CHAPTER 5: Validating the domestic fowl as a model to investigate the pathophysiology of diclofenac in Gyps vultures

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VALIDATING THE DOMESTIC FOWL AS A MODEL TO INVESTIGATE THE PATHOPHYSIOLOGY OF DICLOFENAC IN GYPS VULTURES

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5.1 Abstract

Diclofenac has recently been identified as a cause of the widespread vulture decline on the Indian subcontinent. Although the clinical signs and pathology have been described, the pathophysiology of toxicity remains unexplained. In the following study we attempt to validate the domestic fowl as a model, to allow for the further characterisation of diclofenac's mechanism of toxicity. In a lethal dose study, diclofenac was shown to have an approximate intramuscular LD_{50} of 9.8 mg/kg in 18 week old layers. Signs of toxicity in the affected birds were severe depression that persisted from 24 hours post dosing to death with corresponding increased plasma uric acid concentrations. Post-mortem examinations showed signs of gout with deposits of urates (tophi) in the kidneys, liver, heart and spleen. The pharmacokinetics after both the intramuscular and oral route showed that diclofenac had a short half-life of elimination of approximately one hour, a volume of distribution of 0.09 to 0.24 L/kg and relative oral bioavailability of 50% compared to intramuscular administration. With the similarity in the clinical signs, necropsy findings, histopatological lesions and clinical pathological changes, the fowl may be used in further studies to characterise the mechanism of toxicity of diclofenac. However, due to the large difference in susceptibility of the fowl, it is not a suitable model to simulate the dose-response relationship of the vulture to the other non-steroidal anti-inflammatory drugs.

Key Words: Vulture, *Gyps africanus*, Diclofenac, Pharmacokinetics, LD_{50}, hyperkalaemia

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5.2 Introduction

Three species of vultures endemic to South Asia are in grave danger of extinction across the Indian subcontinent. Populations of the Oriental white-backed vulture (*Gyps bengalensis*), long-billed vulture (*G. indicus*) and slender-billed vulture (*G. tenuirostris*) have declined by more than 97% in India and Pakistan, with the result that all three species have now been classified by the IUCN, The World Conservation Union, as Critically Endangered (Prakash *et al.*., 2003). Although the devastation was first noticed in the late 1990s, it was not until 2004 that Oaks *et al.*. linked the vulture deaths to the veterinary use of diclofenac, a non-steroidal anti-inflammatory drug (NSAID) through drug exposure in their food source (Oaks *et al.*., 2004). This toxicity has since been reproduced under controlled experimental conditions in both captive Oriental and African white-backed vultures (Oaks *et al.*., 2004; Swan *et al.*., 2006b).

When exposed to diclofenac, the vultures all demonstrated similar clinical signs. The birds became depressed at approximately 24h after exposure, and became progressively more depressed until they succumbed 36 to 48h post-exposure (Swan *et al.*., 2006b). Similar signs have also been described in the field where sick birds were observed to be very depressed in their nests and "literally fall over dead" (Prakash *et al.*., 2003). The post mortem examinations were also very similar in both the tested and naturally poisoned birds with signs of a severe diffuse visceral gout and concurrent severe bilateral nephrosis being present (Meteyer *et al.*., 2006).

Although the cause of toxicity has been clearly linked to the veterinary use of diclofenac the mechanism of drug toxicity remains unclear (Meteyer *et al.*., 2005). At present numerous mechanisms of toxicity have been described for diclofenac in mammals and range from simple COX mediated ischemic necrosis to caspase induced liver failure. Although the toxicity seen may be as a result of inhibition of the COX enzyme, meloxicam, also a NSAID, showed no ill effects in the *G. africanus, G. bengalensis and G indicus* vultures when administered as either a formulated drug or as residues in meat from treated cattle (Swan *et al.*., 2006a). In people

meloxicam and diclofenac are both considered more cyclo-oxygenase (COX) II selective in their mechanism (Burke *et al.*., 2005). Pharmaceutically the only difference between the two molecules is their actual structure which tends to suggest that toxicity may be related to receptor binding affinity or perhaps even the plasma pharmacokinetics.

The aim of this study was to validate the fowl as a model for the study of diclofenac-induced intoxication in vultures to allow for future evaluations of the mechanism of toxicity. In addition to comparing clinical signs of toxicity, the pharmacokinetics, approximate lethal dose and changes in blood chemistry were compared between the two species.

5.3 Material and Methods

5.3.1 *Animals*

5.3.1.1 Husbandry

Leghorn layers were selected for this study because of easy availability. The layers were reared by a commercial rearer until 18 weeks of age after which they were allowed a week of acclimatisation, prior to commencing the study. They were housed in poultry houses of the Poultry Reference Laboratory, at the Faculty of Veterinary Science (Onderstepoort, Pretoria) and fed a commercial layer diet with water available *ad libitum*. Housing involved natural ventilation and an artificial light source of 18 hours. For the first two toxicity studies the fowls were housed in individual pens and wire cages, respectively. For the pharmacokinetic studies they were housed in layer cages in groups of three. For all studies the individual fowls were marked with an easily identifiable ring band with a unique colour code.

5.3.1.2 Intramuscular toxicity study

Thirty layers at the beginning of their production cycle were injected intramuscularly in the pectoral muscles. The layers were divided into groups (n=6) and were administered incremental doses of 0.6, 1.25, 2.5, 5 and 10 mg/kg. Dosing was staggered across 5 weeks

with one group treated per week. A non-treated control group was included for each dose level.

5.3.1.3 Pharmacokinetic study

Eighteen layers were used in a pharmacokinetic study and were initially dosed by the oral route, then the intramuscular route after a washout period of two weeks. Prior to the intramuscular study the animals were deemed clinically healthy and had normal serum uric acid concentrations. Diclofenac was administered at a dose of 0.8 mg/kg, the dose reported to kill African white-back vultures at probability of > 80% (Swan *et al.*., 2006b). According to our probit analysis of the toxicity data, the dose had a less than 10% possibility of inducing toxicity in fowls.

5.3.1.4 Blood collection

Blood was collected from the wing vein using a 21G needle and 2.5 ml syringe (Braun). Collected samples were immediately transferred into either plain or heparinised vacutainer tubes for serum chemistry analysis and diclofenac quantification, respectively. For the pilot and intramuscular toxicity study, samples for clinical pathology and diclofenac plasma concentrations were collected prior to dosing, 24 hours and at 48 hours post-dosing or death.

5.3.2 *Clinical Pathology*

The electrolytes Na^+, K^+ and blood pH were measured with a blood gas analyser (Rapidlab 34E Chiron diagnostics, Bayer SA). Uric acid and calcium (Ca^{2+}) were measured with a Nexet Chemistry Analyser (Alfa Klasserman, Bayer SA).

Serosal deposits of the white amorphous material were collected and tested for the presence of uric acid using the murexide reaction (Lumeij, 1994). For this reaction egg albumin (sulphur containing proteins) was used as a negative control (yellow) and urine urate deposits as a positive control (mauve).

The total accumulative increase in serum uric acid was calculated by plotting serum uric acid concentrations against time. The area under the curve was determined using the trapezoidal method (Toutain *et al.*., 2004).

5.3.3 *Pathology and Histopathology*

Lesions seen at necropsy were recorded. Tissue samples collected for residue analysis (liver, kidney, heart, brain, muscle) were frozen or preserved in buffered formalin for histopathology (liver, kidney, heart, spleen, shell gland, lungs). Samples collected in formalin were trimmed, embedded in paraffin, sectioned and stained with hematoxylin and eosin using standard methods.

5.3.4 *Residue Analysis*

Tissues harvested from the dead birds dosed at 10 mg/kg were analysed for the concentration of the diclofenac parent molecule in the tissues. Organs were stored at -30°C until analysed. Half a gram of defrosted tissue was homogenised, using a scalpel blade, and subsequently extracted with 2 ml acetonitrile, using a multitube vortex for ten minutes (Swan *et al.*., 2006a). The mixed samples were centrifuged at 4500 x G for 10 minutes, the supernatant was decanted, dried under a steady stream of nitrogen in a water bath at 50°C and stored at -25°C until analysis by high performance liquid chromatography (HPLC).

5.3.5 *Pharmacokinetic Analysis*

5.3.5.1 Model Parameters

For this study the third group of fowls were bled using a dose pooling method, where three birds were grouped to provide a single complete pharmacokinetic profile (Animal unit)(Ette *et al.*., 2004a; Ette *et al.*., 2004b). Each bird assigned to the animal unit was treated with drug at 0h and thereafter bled in rotation. A total of six profiles were used (18 birds). Blood samples were collected at 30, 45 minutes and at 1, 2, 3, 4, 5, 7, 9, 12 and 24 hrs for each profile. An

additional 5 minute bleed was included for the intramuscular profile. The same group of fowls were used for both profiles to allow intra and inter-group variations to be carried across both profiles. The birds were allowed a two week acclimatization period prior to conducting the oral study. A two week washout period was allowed prior to characterising the intramuscular profile. Compartmental pharmacokinetic analysis was undertaken using WinNonLin Ver 4.2 (sponsored by the PharSight Corporation.)

5.3.5.2 Sample preparation

Blood samples were centrifuged at 2800 x G for 15 minutes within 1 hour of sample collection. Sample volumes of 1.5 ml plasma were pipetted into 10ml tubes; 3ml diethyl ether was added to each tube, followed by 3ml of 0.3 M potassium dihydrogen phosphate, pH 3.5. The tubes were capped and the contents mixed for two minutes on a multi-tube vortex mixer, and centrifuged for 10 minutes at 2800 x G. The test tubes were inserted in an ice bath (methanol/solid carbon dioxide) to solidify the aqueous phase, and the organic layers decanted into 10 ml glass tubes. The organic layer was evaporated to dryness for 30 minutes at 50 ºC under a mild flow of nitrogen. The residue was dissolved in 400 µl mobile phase and transferred to the inserts, fitted in the vials, and the caps screwed on (Odensvi *et al.*., 1995).

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5.3.5.3 High performance liquid chromatography

A Beckman System Gold HPLC consisting of an autosampler module 507, programmable solvent module 126, diode array detector module (DAD) 168, and 32 Karat™ software package, was used (Beckman Instruments, Fullerton, California, USA). Separation was achieved with a Synergi Max-RP column (80A, 150 x 4.6 mm, 4; Phenomenex, Torrance, California, USA). The mobile phase consisted of 0.05M sodium dihydrogen phosphate (pH=4.85 to 4.89):CH_3CN, 42.5:57.5. 100 µl of the reconstituted samples were injected onto the HPLC column at 1 ml/min in an isocratic run. Detection of diclofenac and flunixin (internal standard) was carried out at 275 nm. The total runtime per sample was 8 minutes with retention times as follows: flunixin at 3.9 min, diclofenac at 4.9 min. Control values showed a mean % CV of 0.156 and regression coefficients greater than 0.99 for each

analytical run. The LLQ was established at 0.1µg/ml and a linear relationship between concentration and peak area was demonstrated for the total concentration range between 0.1 and 20µg/ml (Odensvi and Johansson, 1995).

5.3.6 *G. africanus results*

Stored serum samples collected during the diclofenac toxicity study were analysed for the concentration of potassium using the same method described above (refer to 5.3.2). Diclofenac plasma concentrations were re-analysed using the same pharmacokinetic model described above (Swan *et al.*., 2006b). To obtain a complete profile, the Cmax was set to 5 hours as this was the maximum concentration observed in each bird.

5.4 Results

5.4.1 *Clinical Signs*

Thirty birds in groups of six were exposed to five different doses of diclofenac. In total 9 birds died from severe toxicity while the tenth bird, at the highest dose, showed signs of toxicity but eventually recovered. None of the control birds died. Two birds died in each of the 1.25, 2.5 and 5 mg/kg group with the three remaining birds dying in the 10 mg/kg group. The sick birds (n=10) were reluctant to move, perched in one corner of their cages, appeared to be asleep and had stopped eating (i.e. were severely depressed). The birds initially responded to external stimuli such as touch and noise by “waking up” but within a minute became depressed once again. This depression on average occurred at 24h post-dosing and progressively became more severe. Prior to death, the affected birds appeared to be comatose and could not be roused. In the birds that were producing eggs, at 24 hours the eggs were malformed and very thin shelled. Although the shell weight had decreased, the ratio of shell weight:thickness remained constant.

5.4.2 *Clinical Pathology*

Uric acid, Ca^{2+}, Na^{+} and K^{+} were within the normal range at 24h for that published for the fowl (Ross *et al.*., 1978). After 24h, only plasma uric acid concentrations were extremely high (7.662 ± 2.54 µg/ml) for the 9 birds that eventually died. For the period from dosing to mortality the dead birds exhibited concentrations of uric acid of 234 ± 34 h*µg/ml. The sick bird that recovered was exhibited 157 h*µg/ml uric acid. The blood pH at 24 h was 7.44 but decreased to ± 6.7 at death. The drop in pH had a corresponding increase of plasma potassium exceeding 15mmol/l (25.3 ± 10 mmol/l).

5.4.3 *Necropsy*

Severe visceral gout and severe diffuse nephrosis was evident in only the birds that died. The birds in the 5 mg/kg and 10 mg/kg dose group also had signs of articular deposition of a white amorphous substance. In the birds necropsied within minutes of dying, the diffuse white substance within the pericardium was in a liquid form and solidified as the carcass cooled to room temperature. Samples of the white material on the viscera were analysed for the presence of uric acid. For the majority of samples collected, a reaction was achieved for the presence of sulphur containing protein and not uric acid. Only one sample analysed from the highest dose group eventually tested positive for uric acid. The specific sample was from the pericardial effusion. When this sample was allowed to cool, it finally crystallised and supported the histopathological diagnosis of urate tophi. No signs of pathology were evident in the healthy birds euthanized five days after dosing.

5.4.4 *Histopathology*

The cortical architecture of the kidney showed marked disruption due to the presence of tophi containing both spiculate as well as globloid urate forms which caused both cell necrosis and tubule loss. In most cases massive heterophil infiltration into the interstitium of the cortex as well into the tubule and collecting ducts was present.

Renal necrosis was, however, most severe in the birds that had died in the 10 mg/kg group and was characterized by massive urate crystal precipitation with necrosis of the adjacent structures. In most cases, although the outline of the tubules was still apparent, most of the tubular structure was lost due to the presence of large aggregates of urates (Figure 5-1). In two of the birds the tophi had multinucleate giant cells at their periphery. Some areas of the interstitium were associated with macrophage and lymphocyte infiltrate and possible early fibroblast proliferation.

The liver was also severely affected, and was characterized by small tophi associated with hepatocyte necrosis. In most cases the tophi were associated with an inflammatory reaction of both heterophils and round cells. In some cases the tophi consisted of central aggregates of urate spicules with minimal infiltration of inflammatory cells at the periphery.

The shell gland had scattered tophi present within the gland resulting in local necrosis of the glands as well as the overlying epithelium. The heart, lungs and spleen contained scattered tophi within the parenchyma which was associated with mild to more extensive cell necrosis. At times aggregates consisted of only urate deposits which had not yet become tophi.

The diclofenac plasma concentrations measured in the dosed fowls were very variable. Only seven fowls, from the 30 treated, had detectable drug concentrations at 24 h post dosing, with a mean of 0.20 ± 0.05µg/ml. Only one fowl (5 mg/kg group) had detectable drug concentration at 48 h which translate to a half-life of 14 h. None of the other animals, sick or healthy, had any detectable drug at the last sampling point indicating a half-life under 5h (± 5 half-lives).

There was no relationship between animals demonstrating plasma concentrations at 24h and the occurrence of toxicity. Only two of the dead animals had detectable tissue drug concentrations in either the kidney or liver. The specific tissue partition co-efficient could not be determined due to the absence of a relationship with diclofenac plasma tissue concentrations.

5.4.5 *Pharmacokinetic (PK) Analysis*

All six animal units were best analysed using a one compartmental open model. The PK parameters obtained is listed in the Table 5-1 for both routes and the average plasma profile in Figure 5-2. The drug showed a relatively short half-life of elimination of 0.6 ± 0.19 h following intramuscular administration and 0.89 ± 0.36 h following oral administration. None of the animals in this phase of the study showed any signs of toxicity.

5.5 Discussion

5.5.1 *Comparison Between Fowls and G. Africanus Vultures*

The clinical signs and increase in uric acid concentrations evident in the fowls succumbing to toxicity was similar to the signs reported by Swan *et al.*. (2006b) for *G. africanus*. Similarity in total uric acid exposure between fowls that had died and those of the vultures (calculated from the results presented by Swan *et al.*. 2006b) were observed. The total uric acid exposure for vultures was calculated as 219 ± 1 mmol/L/h and suggests that the extent of uric acid exposure is directly related to mortality (Figure 5-3).

A terminal increase in potassium concentrations evident in the dead fowl's plasma prompted re-analysis of stored samples from the *G. africanus* vultures for comparison (Swan *et al.*., 2006b). A corresponding increase in the plasma potassium concentrations was present in vultures. In the past it has been suggested that a terminal increase in potassium was the cause of death in gouty fowls (Lumeij, 1994). This study therefore supports this supposition that the

cause of death was potassium related. Hyperkalaemia is a known cause of bradycardia in birds (Zandvliet, 2005).

On comparison the pathological changes observed in the Leghorn layers following diclofenac exposure were similar to those reported in the Gyps species, i.e. diffuse visceral gout to severe nephrosis (Oaks *et al.*. 2004, Meteyer *et al.*. 2005, Swan *et al.*. 2006b). Differences were, however, present on histopathological examinations. The fowl kidneys and other organs in the layers appeared to be more severely affected than in the vulture. In addition the tophi in the layers were associated with a fairly marked cell infiltration. In vultures, minimal (or more usually) no cellular response was evident. This tends to suggest that the period of time from tophi formation to death was more prolonged in the layers providing more opportunity to launch a cellular response. However, the average time to death between vultures and layers were very similar (42 hours in vultures and 44.5 ± 6.6 hours in fowls).

This toxicity study confirmed the greater tolerance of fowls to diclofenac poisoning compared to Gyps vultures. A probit analysis (SPSS 13, SPSS Inc.) of the toxicity data showed a LD_{50} of 9.8 mg/kg for the intramuscular dose, which is markedly higher that the oral LD_{50} calculated for *G. bengalensis* of 0.098 to 0.225 mg/kg (95% CI 0.027 – 0.351 mg/kg)(Swan *et al.*., 2006b). Although the routes of exposure were different, the pharmacokinetic data generated allows for a better comparison. The bioavailability of diclofenac in fowls following oral administration was 50% compared to the intramuscular route with a relative bioavailability of 50%. This indicates that the oral LD_{50} in the layers could be as high as 19.6 mg/kg on the assumption that the dose-response relationship is linear. The intramuscular pharmacokinetic data does, however, have to be interpreted with care, as the same fowls were used for both phases of the study. As such it is possible that mild, clinically overt, cellular toxicological changes induced by the oral dosing may have been carried through to the intramuscular phase.

Although only an incomplete diclofenac plasma concentration versus time profile is available for diclofenac in vultures, the two *G. africanus* vultures dosed at 0.8 mg/kg still had a five fold higher plasma concentration at 5 hours than fowls. The natural logarithmic (Ln) transformed data (Figure 5-4) generated by Swan *et al.* (2006b), suggests that the time to maximum

concentration (Tmax) for vultures is approximately 5 hours as the graph is no longer linear at this point. By assuming that 5 hours is the Tmax we estimate an AUC of 34 and 133ugml/h for vultures 1 and 2, respectively (Table 5-2). When comparing the overall extent of absorption (AUC), at 0.8 mg/kg, the *G. africanus* vultures had a greater overall exposure than any of the fowls treated, even though the vultures were treated orally and the fowls intramuscularly (Swan *et al.*, 2006b). With the AUC being a measure of the extent of absorption, it clearly evident that diclofenac is absorbed to a greater degree in the vulture than the fowl.

The other major pharmacokinetic difference observed was in the biological half-life of diclofenac between fowls and vultures. Biological half-lives of 14 and 18 hours were reported for the two vultures (Table 5-2)(data from Swan *et al.*, 2006b). This was markedly different to fowls which had a half-life of approximately 1h. These differences were also reflected in the tissue concentrations of diclofenac, which was present in all the major organs (liver, kidney, heart, brain and muscle) of the orally dosed *G. africanus* vultures and almost completely below the level of quantification in the intramuscularly dosed fowls, even at the maximum dose. The volume of distribution was much smaller in vultures than for fowls, while the fowls had a volume of distribution more typical of that described in mammals (Busch *et al.*., 1998). Clearance was similarly lower in vultures than for fowls. This possibly suggests a larger degree of plasma protein binding in the vulture with a higher concentration of drug remaining in the plasma.

When comparing intestinal absorption, the absorption half-life appeared to be much longer in the vultures than in fowls, and is similar to the elimination half-life found in fowls. It may therefore be plausible that flip-flop kinetics occurs in vultures with diclofenac being slowly released from the crop over time. This hypothesis is supported by results from another pharmacokinetic study in fowls that showed the absorption of ibuprofen was delayed when the drug was deposited into the crop instead of the proventriculus (Vermeulen *et al.*., 2001).

5.5.2 *Comparison of Results to That Presented by Reddy et al. (2006)*

The 33% mortality seen at the 5mg/kg dose was similar to 40% described by Reddy *et al.*.(2006) However, the necropsy and histological findings are very different to that discussed in their report. In contrast we found that toxicological changes in the fowls were in-fact similar to those for the vultures as discussed above. Reddy *et al.*, also discussed the changes as being renal necrosis accompanied by tubular fibrosis. The presence of fibrosis indicates a more long-standing condition which is probably due to the long survival time experienced. The lack of renal fibrosis in our study is more in line with the findings by Oaks *et al.*. (2004) in which no signs of fibrosis or chronic changes were found. Further studies will be needed to investigate why the pathology of the acute deaths differed to such an extent between the trials.

5.5.3 *Suggestive Pathophysiology of the Clinical Signs*

The clinical signs seen in all the birds may be explained by the changes in the clinical pathology. The metabolic acidosis (pH of 6.5) can be linked to an increase in plasma uric acid levels and may be further exacerbated by the inability of the damaged proximal convoluted tubule to conserve bicarbonate (Seifter, 2004). At 24 hours this is countered by the natural buffering systems as seen with the normal blood pH (Campbell, 2004). The pH of 6.5 at the point of death indicates a progressive drop in the blood pH from 24 hours to the time of death.

Acidosis also explains the progressive increase in CNS depression in that depression corresponded to the initial increase in uric acid and progressively became more severe until death. The clinical signs of acidosis in cattle provides further credence to this theory as one of the major signs seen in these animals is initial depression which progressively becomes more severe until the animal dies (Blood *et al.*., 1989). Similarly severe plasma acidosis is characterised by depression in people (Seifter, 2004).

The actual cause of death in the birds is once again most likely attributable to the terminal hyperkalaemia. The massive potassium increase would lead to cardiac failure and death as

potassium is very finely controlled in all animals. An increase in potassium will lead to an abnormal cardiac rhythm by altering the T-wave and at high levels will lead to fatal cardiac arrhythmias (Sturkie, 1986; Zandvliet, 2005).

5.6 Conclusion

The clinical signs of severe depression and death in the fowls and *G. africanus* vultures appeared to be directly related to the increase in uric acid in the plasma and possibly to hyperkalaemia. With the cause of death being clearer, it may be possible to treat affected birds. From the one fowl that recovered, we know that diclofenac toxicity is not uniformly lethal once clinical signs develop. In addition to gavaging large quantities of water to flush out the effected kidneys, other treatments may be equally as effective. At this stage we would suggest an immediate correction of the acid base status of the animal. If stabilised it should prevent the terminal increase in potassium while treatment with a hydrochlorthiazide diuretic may also enhance the excretion of potassium in the urine thereby preventing the lethal hyperkalaemia (Sturkie, 1986) assuming that the potassium exchange in the distal tubules is still functional.

With dose being the only major difference in the toxicity of the compound, it is proposed that fowls could be used as a model for further studies into the pathophysiology of diclofenac induced intoxication in vultures. The difference in dose does, however, imply that fowls are not suitable as a model to determine the susceptibility of the vulture to other veterinary NSAIDs. With the present study evaluating only the acute toxic effect of diclofenac in fowls it will be of benefit to study the toxic effects of diclofenac at reduced doses following chronic exposure.

5.7 Acknowledgements

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5.8 References

1. Blood, D.C., and Radostits, O.M., 1989. *Veterinary Medicine. A textbook of diseases of cattle, sheep, pigs, goats and horses*. Bailliere Tindall, London.

2. Burke, A., Smyth, E.M., and Fitzgerald, G.A., 2005. Analgesic and Antipyretic Agents; Pharmacotherapy of Gout. In Brunton, L., Lazo, J., Parker, K., Buxton, I. and Blumenthal, D., (Eds.), Goodman & Gilman's The Pharmaceutical Basis of Therapeutics, McGraw Hill, United States of America, pp. 671-716

3. Busch, U., Schmid, J., Heinzel, G., Schmaus, H., Baierl, J., Huber, C., and Roth, W. (1998). Pharmacokinetics of meloxicam in animals and the relevance to humans. Drug Metab. Dispos. 26, 576-84.

4. Campbell, T.W., 2004. Blood chemistry of lower verterbrates. 55'th Annual meeting of the Ameican College of Veterinary Pathologists (ACVP) & 39'th Annual meeting of the American Society of Clinical Pathologists (ASVCP) Middleton, USA

5. Ette, E.I., and Williams, P.J., 2004a. Population Pharmacokinetics I: Background, Concepts, and Models. Ann. Pharmacother. 38, 1702-1707.

6. Ette, E.I., and Williams, P.J., 2004b. Population Pharmacokinetics II: Estimation Methods. Ann. Pharmacother. 38, 1907-1915.

7. Lumeij, J.T., 1994. Nephrology. In Ritchie, B.W., Harrison, G.J., and Harrison, L.R. (Eds.), Avian medicine: Principles and application. Wingers, Florida. pp. 538-555.

8. Meteyer, C.U, Rideout, B.A, Gilbert, M, Shivaprasad, H.L, and Oaks, J.L., 2005. Pathology and proposed pathophysiology of diclofenac poisoning in free-living and experimentally exposed oriental white-backed vultures (*Gyps bengalensis*). J. Wildl. Dis. 41, 707-16 .

9. Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., and Meteyer, C.U., 2004. Diclofenac residues as the cause of vulture population declines in Pakistan. Nature. 427, 630-633.

10. Odensvi, K., and Johansson, I.M., 1995. High-performance liquid chromatography method for determination of flunixin in bovine plasma and pharmacokinetics after single and repeated doses of the drug. Am. J. Vet. Res. 56, 489-495.

11. Prakash, V., Pain, D.J, Cunningham, A.A., Donald, P.F., Prakash, N., 2003. Catastrophic collapse of Indian white-backed Gyps bengalensis and longbilled Gyps indicus vulture populations. Bio. Con. 109, 381-390. .

12. Ross, J.G., Christie, G., Halliday, W.G., and Jones, R.M., 1978. Haematological and blood chemistry "comparison values" for clinical pathology in poultry. Vet. Rec. 102, 29-31.

13. Reddy, N.C.P, Anjaneyulu, Y., Sivasankari, B., and Rao, K.A., 2006, Comparative toxicity studies in birds using nimesulide and diclofenac sodium. Environ Toxicol Pharmacol. 22, 142-147

14. Seifter, J.L., 2004. Acid-Base disorders. In Arend, W.P., Drazen, J.H., Ill, G.N., Riggs, R.C., Owell, D.W., and Cheld, W.M. (Eds.), Cecil Textbook of Medicine. W. B. Saunders Company, Philadelphia. pp. 688-695.

15. Sturkie, P.D., 1986. Avian Physiology. Springer Verlag, New York.

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

17. Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A., Duncan, N., Meharg, A., Oaks, J.L., Parry-Jones, J., Schultz, S., Taggart, M. A., Verdoorn, G.H., and Wolter, K. (2006b). Toxicity of diclofenac in Gyps vultures. Biol. Lett. 2, 1-4.

18. Toutain, P.L., and Bousquet-Mlou, A., 2004. Bioavailability and its assessment. J. Vet. Pharmacol. Ther. 27, 455-466.

19. Vermeulen, B., and Remon, J.P., 2001. The oral bioavailability of ibuprofen enantiomers in broiler fowls. J. Vet. Pharmacol. Ther. 24, 105-109.

20. Zandvliet, M.M., 2005. Electrocardiography in psittacine birds and ferrets. Seminars in Avian and Exotic Pet Medicine. 14, 34-51.

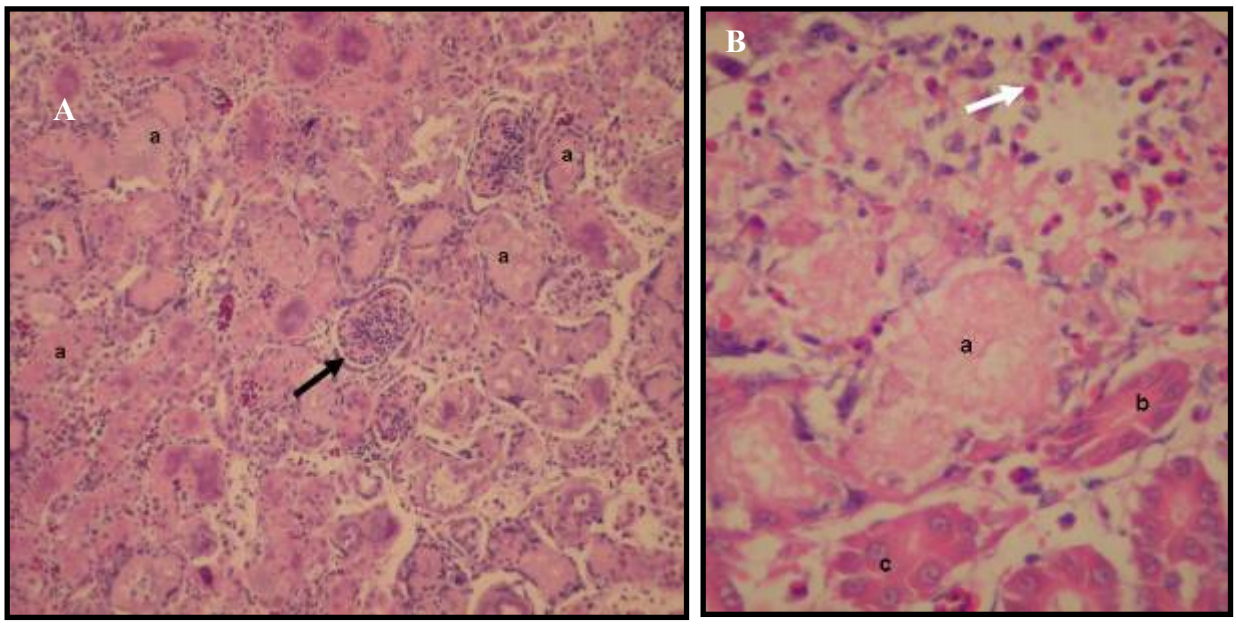

Figure 5-1: Kidney. A: HE x100 B: HE x 300 from a fowl dosed at 10 mg/kg. Marked tubular damage with complete destruction of the tubular structure (a), tubule with minimal damage (b) and normal tubule (c). Heterophil infiltration varied (white arrow). Unaffected mammalian glomerulus (black arrow).

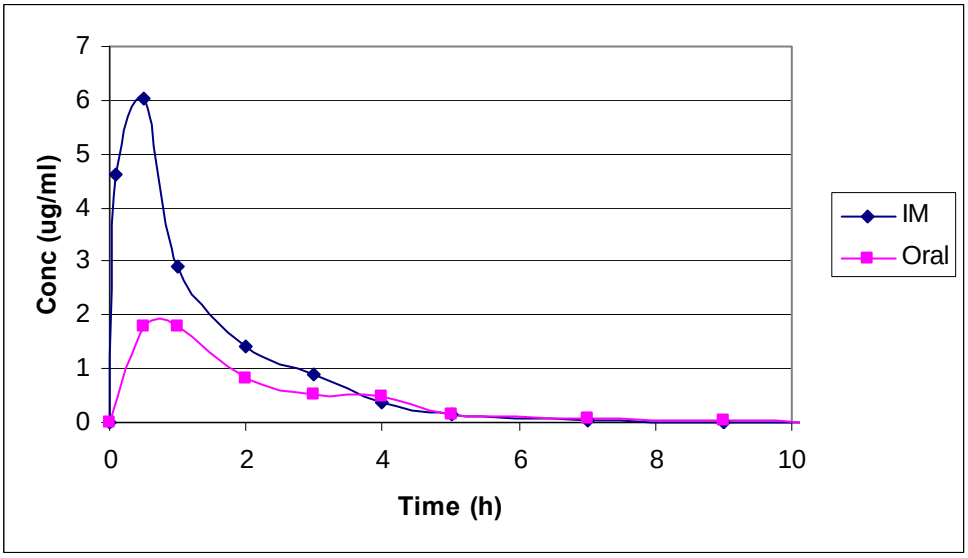

Figure 5-2: Mean diclofenac plasma concentration versus time profile for the oral and intramuscular route of administration

Table 5-1: Pharmacokinetic parameters following oral and intramuscular administration of diclofenac at 0.8mg/kg to fowls

Units		Intramuscular			Per Os		
		Mean	**SD**	**%CV**	**Mean**	**SD**	**%CV**
AUC	h*µg/ml	8.51	1.98	23.22	4.33	0.74	17.07
Cl/F	ml/h/kg	98.42	23.21	23.58	189.45	33.16	17.5
Cmax	µg/ml	6.79	0.87	12.78	2.11	0.63	29.98
A	1/hr	9.71	3.12	32.12	4.37	3.93	89.84
Alpha-HL	h	0.08	0.02	27	0.31	0.23	74.31
B	1/h	1.15	0.42	36.91	0.87	0.28	32.57
Beta-HL	h	0.66	0.20	30.02	0.89	0.37	41.37
Tmax	h	0.26	0.04	16.33	0.63	0.28	44.24
Vd/F	L/kg	0.09	16.00	17.89	0.24	85.23	35.73

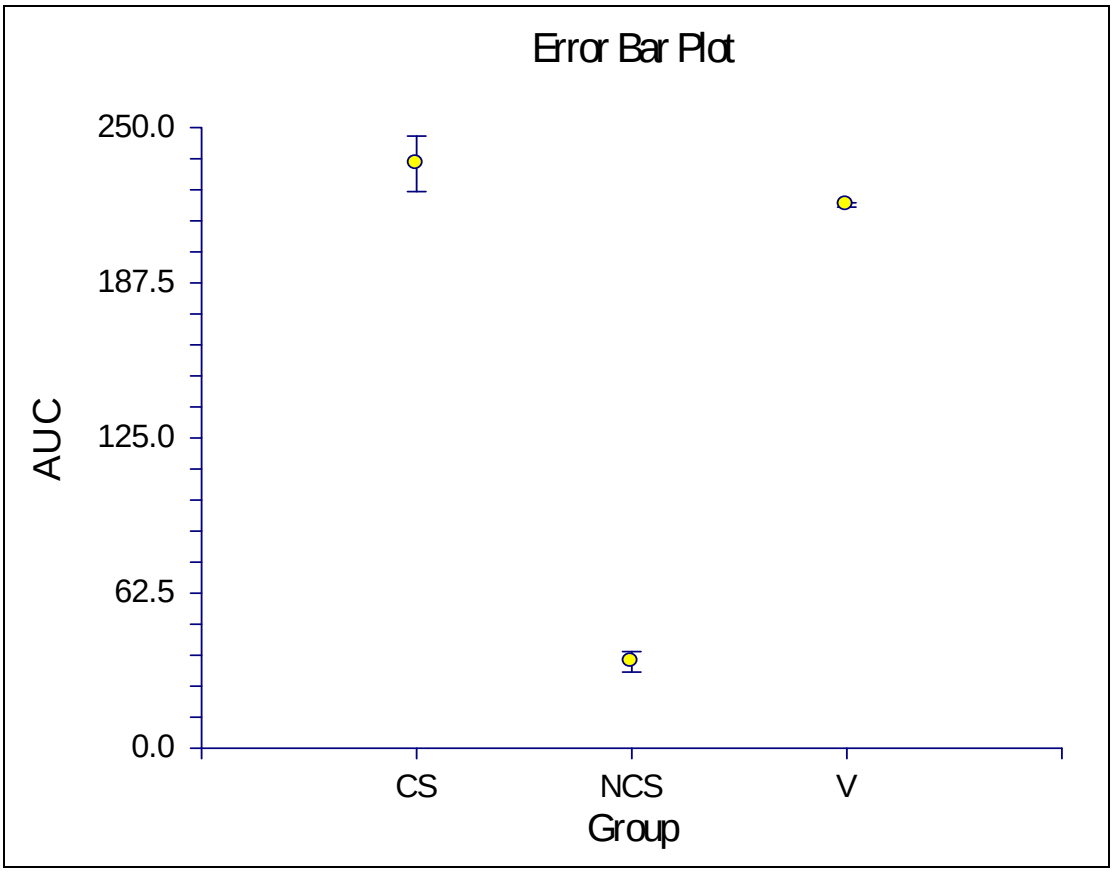

Figure 5-3: Mean uric acid levels for the bird that died (CS), treated birds that survived (NCS) and the two *G. africanus* vultures (V)

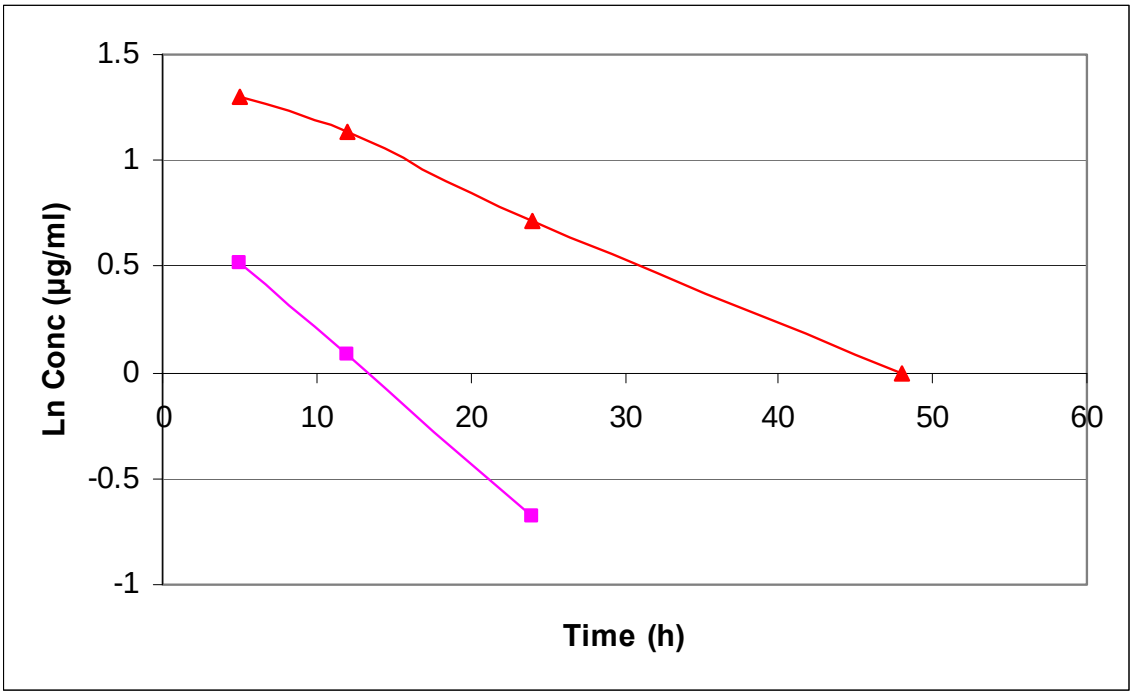

Figure 5-4: Semi-logarithmic plasma profiles for diclofenac from the two treated vultures (courtesy of Swan *et al.*, 2006)

Table 5-2: Estimated PK parameters of two dosed vultures. Values were calculated on the assumption of Cmax being achieved at 5h

Parameter	**Units**	**Vulture 1**	**Vulture 2**
AUC	ug/ml/h	34.09	133.44
Cl/F	ml/h	0.02	0.01
Cmax	ug/ml	1.76	3.68
K01	h^{-1}	0.85	0.63
K01-HL	h	0.81	1.11
K10	h^{-1}	0.06	0.03
K10-HL	h	10.90	21.39
Tmax	h	3.29	4.99
Vd/F	L/kg	0.0004	0.0002