	16.40	456.75	236.57	311.38
	AUMCtot	**µg/mL*(h)²**	108.83	1091.12	599.98	694.58
	t½	**h**	9.67	16.86	13.26	5.08
	MRT	**h**	14.00	25.81	19.90	8.35
	Clearance	**L/h*kg**	1.48	0.27	0.88	0.85
	Vz	**L/kg**	20.63	6.61	13.62	9.91

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	Carprofen (11.5 mg/kg)					
	Parameter	Unit	Bird 1	Bird 2	Average	SD
Compartmental results	Ka		1.11	0.61	0.86	0.36
	t½ absorption	h	0.62	1.14	0.88	0.37
	Kel		0.07	0.04	0.05	0.02
	t½ elimination	h	10.37	17.96	14.16	5.37
	A	ng/mL	608.40			
	Alpha		0.98			
	t½ alpha	h	0.71			
	B	ng/mL	473.77	1654.32	1064.05	834.78
	Beta		0.07	0.04	0.05	0.02
	t½ beta	h	10.37	17.96	14.16	5.37

Table 4-10: Non-compartmental and compartmental results for flunixin

	Flunixin (1 mg/kg)					
	Parameter	Unit	Bird 3	Bird 4	Average	SD
Non-compartmental results	Cmax	ng/mL	361.59	310.20	335.89	36.34
	Tmax	h	0.47	0.43	0.45	0.02
	AUClast	µg/mL*h	0.58	0.98	0.78	0.28
	AUCextra	µg/mL*h	0.00	0.00	0.00	0.00
	AUCtot	µg/mL*h	0.58	0.98	0.78	0.28
	%AUCextra	%	0.03	0.02	0.02	0.01
	Lz	1/h	0.63	0.27	0.45	0.26
	AUMClast	µg/mL*(h)²	0.71	3.02	1.87	1.63
	AUMCextra	µg/mL*(h)²	0.002	0.006	0.004	0.003
	AUMCtot	µg/mL*(h)²	0.72	3.03	1.87	1.64
	thalf	h	1.10	2.58	1.84	1.05
	MRT	h	1.24	3.10	2.17	1.31
	Clearance	L/h*kg	1.74	1.02	1.38	0.50
	Vz	L/kg	2.75	3.82	3.29	0.75
Compartmental results	Ka	h^{-1}	10.29	3.75	7.02	4.62
	t½ absorption	h	0.07	0.18	0.13	0.08
	Kel	h^{-1}	0.43	0.24	0.34	0.13
	t½ elimination	h	1.60	2.86	2.23	0.89
	A	ng/mL	41.53	363.10	202.31	41.53
	Alpha	h^{-1}	0.43	0.59	0.51	0.43
	t½ alpha	h	1.60	1.17	1.39	1.60
	B	ng/mL	516.56	81.29	298.93	516.56
	Beta	h^{-1}	1.11	0.24	0.68	1.11
	t½ beta	h	0.62	2.86	1.74	0.62

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Table 4-11: Non compartmental and compartmental results for PBZ

	PBZ (1.70 mg/kg)					
	Parameter	**Unit**	**Bird 5**	**Bird 6**	**Average**	**SD**
Non-compartmental results	**Cmax**	**ng/mL**	9400.00	12900.00	11150.00	2474.87
	Tmax	**h**	9.00	1.62	5.31	5.22
	AUClast	**µg/mL*h**	311.92	214.78	263.35	68.69
	AUCextra	**µg/mL*h**	136.28	21.72	79.00	81.01
	AUCtot	**µg/mL*h**	448.21	236.49	342.35	149.70
	%AUCextra		30.407	30.41	9.18	19.80
	Lz	**1/h**	0.03	0.07	0.05	0.03
	AUMClast	**µg/mL*(h)²**	6310.14	3108.08	4709.11	2264.20
	AUMCextra	**µg/mL*(h)²**	11826.90	1032.86	6429.88	7632.54
	AUMCtot	**µg/mL*(h)²**	18137.10	4140.94	11139.02	9896.78
	thalf	**h**	26.79	10.64	18.72	11.42
	MRT	**h**	40.47	17.51	28.99	16.23
	Clearance	**L/h*kg**	0.004	0.007	0.005	0.002
	Vz	**L/kg**	0.15	0.11	0.13	0.03
Compartmental results	**Ka**		1.43	1.10	1.27	0.24
	t½ absorption	**h**	0.48	0.63	0.56	0.10
	Kel		0.016	0.010	0.013	0.004
	t½ elimination	**h**	44.63	71.74	58.19	19.17
	A	**ng/mL**		7567.05		
	Alpha			0.01		
	t½ alpha	**h**		71.74		
	B	**ng/mL**	8871.64	17061.60	12966.62	5791.18
	Beta		0.02	1.21	0.61	0.84
	t½ beta	**h**	44.63	0.57	22.6	31.15

4.6 Biotransformation pathways

The glucuronide metabolite was present for both flunixin and carprofen. Additional phase 1 metabolites could not be identified. The retention times and intensity (CPS) of the data is presented in Table 4-12, glucuronidation was identified as a mass transition of +176 Daltons.

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Table 4-12: Glucuronidation retention times and CPS for birds 1 to 4.

Drug	Bird	Sample Time Point	Retention Time (mins)	Intensity, CPS
Carprofen	Bird 1	7h	4.43	3.0e6
		9h	4.43	3.2e7
		12h	4.40	1.2e7
		24h	4.43	2.7e7
		32h	4.41	2.5e7
	Bird 2	12h	4.40	5.4e6
Flunixin	Bird 3	7h	4.44	1.7e7
	Bird 4	3h	4.39/4.47	2.2e7
		5h	4.39	1.6e7

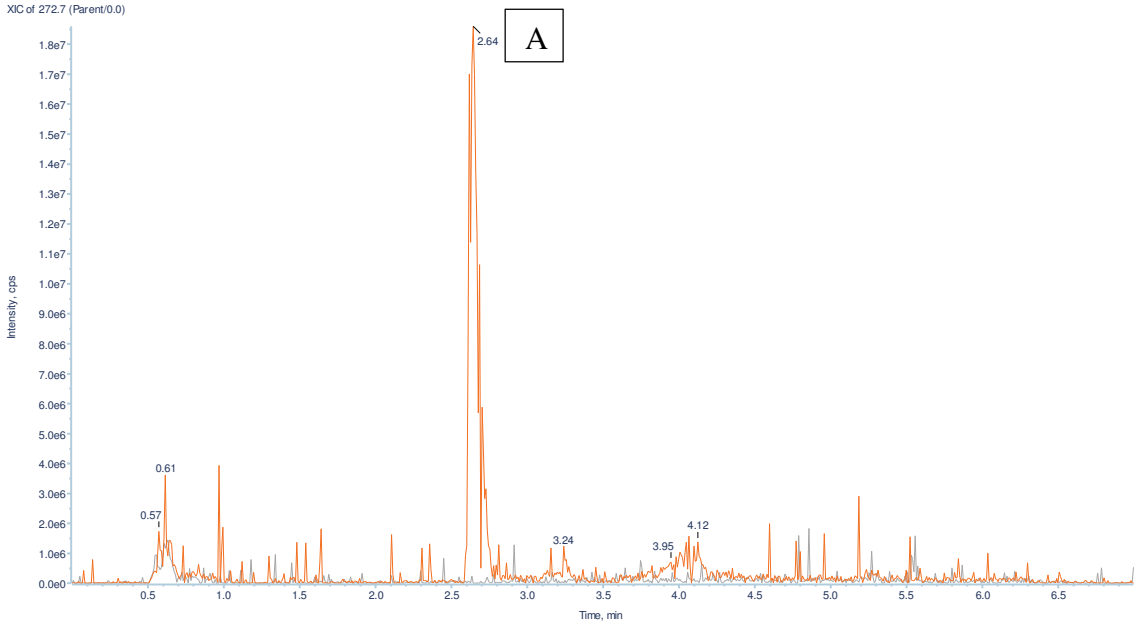

Figure 4-14: Chromatogram of parent molecule (carprofen) in bird 1 from the 9 h sample, peak indicated (A)

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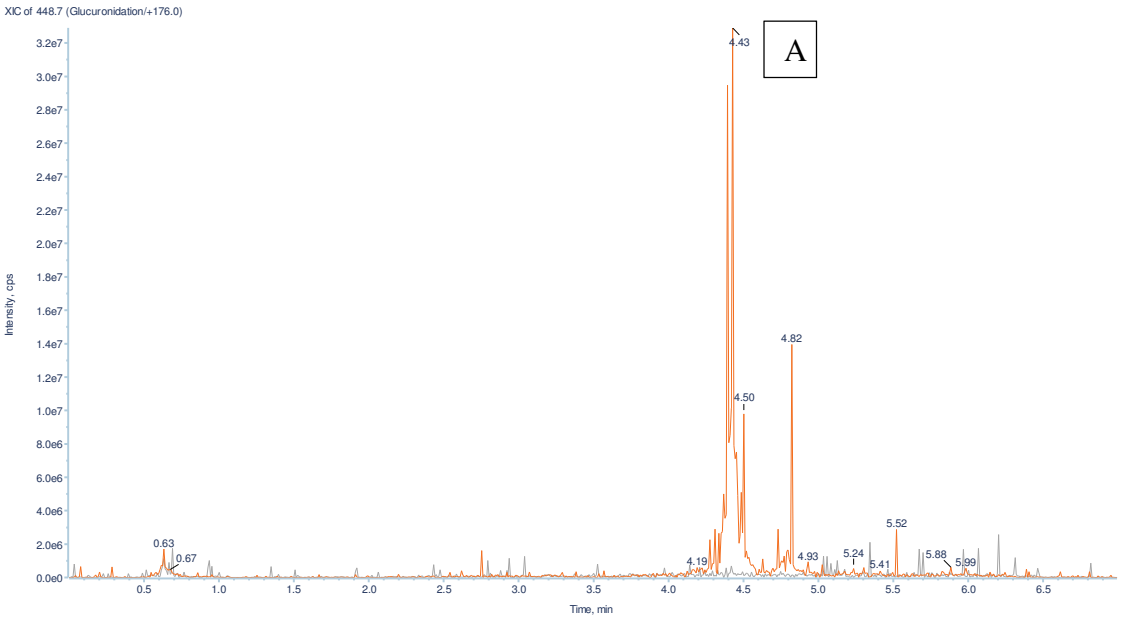

Figure 4-15: Chromatogram of glucuronidation present in bird 1 at 9 h, peak indicated (A)

4.7 Hysteresis plots

Hysteresis plots of uric acid concentration versus drug concentration only fell outside of the population reference ranges for flunixin for one time point (9 h) after which it returned to within the reference range (Figure 4-16; Figure 4-17; Figure 4-18). However, the same plots graphed for ALT, showed serum ALT activities above that of the population ranges for all three of the study drugs. Interestingly the elevated ALT serum activity for flunixin was only evident once the drug plasma concentration approached zero.

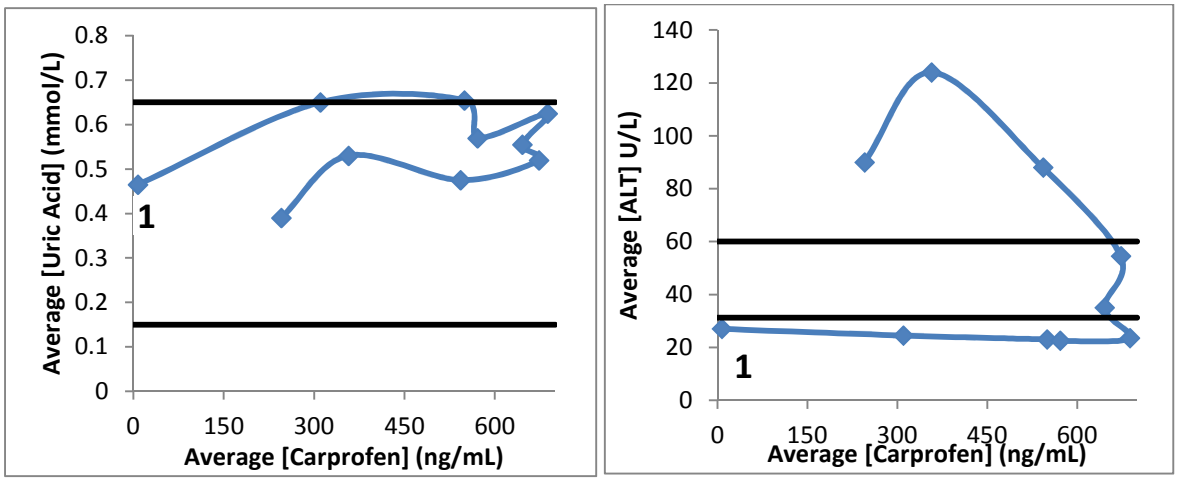

Figure 4-16 a and b: Average UA and ALT concentration graphed as a function of the average drug concentration over time for carprofen (Numbers indicate the direction of the plot i.e. 1 is the starting point)

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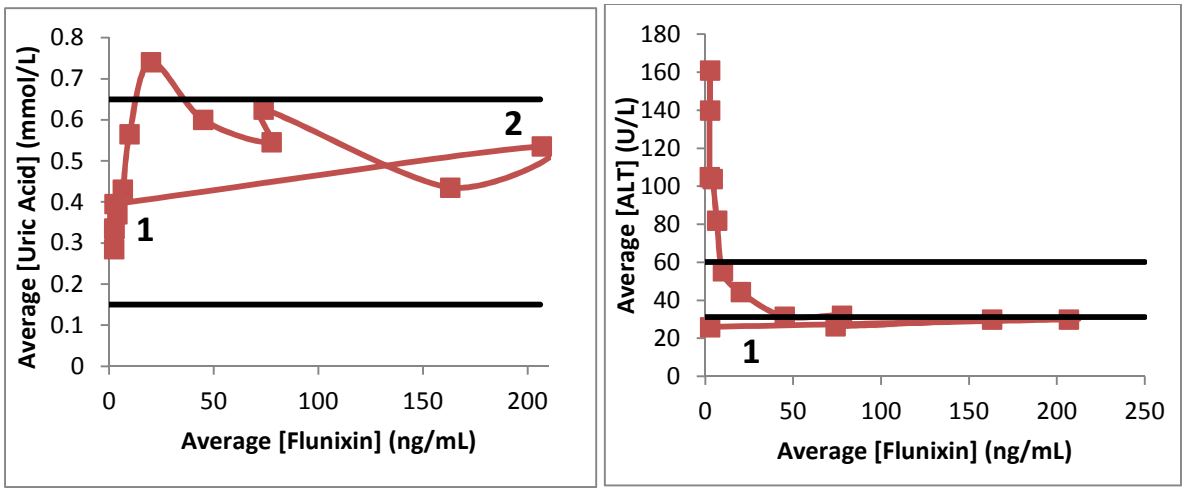

Figure 4-17: Average UA and ALT concentration graphed as a function of the average drug concentration over time for flunixin (Numbers indicate the direction of the plot i.e. 1 is the starting point.)

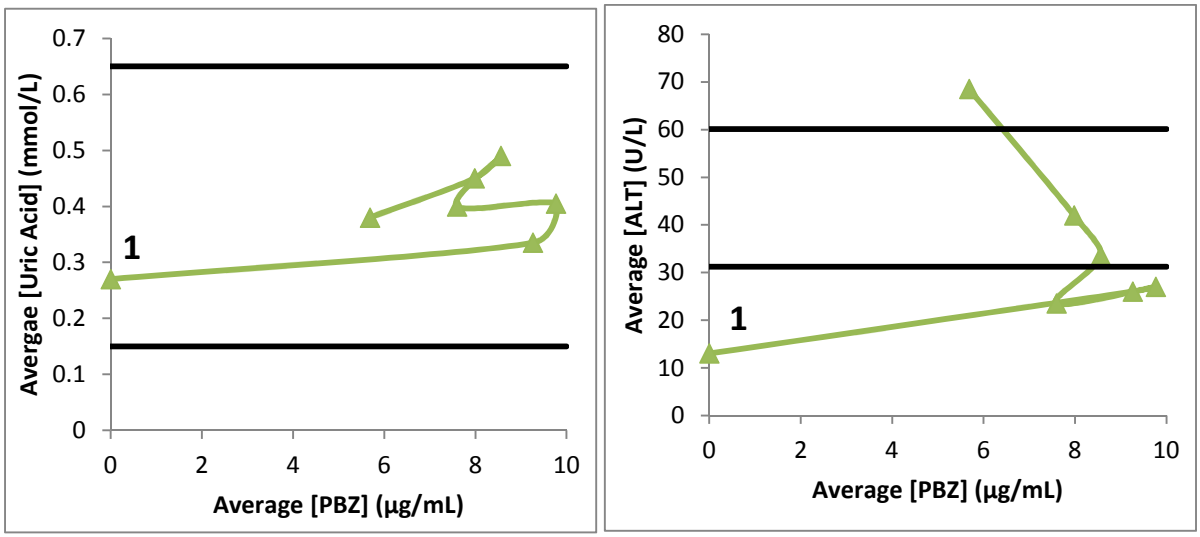

Figure 4-18: Average UA and ALT concentration graphed as a function of the average drug concentration over time for PBZ (Numbers indicate the direction of the plot i.e. 1 is the starting point.)

Data obtained from previous studies related to CV and NSAID toxicity, in which the Uric acid concentration versus drug plasma concentration were graphed in a similar fashion for meloxicam (Figure 4-19a), diclofenac (Figure 4-19b) and ketoprofen (Figure 4-20) was obtained for comparison purposes. UA remained within the reference ranges for all concentrations of meloxicam. Increasing diclofenac and ketoprofen concentrations caused an increase in UA over that of the population interval. Diclofenac, albeit only 1 bird's results, produced a 21.3 fold increase in UA and the death of the bird. A similar profile is evident for

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ketoprofen, however with a 17.7 fold increase resulting in death and a 7.3 fold increase survival of the birds.

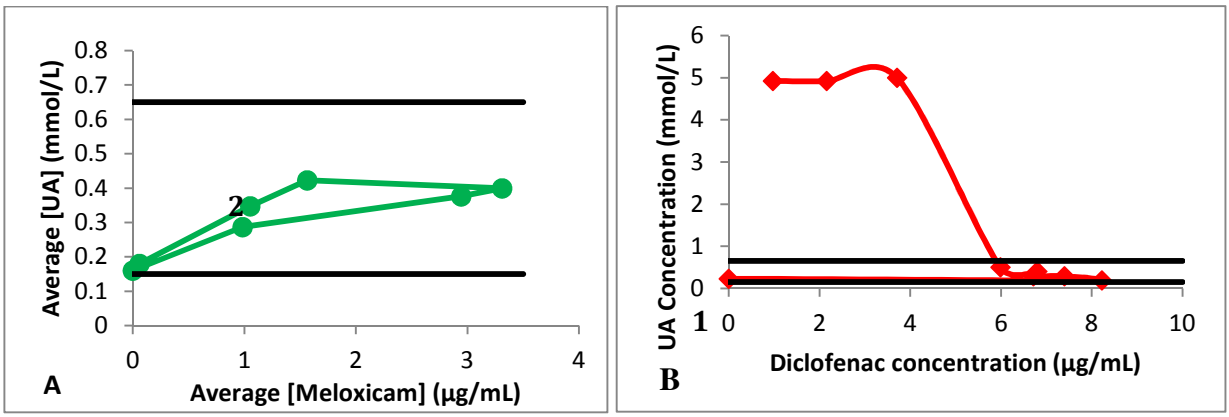

Figure 4-19: Average UA (mmol/L) concentration versus average meloxicam (µg/mL) concentration (n=4) (A) and diclofenac (n=1) (B) (Numbers indicate the direction of the plot i.e. 1 is the starting point)

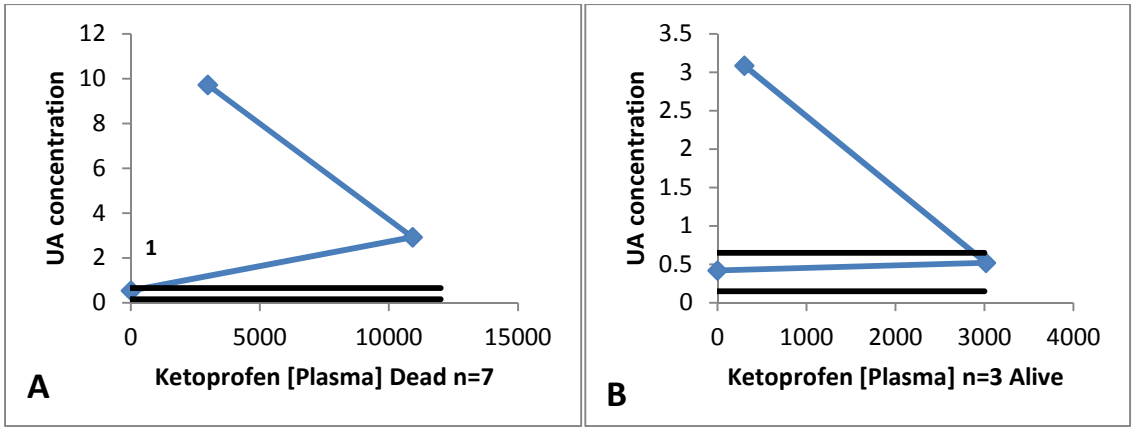

Figure 4-20: Average UA (mmol/L) concentration graphed as a function of average ketoprofen (µg/mL) plasma concentration. Graph A indicates the trend in 7 birds which died and graph B the trend in the 3 birds that survived. (Numbers indicate the direction of the plot i.e. 1 is the starting point)

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5 DISCUSSION

5.1 Toxicity

This study made use of only 2 birds per drug. This was based upon the methodology of Swan et al, 2006, in which the LD50 of diclofenac in the WBV was established using a probit analysis. The specific parameters monitored were changes in habitus, clinical pathology and plasma drug concentration over time as previously used in NSAID toxicity studies in CVs (Naidoo et al., 2008; Naidoo & Swan, 2009; Naidoo et al., 2010). NSAID have been shown to be nephrotoxic in Griffon Vultures, consequently clinical pathology markers for renal failure were monitored (Naidoo & Swan, 2009). Markers for hepatotoxicity were included.

5.1.1 Clinical signs and necropsy

No mortalities or post-mortem changes were seen in any of the study individuals, thereby indicating that the tested NSAIDs were not as toxic as ketoprofen and diclofenac. Despite the lack of overt toxicity, clinical signs of lethargy and depression were noted in the 1/2 carprofen treated birds, 2/2 flunixin treated birds, 1/2 PBZ treated birds and 0/2 control birds which could be indicative of general toxicity. This was in some cases different and other similar to what was previously described in the literature for other bird species.

- **Carprofen:** In avian species, toxicity has been tentatively documented in raptor species although the mechanism of such toxicity has not been clarified, with 5/40 cases of mortality in Gyps vultures and other species (Cuthbert et al., 2007). Mortalities have also been reported following oral repeated dose use for arthritis in backyard chickens at doses of 3-4 mg/kg on days 7, 10 and 11 post treatment (Hadipour et al., 2011). In contrast studies evaluating the pharmacodynamic aspects of various doses of carprofen there was no mention of toxic effects or death in the domestic fowl (*Gallus gallus domesticus*) or at 3 mg/kg intramuscularly in Hispaniola Parrots (*Amazona ventralis*) (McGeown et al., 1999; Danbury et al., 2000; Paul-Murphy et al., 2009).

- **Flunixin:** Flunixin was shown to be toxic in broilers at 0, 1.25, 2.5, 5 and 10 mg/kg body (IM daily for four days) with clinical signs analogous to those of diclofenac

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toxicity in vultures, namely stiff gait, anorexia, depression, lethargy and coma (Ramzan et al., 2012). Mortality rates in the 2.5, 5 and 10 mg/kg groups were 20, 40 and 60% respectively within 48 – 72 hours of administration. Pathological lesions of visceral gout were confirmed by histopathology findings of urate tophi in the tissues. Concurrent elevation of serum uric acid and creatinine concentrations were also associated with the drug's toxicity. Therapeutic use of the drug in scavenger birds has been associated with mortalities with renal disease and gout in 7/23 cases in Gyps vultures and other species (Cuthbert et al., 2007). When administered to Patagonian Conure (*Cyanoliseus patagonus*) and budgerigars (*Melopsittacus undulatus*) at 5mg/kg IV, 1 budgie became lethargic and depressed 15 min following administration (Musser et al., 2013b). When administered to Mallard Ducks (*Anas platyrhychos*) at 5mg/kg no pathological or clinical signs of concern were noted aside from muscle necrosis at the injection site (Machin et al., 2001). Flunixin has been associated with renal ischemia, necrosis and gout in Siberian Cranes (*Grus leucogeranus*) (5mg/kg), Whooping Cranes (*G. americana*), and re-crowned cranes (*G. japonensis*) (Clyde & Paul-Murphy, 1999) and dose dependant necrotising glomerulitis tophi in renal tubules and visceral gout in Bobwhite Quails (*Colinus virginianus*) (Klein et al., 1994). The lack of overt toxicity and mortality in this study was therefore unanticipated, however these studies utilised repeated dosing.

- **Phenylbutazone:** Two cases of raptor mortality with renal disease and gout have been reported for PBZ (Cuthbert et al., 2007). At 50 mg/kg and 100 mg/kg administered intramuscularly twice a day to broilers, PBZ was found to be toxic without UA being elevated, or with post-mortem lesions being present in the kidney up to 9 days post treatment. The study did demonstrate hepatotoxicity with elevations in AST, ALT and ALP serum activities as well as post mortem lesions in the liver (Awan et al., 2003).

The clinical signs of lethargy and depression, although non-specific in nature, were similar to those seen in *Gyps* vultures when other NSAIDs were administered, which induced toxic responses.

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- **Ketoprofen:** When ketoprofen was administered to CV's at 5 mg/kg PO, toxicity described as a prone position was noted and death followed within 48 h of dosing. Extensive visceral gout was confirmed histologically with tophi in the kidneys, liver, spleen and lungs (Naidoo et al., 2010). In comparison, administered to broilers and mallard ducks, toxicity was not clinically evident and confirmed with normal organ architecture on histological examination (Machin et al., 2001; Mohan et al., 2012).

- **Diclofenac:** Diclofenac administered to WBV exhibited toxicity within 24 h described as signs of lethargy and neck drooping and death followed within 42 h (Swan et al., 2006a). In another study, diclofenac administered at 0.8 mg/kg IV to CV induced toxicity described as depression viz. loss of appetite, failure to drink, dehydration and drooping neck within 24 h and death followed within 48 h. Concurrent pathology of urate tophi deposition on and within the renal tissue, liver, spleen and lungs was confirmed, associated with necrosis and cell injury to renal tubular cells and hepatocytes (Naidoo et al., 2009). Similar clinical signs were observed, viz. lethargy, depression, anorexia, dullness and sunken eyes in broilers administered diclofenac with histopathology corresponding to that of the CV study (Mohan et al., 2012).

5.1.2 Clinical pathology

In general clinical pathology provides a good tool for the evaluation of organ function and/or dysfunction, especially when used in hospital situations. For this study analysis, was undertaken almost 2 years after collection of banked samples. While it may be argued that sample degradation could have resulted, it must be taken into consideration that other studies, albeit for human samples, have shown that serum stored at -25°C for 2 years can still produce reliable results (Gislefoss et al., 2008) for Na (-0.1%), Ca (+6.4%), UA (+4.2%), and K (+19.9%) and (Gislefoss et al., 2009) for ALB (-5.5%) however not for ALT (-41%) (Values in parenthesis indicate the percentage increase or decrease in concentration following storage). Although K increased by 19.9% after 2 years of storage, this was attributed to the difference in clotting time during pre-storage sample handling. When comparing the 2 and 25 year values (Giselfoss et al., 2008), K was noted to be more stable (+5.4% attributed to evaporation). While we cannot be certain of the effect of storage for this study, the samples were never

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defrosted and re-frozen during the 2 year storage period. In addition we believe that the presence of the control group, and concentration versus time samples with baseline values for all samples, provides for proper interpretation as the focus is change over time in comparison to the control group.

For this study parameters related to kidney and hepatic function were focused upon as previous studies have indicated signs of nephrotoxicity and/or hepatotoxicity in CV (Naidoo et al., 2008; Naidoo & Swan, 2009; Naidoo et al., 2010). While numerous changes were evident in evaluated parameters, these changes were only considered significant if the change seen was consistent over more than one time point, with both animals being affected in a treatment group. Using these criteria, clinically important changes were noted for the parameters ALT, ionised calcium, sodium and uric acid.

5.1.2.1 *Ionised Calcium*

The ionised calcium concentrations were outside the reference range for all the treated birds. With a similar trend being evident for the control birds, this change is not considered to be of toxicological significance. While the reason for the major difference to the previous published reference interval is unknown, the difference may be due to degradation of the sample during the lengthy storage time or perhaps a difference in gender as this study made use of only female birds. For the former, Gislefoss *et al* (2008) found that total Ca was increased by 6.4% in human serum samples stored for 2 years, however this difference was thought to be due to evaporation (Gislefoss et al., 2008). From literature, the only other reported reason for an increase in plasma calcium concentrations in birds has been reported during egg production, with a variation in ionised calcium of 0.4mm/l just prior to and after oviposition (Luck & Scanes, 1979).

5.1.2.2 *Sodium*

After the 12 hour sample carprofen and flunixin treated birds had sodium concentrations higher than the control, with PBZ being similarly raised from 24 h. Due to the stability of sodium in stored serum (Gislefoss et al., 2008), it is unlikely that the sample underwent degradation during storage. It is presumed this effect is due to control bird 8's lowered Na after the 5 h

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sample (104.8 – 143.6 mmol/l) compared to the relatively higher values of bird 7 (142.1 – 149.2 mmol/l) and not from the results of pathogenic changes.

5.1.2.3 *Uric Acid*

Uric acid concentration was specifically monitored as it was the most indicative parameter for both ketoprofen and diclofenac toxicity. From literature, uric acid in avian species is a major waste product of protein metabolism (i.e. nitrogen elimination in the body) that is produced in the liver and eliminated exclusively by renal tubules as a function of blood flow via active transport systems in both the basolateral (Organic anion transporters - OAT) and apical cell membranes (p-glycoprotein pump - MRP) (Jones, 1999; Inui et al., 2000; Bataille et al., 2008). Unlike humans, reabsorption of UA by a mediated apical transporter (Uric acid reabsorption transporter 1 – URAT1 which is a member of the OAT family) does not play a role in urinary UA excretion in chickens (Berger et al., 1960; Roch-Ramel & Guisan, 1999). Birds with renal failure induced by tissue damage, are typically unable to excrete uric acid via renal tubular secretion. Reabsorption, absent in chickens, is unable to compensate for this failure with resultant elevated plasma concentrations (Leirz, 2003). Once the plasma's capacity to dissolve uric acid is exceeded (i.e. UA levels above 6.8 mg/dL), crystallisation of the uric acid results with the resultant clinical/pathological sign being gout (Smith et al., 2011). In ketoprofen and diclofenac exposed birds, toxicity was characterised by severe renal damage with concurrent increased plasma uric acid concentration and gout (Naidoo et al., 2009; Naidoo et al., 2010).

For this study all the treated birds showed an increase in uric acid concentration, which decreased to pre-treatment concentrations by 48 hours, in the absence of concurrent histological lesions of renal damage or changes in serum potassium concentrations. This increase in uric acid concentration (i.e. mild inhibitory effect on uric acid excretion) for PBZ was an unexpected finding, as it has been reported as having uricosuric activity in humans (Burns et al., 1958), although increased UA concentration has been noted in chickens (Berger et al., 1960). The change in uric acid concentration, in general, was also within the population reference interval for the monitoring period. As a result it is concluded that the raised UA is more likely from reversible inhibition of UA excretion and not toxicity. The latter has been

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well described in human literature whereby the NSAIDs are known to interact with tubular uric acid transporters in a reversible manner (Nozaki et al., 2007).

To further demonstrate that the change in uric acid concentration was due to reversible inhibition, hysteresis curves for these drugs were compared to curves plotted from information published on diclofenac, meloxicam and ketoprofen, with drug concentration being the independent variable and uric acid concentrations being the dependent variable. All three drugs in the current study resembled the hysteresis plots of meloxicam with the return to baseline concentrations corresponding with the absence of the drug i.e. reversible effect. The curves for flunixin and PBZ in addition could be described as counter-clockwise hysteresis which is characteristic of an inhibitory effect with a deep effect compartment, namely that the effect is still evident after plasma concentration decline (Ing Lorenzini et al., 2012). The latter may be indicative of a slower redistribution of drug into the central compartment from the effect site or more likely a lag phase to reactivation of the transporter. The profile for carprofen indicated a clockwise curve which suggests a shallow effect compartment (rapid re-distribution) or immediate reactivation of the channels on drug removal from the effect site (Derendorf & Meibohm, 1999). In contrast diclofenac and ketoprofen (dead birds) showed a continued increase in plasma uric acid concentration despite declining drug concentrations, albeit with initial counter-clockwise activity.

While speculative, the hysteresis curves seen may offer an explanation on the mechanism of toxicity of the NSAIDs in birds. In man, the uricosuric drugs bring about their effect by inhibiting the basolateral (OAT1, OAT3) and apical (MRP, URAT 1) uric acid transporters, which results in an initial build-up of uric acid in the blood, with subsequent greater glomerular excretion (Roch-Ramel & Guisan, 1999). Under normal circumstances, this increase in tubular uric acid would undergo tubular reabsorption via the apical URAT 1 pump (Perez-Ruiz et al., 2012). With the inhibition of the URAT1 pump, tubular uric acid is not reabsorbed resulting in a decrease in the plasma uric acid concentrations. With the absence of the URAT-1 transporter in the bird (Bataille et al., 2008), it is possible that the different drugs have their effects on different transporters. The inhibition of the OAT channel would result in an immediate increase in plasma uric acid as seen with PBZ and flunixin as it would fail to enter the cell, while the inhibition of the MRP would result in initial increase in uric acid build-up (storage) within the

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cell in a lag phase prior to cellular death and subsequent release of this stored uric acid into the plasma similar to that seen with aminoglycoside nephrotoxicity and possibly even diclofenac and ketoprofen toxicity (Giguere et al., 2006). As a result it may be interesting to establish the effects of these drugs on cloned vulture uric acid transporters.

5.1.2.4 *Alanine transferase*

In mammalian species, ALT is an enzyme found in the cytosol of hepatocellular cells. When acute cellular injury results, these enzymes leak into the vasculature, increasing serum activities (Bain, 2011). ALT, bound to the plasma membrane, can also be increased in states of enzyme induction e.g. drug / hormonal effects (Bain, 2011). Although ALT in many mammalian species is indicative of hepatocellular damage, in psittacine species ALT activity may be increased due to damage to the liver, heart, skeletal muscle, lung or intestine (Jones, 1999). In contrast to many mammalian species, the highest tissue distribution of ALT is also found in the kidney of domestic fowls (Lumeij & Westerhof, 1987). However in a later study the same author established increased ALT activity in racing pigeons (*Columba livia*) with induced hepatic necrosis confirmed histologically and no pathology in the kidneys (Lumeij et al., 1988). As a result an increase in ALT activity needs to be looked at in conjunction with other changes when deciding if the increase is renal or hepatic in origin. In vultures, Swan et al., (2006a); Naidoo et al., (2009) and Naidoo et al., (2010); demonstrated an increase in ALT activity with concurrent hepatocellular damage evident histologically.

For this study elevated ALT activities were noted in the flunixin, carprofen and PBZ treated groups. The magnitude of the ALT elevation was similar to the 3 to 7 times increase seen in diclofenac toxicity in griffon vultures (Swan et al., 2006a). While the tissue associated with this increase is speculative, the most likely tissue responsible for this increase is a mild liver injury or induction of liver enzymes (Boone et al., 2005), as the clinical pathology did not indicate overt renal toxicity nor was there any indication of any renal toxicity on histopathology. It is important to note that ALT activities may have been compromised by the two year storage time, this is further discussed in section 5.1.2.

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- **Carprofen:** The effect of carprofen appeared to be reversible as activities had already started declining by 48 h, whereas the other two drugs at 32 and 48 h still had the elevated ALT activities. In dogs (*Canis lupus familiaris*) ALT activity is elevated within 12 hours after a hepatic insult, peaking 1-2 days later and slowly returning to baseline over a 2 – 3 week period due to a clearance half-life of 60 hours (Bain, 2011). This exact pattern was evident for carprofen, albeit with a much quicker decline. Carprofen also appeared to be less toxic in the CV, compared to the pigeon at doses of 2, 5 and 10 mg/kg for 7 daily treatments where the drug was shown to induce a statistically significant increase in ALT activity as well as histologic lesions in the liver (Zollinger et al., 2011). In a study comparing 2.2 mg/kg and 4.4 mg/kg treated dogs, an upward trend in ALT activity was noted in the higher dose group, albeit not statistically significant or outside reference ranges (Kennerman & Polat, 2006). Based on the absence of histopathological lesions in any of the carprofen treated birds, it is speculated that the elevated ALT activity in the CVs was related to the use of a NSAID albeit in a nontoxic manner. However, with this study relying on a single dose, the question of repeat dose administration is of concern i.e. if the animals were to be exposed to repeat doses of these NSAIDs, it is possible that toxicity may result.

- **Flunixin:** The increasing activities of ALT over time reported for the CV differed to that for broilers at 1.25, 2.5, 5 and 10 mg/kg where no significant difference in ALT activities was detectable. Interestingly the elevated ALT activity versus drug concentration for flunixin was only present when the drug plasma concentration approached zero, possibly due to release of induced enzymes, or perhaps even tissue damage becoming evident later due to other latent mechanisms. With the concurrent spike in UA, it is possible that the raised ALT relates to renal tissue and not hepatic damage. This theory could not be corroborated on pathology.

- **Phenylbutazone:** The increase in ALT activities for the PBZ group was as expected as similar changes, albeit with concurrent pathological change, were seen in chickens treated with the drug (Awan et al., 2003). As a result it is speculated that repeat dose exposure would most likely induce more severe pathological changes.

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5.2 Pharmacokinetics

Signal to noise ratios for all three methods were well above the recommendations as laid out in the VICH guidelines for analytical reports (VICH, 1998), indicating that the limit of detection and limit of quantitation was potentially lower than the lowest concentration used to create the calibration curve. Despite all the calibration curves being linear throughout the concentration ranges of relevance, the accuracy of the method for carprofen and flunixin at the lower concentrations was not acceptable. Ideally the calibration curve for both should have been repeated to include concentrations within 80 – 120% of the test concentration range. Unfortunately this error was only discovered two years after the analysis was completed, and repeat analysis was not possible due to unavailability of the necessary equipment. This has led to data which is inaccurate at lower concentrations. The data however is still valuable to provide an indication of the pharmacokinetic profile that could be expected, and any further studies can be used to confirm this pharmacokinetic data.

Ideally vulture plasma should have been used for the calibrations curves to effectively counter possible matrix effects influencing the analysis, however this was not possible due to the limited number of birds available of this endangered species. No studies investigating the different constituents of chicken vs. vulture plasma could be found however chicken plasma was used in published methods (Naidoo et al., 2007). Chicken plasma was the closet avian species plasma that was readily available at the time of the study. As a result the concurrent internal standards were run to indicate whether or not the recovery of NSAID from the vulture samples was hampered by interference from plasma constituents. The calibration curve for carprofen and flunixin did not contain an internal standard, this oversight was corrected when the additional PBZ analysis was performed. Internal standards for carprofen and flunixin were within 2 standard deviations of the mean for all time points except for Bird 1 at 0 h and Bird 4 at 0.43h, which were below two standard deviations of the mean. Extraction of the analyte was therefore considered to be adequate.

Phenylbutazone extraction using the online column extraction method failed, possibly due to very high protein binding or inadequate extraction of the analyte due to saturation of the extraction column with compounds of higher affinity for the column. Another theory postulated

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that a constituent of vulture plasma may interfere with the extraction process of PBZ. Although all 3 NSAIDs are highly protein bound, it is not certain why PBZ was the only one to exhibit this problem. The AUC_{extra} for Bird 5 was 30%, therefore the curve was not adequately captured and the kinetic data is incomplete.

The profiles attained for flunixin and PBZ were similar between the two treated birds. This was not the case for carprofen where bird 2's C_{max} was double that of Bird 1, while AUC_{last} had a fivefold increase with a concurrent larger MRT and $T_{1/2}$. It should also be noted that this bird was also the one that showed signs of depression. With both birds known to have received the same dose, the only reason for this difference would be difference in metabolic capacity either as a result of individual variation or age related changes viz. Bird 1 (with the lower peak) was a fledgling at the time of the study whereas bird 2 was between 1 – 3 years old. This effect was seen in the vultures treated with ketoprofen (Naidoo et al., 2010) and the chickens treated with diclofenac (Naidoo et al., 2007), also once again speculated to arise from a difference in metabolic capacity. One important difference that is readily evident from the different profiles, is the higher net exposure of bird 2 to carprofen indicating that the difference is most likely at the level of pre-systemic elimination.

As only oral doses were examined absolute bioavailability could not be determined. Using compartmental modelling, the absorption half–life for flunixin and PBZ was rapid at 0.13 and 0.56 h respectively - while carprofen was slower at 0.88 h. Similar to flunixin and PBZ, meloxicam administered orally to vultures had an absorption half-life of 0.33 h (Naidoo et al., 2008). The CV absorption half-life of PBZ is unlike that published for cattle (*Bos Taurus*) dosed orally with PBZ, which had a slow absorption half-life of 2.4 h (Arifah & Lees, 2002). Absorption in the oesophagus / buccal cavity is unlikely due to the keratinisation of the epithelium as seen in the white back griffon vulture (Houston & Cooper, 1975). In light of this and the relatively short absorption half-lives, transit to the stomach was considered to be short. The PBZ profiles were also characterised by a secondary peak. From general pharmacokinetic principles, a degree of enterohepatic recirculation is present for this drug.

Carprofen and flunixin were characterised by a large volume of distribution with average values being above 3L/kg. In contrast PBZ was characterised by a small volume of distribution.

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For carprofen the volume of distribution (13.62 L/kg) was substantially larger than that described for dogs at 0.09- 0.25 l/kg, (McKellar et al., 1990) and horses (*Equus ferus caballus*) at 0.25 L/kg (Lees et al., 1994) when administered at a dose of 0.7 mg/kg IV. The same can be said for flunixin as the Vd in Ostriches (*Struthio camelus*) at a dose of 1.1 mg/kg IV was substantially lower at 0.13 L/kg (Baert & De Backer, 2002; Baert et al., 2002). While the reason for this difference cannot be ascertained from just the oral profile, the results do suggest that a substantial first-pass effect is present for the NSAIDs.

The volume of distribution of PBZ was similar to that published in Holstein bulls, at 0.134 +- 0.021 L/kg following a dose of the 10 mg/kg PO (Williams et al., 1990). The drugs was characterised by an unexpectedly high C_{max}, which was only previously reported in the goat at 27.23 ug/ml, 3.47 h after oral dosing at 4.4 mg/kg (Cheng et al. 1997). It is difficult to ascertain why the vultures had a higher C_{max} than that seen in other species. One possible explanation for the larger extent of absorption would be limited pre-systemic metabolic capacity as evident with ketoprofen i.e. saturation of the pre-systemic elimination pathways had resulted. Further support for this can be seen with the longer elimination half-life of 16 hours in the goat in comparison with the much shorter half-life of 4 to 6 hours in the dog. Another possible reason is enzyme inhibition either in the intestines and/or liver, as PBZ is a known inhibitor and substrate of the CYP2C9 enzyme (Kirchheiner & Seeringer, 2007). Nonetheless, as mentioned above, an intravenous profile will be beneficial to establish the validity of these assumptions.

The half-life of elimination of carprofen and phenylbutazone were both long and above 12 h, while flunixin was characterised by a relatively short half-life. The half-life of carprofen was not dissimilar to that described in the horse at 18.1 h (Lees et al., 1994), although it was larger than the range of 3.2 to 11.77 h reported in the dog (McKellar et al., 1990). While we consider the half-life of PBZ, to be long, it should be noted that this was substantially lower than the half-life of 70h for people (Aarbakke, 1978) and 62.6 h for Holstein cattle (Williams et al., 1990) when administered orally. Flunixin's half-life was similar to that described in other birds at, 0.62 h, 0.43 h, 0.54 h and 0.17 h for, pigeons, mallard duck, turkey and ostrich respectively (Baert & de Bekker 2003) and at 0.72 h and 0.91 h for budgerigars and conures (Musser et al., 2013a). Based on the half-lives, flunixin (1.84 h) is the least likely to be accumulative on repeat administration while both carprofen and PBZ (13.2 and 18.7 h) could result in toxicity with

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repeat administration. The likelihood of such repeat administration is minimal. The animal would need to feed on another contaminated carcass within 66 hours (carprofen) or 93.5 hours (PBZ) of the initial drug intake for repeat exposure to be relevant. That said, with the exact mechanism of toxicity being unknown, this is not a guarantee.

5.3 Metabolite information

A glucuronide metabolite was present for both carprofen and flunixin, in similarity to meloxicam in the CV (Naidoo et al., 2008), carprofen in man, dogs and rats (Rubio et al., 1980) and flunixin in dogs (Brady et al., 1998). The overall importance of glucuronidation as a general metabolic pathway for the three study drugs in birds is not known. Although, it can be confirmed that none of the 3 domestic birds (chicken, duck and turkey) were deficient in the capacity to glucuronidate (Short et al., 1988). As a result is has been speculated that toxicity due to Phase II metabolism glucuronidation deficiencies, as seen in cats with acetaminophen, are unlikely in these species (Riviere, 2011). The same assumption can probably be applied to the CV, although it is impossible to postulate whether zero-order kinetics will result.

5.4 Handheld vs automated analyser UA readings

According to the manufacturer's user guide, the handheld meter was plasma referenced using the EPAC6140 (Eppendorf, Hamburg, Germany), included UA Plus 1661868 Kit (ROCHE, Postfach, Switzerland). The specimens ranged from 3.7 mg/dL to 19.2 mg/dL, as measured by the EPAC 6140. A linear regression line with intercept 0.32 mg/dL, slope 0.986 and correlation coefficient of 0.952 indicated adequate accuracy between the methods. The method was precise when tested at four concentrations between 5.4 – 18.4 mg/dl (n=20) with a %CV <3.6% and SD between 0.1 and 0.7 mg/dL. Validation of the meter was adequately demonstrated in people, therefore, it is reasonable to assume that the meter may be of benefit in animal patients. The comparison between the handheld UA meter and the automated analyser results yielded no obvious relationship when graphed, however, on a Pearson correlation a weak statistically significant correlation could be found. It appears that the handheld meter was not sensitive at values less than 0.33 mmol/l. However, with this being a pilot study, the information gathered

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is sufficient to warrant further investigation with the following points being given consideration:

- More replicates and samples are required together with repeat analysis at every time point sampled. This will indicate the accuracy and precision of the method.
- A stronger study design correcting for temperature and time of day. The meter is only rated to operate at room temperatures of 18 – 38°C. The meter self-tests the room temperature, however testing of avian blood with body temperatures above 40°C may adversely affect test accuracy. Time of day corrections will provide an indication of intra-day and inter-day precision of the test.
- It may also be of value to demonstrate a correlation between samples spiked with known concentrations prior to the use of natural samples. Known concentrations provide a comparison tool for accuracy, mixed into blank serum samples matrix effect on test results can be established.
- Other points that could have resulted in the difference may be the use of stored samples for laboratory analysis and fresh samples on the meter. It may be worthwhile to look at freeze thaw stability of uric acid in vulture samples. However, we consider this unlikely as medical samples stored for 2 years resulted in minimal degradation. In addition for this study, the analysis from the clinical pathology analyser was higher than the meter for 9 of the readings and lower for 6 of the readings, suggesting that stability/degradation is not the reason for the differences seen.
- More information about the sample to be tested should be investigated. It is possible that the different materials tested viz. whole blood on the handheld meter and serum on the Roche equipment, may have influenced the results. In the initial use of the meter this was not given consideration as the same comparisons in people yielded no differences (UASure ® user manual).
- Receiver operating characteristic (ROC) maximise the sensitivity and specificity of a diagnostic tool (Greiner, 1995; Greiner et al., 1995). It would be beneficial to perform this analysis on the results of the handheld meter, using the Roche equipment as a gold standard and determine the cut-off values of the handheld meter. The current study sample size was insufficient for such purposes.

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6 GENERAL CONCLUSION

The hypothesis namely "Carprofen (11.5 mg/kg), flunixin (1 mg/kg) and phenylbutazone (1.7 mg/kg) is fatal to Cape Vultures following single acute oral exposure", was rejected.

Based on the information collected, the following conclusions are made:

- **Carprofen:** Birds were exposed to carprofen at the maximum recommended clinical dose of 10 mg/kg which is nine times the expected cattle tissue residue concentration. One of the treated birds showed signs of depression, in conjunction with an increased long half-life. The drug had minimal effects on the other clinical pathology parameters, with ALT activities showing the biggest change. However unlike the other two drugs, ALT activities returned to normal levels by the study end. Based on this result, it suggested that this product may be of clinical benefit in the vulture, but as a single treatment. This drug should also be safe for exposure in the food chain, as it is unlikely that vultures will be exposed to the drug often enough for it to be cumulative. It should also be noted that the vultures were exposed to a much higher dose than is expected to be found in a carcass with the currently marketed formulations. Further information regarding carprofen residues in carcasses shortly after administration at the injection site and within companion animals is needed to further evaluate this claim.
- **Flunixin:** Birds were exposed to flunixin at double the expected cattle tissue residue concentration at 1.0 mg/kg. With both the treated birds showing signs of depression, in combination with the elevated ALT activity, we suspect that drug will be toxic in the general vulture population. While the drug did not have the same effect on uric acid as diclofenac thereby indicating a decreased likelihood of nephrotoxicity, hepatotoxicity is the main concern. Clinical use or its presence in the food-chain should therefore be avoided.
- **Phenylbutazone:** Birds were exposed to PBZ at double the expected cattle tissue residue concentration at 1.7 mg/kg. While only one bird showed signs of depression, this drug was characterised by a long-half life with indication of enzyme inhibitory activity. In addition, the drug resulted in a time-dependent increase in serum ALT activity. Based on this, the drug may be hepatotoxic in vultures. Clinical use or its presence in the food-chain should therefore be avoided.

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6.1.1 Drawbacks of the study

- The sample size of this study was a major drawback for the effective interpretation of the results. In addition no statistical inference could be made with such small sample sizes. However, with the conservation status of this species the use of a larger number of samples could not be justified. The most important information, namely overt toxicity and indication of repeated dose study necessity, could be adequately obtained with the small sample size.

- A further matter of concern with this study was the delay in performing the clinical pathology analysis on the samples, the effect on the results for Ca, K, Na, UA and ALB however was deemed minimal due to published data on serum bank analyte stability. It is important to note that ALT activities may have been compromised by the two year storage time.

- The methods used to determine drug concentrations did not prove to be accurate nor precise at lower concentrations, although the detection and quantitative limits were good and calibration curves linear It is possible this is due to differences in chicken and vulture plasma. The method was considered adequate for the purpose.

- Clinical monitoring of the birds was performed by three different observers, it may have been beneficial to set up a score sheet / descriptions of conditions beforehand to create more homogenous clinical scoring data.

6.1.2 Future trends

- From the results, it is suggested that carprofen can be investigated as the next potentially vulture safe drug. Studies that need to be undertaken should initially expose a larger number of birds to confirm this finding. A repeat dose study should also be undertaken to establish the safety profile of the drug. If possible further pharmacokinetic studies should be undertaken.

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- Based on the differences in the profiles achieved for the three NSAIDs, it would appear that metabolic capacity is variable amongst the individuals. With this difference most likely being different at the CYP level, it may be helpful to characterise the CYP enzyme system responsible for the metabolism of these NSAIDs in conjunction with quantification of the metabolites.

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