The use of toxicokinetics and exposure studies to show that carprofen in cattle tissue could lead to secondary toxicity and death in wild vultures

Naidoo, V.; Taggart, M. A.; Duncan, N.; Wolter, K.; Chipangura, J.; Green, Rhys E.; Galligan, Toby H.

Published in:
Chemosphere

Publication date:
2018

The use of toxicokinetics and exposure studies to show that carprofen in cattle tissue could lead to secondary toxicity and death in wild vultures

V Naidoo[1,2]; M.A. Taggart[3]; N Duncan[1]; K Wolter[4]; J Chipangura[2]; R.E. Green[5, 6]; T.H. Galligan[6]

[1] Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria.
[2] Biomedical Research Centre, Faculty of Veterinary Science, University of Pretoria
[3] Environmental Research Institute, University of the Highlands and Islands, Castle St, Thurso, Scotland, KW17 7JD
[4] VulPro, Plot 121, Rietfontein, 0048, South Africa
[5] RSPB Centre for Conservation Science, David Attenborough Building, Cambridge, Cambridgeshire, CB2 3QZ, UK
[6] Conservation Science Group, Department of Zoology, University of Cambridge, David Attenborough Building, Cambridge, Cambridgeshire, CB2 3QZ, UK

Abstract

Veterinary medicines can be extremely damaging to the environment, as seen with the catastrophic declines in *Gyps* vulture in South Asia due to their secondary exposure to diclofenac in their primary food source. Not surprisingly, concern has been raised over other similar drugs. In this study, we evaluate the toxicity of carprofen to the *Gyps* vulture clade through plasma pharmacokinetics evaluations in *Bos taurus* cattle (their food source) and *Gyps africanus* (a validated model species); tissue residues in cattle; and the effect of carprofen as a secondary toxicant as both tissue-bound residue or pure drug at levels expected in cattle tissues. Carprofen residues were highest in cattle kidney (7.72 ± 2.38 mg/kg) and injection site muscle (289.05 ± 98.96 mg/kg of dimension of 5x5x5 cm). Vultures exposed to carprofen as residues in the kidney tissue or pure drug equivalents showed no toxic signs. When exposed to average injection site concentrations (64 mg/kg) one of two birds died with

evidence of severe renal and liver damage. Toxicokinetic analysis revealed a prolonged drug half-life of 37.75 h in the dead bird as opposed to 13.99 ± 5.61h from healthy birds dosed intravenously at 5mg/kg. While carprofen may generally be harmless to *Gyps* vultures, its high levels at the injection site in treated cattle can result in lethal exposure in foraging vultures, due to relative small area of tissue it is found therein. We thus suggest that carprofen not be used in domesticated ungulates in areas where carcasses are accessible or provided to vultures at supplementary feeding sites.

Introduction

The harm that veterinary medicines can cause to the environment, not only directly via spillage, runoff and poor disposal practice, but also indirectly via excreta and residues in animal tissue has only recently been acknowledged (Arnold et al., 2014, Margalida et al., 2014, Kuster and Adler, 2014). Of these pathways, residues of veterinary medicines in animal tissues have usually been considered only of importance to human health. However, veterinary medicine residues in animal tissues can be toxic to any species consuming them, particularly if that species is highly intolerant to the medicine concerned or is exposed to said medicine at high concentrations or for prolong periods.

One veterinary medicine that is known to harm wildlife is the non-steroidal anti-inflammatory drug (NSAID) diclofenac. Recognised as an environmental toxicant, diclofenac entering waterways can cause toxicity in fish (Schwaiger et al., 2004). However, the greatest impact diclofenac has had on wildlife is as the primary cause of population declines in several species of *Gyps* vulture resident to South Asia (Oaks et al. 2004, Green et al., 2004, 2007; Prakash et al., 2007). *Gyps* vultures are highly intolerant to diclofenac, showing acute renal failure and death at doses at or below 0.8 mg/kg body weight (Oaks et al., 2004, Swan et al., 2006). Vultures are exposed to diclofenac residues in tissues of livestock that have been treated shortly before death and then left for vultures to consume. The use of diclofenac in palliative care of cattle among Hindu communities and the fact that cattle constitute the primary source of food for vultures in South Asia, resulted in population declines in vultures of up to 99.9% (Prakash et al., 2007).

Experiments have shown that a second NSAID, ketoprofen, is also toxic to vultures (Naidoo et al., 2010a, Naidoo et al., 2010b), whereas a third NSAID, meloxicam, is not (Swan et al., 2006, Swarup et al., 2007). All three NSAIDs belong to different chemical

61 classes and the reason that two of these three are toxic to vultures remains unknown.
62 However, vulture intolerance may be linked to zero-order elimination (Naidoo et al., 2010b).
63 More importantly, with toxicity resulting from oral exposure, it is suspected that metabolic
64 capacity at the level of the pre-systemic metabolic system drives exposure and subsequent
65 toxicity. As a result, much concern has been raised over the large number of untested NSAID
66 compounds available for veterinary use in South Asia and other regions where *Gyps* vultures
67 range (i.e., Africa and Europe). One of these untested NSAIDs is carprofen, which is widely
68 used in veterinary medicine and is recommended for use in many taxa, including raptors
69 (Richman and Hayward, 2011).
70 A recent study exposed two Cape griffon (*Gyps coprotheres*) to carprofen by the oral
71 route at the recommended veterinary dose of 10 mg/kg body weight (Fourie et al., 2015).
72 While no overt toxicity was detected, the two vultures had substantially different
73 pharmacokinetic profiles, suggesting that zero-order metabolism and the potential for toxicity
74 in susceptible *Gyps* vulture individuals is possible. Further, concentrations of carprofen in
75 animal tissues consumed by vultures may be greater than the concentration of carprofen in
76 the therapeutic dose tested.
77 For this study, we further explore the toxicity of carprofen to *Gyps* vultures. We
78 report studies of the pharmacokinetics and maximum residue levels of carprofen in *Bos*
79 *taurus* cattle; calculate the maximum residue exposure (MRE) for carprofen to wild *Gyps*
80 vultures; and harvest carprofen-rich cattle tissues to provision *Gyps* vultures under controlled
81 experimental conditions. Unfortunately, with clear evidence of toxicity in vultures currently
82 being limited to *Gyps* (although other old world species may also be vulnerable), the only
83 method available for elucidating the toxic potential of various NSAIDs is to undertake in vivo
84 toxicity studies with a *Gyps* species. However, studies are enhanced when combined with the

pharmacokinetic/toxicokinetic evaluation of the drug under investigation to determine if it is characterised by a prolonged half-life of elimination.

Methods

Research permission

Permission to undertake the study was given by the Research Committee of the Faculty of Veterinary Sciences, University of Pretoria, and the Animal Ethics Committee of the University of Pretoria (V062-13). Permission to work on an endangered species (*Gyps africanus*) was approved by the South African Department of Environmental Affairs (Permit 07137).

Cattle test subjects

Bos taurus cattle were used in a pharmacokinetics study and a tissue residue study. Four female mixed-breed cattle 15-18 months of age were used in the pharmacokinetics study; and four female Friesian cattle 9 months of age were used in the tissue residue study. Cattle were acquired from commercial livestock owners in Pretoria, South Africa. Initially, they were kept under non-treatment quarantine in outdoor camps of the University of Pretoria for six of seven weeks prior to experimentation to ensure they were free of NSAIDs and other drugs. Then cattle were moved to temperature-controlled stables at the Biomedical Research Centre, University of Pretoria, for the last week before experimentation. Cattle were given food, water, bedding and exercise daily. During the acclimatisation period each animal received a complete veterinary examination and was deemed fit and healthy. Throughout the study, each animal was monitored by a veterinarian or animal technician for signs of adverse drug reactions, but none were detected. At the completion of the pharmacokinetics study, the cattle

were returned to their owners with an enforced three-month withdrawal period. At the completion of the tissue residue study, the cattle were terminated within the pathology facilities.

Vulture test subjects

White-backed vultures (*Gyps africanus*) were used in a pharmacokinetics study (n = 6) and toxicity study (n = 8). This species was selected as it is a validated surrogate species for Asian *Gyps* vultures in NSAID toxicity studies, having been found to be killed by low oral doses of diclofenac (Swan et al. 2006). Some of the vultures used in the pharmacokinetic study were also used in the toxicity study. All vultures originated from the wild, each had been rescued and treated for illness and injuries at VulPro, South Africa, within six months of the start of the experiment. During that time, the vultures were kept in large communal aviaries at VulPro, where they were provided with water, food (every three days), perches and the room for short flapping flight. Each received a complete veterinary examination and was deemed fit and healthy. Three days prior to experimentation, vultures were moved to individual holding aviaries (dimensions: 3 x 2 x 2 m) also at VulPro. At the completion of both studies, the vultures were returned to large communal aviaries.

Pharmacokinetic study in cattle

On the first day of this study, each cow was weighed (mean ± SD = 413.25 ± 40.6 kg); and a 10 ml sample of blood was collected from its jugular vein into an evacuated heparinised tube. Next, each cow was treated with carprofen (Norocarp, Norbrook South Africa) at the standard cattle dose of 1.4 mg/kg bw and further blood samples were collected in evacuated heparin tubes at 0.25, 0.75, 1.50, 2.00, 3.00, 5.00, 7.00, 9.00, 12.00, 24.00, 36.00, 48.00,

96.00 and 120.00 h after treatment. Within 2h of collection, all blood samples were centrifuged at ~3,000 *g* for 15 min and the supernatant (plasma) was transferred to a glass screw-top vial and immediately frozen at -80 °C.

Pharmacokinetic study in Gyps *vulture*

Vultures (n = 6) were assigned to three groups of two birds each, with each pair being subsequently treated with carprofen (Norocarp) at 5 mg/kg bw by the intramuscular injection; intravenous injection or by oral gavage. Blood samples (5 ml) were collected from the tibiotarsal vein into sterile syringes before treatment and at 1.00, 2.00, 4.00, 6.00, 8.00, 12.00, 24.00, 36.00 and 48.00 h after treatment. Immediately after collection, half of each sample was transferred into evacuated heparinised tubes for carprofen analysis, and, the second half was transferred into non-treated evacuated tubes for biochemistry analysis. Samples were processed as for the cattle pharmacokinetic study and stored at -30°C.

Tissue residue study in cattle

On the first day of this study, each of the four study animal was weighed (mean ± SD = 207.25 ± 31.89 kg) and treated with carprofen (Norocarp) at 2.8 mg/kg bw in a single dose. The reason for doubling the dose was to simulate the behaviour of veterinarians and livestock owners in South Asia. Measured NSAID residues in tissues of dead cattle indicate that they have often received much more of the drug than the recommended dose of NSAIDs in palliative care of cattle (see Taggart et al. 2007). At 12 h after treatment, which corresponded to the mode T_{max} (as informed by the pharmacokinetic study undertaken previously), the cattle were slaughtered by captive bolt and pithing. The liver, kidneys, muscle from the hind leg, omental fat and muscle from the injection site in the neck (i.e., a 5 cm area surrounding

the injection site, to a depth of 5 cm) were harvested. Three replicate sub-samples were taken of each tissue (except for the injection site) and stored at -30 ^{o}C prior to analysis. Remaining bulk tissue was also stored at -30^{o}C for the final phase of the study which involved feeding vultures with harvested tissues.

Toxicity study in Gyps *vultures*

Toxicity was determined with a four-phase cross-over study. Each phase was separated by two weeks when vultures were returned to their communal aviaries. Vultures were randomly assigned to one of two balanced groups: Group A (n = 4); and Group B (n = 4). In Phase 1, Group A were given approximately 1 kg of kidney tissue rich in carprofen residues from the cattle slaughtered in the residue study (a single vulture received kidney tissue from a single cow). Group B were given approximately 1 kg of cattle kidney tissue which was purchased from a commercial butchery. According to South African legislation, butchered meat should comply with Codex Alimentarius standards for food safety and, therefore, unlikely to be contaminated with NSAIDs.

In Phase 2, Group B were given muscle tissue rich in carprofen residues (harvested from previously dosed cattle); and Group A were given pig muscle tissue acquired from a commercial piggery that confirmed that no NSAIDs were used in the pigs prior to death. For both Phases 1 and 2, the carprofen-rich tissues were frozen at -30 ^{o}C after harvesting and then thawed in a water bath at room temperature the night before use. Carprofen-free tissues were purchased fresh and held refrigerated at 4 ^{o}C overnight before use.

In Phase 3, Group A were given carprofen orally at a level based on the theoretical maximum residue exposure (MRE) calculated from the cattle kidney concentrations (see below); and Group B were given water.

In Phase 4, two vultures randomly selected from Group B were given carprofen orally at the average concentration found within the injection site tissues; and two vultures randomly selected from Group A were given carprofen orally at the MRE calculated from the cattle kidney concentrations. The use of two vultures for toxicity prediction, was as previously validated by Swan et al (2006b).

Blood was sampled from all vultures in Phase 3 and Phase 4 before and at 2.00, 8.00, 24.00 and 48.00 h after treatment for toxicokinetic and clinical pathology analyses as described above. Sampling was not attempted in Phase 1 and Phase 2 as it might have resulted in vultures regurgitating tissues. For comparative purposes, the expected blood parameter ranges for normal vultures was estimated only from samples taken from the control group in Phase 3.

Plasma and tissue analysis

All cattle and vulture plasma and tissue samples were couriered on dry ice to the Environmental Research Institute (ERI), United Kingdom. A subsample of each (0.3 ml for plasma or 0.5 g for tissue) was extracted using 2.5 ml of HPLC grade acetonitrile. For plasma this was done by vortex mixing (20 s), then setting to stand at room temperature (600 s), then vortex mixing (20 s), and finally centrifuging at 2000 rpm (671 *g*; 600 s). For the tissue this was done by homogenising with a VDI 12 (VWR) homogeniser, then centrifuging as per the plasma. The supernatant was, in both cases, syringe filtered (0.2 µm HDPE disposable) directly into an amber 2-ml screw top LC vial. These extracts were held at -20 ºC until analysis. Carprofen concentration was determined using a liquid chromatography electrospray ionisation triple quadrupole mass spectrometry system (LC-ESI-MS/MS) utilising a methodology adapted after Taggart *et al.* (2009). Carprofen was determined in

205 negative ion mode utilising a parent target mass of 272 m/z and a daughter ion of 228 m/z (in
206 MRM mode). Mean recovery of carprofen spiked in triplicate into blank plasma and liver
207 tissue at three different concentration levels and extracted as above was 79.4% and 70.6%
208 respectively (n = 9 in both cases) and the limit of quantification for the analysis was 4 ng/ml
209 and 10 ng/g for plasma and tissue respectively. Final concentrations were calculated
210 following correction for these extract recovery levels.
211
212 *Pharmacokinetics/Toxicokinetic evaluation*
213 For both cattle and vulture plasma samples, pivotal parameters were ascertained by non-
214 compartmental modelling in Kinetica 5.1 (Thermo 2012). The maximum plasma
215 concentration (C_{max}) and the time to reach it (T_{max}) were read from the plasma concentration
216 versus time profile. The last quantifiable time point C_{last} and the linear trapezoidal rule was
217 used to calculate the area under the curve (AUC_{last}) and the area under the moment curve
218 ($AUMC_{last}$) as ($AUC_{last} = \Sigma([T_{last}-T_{last-1}]*[C_{last}+C_{last-1}]/2)$) and ($AUMC_{last} = \Sigma([T_{last}-T_{last-1}]*[T_{last}*C_{last}+T_{last-1}*C_{last-1}]/2)$), respectively. The elimination rate constant (λ) was calculated
220 by ordinary least squares regression of the terminal three points of the curve after natural
221 logarithmic transformation; and subsequently, the half-life of elimination (T_{half}) was
222 calculated as $\ln(2)/\lambda$. The mean residence time (MRT) was calculated as $AUMC/AUC_{last}$ and
223 the area under curve to infinity (AUC_{inf}) was calculated as $AUC_{last} + C_{last}/\lambda$. For carprofen
224 administered intramuscularly and orally, the apparent volume of distribution (V_z/F) was
225 calculated as dose/($AUC_{last}*\lambda$), the apparent volume of distribution at steady state (V_{ss}/F) was
226 calculated as (dose*MRT)/AUC_{last} and the apparent clearance (Cl/F) was calculated as
227 dose/AUC_{last}; and for carprofen administered intravenously, the actual volume of distribution
228 (Vz), actual volume of distribution at steady state (V_{ss}) and actual clearance (Cl) were

229 calculated by first finding the fraction of absorption (F) and dividing this by the apparent
230 measures of these parameters. F was calculated as a bird's extravascular AUC_{inf} divided by
231 the pooled AUC_{inf} from the intravenous profile. All parameters are presented as geometric
232 means with standard deviations.
233
234 *Calculation of maximum residue exposure*
235 To calculate the maximum residue exposure (MRE) we followed the method outlined by the
236 European Medicines Agency (EMA) for establishing withdrawal periods (The European
237 Agency for the Evaluations of Medicinal Products, 1995). Residue depletion in tissues
238 follows a one compartmental model and thereby can be described by one exponential term.
239 The first order kinetic equation for residue concentration at time *t* is $C_0*e^{-\lambda t}$, where C_0 is the
240 hypothetical concentration at time 0 and λ is the elimination rate constant estimated from an
241 ordinary least squares regression as described above. We took the MRE to be the carprofen
242 concentration below which concentrations of 95% of a population of cow tissues of a
243 particular type would be expected to lie, with 95% confidence. We based this calculation
244 upon the average C_t. Therefore, MRE values in mg/kg were calculated as $M_i+SD_i*5.144$,
245 where M_i and SD_i were the mean concentration and its standard deviation in tissue type *i*.
246 The multiplier 5.144 is the value for a sample size of four cattle and was taken from the table
247 of factors of Hahn and Meeker (2011). Oral exposure in mg for vultures in Phase 3 and Phase
248 4 of the toxicity study were calculated as $(MRE_{kidney}*1.00)/4.50$, where MRE_{kidney} was the
249 maximum residue exposure for kidney tissue, 1.00 was a rough estimate of the amount of
250 edible tissue in kilograms that a vulture would consume in a large meal (see Swan et al. 2006)
251 and 4.5 was a rough estimate of the mean body weight in kilograms of a white-rumped
252 vulture *G. bengalensis* (the smallest *Gyps* species). Since, the species of *Gyps* vulture used in

this study were larger than 4.5 kg, they received a greater exposure to carprofen than that calculated using MRE_{kidney} (see Results). We used the MRE calculated for kidney tissue as this gave the greatest MRE value apart from that given by the muscle at the injection site (see Table 1). We did not use the MRE calculated for the muscle at the injection site as this gave a value that was extremely high and one that would most certainly kill vultures (see Table 1); therefore, we opted to test the average concentration in the muscle at the injection site.

Clinical pathology

Serum samples were analysed by the Veterinary Clinical Pathology Laboratory of the University of Pretoria using the Cobas Integra 400 (Roche Diagnostics) for activities of alanine transferase (ALT) and alkaline phosphatase (ALP); and concentrations of potassium (K), sodium (Na) and uric acid (UA). Due to limited sample volume, some samples (n = 4) for clinical pathology parameters were evaluated by a second commercial accredited laboratory in the United Kingdom (using plasma samples remaining following carprofen residue analysis). Changes in the measured clinical pathology parameters were considered significant if the changes at a particular time were either different to a control group of vultures or were different to the baseline value for a given individual vulture.

Results

Pharmacokinetic study of carprofen in cattle

The pharmacokinetic profiles (Supplementary Figure 1) and parameters (Supplementary Table 1) were fairly consistent among the six cattle treated at 1.4 mg/kg, with the exception of T_{max} and C_{max}, both of which showed considerable variability. Because of this variability,

277 we used the mode, not the mean T_{max} as the time at which cattle were to be slaughtered in the
278 tissue residue study.
279
280 *Pharmacokinetics of carprofen in Gyps vultures*
281 For this study vulture in pairs were treated with carprofen at 5mg/kg by the oral,
282 intramuscular or intravenous route. The pharmacokinetic parameters obtained are presented
283 in Table 1. Carprofen was well tolerated in all exposed vultures. The intravenous and
284 intramuscular profiles were very similar, as a result of very rapid absorption from muscle
285 tissue. The oral profile differed from these profiles in respect to absorption, with half as much
286 carprofen being absorbed, but not in respect to elimination which was of a similar rate despite
287 lower absorption. Although the profiles were not from the same birds, the population absolute
288 bioavailability was roughly estimated at 100% for the intramuscular route and 42% for the
289 oral route.
290
291 *Tissue residue study of carprofen (*2.8 mg/kg bw *) in cattle*
292 The concentrations of carprofen present in the liver, fat (omental), kidney, muscle
293 (quadriceps), and muscle at injection site were 5.75±1.49, 3.74±2.50, 7.72±2.38, 6.03±1.59
294 and 289.05±286.05 mg/kg respectively (Supplementary Table 2, for individual animal data
295 and variation of concentrations between animals). Mean carprofen concentration at the
296 injection site was 37 times greater than the next highest mean concentration found in the
297 kidneys and 48 times greater than the mean concentration found in the hind leg muscle. As a
298 result of the extremely high concentration at the injection site, we used both the MRE
299 calculated for kidney and the mean injection site concentration in the toxicity study.
300

Toxicity study of carprofen in Gyps *vultures*

In general, the four vultures per group were reluctant to eat kidney or muscle tissue provided in Phase 1 and Phase 2, respectively. As this was the case for both carprofen-rich and carprofen-free tissues, this was likely to be because individual housing of vultures prevented competitive feeding cues and caused a certain level of stress. The result was rather low dose levels across the two phases (0.12 to 0.90 mg/kg bw; Supplementary Table 3). The highest exposures for Phase 1 (kidney tissue) and Phase 2 (muscle tissue) were 0.87 and 0.90 mg/kg bw, respectively. No observable or clinical signs of toxicity were evident in any of the treated animals.

Four vultures in Phase 3 and two vultures in Phase 4 were given an oral dose of carprofen at 4.4 mg/kg bw, which equated to a range of exposures between 23.10 and 37.62 mg (Supplementary table 4). No observable or clinical signs of toxicity were evident in any of these treated animals from phase 3 at 4.4. mg/kg. Carprofen was characterized by a mean T_{half} of 10.29 ± 5.03 h (Table 2). All the pharmacokinetic parameters showed substantial variability (CV > 30%; Table 4). AUC_{last} was more variable than C_{max} and T_{max}, which indicated that both absorption and elimination profiles among vultures differed substantially (Supplementary Figure 2). The estimated fraction of absorption also ranged widely (25 to 103%), which is an indicator of pre-systemic elimination, which in turn is indicative of substantial inter-subject variation in metabolic capacity.

Two vultures in Phase 4 were given an oral dose of carprofen at 64.0 mg/kg bw, which equated to the average carprofen concentration at the injection site of cattle in the tissue residue study (289.05 mg/kg) and exposures of 345.60 and 387.20 mg (Supplementary Table 4). The vulture exposed to the greater amount of carprofen (387.20 mg) showed severe signs of depression (i.e., head drooping, reluctance to move, poor response to external

stimuli) approximate 48 h post-exposure. This vulture succumbed to toxicity at 52 h post-exposure. The vulture exposed to the lesser amount of carprofen showed no observable signs of toxicity. On clinical pathology, this vulture showed a moderate increase in ALP compared to the range for non-treatment vultures; whereas, the vulture that died showed a large increase in uric acid concentrations compared to the range for non-treatment vultures (Figure 1)(The calculated healthy values are applicable to the study population are presented in Supplementary Table 5). The uric acid concentrations in the vulture that died represented a 27-fold increase over the pre-treatment concentrations; and more than a 7-fold increase over the non-treatment maximum concentration (Figure 1). Similarly, ALT concentration in the vulture that died was above the range for non-treatment vultures from 24 h onwards and showed a marked increase at 48 h. In terms of pre-treatment concentration, the change in ALT represented a 2.5- and 15- fold increase at 24 and 48 hours. Finally, potassium (but not sodium) concentration in the vulture that died showed a moderate increase at 48 h post-exposure (Figure 1).

For a terminal blood sample revealed extremely high uric acid (15.20 mMol/L), ALT (813.00 U/L) and potassium (10.10 mMol/L) levels in the bird that died. On necropsy, the most significant findings were in the kidneys, liver, spleen and lungs. The kidneys revealed widespread dilatation of tubules with loss of the cuboidal lining cells and their replacement with an amorphous pink material in which pyknotic cell debris was entrapped (Figure 2). Radiating aggregates of purple crystalline spicules were also present in many of these tubules. Less affected tubules showed increased eosinophilia of the cytoplasm of the lining cells, varying degrees of shrinking and basophilia of the nuclei and even desquamation of the cells themselves from the basement membrane. Many of these tubules also contained varying amounts of crystalline urates. A small number of heterophils were present at the periphery of

some of the damaged tubules. Globular urates were also present in the lumens of some of the tubules. The liver and spleen had large multifocal areas of necrosis associated with uric acid crystal formation, and also associated with a small number of inflammatory cells, mainly heterophils; while the lungs had very small foci scattered within the parenchyma involving only a few cells.

Comparing the toxicokinetic parameters of the two vultures dosed at 64.0 mg/kg, C_{max} was 17% greater in the individual that survived than the individual that died; but AUC_{last} and T_{half} were 89 and 332% greater, respectively, in the individual that died than the individual that survived (Table 3). The bird that died was the adult. The extremely long T_{half} in the vulture that died was important, as was the prolonged period during which carprofen concentrations in its plasma remained around 30 μg/mL (i.e., for more than 20 h) before declining at a very slow rate (Figure 3). This profile indicates zero-order metabolism. Again, as for the pharmacokinetic studies all evaluated parameters showed substantial variability (CV > 30%) with AUC_{last} being more variable than C_{max} and T_{max} (Table 3).

While not planned, an interesting additional result was observed for vultures G31917 and G30796. G31917 was treated orally at 4.4 mg/kg bw in the toxicity study and intramuscularly at 5 mg/kg bw in the pharmacokinetics study, with a resultant AUC_{last} of 170.82 and 579.63 μg/mL*h, respectively. G30796 was treated orally at 64.0 mg/kg bw orally (and survived) and intramuscularly at 5 mg/kg, with a resultant AUC_{last} of 650.16 and 690.79 μg/mL*h, respectively. These findings yielded relative bioavailabilities of approximately 28% and 8% for G31917 and G30796, respectively, for dose normalised parameters; and indicated a substantial first pass effect present in both vultures (Figure 4).

Discussion

373 Carprofen is a non-steroidal anti-inflammatory drug from the carboxylic acid group (Riviere
374 and Papich, 2013). Like other NSAIDs, carprofen is believed to function via the inhibition of
375 cyclooxygenase (COX) enzyme systems. The drug is widely used globally as a veterinary
376 medicine for the modulation of pain and inflammation in pets, horses and production animals.
377 In South Africa, carprofen is mainly used under intensive farming conditions as adjunct
378 therapy for the control of acute inflammation associated with respiratory disease (Carrington
379 et al., 2013). Carprofen is also recommended as a drug of choice for the management of pain
380 and inflammation in raptors and other birds, as an off-label indication (Carpenter, 2005).
381 For this study, we set out to ascertain the safety of carprofen in a susceptible vulture
382 species using the same parameters from previous vulture toxicity studies. The study model
383 used, assumes a worst case scenario of the double dosing of cattle recently prior to its death,
384 and a vulture being exposed to the highest concentration in a particular tissue in a said meat
385 as a kilogram meal. For this study we were able to demonstrate that carprofen is generally
386 harmless to *Gyps* vultures at concentrations present in most contaminated animal tissues.
387 However, this was not the case when considering the high concentrations present at injection
388 sites. If a vulture were to consume muscle tissue from the injection site from a cow treated
389 with carprofen just prior to its death, then it could ingest a very high concentration of the drug
390 and could die as a result. The reason for the high concentration of carprofen at the injection
391 site appears to be slow absorption of the drug. Yet, only two of the four cattle dosed with
392 carprofen showed very high concentrations at the injection site. Further, only one of the two
393 *Gyps* vultures exposed to concentrations based on injection site results died. The reason for
394 this is probably individual variation in tolerance and zero-order elimination, as is the case for
395 *Gyps* vultures exposed to diclofenac and ketoprofen (see below) (Naidoo et al., 2009, Naidoo
396 et al., 2010a). Individual variation among cattle in terms of carprofen absorption rate and

among *Gyps* vultures in terms of carprofen toxicity, make it difficult to determine what impact carprofen contamination of cattle carcasses (left to scavengers) would have on *Gyps* vulture populations. Nevertheless, our data suggest that a single *Gyps* vultures feeding on a cattle carcass rich in carprofen residues could die if they consume the entire area of drug depot.

Implications for Gyps *vulture conservation*

Two cattle in the tissue residue study had concentrations of carprofen at the injection site of >511.00 mg/kg, but the other two cattle had concentrations of carprofen at the injection site of <55.00 mg/kg (almost a ten-fold difference). High inter-animal variability was seen in all tissue types (CV > 25%), but was greatest at the injection site (99%). This phenomenon was unlikely solely due to sampling error, since carprofen concentrations in replicate samples from each individual cow were similar (average intra-animal %CV was 48% and higher than for all other tissues). Given that the proportion of edible tissue on a typical carcass that may contain lethal levels would be expected to be very small (i.e., solely that which immediately surrounds the injection site), the risk associated with the use of carprofen in cattle would be smaller than that for diclofenac and ketoprofen.

Nonetheless the potential does exist that at a single feeding, a single *Gyps* vulture could consume the entire carprofen-rich injection site tissue and succumb from toxicity. While the method we've selected is highly conservative in estimating exposure and safety based on an unlikely exposure to 1kg of carprofen rich tissue, the relative exposure can be further evaluated in terms of the MRE. According to the prescribed EMA method, the safety of the drug at the injection site is further considered in terms of potential exposure to 300g of tissue rich in said residue. If the latter method is taken consideration, in conjunction with the

high concentration of 559 mg/kg found in the one cow, a bird would have been exposed to dose of 167 mg/kg. If the said bird was at the lower end of the weight range for the white-backed vultures, a bird of 3.5 kg would be exposed to 47.7 mg/kg, which is not substantially lower than the test dose of 64 mg/kg, which shows the model used while being conservation is not inaccurate.

In South Asia carprofen is marketed for use in dogs only, where the recommended dose (2-4 mg/kg) is within the range that *Gyps* vultures can tolerate. However, the misuse of diclofenac intended for human use in livestock is rampant in South Asia; and therefore, the misuse of carprofen in livestock is possible. That said, a series of surveys of domesticated ungulate carcasses left for scavengers across India (the largest country and user of NSAIDs in South Asia), between 2004 and 2010, have not (to date) encountered any detectable carprofen contamination (Taggart et al., 2007, Taggart et al., 2009, Cuthbert et al., 2011). The combination of the particular lethality of carprofen and its apparently negligible use in livestock in South Asia suggests that it is unlikely to present a significant threat to *Gyps* vultures there at the current time. That is not to say that carprofen use might not impede recovery of South Asia's critically endangered *Gyps* vultures if regional governments do not control its use appropriately.

A further consideration is the availability of carprofen in cattle injectable formulations in South Africa and other countries where other species of threatened vultures are found. We would strongly recommend that carprofen be restricted for use in the intensive farming sector, where incidence of respiratory (and other) disease are more likely to occur and carcasses are disposed of by means other than provision to vultures at feeding sites (i.e., incineration and burial). Furthermore, while it would be tempting to remove injection site

445 tissue from cattle carcasses before provision at feeding stations, this relies on knowing
446 exactly how many doses of the drug the animal had been given prior to death and exactly
447 where on the animal these doses had been administered. Lastly, the use of carprofen in cattle
448 kept under extensive farming conditions should be precautionary stopped. Further
449 consideration can perhaps also be given to the development of oral formulations of carprofen
450 for use in cattle in these areas.
451
452 *Insights into NSAID toxicity in* Gyps *vultures*
453 The vulture that died in this study was exposed to 387.20 mg of carprofen, while another
454 vulture that survived was exposed to 345.60 mg of carprofen. Substantial differences in
455 individual intolerance of NSAIDs were also seen for both diclofenac (Oaks et al. 2004) and
456 ketoprofen (Naidoo et al. 2010). Carprofen toxicity in *Gyps* vultures also resembles that of
457 diclofenac and ketoprofen in respect to toxicokinetics, specifically: zero-order elimination;
458 and the subsequent extended elimination profile. The vulture that died from a high dose of
459 carprofen clearly showed zero-order elimination – that is, a constant amount of drug
460 eliminated per unit time (see linear elimination in Figure 5); whereas, the vulture that
461 survived from a high dose of carprofen clearly shows first-order elimination – that is, a
462 constant proportion of drug eliminated per unit time (see exponential elimination in Figure 5).
463 Zero-order elimination suggests limited enzyme capacity for processing a given drug, which
464 results in the accumulation of that drug in the body and thereby toxicity. The effect of zero-
465 order elimination was also associated with elimination and not absorption, as the vulture that
466 died from a high dose of carprofen showed greater values for AUC, T_{half} and MRT than the
467 vulture that survived from a high dose of carprofen; but C_{max} and T_{max} was similar between
468 the two vultures. Further, AUC, T_{half} and MRT for the vulture that survived were within the

range of values for those parameters among the vultures given a small dose of carprofen (i.e., 4.4 mg/kg). While still speculative, we believe that the point of capacity limitation lies with the hepatic cytochrome P450 (CYP) enzymes concentrations and or ratios. Also with the bird dying being an adult, this would indicate that the constraints present in metabolism is not due age-specific limitations in metabolism, but rather individual limitations in metabolic capacity.

In this study, toxicity was monitored through clinical, clinical pathology, toxicokinetics and necropsy following previous toxicity studies in vultures (Oaks et al., 2004, Swan et al., 2006, Naidoo et al., 2010b). The vulture that died showed general depression and a reluctance to respond to stimuli. Clinical pathology showed clear progression of disease: plasma uric acid concentration exceeding the normal range at 24 h, indicative of kidney damage; and plasma ALT concentration also exceeding the normal range at 48 h, indicative of substantial damage to other tissues. Death probably resulted from the increase in plasma potassium concentration and cardiac toxicity, which has been shown to be compensatory to an increase in plasma uric acid concentration (Lumeij, 1994). This progression of disease is very similar to that seen in vultures exposed to diclofenac (Oaks et al., 2004) and ketoprofen (Naidoo et al., 2010b), suggesting that all three drugs have the same mechanism of toxicity. While the mechanism of toxicity still remains elusive, cascading kidney toxicity is either secondary to hypoperfusion (Meteyer et al., 2005) or results from alteration in the renal ability to excrete uric acid (Naidoo and Swan, 2009).

Innovations for NSAID safety testing in Gyps *vultures*

The method followed here builds on the methods used in previous NSAID toxicity tests in *Gyps* vultures. It incorporates studies in cattle to determine a more accurate measure of the maximum exposure to vultures through the examination of the pharmacokinetic profile in

493 cattle. Previous toxicity studies on diclofenac, meloxicam and ketoprofen relied on data from
494 residue depletion studies published by the European Medicines Agency to estimate when
495 cattle are likely to have reached their maximum residue concentrations in tissues and what
496 tissue types have the highest maximum concentration at those times. Residue depletion
497 studies are not necessarily accurate at determining such parameters because cattle are
498 slaughtered at predetermined times and often large time intervals are used that may not
499 correspond to true maxima. Analysing a time series of plasma samples for C_{max} and T_{max} is a
500 more accurate alternative because repeated measurements can be made on the same
501 individual. Importantly, observed drug concentrations in plasma are proportional to those
502 expected in tissues. Accurate C_{max} and T_{max} lead to accurate maximum tissue residue levels,
503 which in turn lead to an accurate measure of maximum exposure.
504 Note that published pharmacokinetics studies in cattle on the given drug should be
505 used with caution as these may use animals or methods of administration not entirely
506 appropriate for a toxicity test in *Gyps* vultures. This was the case for carprofen where two
507 studies had previously examined carprofen pharmacokinetics in calves (3-4 months of age)
508 using intravenous injection and showed considerably different parameters to those we found
509 here (Delatour et al., 1996, Brentnall et al., 2013). It may be that there are important age
510 related differences in carprofen pharmacokinetics in cattle. This is important because cattle
511 carcasses contaminated with NSAIDs and left for vultures in South Asia (and probably South
512 Africa) are far less likely to be those of calves (Taggart et al., 2007).
513 MRE, developed by the EMA, was used as the worst case scenario exposure for
514 vultures. The philosophy of the method is based on chemical safety principles that protect
515 people from potentially dangerous residues in their food. The use of the upper tolerance value
516 takes into account the worst case exposure resulting from linear tissue depletion by

converting tissue residue results from the test population (of small sample size) into a value that would cater for the potentially large inter-subject differences in the various cattle breeds available. As a result, our estimate of exposure for the non-injection site scenario best reflects the true worst case exposure for wild vultures, if cattle are treated at doubled the dose recommended. Further caution will need to apply if higher doses or more frequent administrations occur. The method adopted for this study was also stricter than that previously applied in the testing of meloxicam and ketoprofen, as both those studies simply doubled the potential tissue residue concentrations. In this study, double the tissue residue concentration in all tissue types was lower than the MRE (e.g. ~15 mg/kg vs. 20 mg/kg for the kidneys).

Finally, this method examined the concentration of the tested drug at the injection site. The injection site has also been considered as an area of potential concern in human food safety, as it is known that drugs may persist at elevated levels at such sites. The importance of this is best emphasised by the EMA's own guidelines, that suggest that the injection site residue concentration should be substituted for the wider muscle concentration (when higher) to also take into account the worst case scenario of a person accidentally being exposed to meat sourced from the injection site. While we expected (in general) a difference in injection site and muscle tissue concentrations, since termination was at plasma T_{max}, the substantial difference observed here was certainly not expected. In addition, such a difference was contrary to findings in toxicity studies for meloxicam and diclofenac, where the concentrations at the injection site was lower than that in muscle away from the injection site (EMA public safety information; The European Agency for the Evaluation of Medicinal Products, 2003 and The European Agency for the Evaluation of Medicinal Products, 1999). Our findings thus highlight the potential danger of injection site tissue from animals treated

541 with NSAIDs, especially if more than one dose has been administered at the same site.

542

543 **Acknowledgements**

544 We like to thank the staff of the University Pretoria Biomedical Research Centre and the

545 staff and volunteers of the VulPro for making this study possible. The study was funded

546 partly by the Royal Society for the Protection of Birds and the Chester Zoo.

547

548 **References**

549 1. Arnold KE, Brown AR, Ankley GT, Sumpter JP (2014) Medicating the environment:
550 assessing risks of pharmaceuticals to wildlife and ecosystems. Philos Trans R Soc
551 Lond B Biol Sci 369: 20130569.

552 2. Brentnall C, Cheng Z, McKellar Q, Lees P (2013) Influence of oxytetracycline on
553 carprofen pharmacodynamics and pharmacokinetics in calves. J Vet Pharmacol Ther
554 36: 320-328.

555 3. Carpenter J (2005) Exotic Animal Formulary Avian and Exotic Pets (ed 3). St. Louis,
556 MO, Saunders.

557 4. Carrington C, du Plessis A, Naidoo V (2013) IVS Desk Reference, MIMS,
558 Saxonworld

559 5. Cuthbert RJ, Dave R, Chakraborty SS, Kumar S, Prakash S, Ranade SP, Prakash V
560 (2011) Assessing the ongoing threat from veterinary non-steroidal anti-inflammatory
561 drugs to Critically Endangered Gyps vultures in India. Oryx 45: 420-426.

562 6. Delatour P, Foot R, Foster A, Baggo D, Lees P (1996) Pharmacodynamics and
563 chiralpharmacokinetics of carprofen in calves. Br Vet J 152: 183-198.

564 7. Fourie T, Cromarty D, Duncan N, Wolter K, Naidoo V (2015) The Safety and
565 Pharmacokinetics of Carprofen, Flunixin and Phenylbutazone in the Cape Vulture
566 (Gyps coprotheres) following Oral Exposure. PloS One 10: e0141419.

567 8. Green RE, Newton I, Shultz S, Cunningham AA, Gilbert M, Pain DJ, Prakash V
568 (2004) Diclofenac poisoning as a cause of vulture population declines across the
569 Indian subcontinent. J Appl Ecol 41: 793-800.

570 9. Green RE, Taggart MA, Senacha KR, Raghavan B, Pain DJ, Jhala Y, Cuthbert R
571 (2007) Rate of decline of the oriental white-backed vulture population in India
572 estimated from a survey of diclofenac residues in carcasses of ungulates. PLoS One 2:

573 e686.

574 10. Hahn GJ, Meeker WQ (2011) Statistical Intervals: A Guide for Practitioners. John
575 Wiley & Sons, United States.

576 11. Kuster A, Adler N (2014) Pharmaceuticals in the environment: scientific evidence of
577 risks and its regulation. Philos Trans R Soc Lond B Biol Sci 369: 20130587.

578 12. Lumeij JT (1994) Nephrology. Avian medicine, principles and application. Lake
579 Worth, USA: Wingers Publishing Inc, pp.538-555.

580 13. Margalida A, Bogliani G, Bowden CG, Donazar JA, Genero F, Gilbert M, Karesh
581 WB, Kock R, Lubroth J, Manteca X, Naidoo V, Neimanis A, Sanchez-Zapata JA,
582 Taggart MA, Vaarten J, Yon L, Kuiken T, Green RE (2014) Science and regulation.
583 One Health approach to use of veterinary pharmaceuticals. Science 346: 1296-1298.

584 14. Meteyer CU, Rideout BA, Gilbert M, Shivaprasad H, Oaks JL (2005) Pathology and
585 proposed pathophysiology of diclofenac poisoning in free-living and experimentally
586 exposed oriental white-backed vultures (Gyps bengalensis). J Wildl Dis 41: 707-716.

587 15. Naidoo V, Wolter K, Cuthbert R, Duncan N (2009) Veterinary diclofenac threatens
588 Africa's endangered vulture species. Regul Toxicol Pharm 53: 205-208.

589 16. Naidoo V, Swan GE (2009) Diclofenac toxicity in Gyps vulture is associated with
590 decreased uric acid excretion and not renal portal vasoconstriction. Comp Biochem
591 Phys C 149: 269-274.

592 17. Naidoo V, Venter L, Wolter K, Taggart M, Cuthbert R (2010a) The toxicokinetics of
593 ketoprofen in Gyps coprotheres: toxicity due to zero-order metabolism. Arch Toxicol
594 84: 761-766.

595 18. NaidooV, Wolter K, Cromarty D, Diekmann M, Duncan N, Meharg AA, Taggart MA,
596 Venter L, Cuthbert R (2010b) Toxicity of non-steroidal anti-inflammatory drugs to
597 Gyps vultures: a new threat from ketoprofen. Biol Lett 6: 339-341.

598 19. Oaks JL, Gilbert M, Virani MZ, Watson RT, Meteyer CU, Rideout BA, Shivaprasad
599 H, Ahmed S, Chaudhry MJI, Arshad M (2004) Diclofenac residues as the cause of
600 vulture population decline in Pakistan. Nature 427: 630-633.

601 20. Prakash V, Green R, Pain D, Ranade S, Saravanan S, Prakash N, Venkitachalam R,
602 Cuthbert R, Rahmani A, Cunningham A (2007) Recent changes in populations of
603 resident Gyps vultures in India. J Bombay Nat Hist Soc 104: 129-135.

604 21. Richman L, Hayward G (2011). Fowler's zoo and wild animal medicine: current
605 therapy. Elsevier Sanders, Missouri.

606 22. Riviere JE, Papich MG (2013) Veterinary Pharmacology and Therapeutics. John
607 Wiley & Sons, United States.

608 23. Schwaiger J, Ferling H, Mallow U, Wintermayr H, Negele R (2004) Toxic effects of

the non-steroidal anti-inflammatory drug diclofenac: Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat Toxicol 68: 141-150.

24. Swan G, Naidoo V, Cuthbert R, Green RE, Pain DJ, Swarup D, Prakash V, Taggart M, Bekker L, Das D (2006a) Removing the threat of diclofenac to critically endangered Asian vultures. PLoS biology 4: e66.

25. Swan GE, Cuthbert R, Quevedo M, Green RE, Pain DJ, Bartels P, Cunningham AA, Duncan N, Meharg AA, Oaks JL, Parry-Jones J (2006b). Toxicity of diclofenac to Gyps vultures. Biol lett *2*: 279-282.

26. Swarup D, Patra R, Prakash V, Cuthbert R, Das D, Avari P, Pain D, Green R, Sharma A, Saini M (2007) Safety of meloxicam to critically endangered Gyps vultures and other scavenging birds in India. Anim Conserv 10: 192-198.

27. Taggart M, Senacha K, Green R, Jhala Y, Raghavan B, Rahmani A, Cuthbert R, Pain D, Meharg A (2007) Diclofenac residues in carcasses of domestic ungulates available to vultures in India. Environ Int 33: 759-765.

28. Taggart MA, Senacha KR, Green RE, Cuthbert R, Jhala YV, Meharg AA, Mateo R, Pain DJ (2009) Analysis of nine NSAIDs in ungulate tissues available to critically endangered vultures in India. Environ Sci Technol 43: 4561-4566.

29. The European Agency for the Evaluation of Medicinal Products, 2003. Diclofenac Summary Report. EMEA/MRL/885/03-Final.

30. The European Agency for the Evaluation of Medicinal Products, 1999. Meloxicam Summary Report 2. EMEA/MRL/571/99-Final.

31. The European Agency for the Evaluations of Medicinal Products, 1995. Approach towards harmonisation of withdrawal periods. EMEA/CVMP/036/95, 1-37.

Table 1: Pharmacokinetic parameters obtained using non-compartmental modelling for six adult *G. africanus* treated with carprofen at 5 mg/kg body weight via intravenous injection (n = 2), intramuscular injection (n = 2) and oral gavage (n = 2). Each vulture has a unique code. Also shown are the geometric mean and standard deviation (SD).

Table 2: Pharmacokinetic parameters obtained using non-compartmental modelling for six adult *Gyps* vultures (*G. africanus*) treated with carprofen at 4.4 mg/kg via an oral gavage in Phase 3 and Phase 4 of a toxicity study.

Table 3: Toxicokinetic parameters obtained using non-compartmental modelling for two adult *Gyps* vultures (*G.*) treated with carprofen at 64 mg/kg via an oral gavage in Phase 4 of a toxicity study. Note: G31961 died.

Supplementary Table 1: Pharmacokinetics parameters obtained using non-compartmental modelling for four mixed-breed female *Bos taurus* cattle (15-18 moa) treated with carprofen (Norocarp) at 1.4 mg/kg body weight. Also shown are the geometric mean, standard deviation (SD) and coefficient of variation (CV) as a percentage.

Supplementary Table 2: Mean tissue concentrations (mg/kg) of carprofen from three samples from four female Friesian *Bos taurus* cattle (9 months of age) treated with Norocarp at 2.8 mg/kg body weight. Also shown are the arithmetic means and standard deviations (SD) of the animal means and maximum residue exposure (MRE).

Supplementary Table 3: Carprofen dose and exposure to *Gyps* vultures in Phase 1 (kidney) and Phase 2 (muscle) of the toxicity study. Also shown are individual codes, vulture body weights, amount of tissue consumed and concentration of carprofen used to calculate dose and exposure.

Supplementary Table 4: Carprofen dose and exposure to *Gyps* vultures in Phase 3 (dose = 4.4mg/kg) and Phase 4 (64 mg/kg) of the toxicity study. Also shown are individual codes and vulture body weights.

Supplementary Table 5: Descriptive statistics for biochemical parameters from samples of *Gyps* vulture plasma collected in the absence and presence of carprofen. Parameters in the absence of carprofen were obtained from the control groups and samples evaluated prior to treatment from the descriptive pharmacokinetic phase of the study.

675 Table 1: Pharmacokinetic parameters obtained using non-compartmental modelling for six adult *G. africanus* treated with carprofen at 5 mg/kg
676 body weight via intravenous injection (n = 2), intramuscular injection (n = 2) and oral gavage (n = 2). Each vulture has a unique code. Also
677 shown are the geometric mean and standard deviation (SD).

Parameter	Unit	Intravenous				Intramuscular				Oral			
		G31980	G31943	G.Mean	SD	G31917	G30796	G.Mean	SD	G32857	G31997	G.Mean	SD
C_{max}	µg/mL	27.79	38.09	32.54	7.28	30.76	34.34	32.50	2.53	14.65	17.53	16.03	2.04
T_{max}	h	1.00	1.00	1.00	0.00	3.00	2.00	2.45	0.71	1.00	1.00	1.00	0.00
AUC_{last}	µg/mL*h	658.43	543.84	598.39	81.03	690.98	579.63	632.86	78.73	153.53	344.94	230.12	135.35
AUC_{inf}	µg/mL*h	133.31	24.61	57.28	76.86	40.89	15.51	25.18	17.95	3.09	48.32	12.23	31.98
λ	h^{-1}	0.04	0.07	0.05	0.02	0.07	0.08	0.08	0.01	0.09	0.04	0.06	0.03
$AUMC_{last}$	µg/mL*h^2	11256.70	7271.06	9047.00	2818.27	10918.40	8027.24	9361.87	2044.36	1867.96	6133.33	3384.79	3016.07
T_{half}	h	18.51	10.58	13.99	5.61	10.43	8.17	9.23	1.60	7.92	16.00	11.25	5.71
MRT	h	26.80	15.53	20.40	7.97	18.44	15.05	16.66	2.40	13.10	24.33	17.85	7.94
Cl/F^*	L/h*kg	0.01	0.01	0.01	0.00	0.01	0.01	0.01	0.00	0.03	0.01	0.02	0.01
V_z/F^*	L/kg	0.17	0.13	0.15	0.02	0.10	0.10	0.10	0.00	0.36	0.29	0.33	0.05
V_{ss}/F^*	L/kg	0.17	0.14	0.15	0.02	0.13	0.13	0.13	0.00	0.42	0.31	0.36	0.08
F	%	-	-	-	-	114.95	96.42	105.28	13.10	25.54	57.38	38.28	22.52

678 *For intravenous injection these are Cl, Vz and Vss (see Methods)
679
680

Table 2: Pharmacokinetic parameters obtained using non-compartmental modelling for six adult *Gyps* vultures (*G. africanus*) treated with carprofen at 4.4 mg/kg via an oral gavage in Phase 3 and Phase 4 of a toxicity study.

Parameter	Units	Vulture						Geometric Mean	SD	CV (%)
		G19042	G30404	G31917	G31977	G30402	G30258			
C_{max}	µg/mL	21.06	30.28	12.76	12.39	21.82	8.91	16.42	7.95	48.43
T_{max}	h	2	2	2	2	2	2	2.00	0.00	0.00
AUC_{last}	µg/mL*h	270.21	481.09	170.82	124.75	548.61	131.12	241.68	184.67	76.41
AUC_{inf}	µg/mL*h	273.51	511.37	171.43	125.33	677.27	139.78	256.45	227.51	88.71
λ	h^{-1}	0.08	0.05	0.11	0.1	0.04	0.06	0.07	0.03	40.80
$AUMC_{last}$	µg/mL/h^2	3317	7713	2038	1360	10182	1899	3333.07	3651.14	109.54
T_{half}	h	8.39	13.31	6.59	6.87	19.78	11.88	10.29	5.03	48.86
MRT	h	12.93	19.45	12.11	11.15	29.57	17.62	16.14	6.91	42.83
Cl	L/h*kg	0.02	0.01	0.03	0.04	0.01	0.36	0.03	0.14	448.71
V_z	L/kg	0.22	0.17	0.25	0.35	0.19	6.13	0.40	2.41	609.02
V_{ss}	L/kg	0.24	0.17	0.31	0.39	0.19	6.3	0.43	2.47	580.32
F	%	51.08	90.94	32.29	23.58	103.71	24.79	45.69	34.91	76.41

685 Table 3: Toxicokinetic parameters obtained using non-compartmental modelling for two adult *Gyps* vultures (*G.*) treated with carprofen at 64
686 mg/kg via an oral gavage in Phase 4 of a toxicity study. Note: G31961 died.

Parameter	Units	Vulture		Mean	SD	CV (%)
		G30796	G31961			
C_{max}	µg/ml	40.37	33.70	36.88	4.72	12.79
T_{max}	h	2.00	2.00	2.00	0.00	0.00
AUC_{last}	µg/ml*h	650.16	1231.27	894.72	410.91	45.93
AUC_{inf}	µg/ml*h	667.34	2064.18	1173.67	987.72	84.16
λ	h^{-1}	0.07	0.02	0.04	0.04	94.49
$AUMC_{last}$	µg/ml/h^2	8622.00	27113.00	15289.48	13075.11	85.52
T_{half}	h	8.74	37.75	18.16	20.51	112.93
MRT	h	14.48	54.48	28.09	28.28	100.70
Cl	L/h*kg	0.01	0.02	0.01	0.01	50.00
V_z	L/kg	0.08	1.32	0.32	0.88	269.82
V_{ss}	L/kg	0.10	1.32	0.36	0.86	237.44
F	%	91.02	209.80	138.19	83.99	60.78

687 F: For the calculation of the fraction of absorption, plasma concentrations were normalised to 1 mg/kg exposure for the birds dosed orally.

688

689

690

691

692

693

Figure 1: Change in concentrations of uric acid (A), ALT (B), ALP (C) and potassium (D) concentrations in the two bird exposed to carprofen at 64 mg/kg. Bird G31961 (triangle) died from exposure

Figure 2: Histopathology slide from the bird that died. Evident are widespread dilation of tubules with loss of the cuboidal lining cells and replacement with an amorphous pink material in which pyknotic cell debris was entrapped

Figure 3: Plasma concentration versus time profile for the two birds treated with carprofen at 64 mg/kg

Figure 4: Illustration of the individual variability in oral bioavailability. In all case the plasma concentration versus time profiles have been equalized to 4.4. mg/kg, the lowest dose administered. Bird 31917 (A), which was dosed intramuscular (5mg/kg, before equalization) and orally (4.4 mg/kg) had a fraction of absorption of approximately 27.84%; while bird 30796 (B) dosed intramuscular (5mg/kg, before equalization) and orally (64 mg/kg, before equalization) had a fraction of absorption of only 8%.

Supplementary Figure 1: Individual (A) and arithmetic mean (B) plasma concentration versus time profiles for the cattle treated with carprofen at 1.4 mg/kg

Supplementary Figure 2: Plasma concentration versus time profile for birds exposed to carprofen (5mg/kg) by the iv (A), oral (B) or intramuscular route (C)

Supplementary Figure 3: Individual (A) and arithmetic mean (B) plasma concentration versus time profiles for the vultures treated with carprofen at 4.4 mg/kg by the oral route (n=6)

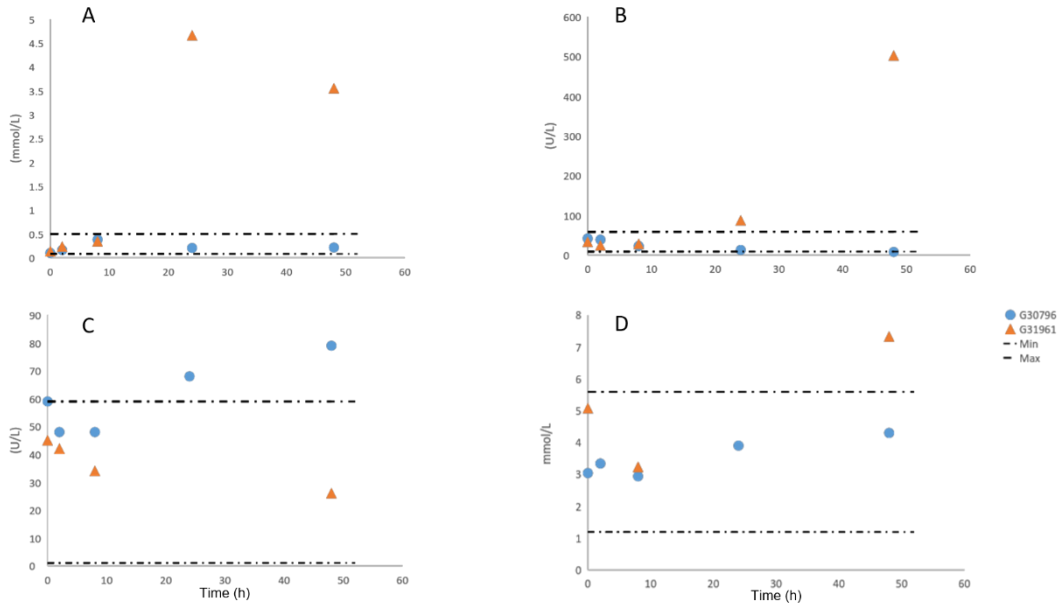


Figure 1: Change in concentrations of uric acid (A), ALT (B), ALP (C) and potassium (D) concentrations in the two bird exposed to carprofen at 64 mg/kg. Bird G31961 (triangle) died from exposure

724

725

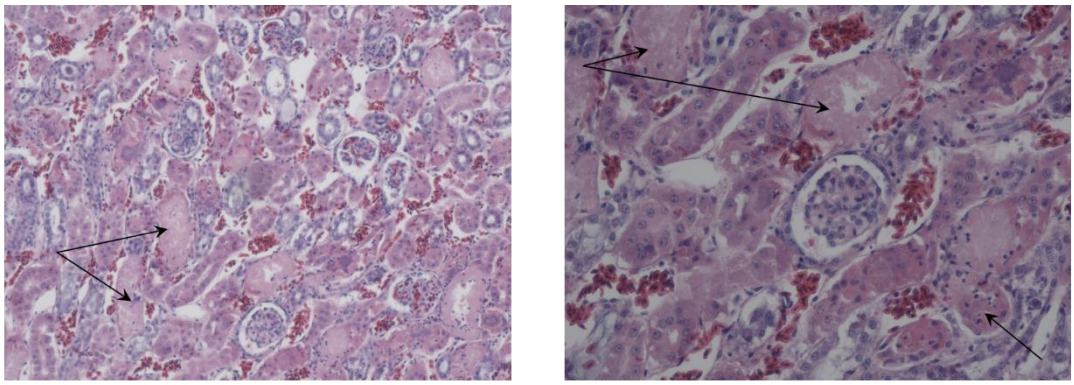


Figure 2: Histopathology slide from the bird that died. Evident are widespread dilation of tubules with loss of the cuboidal lining cells and replacement with an amorphous pink material in which pyknotic cell debris was entrapped (arrows)

726

727

728

729

730

731

732

733

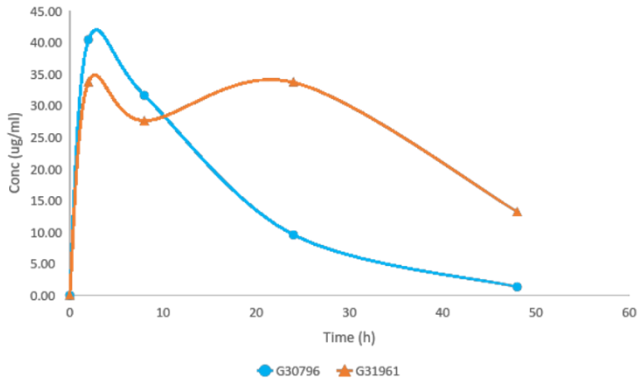


Figure 3: Plasma concentration versus time profile for the two birds treated with carprofen at 64 mg/kg

734

735

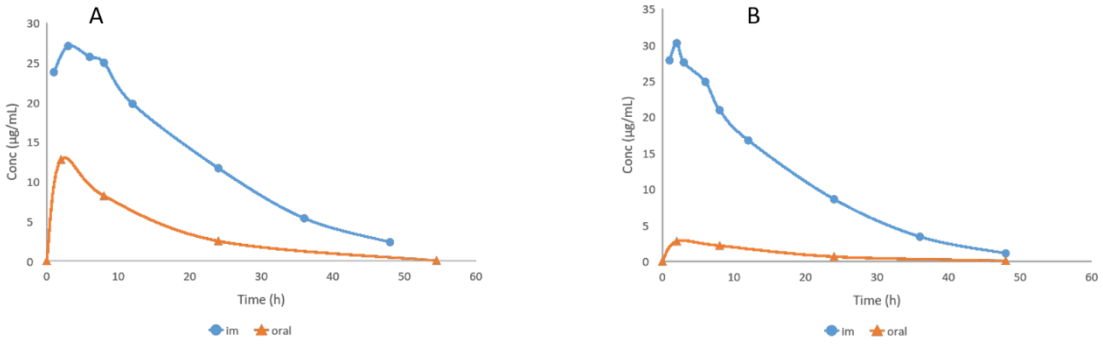


Figure 4: Illustration of the individual variability in oral bioavailability. In all case the plasma concentration versus time profiles have been equalized to 4.4. mg/kg, the lowest dose administered. Bird 31917 (A), which was dosed intramuscular (5mg/kg, before equalization) and orally (4.4 mg/kg) had a fraction of absorption of approximately 27.84%; while bird 30796 (B) dosed intramuscular (5mg/kg, before equalization) and orally (64 mg/kg, before equalization) had a fraction of absorption of only 8%.

736

737

738

739

740

741

742

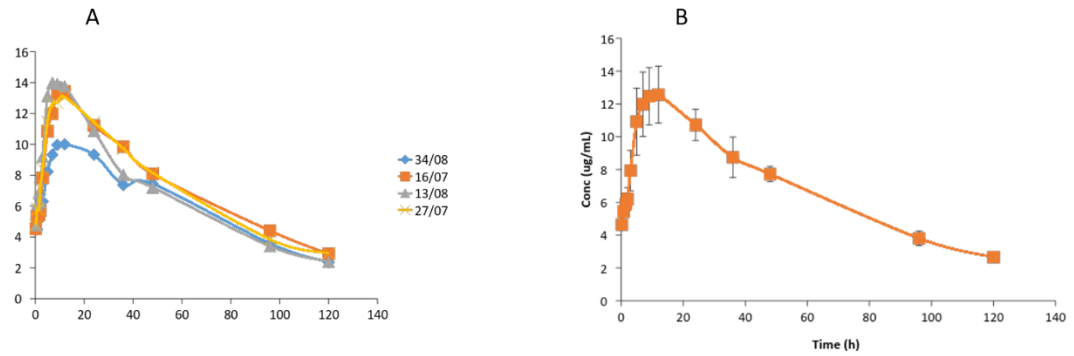
743

744

745

746

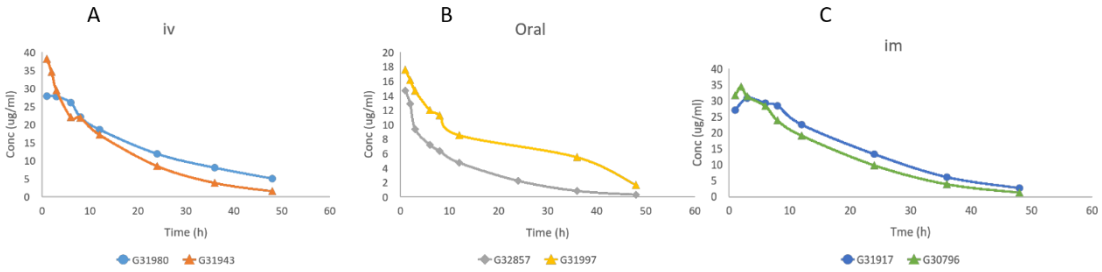
747



Supplementary Figure 1: Individual (A) and arithmetic mean (B) plasma concentration versus time profiles for the cattle treated with carprofen at 1.4 mg/kg

748

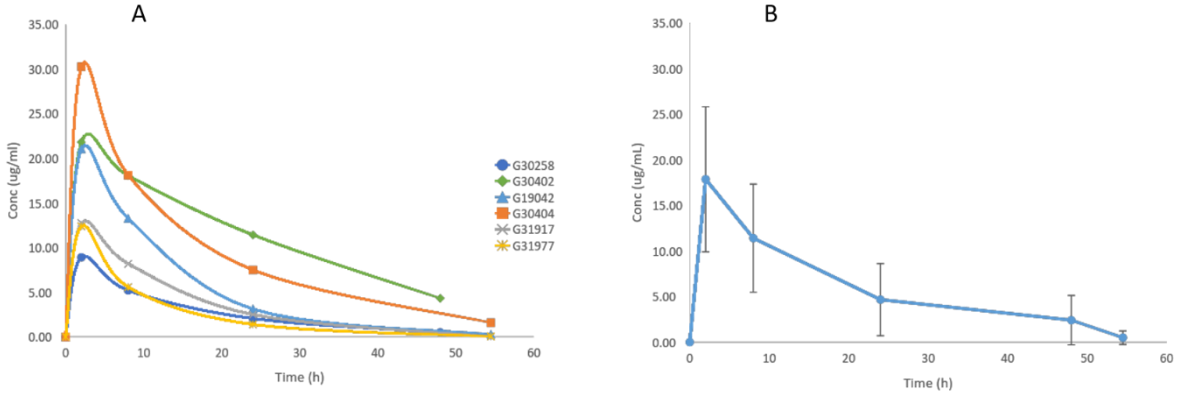
749



Supplementary Figure 2: Plasma concentration versus time profile for birds exposed to carprofen (5mg/kg) by the iv (A), oral (B) or intramuscular route (C)

750

751



Supplementary Figure 3: Individual (A) and arithmetic mean (B) plasma concentration versus time profiles for the vultures treated with carprofen at 4.4 mg/kg by the oral route (n=6)

752

753

754

755

Supplementary Table 1: Pharmacokinetics parameters obtained using non-compartmental modelling for four mixed-breed female *Bos taurus* cattle (15-18 moa) treated with carprofen (Norocarp) at 1.4 mg/kg body weight. Also shown are the geometric mean, standard deviation (SD) and coefficient of variation (CV) as a percentage.

Parameter	Unit	Cow Code				Geometric mean	SD	CV (%)
		34/08	16/07	13/08	27/07			
C_{max}	µg/mL	10.00	13.40	14.00	13.10	12.52	1.79	14.29
T_{max}	h	9.00	12.00	7.00	12.00	9.76	2.45	25.10
AUC_{last}	µg/mL*h	737.40	891.90	814.90	876.05	827.78	70.13	8.47
AUC_{inf}	µg/mL*h	893.75	1108.75	970.45	1071.57	179.20	97.67	16.90
λ	h^{-1}	0.02	0.01	0.02	0.01	0.01	0.00	5.26
$AUMC_{last}$	µg/mL*h^2	33811	40339	34112	38404	36561	3224	8.82
T_{half}	h	44.58	49.98	45.27	47.96	46.90	2.49	5.32
MRT	h	70.07	73.95	64.85	70.36	69.73	3.75	5.37
Cl/F	L/kg*h	0.00	0.00	0.00	0.00	0.00	0.00	9.92
V_z/F	L/kg	0.22	0.20	0.20	0.19	0.20	0.01	5.03
V_{ss}/F	L/kg	0.24	0.20	0.20	0.20	0.21	0.02	8.71

Supplementary Table 2: Mean tissue concentrations (mg/kg) of carprofen from three samples from four female Friesian *Bos taurus* cattle (9 months of age) treated with Norocarp at 2.8 mg/kg body weight. Also shown are the arithmetic means and standard deviations (SD) of the animal means and maximum residue exposure (MRE).

Tissue	Cow Code				Mean	SD	CV (%)	Maximum Residue Exposure
	1356	1357	1342	1343				
Liver	4.04	7.58	5.25	6.12	5.75	1.49	25.91	13.41
Fat (omental)	2.19	3.58	1.87	7.32	3.74	2.50	66.84	16.59
Kidney	5.28	6.55	8.26	10.79	7.72	2.38	30.83	19.97
Muscle (quadriceps)	5.62	6.32	4.19	8.00	6.03	1.59	26.37	14.19
Muscle at injection site	54.19	29.98	559.58	511.50	289.05	286.05	98.96	1759.98

The maximum residue exposure (MRE) was calculated at the 95% upper population tolerance at 95% confidence as per the regulatory standard (EUDRALEX)

Supplementary Table 3: Carprofen dose and exposure to *Gyps* vultures in Phase 1 (kidney) and Phase 2 (muscle) of the toxicity study. Also shown are individual codes, vulture body weights, amount of tissue consumed and concentration of carprofen used to calculate dose and exposure.

Vulture	Body Weight (kg)	Tissue	Tissue Consumed (kg)	Concentration in Meat (mg/kg)	Exposure (mg)	Dose Consumed (mg/kg)
19042	5.35	Kidney	425	5.28	2.24	0.42
30402	5.95	Kidney	275	8.26	2.27	0.38
31961	6.05	Kidney	490	10.79	5.29	0.87
31977	6.2	Kidney	200	6.55	1.31	0.21
31917	5.25	Muscle	830	4.19	3.47	0.66
30796	5.4	Muscle	835	5.62	4.70	0.87
30258	5.65	Muscle	805	6.32	5.09	0.90
30404	8.55	Muscle	250	4.19	1.05	0.12

771 Supplementary Table 4: Carprofen dose and exposure to *Gyps* vultures in Phase 3 (dose = 4.4mg/kg) and Phase 4 (64 mg/kg) of the toxicity
772 study. Also shown are individual codes and vulture body weights.

Vulture	Body Weight (kg)	Dose Given (mg/kg)	Phase	Exposure (mg)
G31917	5.25	4.4	3	23.1
G31977	6.2	4.4	3	27.28
G30258	5.65	4.4	3	24.86
G30404	8.55	4.4	3	37.62
G19042	5.35	4.4	3	23.54
G30402	5.95	4.4	3	26.18
G30796	5.4	64	4	345.6
G31961	6.05	64	4	387.2

773 Vultures G19042, G30402, G31977, G31917, G30258 & G30404 were adults (older than 5 years); while G31961, G30796 were juvenile (approximately 1 year of age)
774
775

Supplementary Table 5: Descriptive statistics for biochemical parameters from samples of *Gyps* vulture plasma collected in the absence and presence of carprofen. Parameters in the absence of carprofen were obtained from the control groups and samples evaluated prior to treatment from the descriptive pharmacokinetic phase of the study.

Parameter	Unit	In the absence of carprofen					In the presence of carprofen				
		n	Mean	SD	Min	Max	*n*	Mean	SD	Min	Max
Uric Acid	mmol/L	26	0.20	0.09	0.08	0.54	15	0.23	0.09	0.12	0.44
ALT	U/L	26	25.27	11.01	9.00	59.00	14	26.86	10.23	9.00	43.00
ALP	U/L	26	29.15	17.13	1.00	59.00	14	29.71	10.96	17.00	50.00
Na	mmol/L	25	147.63	2.40	144.00	152.40	14	146.90	2.71	141.40	151.40
K	mmol/L	25	3.39	1.06	1.19	5.59	14	2.83	0.68	2.04	4.09