The toxicokinetics of ketoprofen in *Gyps coprotheres*: toxicity due to zero-order metabolism

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Abstract In a safety study, Cape Griffon vultures (*Gyps coprotheres*) were dosed with ketoprofen at single doses of ~1 mg/kg ($n = 5$) and 5 mg/kg ($n = 11$). No toxicity was reported in the 1 mg/kg group, with the AUC_{inf}, V_z and Cl being 10.42 μg/ml h, 0.37 l/kg and 0.10 l/h kg, respectively. Toxicity occurred in the 5 mg/kg group, with 7 of the 11 birds dying. Clinical signs of toxicity included depression, loss of appetite and apparent coma. Animals died within 48 h of dosing. The AUC_{inf}, V_z and Cl in the birds that survived were 52.26 μg/ml h, 0.45 l/kg and 0.10 l/h kg, respectively. The AUC_{inf}, V_z and Cl in the birds those died were 207.90 μg/ml h, 0.26 l/kg and 0.02 l/h kg, respectively. Based on the increase in the AUC_{inf} and C_{max} in the birds that died, we surmise that toxicity resulted from saturation of the metabolic process. While the exact metabolic pathway remains unknown in these vultures, we believe that toxicity may be due to pharmacogenomic differences in the cytochrome P450 pathway.

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Introduction

Diclofenac (2-[2-(2,6-dicholorophenyl amino)phenyl]acetic acid) is an important non-steroidal anti-inflammatory drug (NSAID) in human medicine and is commonly used in the management of pain and inflammation associated with the joints (Burke et al. 2006). Diclofenac is a typical NSAID in that it inhibits the cyclo-oxygense (COX) enzyme system. It is slightly more selective towards the COX II enzyme than to COX I (Burke et al. 2006). Whilst this is important in reducing the incidence of side effects in humans, diclofenac is nonetheless recognised as ulcerogenic, nephrotoxic and hepatotoxic (Burke et al. 2006).

In South Asia, diclofenac is also used in veterinary medicine as an analgesic, anti-pyretic and anti-inflammatory drug in cattle (Oaks et al. 2004). Whilst the drug is probably as effective as many other available NSAIDs (such as ketoprofen, meloxicam, flunixin, carprofen), diclofenac has been very commonly used across the Asian sub-continent due to its widespread availability and cheap price (Oaks et al. 2004; Swan et al. 2006b). Unfortunately, this widespread use is now acknowledged to have led to what may be described as one of the worst environmental catastrophes in the twenty-first century: the complete devastation of resident *Gyps* vulture populations across the Asian sub-continent (Oaks et al. 2004). To date, the once ubiquitous Oriental white-backed vulture (*Gyps bengalensis*) population, which used to number tens-of-millions of birds, has declined by a staggering 99.9% in about 15 years as the result of their exposure to diclofenac residues in the cattle carcasses they commonly fed upon (Green et al. 2007;

Prakash et al. 2007). Further, controlled toxicity studies have now shown that diclofenac is also toxic to three other *Gyps* vulture species (*G. africanus*, *G. coprotheres* and *G. fulvus*) as well as the domestic chicken (*Gallus gallus*), although at a higher dose for the latter (Swan et al. 2006b; Naidoo et al. 2007, 2009). At the same time, it has been clearly shown to be safe at very high doses in Turkey vultures (*Cathartes aura*) (Rattner et al. 2008) and in House crow (*Corvus albus*; unpublished work). Hence, very significant differences in toxicity between different bird species clearly exist.

Since diclofenac was identified as the major cause of *Gyps* declines across the Indian sub-continent in 2004, conservationists have strived to initiate effective conservation measures. Diclofenac manufacture and importation (Kumar 2006) were legally banned for veterinary purposes in the region after meloxicam was identified as a safe alternative (Swan et al. 2006a). Unfortunately, even though veterinary diclofenac is banned, other NSAIDs still pose a potential risk to vulture populations, as no data on their safety are available. In a study by Taggart et al. (2009), ketoprofen ((RS)-2-(3-benzoylphenyl) propionic acid) was found to be present in 0.5% of carcasses available to vultures within India, second only to diclofenac, meloxicam and ibuprofen (Taggart et al. 2009). Although a survey by Cuthbert et al. (2007) showed anecdotal evidence that ketoprofen had been safely used in 12 bird species (>22 individual birds), its safety in vultures remained to be fully characterised (Cuthbert et al. 2007).

In an attempt to investigate the safety of ketoprofen in *Gyps*, we exposed adult captive non-breeding Cape Griffons (*G. coprotheres*) to doses of either 1 mg/kg or 5 mg/kg (*n* = 5 and 11 birds, respectively) of ketoprofen (Naidoo et al. 2010). These doses were based upon the lower and upper ranges of doses recommended for the clinical treatment of birds (Pollock et al. 2005) and were also representative of the range of doses that vultures could receive in the wild if they fed upon carcasses of livestock that had been dosed with ketoprofen in the hours before death (Naidoo et al. 2010). At 5 mg/kg, 7 out of 11 birds died with typical signs previously associated with diclofenac toxicity, i.e. animals became increasingly depressed (from about 24 h) until eventually becoming semi-comatose prior to death (at ~40 h). On necropsy, all birds also showed signs of bilateral severe nephrotoxicity and hepatotoxicity, with diffuse visceral gout. The clinical pathology was also typical of diclofenac toxicity, in that extreme increases in uric acid and plasma ALT activity were observed. As such, it was concluded that ketoprofen is toxic to *Gyps* vultures and should, therefore not be considered a viable safe alternative to diclofenac (Naidoo et al. 2010).

From the ketoprofen study mentioned above, in addition to monitoring the safety of ketoprofen, we also undertook regular post-treatment blood sampling in order to establish the pharmacokinetics of ketoprofen at both doses in the Cape griffon. Here, we present these results as a toxicokinetic study, and specifically focus our discussion of results on the interesting significant difference in pharmacokinetic parameters between birds that died, or survived, at the 5 mg/kg dose.

Materials and methods

Animals and dosing

Sixteen adult Cape Griffon vultures were dosed by oral gavage with ketoprofen at a 1 mg/kg (*n* = 5; Neoprofen, India) or 5 mg/kg (*n* = 11; Ketofen 10%, South Africa) body weight dose, with a 1 ml syringe (Braun). The birds were obtained from the Vulture Programme of the Rhino and Lion Non-profit Organisation. Due to the endangered status of the species, only captive non-releasable and non-breeding birds which failed to establish captive breeding pairs were selected for the study. The birds were housed in individual cages (2 × 3 × 3 m) with hard soil floors and wooden perches. Birds had constant access to water, and were fed 1 kg of porcine meat twice per week. The vultures had not been fed during the 48 h period prior to treatment.

Ketoprofen analysis

Blood samples (5 ml) were collected from the tarsal vein or the wing vein (when necessary), immediately before drug administration and at 5, 30 min, and at 1, 1.5, 2, 6, 8, 10 and 24 h after treatment. Samples were collected into 5 ml syringes (Braun) with a 22G needle, then immediately transferred into heparinised or plain vacutainer tubes. Within 2 h of collection, these blood samples were centrifuged at ≈3,000*g* for 15 min, and the supernatant of each sample was transferred to labelled polycarbonate tubes. All samples were stored at −20°C until they were transferred for analysis on dry ice to the University of Aberdeen, Scotland, UK.

Analysis of ketoprofen was undertaken using a validated LC-ESI/MS methodology (Taggart et al. 2009). Samples of plasma (0.5 ml) were vortex mixed with 2 ml of HPLC grade acetonitrile, then centrifuged at ≈1,000*g* for 5 min. Quantification and confirmation were undertaken at m/z of 253.1 and 197.1, respectively, in negative ion mode following compound separation on an Agilent ZORBAX 300SB-C18 column (4.6 mm × 150 mm, 5 μm). Limits of quantification were equivalent to 12 μg/kg in liver tissue and plasma; and in spiked plasma (crow, *Corvus* sp.), recovery of ketoprofen was 94.2% (±2.3%, *n* = 3).
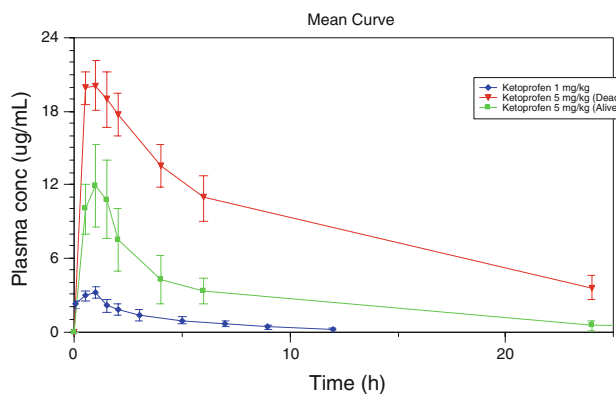
Pharmacokinetic analysis

All curve fitting was undertaken in Kinetica 4.4.1 (Thermo Electron Corporation) using non-compartmental modelling. The elimination rate constant (λ) and the elimination half-life ($T_{1/2}$) were calculated from the terminal phase. The area under the plasma concentration versus time curve (AUC) was obtained using the linear trapezoidal rule, up until the last measurable concentration (C_{last}), with extrapolation to infinity (AUC_{inf}) using the elimination rate constant (C_{last}/λ). Total body clearance (Cl), volume of distribution (V_z), volume of distribution at steady state (V_{ss}) and the mean residence time (MRT) were calculated using standard formulae.

Results

As previously reported (Naidoo et al. 2010), no mortality occurred within the low dose group, while 7 of the 11 birds treated at 5 mg/kg died with the clinical signs described above. Samples were collected from all animals up to 24 h for the 5 mg/kg dose group. Signs of toxicity became evident at ±28 h post-dosing which prompted the termination of the study. All 11 animals treated at 5 mg/kg were immediately taken to the Onderstepoort Veterinary Academic Hospital (OVAH) for emergency treatment. Furosemide, lactated Ringers fluid and sodium bicarbonate were administered. During treatment, animals were clinically depressed, had increased plasma potassium levels and increased uric acid accumulation. Despite treatment, all animals showed depression at the onset of therapy and then died at intervals during the subsequent 12 h. The pharmaco-kinetic study was terminated prior to the initiation of the listed concurrent therapy.

The pharmacokinetic parameters obtained by the non-compartmental analysis are presented in Table 1, and the plasma concentration profile with time is given in Fig. 1. Ketoprofen had a V_z of ∼0.40 l/kg (0.37 and 0.45 l/kg) for both the 1 mg and 5 mg/kg (survived) groups. This was reduced for the 5 mg/kg (died) group to ∼0.26 l/kg. A similar trend was evident in terms of clearance, where, with the 1 and 5 mg/kg (survived) groups, similar figures of approximately of 0.10 and 0.09 l/h kg, respectively, were recorded, but this was substantially reduced to 0.02 l/h kg for the birds that died. The $T_{1/2}$ of ketoprofen at 1 mg/kg was very rapid at 2.66 h, and similar at 3.24 h for the 5 mg/kg (survived) group. The half-life was markedly increased in the birds that died at 7.38 h, i.e. being ∼2.5 times longer.

Fig. 1 Ketoprofen concentrations ± SE in plasma versus time, for vultures treated orally at 1 mg/mg ($n = 5$), 5 mg/kg (died) ($n = 7$; animals that died following dosing) and 5 mg/kg (alive) ($n = 4$, animals that survived dosing)

Table 1 Pharmacokinetic parameters obtained by non-compartmental modelling following the dosing of *G. coprotheres* with ketoprofen at two dose levels

Parameter	1 mg/kg survived		5 mg/kg survived			5 mg/kg died			
	GeoMean	SE	GeoMean	SE	5:1	GeoMean	SE	5:1	5:5
C_{max} (µg/ml)	3.08	0.45	10.77	3.26	3.50	21.00	1.88	6.83	1.95
T_{max} (h)	0.87	0.10	0.71	0.14	0.81	0.79	0.14	0.90	1.11
AUC_{last} (µg/ml h)	9.79	3.23	50.31	17.71	5.14	156.51	33.14	15.99	3.11
AUC_{inf} (µg/ml h)	10.42	3.70	52.26	18.36	5.02	207.90	49.40	19.96	3.98
λ (h^{-1})	0.26	0.04	0.21	0.14	0.82	0.09	0.03	0.36	0.44
$AUMC_{last}$ (µg/ml (h)^2)	28.35	14.40	224.31	141.43	7.91	956.18	396.86	33.73	4.26
T_{half} (h)	2.66	0.46	3.24	1.59	1.22	7.38	1.72	2.78	2.28
MRT (h)	3.49	0.83	4.97	2.05	1.43	10.84	2.44	3.11	2.18
Cl (l/h kg)	0.10	0.05	0.10	0.09	1.00	0.02	0.01	0.25	0.25
V_z (l/kg)	0.37	0.13	0.45	0.10	1.21	0.26	0.03	0.70	0.57
V_{ss} (l/kg)	0.33	0.06	0.48	0.13	1.42	0.26	0.03	0.78	0.55

The ratio represents the quotient for the two dose groups, i.e. 5:1 represents the comparison between parameters at the 5 and 1 mg/kg dose, while 5:5 represents a comparison between animals that died and animals that survived at the 5 mg/kg dose

In terms of the rate and extent of absorption, the C_{max} was threefold higher for the 5.0 mg/kg (survived) group than for the 1 mg/kg group. In contrast, in the birds that died, it was almost 7 times higher at 21.00 µg/ml. As expected, the AUC and AUC_{inf} for the 5.0 mg/kg (survived) group were higher than for the 1 mg/kg group by a factor of around five. In comparison, however, for the birds that died, we noted a threefold to fourfold increase in their exposure in comparison with the birds that survived at 5 mg/kg. Although the high dose group was characterised by a larger AUC, this did not translate into a longer MRT, i.e. the 1.0 and 5.0 mg/kg (survived) groups had similar MRTs. The 5 mg/kg (died) group had a substantially higher MRT, which was most likely related to the greater extent of exposure and the longer observed $T_{1/2}$.

Discussion

While different products were used in the two stages of testing, we consider the different results and mortalities to be a consequence of dose rates, rather than due to any differences in products. The use of different formulation for the 1 and 5 mg/kg dose was unfortunately as a result of non-availability of the Neoprofen for the second part of the study. The use of a different formulation was not considered to be too significant, as both products were in solution, and they would, therefore, be considered bioequivalent. In addition when comparing the pharmacokinetic parameters between groups given 1 and 5 mg/kg (that survived), these groups showed very similar data for all parameters, with the exception of AUC. The AUC and AUC_{inf} showed a fivefold increase (as the dose increased fivefold), implying that the absorption of ketoprofen in birds that lived followed the principles of linear absorption. The latter also demonstrated that the two formulations were absorbed in the same manner, as would be expected from a biopharmaceutical Class I product (Lobenberg and Amidon 2000).

The $T_{1/2}$ of 3.24 h in vultures that survived at the high dose was substantially longer than the 0.5 h reported for Japanese Quail (*Coturnix japonica*), which at present is the only other avian species in which the pharmacokinetics of ketoprofen have been described to the best of our knowledge. Graham et al. (2005) also reported that the $T_{1/2}$ of ketoprofen in the Japanese Quail was similar to the $T_{1/2}$ of salicylic acid, flunixin and meloxicam reported in five other avian species. This was, however, not the case as the $T_{1/2}$ of ketoprofen in our study of *Gyps coprotheres* is sevenfold higher than that found for meloxicam in the same species (0.5 h) (Naidoo et al. 2008a). When we compare the ketoprofen $T_{1/2}$ in birds that died with those that survived in this experiment, the $T_{1/2}$ was ~2.5 times longer in birds that died, implicating the $T_{1/2}$ in toxicity. This would indicate

that metabolism had perhaps reached near-zero- or zero-order in these birds, due to saturation of the metabolic process. This best explains the resultant toxicity, and the changes in pharmacokinetic parameters seen between the birds that survived and died.

Whilst the mechanism behind ketoprofen's toxicity in *G. coprotheres* is unknown, we have previously proposed that diclofenac toxicity is related to the ability of NSAIDs to interfere with uric acid transport at the cellular level (Naidoo and Swan 2009). It is plausible that ketoprofen toxicity occurs in the same way, i.e. that the renal tubular epithelial cells become over-whelmed to such an extent that toxicity results because of oxidative damage. Most importantly, it has also been suggested that differential NSAID toxicity is related to $T_{1/2}$ and Cl, i.e. that diclofenac (which is highly toxic) has a longer $T_{1/2}$, and meloxicam (which is safe) has a very short $T_{1/2}$.

At present, the enzymes which control the metabolism of NSAIDs in *Gyps* vultures are not described. However, in studies on the pharmacokinetics of meloxicam, Naidoo et al. (2008a) described the presence of a glucoronide and hydroxyl metabolite, thereby indicating that the metabolism of meloxicam at least, in *Gyps* vultures, is potentially similar to that found in mammals (Naidoo et al. 2008a; Lees 2009). In the recognised metabolic pathways described for mammals, the presence of the hydroxyl metabolite indicates the involvement of the cytochrome P450 (CYP450) enzyme system, whilst the glucoronide metabolite indicates the involvement of the UDP glycosyltransferase enzyme (Rodrigues 2005). At this stage, it is plausible that either of these enzymatic processes may enforce the rate limiting step in the metabolic transformation of ketoprofen. Evidence for this comes from mammalian metabolic studies in the cat and man.

From veterinary literature, the NSAIDs in particular paracetamol (acetaminophen), has been widely associated with severe toxicity in the cat (Lees 2009). Its toxicity in cats has been fully elucidated and results from deficiencies in phase II glucuronidation enzymes (Court and Greenblatt 1997; Lascelles et al. 2007). With rapid saturation of the limited feline glucoronide enzymatic system, phase I reactive metabolites accumulate within the body. It is these radicals that are then responsible for the toxic effects of paracetamol as they induce severe oxidative damage in the major organ systems. Deficiencies in the CYP450 system in *Gyps* may also explain the toxicity we observe for ketoprofen and diclofenac, as this would allow the accumulation of the primary drugs, which are known to induce free radical cellular damage via the inhibition of the COX pathway or via other mechanisms (Mahmud et al. 1996; Galati et al. 2002).

Of the two enzyme systems mentioned above, deficiencies in the latter (i.e. CYP450 rather than glucuronidation) is more plausible in our opinion, as significant pharmacogenomic

differences have also been described for this system within humans (Rodrigues 2005). CYP450 pharmacogenomic differences within *G. coprotheres* (as opposed to a total deficiency in the glucoronidation pathway) would explain why only 7 of the 11 animals died following dosing at the same level (5 mg/kg). Also, it has been established in humans that the CYP2C9 enzyme is important in the clearance of ibuprofen, indomethacin, flurbiprofen, celecoxib, valdecoxib, lornoxicam, tenoxicam, meloxicamand piroxicam, whilst another (yet to be fully described) CYP450 enzyme seems to be more important in the clearance of sulindac, naproxen, ketoprofen, diclofenac, rofecoxib and etoricoxib. If we consider that meloxicam was rapidly metabolized in *Gyps*, but that both diclofenac and ketoprofen have comparatively longer $T_{1/2}$ (and are both toxic), *G. coprotheres* are perhaps more likely to be deficient in this (yet to be fully described) enzyme system.

The increased ketoprofen $T_{1/2}$ in birds that died occurred alongside increases in the AUC and C_{max}, tends to indicate that ketoprofen undergoes a degree of presystemic elimination. While the absolute/relative bioavailability of ketoprofen has not been established for *Gyps* vultures, Graham et al. (2005) showed that the drug is poorly absorbed in Japanese quail, and that it had an absolute bioavailability of only 24% (Graham et al. 2005). Diclofenac has a relative bioavailability of 50% in chicken and is known to undergo presystemic elimination in *G. coprotheres* (Naidoo et al. 2007, 2009). Assuming ketoprofen and diclofenac share the same enzymatic pathways, it is plausible that ketoprofen also has a substantial first-pass effect in *G. coprotheres* If the hepatic enzyme systems are being saturated as stated above, less of the drug would undergo presystemic elimination, i.e. the extent of absorption would be increased. Since C_{max} and AUC are direct indicators of the extent of absorption, the observed increases in these parameters supports the idea that in birds that died, zero-order metabolism was occurring. Similar increases in AUC and C_{max} have been described for midazolam in humans, and this was reportedly linked to saturation of the presystemic elimination pathways (Bornemann et al. 1985; Thummel et al. 1996).

It is also interesting that we observed significant changes in the Cl of ketoprofen in birds that died (compared to those that did not). While this could simply be linked to changes in $T_{1/2}$, other factors may have played a role. Whilst monitoring the pharmacokinetics of ketoprofen, frequent blood sampling allowed us to make a direct evaluation of renal excretion via the evaluation of uric plasma levels. In birds, uric acid is directly cleared by the kidneys; hence, changes in levels are a good indicator of renal function. Naidoo et al. (2008b) showed that uric acid levels in healthy *Gyps africanus* (in the absence of feeding) should remain constant in plasma. However, in birds that died, despite having not fed before treatment, increases in plasma uric acid concentrations were very evident, which could only have resulted from direct renal tubular epithelial (RTE) cell damage. Whilst RTE cell damage should not directly influence drug clearance (which requires extensive metabolism), studies in humans with renal failure have also shown a direct decrease in ketoprofen clearance (Grubb et al. 1999). The reason for the change in the clearance of ketoprofen during kidney failure is yet to be explained.

Conclusion

This study highlights that yet another NSAID (beyond diclofenac) is inherently toxic towards *Gyps* vultures, and perhaps a wider range of avian species. Ketoprofen can no longer be considered safe towards *Gyps*, and as such cannot (and should not) be used as an alternative within veterinary medicine on the Indian sub-continent to replace diclofenac. The use of ketoprofen as a veterinary NSAID for treating livestock in Southern Africa and Europe also raises concerns over the potential impact of this drug on vulture populations within these regions. Meloxicam now remains the "only" safe alternative available which is accepted as therapeutically effective and safe towards a wide range of avian scavengers. At the same time, this study also highlights the continuing need to test a wider range of available NSAIDs on a wide range of avian scavengers, with *Gyps* vultures being a particular priority.

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