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Title:

Could the environmental toxicity of diclofenac in vultures been predictable if preclinical testing methodology were applied?

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HIGHLIGHTS

- Obtained pharmacokinetic data could not always associate metabolic constraint as the major cause of poisoning.
- The uric acid plasma buffering capacity of a species could be used as a measure of diclofenac's safety.

- Toxicity of diclofenac in vultures is idiosyncratic as the oral LD_{50} is substantially lower than those of other model species.

Abstract

Diclofenac, a non-steroidal anti-inflammatory pharmaceutical agent was responsible for the death of millions of *Gyps* vulture's in the Indian sub-region with the safety of the other non-steroidal anti-inflammatory drugs (NSAIDs) being questionable. With preclinical safety testing not well established for avian species unlike for mammalian and environmental toxicity, we ask the question if a preclinical model could have predicted the toxic effect of the drug. For this study, we test an Organisation for Economic Co-operation and Development (OECD) guideline 223 for assessing the acute toxic potential of pesticides in birds by exposing three avian species to the drug. Exposed Japanese quails (*Coturnix japonica*) and Muscovy ducks (*Cairina moschata*) demonstrated clinical signs and pathology similar to those previously reported in vultures viz. hyperuricemia, depression, death, visceral gout and nephrosis. However, exposed domestic pigeons (*Columba livia domestica*) were insensitive. Following a pharmacokinetic analysis, the drug was well absorbed and distributed in the pigeons with a half-life below 6h. A toxicokinetic evaluation in quails showed poisoning was due to metabolic constraint, with a half-life and mean residence time above 6h and 8h respectively resulting in death. Toxicity seen in the ducks was however not related to metabolic constraint but hyperuricemia as metabolism was rapid [half-life (1-2h) and mean residence time (2-3h)] irrespective of survival or death. Despite succumbing to diclofenac, the established oral median lethal dose (LD_{50}) of 405.42mg/kg and 189.92mg/kg in Japanese quails and Muscovy ducks respectively from this study were substantially higher than those reported for *Gyps* vultures (0.098 mg/kg) which is as a result of

the rapid elimination of the drug from the body in the former species. More importantly, it suggests that these species are not suitable as surrogates for non-steroidal anti-inflammatory drug toxicity testing and that the toxicity of diclofenac in vultures is idiosyncratic most likely as a result of species specific metabolism.

ABBREVIATIONS

NSAIDs: Non-steroidal anti-inflammatory drugs, OECD: The Organisation for Economic Co-operation and Development, EPA: Environmental protection agency, UPBRC: University of Pretoria Biomedical Research Centre, SEDEC: SEquential DEsign Calculator, LD_{50}: Median lethal dose, C_{max}: Maximum plasma concentration, AUC_{last}: Area under curve to the last quantifiable time point, $AUMC_{last}$: The area under the moment curve from the time point zero to the last measured time point thalf ($T_{1/2}$): Half-life, Cl: Clearance, V_d: Volume of distribution, MRT: Mean residence time, Lz: Elimination rate constant, HPLC: High Performance Liquid Chromatography, LOQ: Limit of quantification, LOD: Limit of detection, h: hour, sec: second, AWBV: African White Backed Vulture; CGV: Cape Griffon Vulture.

Keywords: *Gyps* vultures; Diclofenac; LD_{50}; Pharmacokinetics; Acute toxicity.

1 Introduction

The last 10 years of the 20th century saw a tragic population decline of three species of vulture (*Gyps bengalensis*, *Gyps indicus*, *Gyps teniurostris*) endemic to the Southern part of Asia (Green *et al.*, 2004; Oaks *et al.*, 2004). Their population estimated to be more than 60 million in the early 1990's plummeted (Shah, 2010), as it will be later shown, from their accidental exposure to veterinary diclofenac in carcasses of ungulates treated with the drug shortly before death (Oaks *et al.*, 2004). Toxicity in vultures was associated with lethargy and neck drooping within 48 hours of exposure and with an increase in plasma uric acid concentration and terminal hyperkalemia. At post-mortem, there was evidence of renal failure with extensive visceral gout (Naidoo *et al.*, 2009b; Oaks *et al.*, 2004; Swan *et al.*, 2006b). Following diclofenac's devastating effect on the vulture population in the region and its extremely high toxicity (LD_{50} of 0.098mg/kg), the drug was eventually banned in India, Nepal, Pakistan (Prakash *et al*., 2012) and Bangladesh (Balmford, 2013).

The devastating effect of diclofenac was also completely unexpected as the drug is fairly well tolerated in mammalian species (Todd and Sorkin, 1988). This unpredictable toxic effect has since raised numerous questions as to why this specific drug was so toxic, how could a registered drug end up being so toxic, and most importantly could it have been predictable or preventable if more extensive testing was undertaken during product development. At present, while the registration process for medicines may differ slightly per region, the required toxicity testing for veterinary medicines tends to focus on non-clinical mammalian lab animal toxicity, target animal toxicity, and depending on the medicine also aquatic and soil toxicity as a surrogate for determining their environmental impact. Unfortunately little testing is undertaken for avian species unless the veterinary remedy is a pesticide. The reason for the latter is that birds have lower chance of being exposed to substance contaminating the environment, with the notable exception of the pesticides.

For pesticide testing, two commonly used guidelines for assessing the environmental effect of pesticides are the Environmental Protection Agency (EPA) and Organisation for Economic Co-operation and Development (OECD). In these protocols, the Japanese quail/northern bobwhite quail (*Coturnix japonica/Colinus virginianus*), chicken (*Gallus gallus*), mallard duck (*Anas platyrhynchos*) and domestic pigeon (*Columba livia domestica*) are used as indicator species (Kenaga, 1978; EPA, 2012; OECD, 2010) largely due to their availability, adaptation to laboratory conditions and as members of different habitat groups. Other commonly used species, while not expansive, include the zebra finch (*Taeniopygia guttata*), budgerigar (*Melopsittacus undulatus*), house sparrow (*Passer domesticus*) and red-wing blackbird (*Agelaius phoeniceus*).

For the protocol, birds are usually exposed to the said xenobiotic at various doses and data so generated are considered in further extrapolations towards the safety of the xenobiotic to other target avian species. For this study, we ascertain if the use of the OECD models could have been predictive of diclofenac's environmental toxic effects in vultures following single dose exposure or whether toxicity was idiosyncratic. The single dose acute toxicity was an important criterion since toxicity in vultures is known to result from a single meal contaminated with diclofenac. Also, with the chicken being previously tested for its $LD_{50,}$ we focused on the pigeon, quail and duck as the test species.

2 Material and methods

2.1 Housing and care

The study made use of the Japanese quail, Muscovy duck (*Cairina moschata*) and the domestic pigeon. In all cases, the birds were housed in the experimental aviary of the Biomedical Research Centre of the University of Pretoria (UPBRC). Nineteen young-adult birds per species were acquired from commercial farms in Pretoria. Each bird species was housed in groups and individually identified with tags. The birds were allowed at-least a one-week acclimatization period and had free access to fresh commercial feed and municipal potable water. The birds had a daily lightening period of twelve to fourteen hours with controlled room temperature and relative humidity. The study was approved by the University of Pretoria Animal Ethics Committee (V107/16) and followed the South African standard for the care and use of animals for research purposes (SANS10386).

2.2 Animal treatment

The birds were fasted over-night for fifteen hours prior to dosing, but had free access to water. The birds were randomly allocated to a test and control groups comprising fourteen and five birds respectively. The toxicity model followed a stepped process, without the limit test being conducted, as diclofenac was deemed to be of high toxic potential, as directed by the guideline. For the Stage 1 dosing, the LD_{50} was initially estimated from published literature for acute toxicity studies from the rat (*Rattus spp*) (Piao *et al.,* 2006) and chicken (Naidoo *et al.,* 2007). The working LD_{50} was subsequently determined through the dosing of 4 birds with one of four doses extending across this estimated LD_{50}. For Stage 2, the final LD_{50} was determined by dosing 10 birds, each at a different dose, across the predicted LD_{50}. The control groups received vehicles at same dose rate used for test group. The LD_{50} of diclofenac for each of the species was estimated by a probit regression model using the OECD purpose written Microsoft excel work book; SEquential DEsign Calculator (SEDEC) with measure of goodness of fit using Chi-square. The doses used in the study are presented in table 1. The estimates of diclofenac's LD_{50} in some bird species from previous studies were compared for any relationship between body mass and diclofenac's toxicity.

2.3 Monitoring and sampling

Birds were monitored continuously during the first two hours after dosing. Subsequent observation on the day of dosing occurred frequently until nine to twelve hours after dosing. The birds were observed at-least twice daily for a period of fifteen days for signs of toxicity. Blood samples were collected at 0, 2, 8, 24 and 48 hours' post dosing and stored at -25°C for pharmacokinetic and clinical chemistry analysis. Due to the paucity of samples, clinical pathology evaluation was not undertaken in the Japanese quails and domestic pigeons. For the

Muscovy ducks, uric acid, Na^+ and K^+ concentration were assessed using the Cobas Integra 400 plus analyser as per the manufacturer's instruction (Roche diagnostics, Mannheim, Germany). The uric acid concentration per time point for the birds that survived, died and the controls at 24 and 48hours were compared by means of a Kruskal-Wallis test since the data was not normally distributed. Post-hoc analysis was undertaken with a Mann-Whitney Test between these three grouping, with the p-value set to 0.016 after a Bonferroni correction. During the course of the study, all birds were necropsied and gross pathologic lesions recorded. For histopathology, liver, kidney and spleen samples were collected in buffered formalin, sectioned and stained using the standard Haematoxylin & Eosin technique. Tissue samples (liver & kidney) were collected and preserved at -25°C for diclofenac residue analysis.

2.4 Drug analysis

2.4.1 Japanese quail plasma sample preparation

Plasma samples (200µl) were transferred into 2ml tubes and 400µl of diethyl ether was added to each. This was then followed by a 400µl of 0.3M potassium dihydrogen phosphate with pH of 3.5. Sample preparation subsequently followed procedure described by Naidoo *et al.*, (2007).

2.4.2 Muscovy duck and domestic pigeon plasma sample preparation

Plasma samples (200µl) were transferred into 2ml tubes and 40µl of phosphoric acid was added to each. This was then diluted with 200µl deionised water. The tubes were capped and content mixed with a multitube vortex mixer (Vortexer, Heathrow Scientific, Illinois, USA) for 30 secs. The sample mixtures were subsequently loaded onto preconditioned cartridges (1cc 30mg Oasis HLB cartridges from Waters, Miford, MA, USA) and extraction was achieved as described by Suenami *et al.*, (2006).

2.4.3 Liver and kidney sample preparation

For each liver and kidney sample preserved, 500mg of thawed tissue was weighed into a 10ml tube and 2ml of high-performance liquid chromatography (HPLC) grade acetonitrile was added. The mixture was homogenized using a homogenizer (Pro 200, Pro Scientific Inc., Oxford, CT USA) for 1 min and subsequently vortexed for 10 min. The prepared sample was centrifuged at 4500 x g for 10 mins at 4°C and the supernatant filtered using 0.22µm disposable MS® Nylon syringe filter units. The resultant filtrates were stored at -25°C in crimp top vials until analysis using HPLC technique (Taggart *et al.,* 2007).

2.4.4 Separation and quantification of diclofenac sodium using HPLC

The experimental condition described by Naidoo *et al* (2007) for quantifying was followed using a 250 x 4.6mm, 5µ BDS HYPERSIL Phenyl column. The retention times for diclofenac were 3.97, 4.3 and 4.7mins for the pigeons, quails and ducks respectively. The standard calibration curve showed an r^2 value above 0.99 for each run. The limit of detection (LOD) and limit of quantification (LOQ) are 0.195µg/mL and 0.78 µg/mL respectively for the ducks. For the quails the LOD and LOQ are same i.e. 0.39µg/mL, as signal to noise ratio for the lowest standard concentration is far above 10:1. The LOD and LOQ for the pigeons are similarly the same i.e. 1.56µg/mL. The percentage accuracy was 96.7% while mean intra- and inter- day precision were 0.78% and 1.27% respectively for the liquid-liquid extraction technique. The solid phase extraction method produced a mean percentage accuracy of 98.7% and mean intra-day precision of 0.77%. Due to limited plasma volumes, inter-day precision could not be determined.

2.5 Pharmacokinetic analysis

Data generated following HPLC analysis were evaluated by a non-compartmental model technique, using standard equations with the aid of Kinetica 5.0 software package developed by Thermo Scientific. However, due to the detection of diclofenac at only a few sampling time points in the pigeons, pharmacokinetic evaluation for this species was undertaken using the standard formulae of sparse analysis associated with therapeutic drug monitoring (Sojka and Brown, 1986). To compare pharmacokinetic parameters between doses and species, the applicable parameters were equilibrated to 1 mg/kg for the study species as well for published literature (Palatini *et al.,* 1993).

2.5.1 Plasma uric acid saturation assay

Blank plasma samples from the ducks and stored blank plasma samples collected from chicken and Cape vulture (*Gyps coprotheres*) during another study were assessed for Na^+, K^+ (using same method described above) and albumin concentration. Albumin concentrations were assessed using a fully automated electrophoresis system (Pretty, Separation Scientific, Honeydew, South Africa). The maximum saturation with uric acid in plasma samples was ascertained using method previously described above.

3 RESULTS

3.1 Clinical signs

Of the fourteen quail exposed to diclofenac, two birds showed signs of intoxication and died within 48 hours. The first was found depressed and unable to walk just a few minutes prior to death at about 48 hours of dosing. The second bird vomited twice during restraint. At about

twenty four hours of dosing, it was found slightly depressed and separated from the rest of the group. It remained so until it was found dead at about 47 hours after dosing. The resulting oral LD_{50} of diclofenac was estimated at 405.42 mg/kg with a measure of goodness of fit of 4.17.

Following the duck's exposure to diclofenac, five birds succumbed to intoxication and died at about 48-98 hours. Three died naturally while the remaining two were euthanized for humane reasons. On average, the onset of manifestation was about 48-72 hours with birds being depressed, isolated and unable to bear weight. This condition gradually deteriorated to coma and death. Another bird was seen limping about 77 hours after dosing but remained active and alert throughout the study. The resulting oral LD_{50} of diclofenac in the Muscovy duck was estimated at 189.9mg/kg with a measure of goodness of fit of 12.68.

None of the pigeons succumbed following their exposure to diclofenac. However, after the oral gavage many of them were seen regurgitating. Regurgitations and/or attempt to regurgitate occurred within an hour to thirty-one hours after dosing during the course of the study.

3.2 Clinical chemistry of the Muscovy Ducks

Following plasma uric acid analysis, all birds showed uniform concentration at 0 hour of sampling before exposure to the test substance. The control birds showed a uniformly steady concentration throughout the study (i.e. from 0 hour – 48 hour of dosing). For the test birds, there was a progressive and consistent increase in plasma uric acid concentration from 2 hours after dosing up until 24 hours of dosing. At 48 hours after dosing there was a sharp decline (1.04 ± 1.3 mMol/L) in plasma uric acid concentrations in birds that survived, while in the birds that succumbed, there was a further increase (7.96 ± 5.03 mMol/L) in plasma uric acid concentrations

(Figure 1). Birds that succumbed showed a trend for an increase in plasma uric acid concentration from 24h on non-parametric ANOVA (p= 0.34 and 0.14). The 24h and 48h time point plasma uric acid concentrations for the birds that died were significantly different to the control birds on post-hoc evaluation (p=0.016). The 48-hour Na^+ and K^+ concentration were within normal, the only exception is the modest rise in K^+ concentration noticed in one of the birds that succumbed (5.52mMol/L).

To better understand why some of the Muscovy ducks with high uric acid at 24hours may have recovered while all the vultures with high uric acid at the same time died we compared the capacity of duck plasma for uric acid. Evaluations showed that total plasma albumin concentrations in the birds was in the ratio of 3.1: 1.9 : 2 for duck, chicken and vulture respectively. The same trend was evident for the maximum solubility of uric acid in plasma with concentration being 8.06 ± 0.2 mMol/L for duck in comparison to 6.98 ± 0.33 mMol/L for the vulture i.e. vulture plasma had a total lower saturation point for uric acid than the duck.

3.3 Pathology

At necropsy, quail that succumbed had urate crystals on the serosal surfaces of all the abdominal organs. The kidneys were moderately swollen and pale. In one of the birds, tubules were visible as white lines in the cortex. The lungs were mildly congested with marked oedema. Four of the five ducks that succumbed had severely swollen and pale kidneys with collapsed ovaries. Visceral gout was evident with urate crystals even present in the bile of one of the ducks. The fifth had normal kidneys but with a moderate generalised congestion. In addition, it had moderate splenomegaly and some collapsed ruptured ova. None of the pigeons demonstrated gross lesions following oral gavage. No pathologic lesions were seen on the other birds that underwent scheduled euthanasia fifteen days after dosing.

Histologically, there was evidence of tophi in the kidney, spleen and liver of quail that succumbed. The kidneys were more affected with damaged tubules being characterized by the presence of urate aggregates (Figure 2). At some instances, the aggregates had assumed a globular form.

Several of the ducks that succumbed revealed moderate to severe injury to the kidneys with widespread dilatation of tubules associated with loss of the normal cuboidal lining cells. The cells were replaced by a granular pink material in which cell fragments were embedded. Within some of the tubules, radiating spicules of urate crystals could be seen within this pink material filling the damaged tubules. In addition, scattered tubules showed mineralisation of individual epithelial cells and many collecting ducts contained desquamated tubule epithelium admixed with heterophils. The only duck that succumbed, with additional lesions in the liver demonstrated swollen scattered single hepatocytes that were lighter in colour with identifiable small needle-shaped crystals within their cytoplasm. Interestingly, some of the ducks that survived demonstrated significant histological changes. The pigeons however, demonstrated numerous non-specific microscopic changes.

3.4 Pharmacokinetics

In all species, plasma concentrations were at their maximum (C_{max}) at the first sampling at 2 hours. In quails, the area under curve to the last quantifiable time point (AUC_{last}) was not directly proportional to the dosage received. Diclofenac was well distributed after administration, and was characterised by a short half-life ($t_{1/2}$). The $t_{1/2}$ was generally under 6 hours, with the exception of the quails that died. These two birds showed a relatively slow elimination rate

constant (Lz) with correspondent lengthy elimination half-life (above 6h) and mean residence time i.e. MRT (above 8h).

In the case of the ducks, there was evidence of a large variation between individual birds in the metabolism of diclofenac. The C_{max} attained, AUC and $t_{1/2}$ did not clearly differentiate between poisoning and survival. For all the birds dosed, the extent of drug absorption was high with a short $t_{1/2}$ of less than 3 hours and mean residence time within 2-3 hours. Diclofenac was well absorbed and distributed following oral gavage in the pigeon. The biological half-life of elimination was generally below 6 hours. In general the maximum plasma concentration reached and extent of drug absorption was directly proportional to the dosage received.

The pharmacokinetic data equalized to 1 mg/kg is presented in table 2. The result show that the AUC in pied crow (*Corvus albus*), quail, duck and pigeon were relatively lower compared to those of chicken and *Gyps* vulture, while the observed volume of distribution of diclofenac in the chicken and *Gyps* vulture were quite negligible compared to estimates in the crow, quail, duck and pigeon. Bird species used for this study demonstrated a much more rapid clearance (at least 108-fold higher) and a shorter biological half-life of elimination (at least 2-fold lower) compared to *Gyps* vultures. In general, the crow demonstrated the fastest clearance rate with a biological half-life of elimination fairly similar to those of quail, pigeon and duck.

None of the bird species had detectable residues of diclofenac parent molecule in their liver and kidney following HPLC analysis.

4 DISCUSSION

4.1 Clinical signs of toxicity

The quail and duck presented signs similar to those demonstrated by diclofenac exposed *Gyps* vultures and chicken viz. depression and acute death (Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b; Swan *et al.,* 2006b). While quail only showed subtle depression before death at 48 hours of dosing, ducks exhibited a much more delayed episode of depression (approximately 48-72 hours) that progressively cumulated in coma and death. These presentations were thus different from the usual depression evident at 24 hours of dosing, which progressively worsened to coma and death at 48 hours in *Gyps* vultures and chicken (Swan *et al.,* 2006b; Naidoo *et al.,* 2007). In addition, the ducks showed signs of lameness a manifestation of urate crystals deposition in joints (Lumeij, 1994). The pigeons surprisingly remained healthy throughout the study.

4.2 Clinical chemistry

While the increase in plasma uric acid concentration in ducks that died was as expected, surprisingly the ducks were able to withstand concentrations far above those reported to kill *Gyps* vultures/chicken in addition to remaining active and alert for another 12-24h before succumbing. We can only surmise that the increasing plasma uric acid concentrations over time only became of toxic concern when the saturation point was reached as explained by Lumeij, 1994 i.e. the higher buffer capacity of duck plasma allowed for a greater increase and time for the increase in plasma uric acid concentration before precipitation, and subsequent toxicity. Uric

acid is a major by-product of protein metabolism. It's mainly excreted via the para-amino hippuric acid channel in avian species (Rafey *et al.,* 2003; Dudas *et al.,* 2005; Naidoo *et al.,* 2009a). Once its limit of solubility within the plasma is exceeded, urate precipitates are deposited within tissues. The latter was evident in the birds that died as they showed both high plasma uric acid concentrations and tissue damage. This is similar to the effects observed in vultures, as the increase in uric acid was soon followed by death and signs of severe gout (Swan *et al.,* 2006b; Naidoo *et al.,* 2007; Naidoo *et al.,* 2009b). The absence of this effect in birds that demonstrated concentrations of up to 6.3 mMol/L was surprising. This suggests that the birds recovered because saturation of the plasma had not occurred, with the result that there was no uric acid precipitation and irreversible tissue damage. This was supported by the *in vitro* study, which demonstrated a maximum uric acid solubility of 8.06 ± 0.2 mMol/L, an underestimation as one can expect even greater solubility at the higher normal avian body temperature. It should also be noted that the maximum solubility in the vulture was lower at 6.98 ± 0.33 mMol/L, which may explain their sensitivity to the effect of diclofenac.

The decline in uric acid concentration towards normal with subsequent survival of the birds has never been observed in exposed vultures and may indicate a restoration of renal function. This potentially suggests that in birds, the drug induces an increase in plasma uric acid concentrations in an identical manner to humans. From literature, diclofenac is known to inhibit the renal uric acid channels in humans (Khamdang *et al.,* 2002). If this is the case, then inhibition of the channels may be more pronounced in vultures, which may explain their higher sensitivity. However, since some of the birds that recovered did still show signs of pathology, it could also mean that the drug only induced partial renal damage in the said birds, and that the recovery was

from the functional renal reserves which can be as high as 70% or more in some bird species (Lierz, 2003).

While the above changes in uric acid could not be evaluated in the quail due to a paucity in sample volumes, the presence of extensive urate deposit in the carcasses of quails and ducks that succumbed has to have resulted from uric acid accumulation within the plasma (Merriman and Dalbeth, 2011), a common characteristic of diclofenac poisoning in *Gyps* vultures (Oaks *et al.*, 2004; Swan *et al.*, 2006b; Naidoo *et al.*, 2009b) and chicken (Naidoo *et al.*, 2007).

4.3 Pathology

Similarly, as previously reported in chickens and *Gyps* vultures, necropsy in the quails and ducks revealed severe visceral gout associated with renal failure (Naidoo *et al.*, 2007; Oaks et al 2004; Swan *et al.*, 2006b). However, lesions in quails were not as severe as those demonstrated in *Gyps* vulture and chicken as there was no evidence of inflammatory cells involvement which was reported in the latter species. For ducks, there were more severe renal lesions involving the proximal tubules, interstitium, collecting duct and Bowman's capsule associated with inflammatory cells. This would explain the longer duration to death observed in the ducks in comparison to the chicken, quail and vulture, as the birds had sufficient time to initiate an inflammatory response. However, in sharp contrast to the former species, most of the ducks that succumbed had no hepatic or splenic lesion.

The high tolerance exhibited by the pigeons may be due to their inherent metabolic abilities, as the drug was well absorbed and distributed following oral gavage. Despite reacting after dosing, the drug was still detected in plasma up to 24 hours after dosing. Diclofenac is rapidly absorbed

from the GIT (within an hour, in the absence of feed) (Naidoo *et al.,* 2007) and would have been absorbed before the episodes of regurgitation in most of the birds.

4.4 Pharmacokinetics

In an unexpected manner, there was no clear link between obtained pharmacokinetic parameters from birds that survived or died either before or after dose equalization. This was an unexpected finding, as a longer half-life of elimination and thus metabolic constraints was a distinct feature in the vulture. This more than any other finding, indicated how unpredictable toxicity between the different bird species could be.

4.5 Toxicity

The oral LD_{50} of diclofenac in bird species from this study were much higher than those previously demonstrated in *Gyps* vultures and the chicken. While the quail and duck had an estimated oral LD_{50} of 405.42mg/kg and 189.9mg/kg respectively, the pigeon like the crow and turkey vulture (*Cathartes aura*), were insensitive to diclofenac's ill effects (Naidoo *et al.,* 2011; Rattner *et al.,* 2008). This could be due to species peculiar metabolic abilities and/or susceptibility, which tends to suggest that diclofenac's toxicity may not be predictable from one avian species to another. The rapid ability of species used for this study to metabolize and eliminate diclofenac from their systems compared to *Gyps* vulture likely limited their exposure to the drug and increased their chances of survival. This would explain the wide variation in lethal dose between *Gyps* vulture and the other species. A comparison of diclofenac's LD_{50} across these species of bird that demonstrated toxicity showed no direct relationship between toxicity and body mass i.e. *Gyps* vultures (4.5kg) 0.098mg/kg < chicken (1.8kg) 9.8mg/kg < Muscovy-duck (1.8kg) 189.9mg/kg < Japanese-quail (0.3kg) 405.42mg/kg (Swan *et al.,* 2006b;

Naidoo *et al.,* 2007; Present study). The unexpected result was the high LD_{50} in the ducks, which had an LD_{50} that was almost 20-fold higher than the chicken which is of a similar weight to the duck. It is not exactly clear why this was the case. More so, the toxicity in the ducks did not appear to be from any metabolic constraints as the birds that died did not show major differences in metabolism of the drug i.e. the zero-order kinetics and change in half-life seen between surviving and dead/sick vultures exposed to ketoprofen and carprofen (Naidoo *et al.,* 2010a; Fourie *et al.,* 2015) was not evident. This suggests that toxicity in birds may be more mechanistic in that a specific physiological pathway is being inhibited, which may be dependent on biphasic concentrations as opposed to only metabolic capacity.

5 Conclusion

The outcome from this study suggests that bird species used, have very high metabolic capacity with wide variations. With the observed lethal doses being greater than what has been established in *Gyps* vultures, it is safe to conclude that the use of these bird species as models would not have been predictive of diclofenac's devastating effects on *Gyps* vultures in a preemptive manner. The similarities in the clinical manifestations and pathology suggest a common mechanism of toxicity which thus highlights the possibility of using these models in better understanding the pathophysiology behind diclofenac-induced toxicity in vultures.

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Figure captions

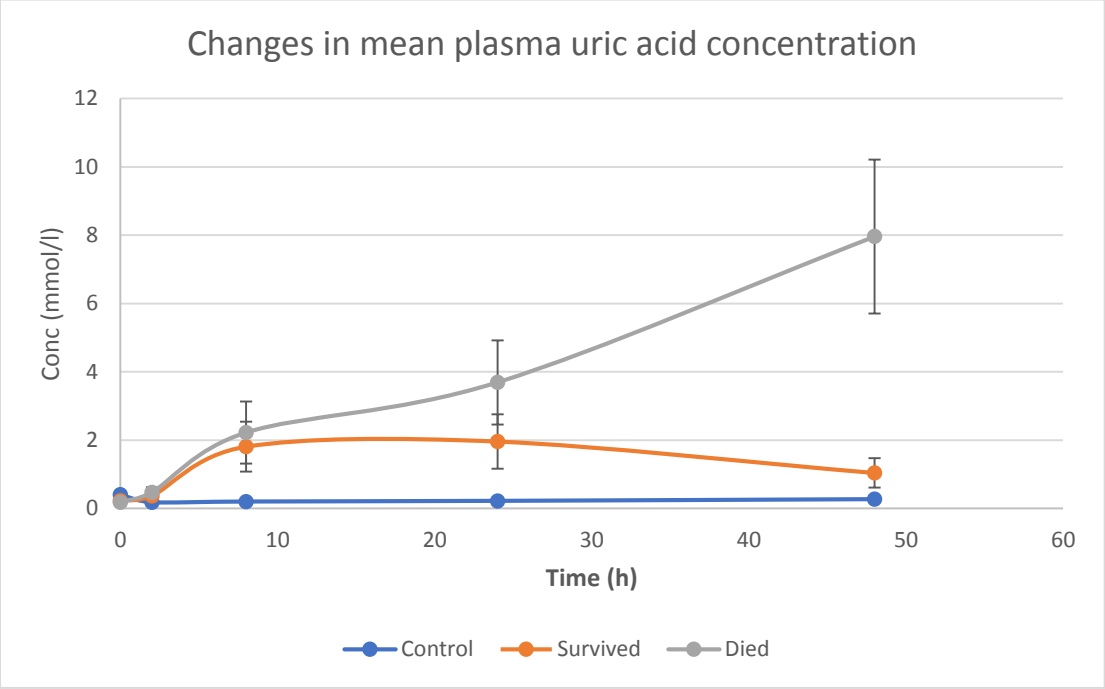

Figure 1. Change in plasma uric acid concentration in Muscovy ducks. The results are pooled for the animals that died (n=5), survived (n=9) and for the control group (n=5). The birds that died all showed a trend for an increase in uric acid concentration from 24h on non-parametric ANOVA (p= 0.34 and 0.14). The concentrations for the birds that died at the 24h and 48h time point were significantly different to the control on post-hoc evaluation (p= 0.016).

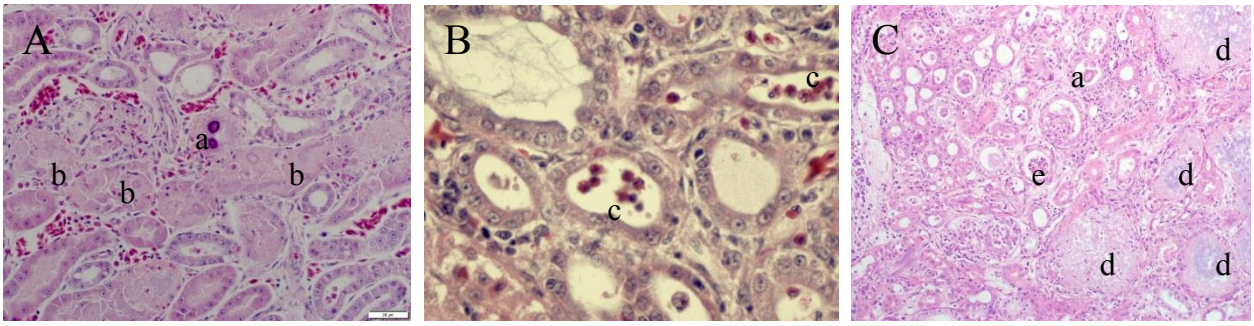

Figure 2. (A)Kidney HE x 20 (B) Kidney HE x 40 (C) Kidney HE x 10 from dosed birds that succumbed. Globoid shaped urate crystal within the renal tubule (a), loss of renal architecture with swollen tubules associated with eosinophilic fragmented cytoplasm and nuclear debris (b), heterophils within the renal tubules (c), obliteration of the Bowman's capsule associated with deposit of urate crystals (d), with cellular cast within the collecting duct (e).

Table

Table 1: Doses used for the determination of the LD_{50} of diclofenac in the three study species

Species	Estimated LD_{50}	Stage 1 doses	Stage one working LD_{50}	Stage 2 doses
Japanese quail	50 mg/kg	7, 26, 96 and 354 mg/kg	184.35 mg/kg	63.2, 80.1 ,102, 129, 164, 208, 263, 334, 424 and 538 mg/kg
Muscovy duck	9.8 mg/kg	1.39, 5.11, 18.8 and 69.3 mg/kg	133.02 mg/kg	45.6, 57.8, 73.4, 93.1, 118, 150, 190, 241, 306 and 388 mg/kg
Domestic pigeon	15.6 mg/kg	2.21, 8.13, 29.9 and 110 mg/kg	211.14 mg/kg	72.3, 91.8, 116, 148, 187, 238, 302, 383, 486 and 616 mg/kg

LD_{50}: Median lethal dose

Table 2. Average equalized pharmacokinetic parameters in bird species following exposure to diclofenac

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

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