

Diclofenac disposition in Indian cow and goat with reference to *Gyps* vulture population declines

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Residual diclofenac in livestock carcasses in India poses a continuing risk to Gyps vultures.

Abstract

Gyps vultures across India are declining rapidly and the NSAID diclofenac has been shown to be the major cause. Vultures scavenge livestock carcasses that have been treated with diclofenac within the days preceding death. We present data on diclofenac disposition in Indian cow and goat, and field data on the prevalence of diclofenac in carcasses in the environment. In the disposition experiment, animals were treated with a single intramuscular injection of diclofenac at 1000 µg kg⁻¹ bw. In cow, diclofenac was detectable in liver, kidney and intestine up to 71 h post-treatment; in plasma, half-life was 12.2 h. In goat, tissue residues were undetectable after 26 h. Prevalence of diclofenac in liver from 36 dead livestock collected in the field was 13.9%. Data suggest that diclofenac residues in Indian cow and goat are short-lived, but diclofenac prevalence in carcasses available to vultures may still be very high.

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1. Introduction

Since the early 1990s *Gyps* vulture populations have collapsed across the Indian subcontinent (Gilbert et al., 2002; Prakash et al., 2003; The Peregrine Fund, 2004). Populations of at least three species are known to have been affected (oriental white-backed *Gyps bengalensis*, long-billed *G. indicus* and slender-billed *G. tenuirostris* vultures) and have declined by more than 97% since 1992 (Prakash et al., 2003; Green

et al., 2004). All three species are now listed as critically endangered by the IUCN – World Conservation Union (Hilton-Taylor, 2000), despite the fact that *G. bengalensis* was, in the previous decade, considered to be the commonest large raptor in the world (Houston, 1985).

Considerable evidence now indicates that the catastrophic vulture decline has been caused by the non-steroidal anti-inflammatory drug (NSAID) diclofenac, which is widely used to treat livestock across the Indian subcontinent (Green et al., 2004; Oaks et al., 2004; Shultz et al., 2004). Vultures are exposed to diclofenac through consuming the carcasses of treated livestock. In experiments, captive *G. bengalensis* died after feeding on tissues of domestic animals that had received a normal veterinary dose of the drug a few hours before death (Oaks et al., 2004). Post-mortem examinations revealed

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that birds had died from renal disease, and all birds showed extensive visceral gout with deposits of uric acid on and within internal organs due to kidney failure. The same clinical signs were found in a high proportion of dead or dying wild vultures in Pakistan, India and Nepal, and all kidney samples analysed from birds with gout contained residues of diclofenac, whereas none of the samples from birds without gout did so (Oaks et al., 2004; Shultz et al., 2004). More recently, diclofenac was found to be lethal to *G. fulvus* and *G. africanus*, and it seems likely that it is equally toxic to all eight *Gyps* species (Swan et al., 2006a).

Despite the mounting evidence regarding the role of diclofenac (Prakash et al., 2005), its importance as the cause of the vulture population crash in India has been questioned (Arun and Azeez, 2004; Changani and Mohnot, 2004). In certain areas of India vultures can still be found in areas where diclofenac is likely to be used (GEER, 2005), although the numbers reported are very small compared to those sighted in the same areas during the early 1990s, despite the intensive survey efforts recently employed. Likewise, other potential agents of decline have also been suggested as likely to reduce the survival and nesting success of vultures (Arun and Azeez, 2004; Changani and Mohnot, 2004). Whilst consideration of alternative or contributory causes remains important, the evidence that diclofenac is the major cause of vulture declines appears overwhelming. In particular, population modelling of vulture demography and mortality indicates that the observed high proportion of deaths of wild vultures attributable to diclofenac constrains the scope for hypothetical causes of decline other than diclofenac poisoning to be no more than minor contributory effects (Green et al., 2004).

Diclofenac is rapidly metabolized in mammals (EMEA, 2004), so livestock would presumably have to die within hours or days of treatment for their tissues to contain sufficiently high concentrations of diclofenac to pose a risk to scavenging vultures. For the domestic cow most common in Europe (*Bos taurus*) the half-life in plasma is 11.3 h (for intramuscular administration; EMEA, 2004) and after 144 h residues in muscle tissue are approximately 1% of levels at 3 h after diclofenac administration (EMEA, 2004). Within the Indian sub-continent domestic and feral ungulate stocks are dominated by Indian cow (*Bos indicus*) and water buffalo (*Bubalus bubalis*), with 185 and 98 million animals respectively estimated in India in 2003 (NDDDB, 2006). Domestic goats (*Capra hircus*) and sheep (*Ovis aries*) with populations of 124 and 61 million respectively (NDDDB, 2006) are the two other most abundant species of livestock. The disposition of diclofenac is unknown within these species, and while *B. taurus* and *B. indicus* are closely related, the pharmacokinetics of NSAIDs can vary greatly between species (Baert and De Backer, 2003; EMEA, 2004). Consequently, despite our knowledge of diclofenac residues in *B. taurus* it is not possible to assume similar results will be found in the main livestock groups occurring within the Indian subcontinent.

If residues of diclofenac persist for longer periods within the tissues of Indian livestock, then the potential risk of vultures encountering a carcass with a lethal dose is greatly

increased. Alternatively, if the disposition in tissue is similar to that found in *B. taurus*, then there would need to be a significant level of livestock mortality in the days immediately post-treatment and a reasonably high number of dosed livestock for there to be sufficient diclofenac within the environment to have driven the observed rate of vulture population decline.

In this study we report on the disposition of diclofenac within the Indian cow and the domestic goat, and secondly, provide initial data regarding the environmental prevalence of diclofenac within livestock carcasses collected from the field.

2. Methods

2.1. Diclofenac residues in tissues of treated livestock

Tissue residue times for diclofenac were studied in the Indian cow and the domestic goat. All cows were obtained from a farm in Kerala State in Southern India, where cattle are kept for milk production and slaughtered for meat. Goats were obtained from Haryana State. Cows were female and ranged from 1 to 7 years of age (mean 3.8 years), with an average mass of 202 kg (range 30–300 kg). Nine animals were in “fair to good” condition, and three were described as being in poor condition. Goats were female and from 2 to 6.5 years old (mean 4.2 years), with an average mass of 22 kg (range 12–28 kg), and all except one animal were in “good” or “fair” condition.

The experimental design was of six treatments with two replicate groups of animals. Cows were slaughtered at (on average) 21 h, 46 h, 71 h, 7 days (167 h) and 14 days (334 h) following treatment with diclofenac. Two further animals were slaughtered without diclofenac treatment as a control. Treatment of goats followed the same experimental protocol, with two control animals and the remaining animals slaughtered at (on average) 4 h, 26 h, 73 h, 7 days (168 h) and 14 days (336 h) post-treatment. Both cows and goats were treated with a single intra-muscular injection of diclofenac (into the neck) at 1000 µg kg⁻¹ body weight (bw). The standard veterinary dose within India would normally involve administering this dosage for a number of consecutive days, but this would commonly depend upon the condition being treated. A proprietary formulation of injectable diclofenac manufactured and sold in India was used (diclofenac sodium 2.5%; 3D Vet, Intas Pharmaceuticals, Ahmedabad, India). All of the cows and goats in the experiment were kept for more than 1 week prior to dosing, to ensure that they were free from any previous NSAIDs that they may have been treated with.

Following slaughter, tissue samples of approximately 25–30 g were immediately taken from the kidney, liver, intestine and muscle and frozen at –20 °C (intestine tissue samples were not taken from the goats). Samples of intestine were taken from the small or large intestine and muscle samples were taken from either the intercostals or gluteal region on the opposite side to the diclofenac injection site.

In order to determine if diclofenac degrades in tissues following death, sub-samples of liver and kidney were taken from the cows and goats slaughtered at 21 and 4 h respectively after treatment (i.e. $n = 2$ tissues for each species at each time point), and these were then kept at ambient temperature, allowing desiccation and decomposition to occur (but excluding insects and other scavengers). The samples were then frozen 24 h and 48 h after death. In order to assess the natural variation in concentration within these tissues, five sub-samples of fresh liver and kidney were also taken from one goat (slaughtered 4 h post-treatment).

During the course of the experiment, blood samples (5 ml) were taken from cows via direct veno-puncture of the jugular vein, using disposable syringes at 15 min, 30 min, 1, 2, 3, 4, 6, 12, 24, 36 and 48 h post-treatment. These samples were immediately centrifuged to separate the plasma (at 1000 × g for 10 min) which was subsequently frozen at –20 °C in Eppendorf vials.

2.2. Carcass sampling (field)

Tissue samples from Indian cow ($n = 30$) and buffalo ($n = 6$) were collected from two carcass dumps, where vultures are known to feed, near the

towns of Bikaner ($n = 15$) and Bayana ($n = 2$) in Rajasthan State, and from a slaughterhouse in the State of Kerala ($n = 13$); samples were also taken opportunistically in the field in the vicinity of Pinjore ($n = 6$) in Haryana State. These sampling sites were identified opportunistically and are not necessarily a representative sample of locations at which livestock tissue is available to vultures. However, neither was there any reason to think that they would have atypical prevalence of diclofenac-treated animals. Liver and kidney were removed from carcasses by skinners working in the carcass dumps/slaughterhouse, and tissue sub-samples of approximately 30 g were then cut from these samples. The selection of dead livestock carcasses was made by skinners and may not have been random. However, there is no reason to believe that there was likely to be a consistent bias with respect to the species, age or condition of dead animals. After collection all liver and kidney tissues were frozen at -20°C .

2.3. Diclofenac extraction and analysis

Extraction of diclofenac from tissues/plasma was achieved using 0.5 g of sample (weighed to an accuracy of ± 0.001 g), extracted using 2 ml of HPLC grade acetonitrile (MeCN). The sample was weighed into a new glass test tube, MeCN added, and the mixture homogenized for 30 s using an Ultra Turrax IKA T8 homogenizer. The mixtures were then centrifuged at $1000 \times g$ for 5 min and the supernatant filtered using disposable PTFE/PE syringe filter units of 0.45 μm . The filtered extract was then stored in crimp top LC vials at -20°C until analysis.

Diclofenac levels were determined by liquid chromatography–electrospray ionisation mass spectrometry (LC-ESI/MS) using an Agilent 1100 series instrument (1946D). The instrument was calibrated using seven standards ranging from 5 to $1000 \mu\text{g l}^{-1}$ in diclofenac concentration, generated using diclofenac sodium salt (Sigma-Aldrich, D6899). The calibration was linear across this range with an r^2 value of at least 0.999. Both a blank and a mid-range standard were analysed every 10 samples in order to monitor for instrumental drift and/or diclofenac carry-over. Diclofenac was monitored by the MS at mass/charge ratios of 294 and 296 (the deprotonated and protonated ions) in the negative ion mode, with the capillary voltage set at 3500 V and the fragmentor voltage at 80 V. The instrument was calibrated against both masses and each sample ran through the instrument twice; this allowed four values for diclofenac to be gained (at 294 and 296 in two runs), the mean of which was taken to be the concentration in the extract. The limit of quantification (LOQ) for this technique (back calculated to wet tissue concentration) was found to be $10 \mu\text{g kg}^{-1}$ and the limit of detection (LOD) was $4 \mu\text{g kg}^{-1}$.

Chromatographic separation was achieved on the LC using a Waters Xterra MS C18 column (3.9 mm \times 150 mm, 5 μm). Samples and standards (20 μl) were subjected to a binary gradient elution profile using 0.1% acetic acid in water (solution A) and 100% MeCN (solution B). The starting conditions were 75% A/25% B for 0.1 min, followed by a 15 min linear gradient from 75% A/25% B to 5% A/95% B, then by a 5 min column wash step of 5% A/95% B, and a 10 min re-equilibration step with 75% A/25% B. The flow rate was set at 0.7 ml min^{-1} , samples were continuously kept cool at 4°C using an autosampler with thermostat, and the column temperature was maintained at 40°C during analysis.

2.4. Pharmacokinetic data analysis

Analysis of diclofenac levels in plasma followed the standard procedure proposed by the FAO-WHO (2005) for pharmaceutical residue levels. Data were analysed by simple-linear regression, with diclofenac values log₁₀ transformed before analysis. The maximum concentration (C_{max}), the time at which this is reached (t_{max}), and the half-life of diclofenac in plasma were calculated using add-in functions for Microsoft Excel (Usansky et al., 2006). It was not possible to use this method to analyse diclofenac levels in tissues as the relationships were not linear. The rate of decline soon after treatment was more rapid than the rate observed later (see Fig. 1). In a combined analysis of data from this experiment and those reported in the EMEA (2004) (Green et al., 2006) it is apparent that the pattern of change of diclofenac concentration in liver, kidney, intestine and muscle of *B. indicus* and *B. taurus* is

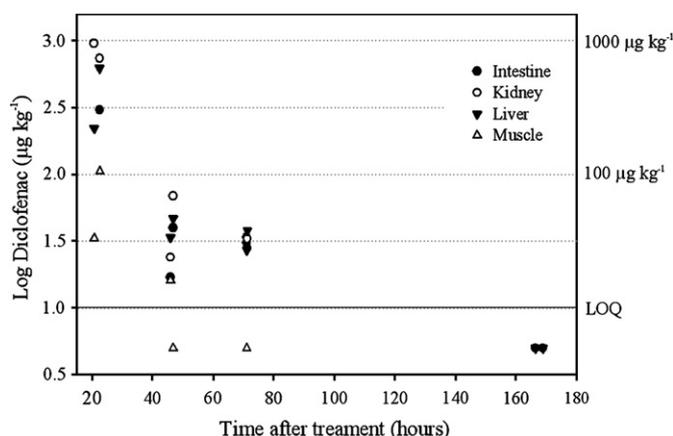


Fig. 1. Disposition of diclofenac in Indian cow (*Bos indicus*) intestine, kidney, liver and muscle tissues; where residues fall below the LOQ, the final time point shown is given as $0.5 \times \text{LOQ}$ ($5 \mu\text{g kg}^{-1}$).

somewhat complex. For this reason, we defer analyses of rates of change in these tissues to Green et al. (2006).

3. Results

3.1. Tissue and blood residues

The five replicate liver and kidney tissue samples taken from one goat indicated relatively low diclofenac variability within organs (10% relative standard deviation (RSD) in kidney, 14% in liver).

Tissue residues in treated goats were below the LOQ of $10 \mu\text{g kg}^{-1}$ in the 26 h post-treatment samples and at all subsequent time points. In the 4 h post-treatment samples, goat kidney residues varied from 170 to $330 \mu\text{g kg}^{-1}$, liver from 35 to $59 \mu\text{g kg}^{-1}$ and muscle from 44 to $465 \mu\text{g kg}^{-1}$ ($n = 2$ in all cases).

In *B. indicus*, tissue residues were detectable above the LOQ in liver, kidney and intestine up to 71 h post-treatment, and in muscle up to 46 h post-treatment (see Fig. 1). Diclofenac remained detectable in plasma up to 48 h post-treatment, beyond which, further samples were not taken (see Fig. 2; $r^2 = 0.76$, $p < 0.0001$). The C_{max} in *B. indicus* was $4.01 \mu\text{g ml}^{-1}$, at a t_{max} of 0.5 h post-dosage, and the half-life was found to be 12.2 h.

The diclofenac concentration in kidney tissue showed no significant post-mortem change for up to 48 h (Fig. 3; tested by one-way ANOVA). The variation in concentrations found in kidney tissues as they aged was on average $10 \pm 8\%$ (average %RSD \pm SD; $n = 4$), and therefore, similar to the level of variation naturally found in different parts of these tissues when fresh. In liver, diclofenac levels appeared to decrease over the first 48 h after death, presumably because of enzyme driven post-mortem degradation (endogenous or otherwise) and/or diclofenac instability. This decrease over time was only significant in goat liver (one-way ANOVA, $p = 0.028$), and the variation in concentration found in liver tissues as they aged was on average $86 \pm 33\%$ (average %RSD \pm SD; $n = 4$). It should be noted that these data were generated using

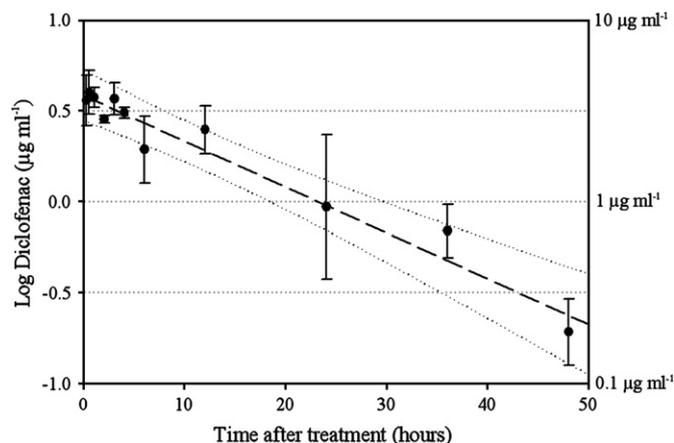


Fig. 2. Disposition of diclofenac in Indian cow (*Bos indicus*) plasma (with regression, 99% CI and standard error bars).

tissue concentrations normalised to 82% moisture content. Obviously, in the field, as tissue such as kidney desiccates, the true concentration per kilogram of tissue will actually tend to increase as moisture is lost.

3.2. Carcass sampling

Of the 36 carcass samples taken, five were found to contain diclofenac concentrations above $10 \mu\text{g kg}^{-1}$ (Table 1). This equates to an overall prevalence of 13.9%, with a 95% binomial confidence interval of 4.7–29.5%. Of these five, two were found in samples taken opportunistically from one buffalo and one Indian cow sampled near Pinjore. The remaining three were all cows from the Bikaner carcass dump. Given that only six samples were taken in and around Pinjore, and 15 were taken at Bikaner, by site the diclofenac prevalence was 33% and 20% respectively.

4. Discussion

The results of this work show that residence times of diclofenac in the tissues of *B. indicus* vary between tissues. Tissues

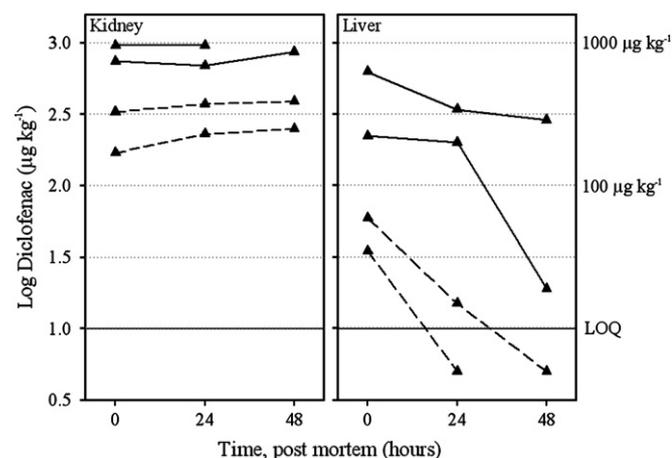


Fig. 3. Residual diclofenac levels in fresh, 24 and 48 h old liver and kidney from both Indian cow (solid lines) and goat (dashed lines).

Table 1

Levels of diclofenac ($\mu\text{g kg}^{-1}$) detected in five ($n = 36$ in total) field collected animals from Pinjore and Bikaner

Site	Species	Liver	Kidney
Pinjore	Buffalo	<LOQ	19
Pinjore	Indian cow	391	285
Bikaner	Indian cow	144	67
Bikaner	Indian cow	5387	6914
Bikaner	Indian cow	361	ns

ns, not sampled. Thirty-one animals were found to have no detectable (<LOQ) diclofenac in the liver or kidney.

with the highest initial post-treatment diclofenac concentrations (kidney, liver and intestine) had the longest residence times, but within 1 week (167 h) of treatment diclofenac concentrations were below the limits of quantification in all tissues. The disposition of diclofenac in *C. hircus* is more rapid, as detectable levels were not found in tissue at or beyond 26 h post-dosage.

The EMEA (2004) describe the disposition of diclofenac in tissues of eight dairy cows (*B. taurus*) and in 16 pigs (*Sus scrofa*) given six daily intramuscular doses of $2500 \mu\text{g diclofenac kg}^{-1} \text{ bw}$. As with *B. indicus* in our study, the EMEA (2004) similarly found the highest concentrations of diclofenac in liver and kidney, with lower concentrations in muscle tissue. In pigs, tissue concentrations were below the LOQ of $5 \mu\text{g kg}^{-1}$ by 1 week post-dosage. In *B. taurus*, at 7 days post-dosage, mean diclofenac concentrations were very low at $25 \mu\text{g kg}^{-1}$, $23 \mu\text{g kg}^{-1}$ and $5 \mu\text{g kg}^{-1}$ in liver, kidney and muscle tissue respectively. Likewise, the disposition of diclofenac in *B. indicus* plasma is also shown to be very similar and as rapid as it is in *B. taurus* and *S. scrofa*. In the above experiment, the EMEA (2004) showed a C_{max} of 4.6 and $4.7 \mu\text{g ml}^{-1}$, at a t_{max} of 3.4 and 0.5 h and with an elimination half-life of 11.3 and 3.4 h for *B. taurus* and *S. scrofa* respectively. Herein we report a comparable bovine half-life of 12.2 h, a slightly lower C_{max} of $4.01 \mu\text{g ml}^{-1}$ at a t_{max} of 0.5 h. Given that the dosage used in this study was $1000 \mu\text{g diclofenac kg}^{-1} \text{ bw}$ it is not unreasonable to expect that the t_{max} would be reached earlier. This indicates that the disposition of diclofenac in Indian livestock species to which vultures will be commonly exposed is not unusual or excessively slow (in comparison to other ungulate species previously studied). Consequently, livestock must die within a few days of dosage with diclofenac to present a significant risk to scavenging vultures.

The EMEA (2004) also report on 16 young cows treated daily by intramuscular injection for 6 days with a $2500 \mu\text{g diclofenac kg}^{-1} \text{ bw}$ dose, and slaughtered at 3, 12, 24 and 144 h post-treatment. Mean diclofenac concentrations fell between 3 and 144 h from 2874 to $27 \mu\text{g kg}^{-1}$ in liver, from 3244 to $21 \mu\text{g kg}^{-1}$ in kidney and from 470 to $5 \mu\text{g kg}^{-1}$ in muscle. Taking data from all tissue types and from both this and the experiments using eight dairy cows mentioned above, diclofenac residues are shown to fall (on average) from 100% at 3 h to 41% at 12, 27% at 24 h, 3% at 96 h and <1% by 144 h in *B. taurus*. Using a dose of $1000 \mu\text{g diclofenac kg}^{-1} \text{ bw}$ we

show that tissue residues in *B. indicus* fall to 17–40, 24–69, 34–47, and $0.016 < 0.010 \mu\text{g kg}^{-1}$ after only 48 h, in intestine, kidney, liver and muscle respectively. Given a 6 day course of $2500 \mu\text{g diclofenac kg}^{-1} \text{ bw}$, EMEA (2004) data shows that in *B. taurus* mean residues fall to 426, 423 and $347 \mu\text{g kg}^{-1}$ after just 24 h, in liver, kidney and muscle respectively. Oaks et al. (2004) gave a $2500 \mu\text{g diclofenac kg}^{-1} \text{ bw}$ dose each day for 3 days to buffalo and goat. Four hours post-treatment, levels in goat kidney, liver and muscle were 940, 220 and $190 \mu\text{g kg}^{-1}$ and in buffalo were 5700, 1500 and $760 \mu\text{g kg}^{-1}$.

Given these figures, it is interesting to note that in 13.9% of field collected samples, detectable levels of diclofenac have been found. This would indicate that diclofenac is being used extensively on the livestock sampled in at least two of the limited number of locations studied. It also seems likely given the liver/kidney levels found that four of the five animals found to contain diclofenac were given a final dose less than 48 h before death. One animal had a very high diclofenac level of $5387 \mu\text{g kg}^{-1}$ and $6914 \mu\text{g kg}^{-1}$ in liver and kidney. This suggests that not only did this animal receive a diclofenac dose within a few hours of death, but that the dose was far in excess of that which is recommended in India. These levels are approximately double that of the 3 h post-treatment levels reported by the EMEA (2004) for *B. taurus* when given six daily doses of $2500 \mu\text{g diclofenac kg}^{-1} \text{ bw}$. This raises the question of whether diclofenac is being administered either by those who are unqualified (farmers, etc.), or by veterinary practitioners who are disregarding the recommended diclofenac dosage for India. Likewise it also suggests that a high proportion of cattle are being given the drug within the days just before death, possibly because veterinary care (and therefore diclofenac) is often increasingly administered to livestock towards the end of their natural life, when animals are in greater need of treatment.

Oaks et al. (2004) has shown that for 10 *G. bengalensis* that consumed doses of $>800 \mu\text{g kg}^{-1}$ of diclofenac when given livestock meat with residues of $6400 \mu\text{g kg}^{-1}$ diclofenac, all died of renal failure soon after. Also, of 10 more birds who consumed doses of $<600 \mu\text{g kg}^{-1}$, three birds died, having apparently consumed just 7, 140 and $550 \mu\text{g kg}^{-1}$. The diclofenac residues reported here, in cattle collected in the field are therefore high enough, in at least one case, to be lethal to *G. bengalensis*.

5. Conclusions

Gyps vultures are extremely susceptible to even very low doses of diclofenac; for example, an LD_{50} for *G. bengalensis* has been estimated as $98\text{--}225 \mu\text{g kg}^{-1} \text{ vulture bw}$ (Swan et al., 2006a). In comparison, the oral LD_{50} for mice, rats, dogs, rabbits and guinea pig is $95,000\text{--}1,300,000 \mu\text{g kg}^{-1}$, $53,000\text{--}1,500,000 \mu\text{g kg}^{-1}$, $59,000 \mu\text{g kg}^{-1}$, $157,000 \mu\text{g kg}^{-1}$ and $1,250,000 \mu\text{g kg}^{-1} \text{ bw}$ respectively (EMEA, 2004), i.e. between 230 and 15,300 times higher. If the field prevalence noted herein (in what is accepted to be a small sample size) is widely replicated across India, as preliminary results

of a far more extensive survey suggest that it is (author's unpublished data), then the situation and outlook for *Gyps* vultures is indeed grave. The prevalence noted here is remarkably high, as is the lower 95% confidence interval of 4.7%. Green et al. (2004) found that if only 0.13 to 0.75% of available carcasses contained a lethal dose of diclofenac, this would be sufficient to have caused, and to continue to cause, the rapid population declines noted in the last decade. Clearly the prevalence of carcasses with detectable diclofenac reported here is not equivalent to the prevalence of carcasses that contain a lethal dose. Even so, the lower 95% confidence limit of 4.7% is more than six times higher than the upper limit (0.75%) of the range of prevalence of carcasses with a lethal dose that is required to cause the observed declines. This indicates that it is quite plausible that there is sufficient diclofenac in the food supply of vultures to have caused the observed population collapse. Further work is now under way to adapt the model of Green et al. (2004) to estimate the rate of vulture population decline expected using the observed prevalence of diclofenac in the carcasses of domestic livestock.

On May 11th 2006, the Drug Controller General (India) ordered the withdrawal of all licenses granted for the manufacture of diclofenac for veterinary use within India. It is now imperative that action is taken across the country to quickly and effectively remove all existing stocks of diclofenac from the inventories of veterinary and associated practices. Meloxicam (another common NSAID) was recently tested on *Gyps* sp., and found not to cause morbidity or death at doses likely to be encountered by wild vultures (Swan et al., 2006b). It has therefore been proposed as a safe alternative drug to diclofenac in areas where vultures are likely to scavenge carcasses of animals treated with NSAIDs.

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