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Metabolism of aceclofenac in cattle to vulture-killing diclofenac

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Keywords: *Gyps*; ecotoxicology; pharmacokinetics; NSAID; non-steroidal anti-inflammatory drugs; vulture declines; threats to vultures; pharmaceuticals in the environment

Running head: Metabolism of aceclofenac to diclofenac

Abstract

The non-steroidal anti-inflammatory drug (NSAID) diclofenac is highly toxic to *Gyps* vultures and its recent widespread use in South Asia caused catastrophic declines in at least three scavenging raptors. The manufacture of veterinary formulations of diclofenac has since been banned across the region with mixed success. However, at least 12 other NSAIDs are available for veterinary use in South Asia. Aceclofenac is one of these compounds and it is known to metabolise into diclofenac in some mammal species. The metabolic pathway of aceclofenac in cattle, the primary food of vultures in South Asia, is unknown. In this study, we give six cattle the recommended dose of aceclofenac (2 mg/kg), collect blood along a time series and undertake a pharmacokinetic analysis of aceclofenac and diclofenac-metabolites in their plasma using liquid chromatography with mass spectrometry. We found that nearly all of the aceclofenac administered to the cattle was very rapidly metabolised into diclofenac. Therefore, treating livestock with pure diclofenac or aceclofenac poses the same risk to vultures. This fact, coupled with the risk that aceclofenac may replace diclofenac in the veterinary market, fortifies the need for an immediate ban on all aceclofenac formulations that can be used to treat livestock. Without such a ban, the recovery of vultures across South Asia will not be successful.

Introduction

The non-steroidal anti-inflammatory drug (NSAID) diclofenac is highly toxic to *Gyps* vultures (Oaks *et al.* 2004; Swan *et al.* 2006a). Widespread use of diclofenac in South Asia to treat livestock – the primary food source – during the 1990s and 2000s caused catastrophic declines in three resident *Gyps* species (Green *et al.* 2004, 2007; Prakash *et al.* 2012) and possibly other birds of prey (e.g. Galligan *et al.* 2014; Sharma *et al.* 2014; Paudel *et al.* 2015) that scavenged on the carcasses of recently treated livestock. The mechanisms of toxicity in *Gyps* vultures remains unknown (but see Hutchinson *et al.* 2014); however, individuals exposed to even low concentrations of diclofenac in the carcasses of animals treated just before death suffer fatal necrosis of the kidneys (Oaks *et al.* 2004; Swan *et al.* 2006).

Beginning in 2006, the governments of India, Pakistan, Nepal and Bangladesh banned the veterinary use of diclofenac to conserve the now critically endangered resident *Gyps* vultures (IUCN Red List 2015). However, these bans have not brought about a complete cessation to diclofenac use (Cuthbert *et al.* 2011, 2014). Compounding this problem, at least another twelve NSAIDs are available for veterinary use across the Indian sub-continent (Cuthbert *et al.* 2011; SAVE unpublished data). Among these, experimental safety-testing has shown that one is vulture-toxic (ketoprofen: Naidoo *et al.* 2010a, b) and another is vulture-safe (meloxicam: Swan *et al.* 2006b; Swarup *et al.* 2007). Two more are almost certainly toxic given that elevated residues of these drugs have been found in dead vultures with clinical signs of NSAID poisoning (flunixin: Zorilla *et al.* 2014; nimesulide: Cuthbert *et al.* 2015).

Sharma (2012) suggested that a fifth NSAID, aceclofenac, was likely to be toxic to vultures, given its 1) close structural and pharmacological resemblance to diclofenac (Fig. 1); and 2) evidence

that diclofenac was a metabolite of aceclofenac in humans and other non-ruminant mammals (e.g. Bort *et al.* 1999; Hinz *et al.* 2003). Aceclofenac is a phenylacetic acid derived NSAID. Like many NSAIDs, the analgesic, anti-arthritic and anti-pyretic properties of aceclofenac are used to treat a wide variety of common ailments in livestock in South Asia. Sharma (2012) concluded that aceclofenac was either likely to be toxic to vultures independently or as a result of its conversion into diclofenac within ruminants which make up the bulk of food for vultures in South Asia.

In this study, we test this latter hypothesis by examining the plasma pharmacokinetics of aceclofenac and its potential diclofenac-metabolites in *Bos taurus* cattle. We then compare our results to existing pharmacokinetic data for diclofenac and comment on the risk posed to vultures by increasing veterinary use of aceclofenac in South Asia.

Methods

Research and animal ethics approval for this study were given by the Research Committee of the Faculty of Veterinary Science, University of Pretoria, and the Animal Ethics Committee, University of Pretoria, respectively.

Most aceclofenac products available in South Asia are oral bolus formulations. We purchased a bolus product in Bhopal, Madhya Pradesh, branded as NBE PLUS, manufactured by Hanuchem Laboratories, Solan, Himachal Pradesh (under licence number: MNB/05/261). Each bolus contained 300 mg of aceclofenac and 1500 mg of paracetamol.

We conducted a closed single dose pharmacokinetic study on six healthy female *Bos taurus* cattle (Friesian) aged >12 months. Cattle were sourced from a single commercial livestock-owner in Pretoria, South Africa. To ensure that the animals were free of any NSAIDs prior to exposure to

aceclofenac, the animals were kept under non-treatment quarantine in outdoor camps of the University of Pretoria for six weeks prior to acclimatisation.

One-week prior to exposure, the cattle were moved into the controlled stables where they were housed in pairs in climate controlled stables on straw bedding at night. Food, water and exercise were provided daily. During the acclimatisation period each animal received a complete veterinary examination and was deemed fit and healthy. Throughout the study, the cattle were observed by a veterinarian or animal technician for signs of adverse drug reactions, but none were detected. After the study, each animal was returned to its owner with an enforced three month withdrawal period.

On the first day of the study, each animal was weighed (mean \pm SD = 323 \pm 93 kg). A 10 ml sample of blood was collected from each animal's jugular vein into an evacuated heparinised tube. Next, all animals were given the aceclofenac bolus (individualised to the animal's weight) orally into the back of the mouth by hand and monitored to ensure that the bolus was swallowed. The recommended single dose for cattle in India was used; this equated to approximately 2,000 μ g of aceclofenac per kg of cattle body weight. The numbers of bolus given per animal were recorded for subsequent dose equalisation of the pharmacokinetic data. Subsequent samples of blood (10 ml) were then collected at eleven time points post administration: 0.25, 0.50, 0.75, 1.00, 1.50, 2.00, 3.00, 5.00, 7.00, 9.00 and 12.00 h. Within 2h of collection, all blood samples were centrifuged at \sim 3,000 *g* for 15 min and the supernatant (plasma) was transferred to a glass screw-top vial and immediately frozen at -80 °C.

Plasma samples were couriered on dry ice to the UK. A subsample of each (0.3 ml) was extracted by: vortex mixing with 2.5 ml of HPLC grade acetonitrile for 20 s; setting to stand at room temperature for 10 minutes; vortex mixing for a further 20 s; and centrifuging at 2000 rpm (671 *g*)

for 10 min. The supernatant was syringe filtered (0.2 μm HDPE disposable) directly into an amber 2 ml screw top LC vial. These extracts were held at $-20\text{ }^{\circ}\text{C}$ until analysis. NSAID and metabolite concentrations were 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). Target analytes determined in negative ion mode were aceclofenac (target mass m/z 352) and the suspected metabolites “diclofenac-metabolite” (masses $294\rightarrow 250$) and “4'-OH-diclofenac-metabolite” (masses $310\rightarrow 266$; see also Support Information). Mean recovery of these compounds spiked into blank plasma at three different concentration levels and extracted as above was $>90\%$ ($n = 12$) for all three compounds. See Support Information for further detail of the analyses.

We used Kinetica 5.1 (Thermo, 2012) to perform non-compartmental modelling for aceclofenac, the diclofenac-metabolite and the 4'-OH-diclofenac-metabolite per animal. We calculated the following parameters from a non-compartmental model: the maximum plasma level (C_{max}) and the time to reach it (t_{max}) read directly from the plasma concentration versus time curve; the elimination constant (λ) using least squares linear regression from the concentrations on the natural logarithmic scale attained for the last three time points for all animals; the area under the plasma concentration versus time curve (AUC_{last}) at the last measured concentration (C_{last}) calculated according to the linear trapezoidal rule ($\text{AUC}_{\text{last}} = (t_{\text{last}} - t_{\text{last-1}}) * [C_{\text{last}} + C_{\text{last-1}}] / 2$); the area under the moment curve ($\text{AUMC}_{\text{last}} = (t_{\text{last}} - t_{\text{last-1}}) * [t_{\text{last}} * C_{\text{last}} + t_{\text{last-1}} * C_{\text{last-1}}] / 2$) used to calculate the mean residence time ($\text{MRT} = \text{AUMC}_{\text{inf}} / \text{AUC}_{\text{inf}}$); the extrapolated concentration for each chemical to infinity ($\text{AUC}_{\text{inf}} = \text{AUC}_{\text{last}} + C_{\text{last}} / \lambda$); and the terminal half-life was calculated as $\ln(2) / \lambda$. All parameters are presented as geometric means with standard deviations; and all comparisons are presented as the difference between geometric means with 95% confidence intervals.

Results

We detected the diclofenac-metabolite at the very first sampling time point (0.25 h) and at every subsequent time point measured (Fig. 2b). The plasma concentration of diclofenac-metabolite was 24-62% less than that of aceclofenac before 2 h (Fig. 2, 3). At 2 h, each chemical was equivalent (aceclofenac versus diclofenac-metabolite: 0.50 vs. 0.49; Fig. 2, 3). After 2 h, the plasma concentration of diclofenac-metabolite was 21-117% greater than that of aceclofenac (Fig. 2, 3). 4'-OH-diclofenac was a minor metabolite in the first 12 h (Fig. 3).

Pharmacokinetic parameters were similar among cattle (Supporting Information).

Diclofenac-metabolite had a mean C_{max} that was 61% greater than and 5 h later than that of aceclofenac (Table 1); however, the difference in mean C_{max} was not statistically significant (geometric mean difference [\pm 95% CI] = 0.71 [0.31, 1.45] $\mu\text{g}/\text{ml}$). The mean area under the curve for diclofenac-metabolite was between 120 and 1163% greater than that of aceclofenac (Table 1), depending on how it was calculated, and statistically significant in all cases (AUC_{last} : 9.52 [5.77, 15.72] $\mu\text{g}\cdot\text{h}/\text{ml}$; $AUMC_{last}$: 77.98 [47.93, 126.47] $\mu\text{g}\cdot\text{h}^2/\text{ml}$; AUC_{inf} : 27.66 [21.87, 33.73] $\mu\text{g}\cdot\text{h}/\text{ml}$). Diclofenac-metabolite had a mean $t_{1/2}$ and mean MRT that were both 44% longer than that of aceclofenac (Table 1); however, the difference in mean $t_{1/2}$ was not significant (1.86 [0.86, 3.46] h), whereas the difference in mean MRT was significant (2.68 [1.24, 4.99] h).

Discussion

Our study confirms the hypothesis (proposed by Sharma, 2014) that aceclofenac is metabolised into diclofenac within cattle. Two hours after administration of aceclofenac, cattle had as much diclofenac-metabolite circulating in their blood as the pure drug, indicating a very rapid conversion

of the aceclofenac into diclofenac. At all subsequent time points tested here, cattle had far higher plasma concentrations of diclofenac than aceclofenac. Therefore, aceclofenac is pro-drug of diclofenac. It follows, that the use of aceclofenac to treat livestock in South Asia is just as dangerous to Critically Endangered *Gyps* vultures as the use of pure diclofenac to do the same.

Our estimate of total exposure of diclofenac-metabolite ($AUC_{inf} = 48 \mu\text{g}\cdot\text{h}/\text{mL}$) is equivalent to that of pure diclofenac in cattle in two previous studies scaled to a 2 mg/kg dose – specifically, EMEA (2003) $AUC = (70/2.5)*2 = 56 \mu\text{g}\cdot\text{h}/\text{mL}$ and Mestrino *et al.* (2006) $AUC_{inf} = 10*4 = 40 \mu\text{g}\cdot\text{h}/\text{mL}$. These are the only studies we know of where the total exposure of diclofenac has been shown. Both delivered diclofenac intramuscularly; however, it is important to note that the methods for calculating AUC differed between all three studies, as such, estimates may be expected to differ slightly.

One potential concern with data obtained in this study was a concentration curve that only extended up to 12 hours. As a result, the ratio of $AUC_{inf}:AUC_{last}$ for the diclofenac metabolite was 62%, and potentially inaccurate. Nonetheless, we believe that AUC_{inf} calculated here is an accurate representation of the total exposure of cattle to diclofenac-metabolite following treatment with aceclofenac as sufficient time points were present to estimate the elimination constant for the diclofenac-metabolite, while its terminal half-life (i.e., $6.09 \pm 3.47\text{h}$) was similar to the reported half-life (i.e., 5.9 hours) reported by the European Medicines Agency (EMEA 2003). With AUC being an indicator of the extent of exposure after drug administration or, in this case, the extent of pro-drug conversion, we estimate that the conversion of 2 mg/kg of aceclofenac could result in a similar or higher extent of exposure as for 1mg/kg diclofenac as a pure active pharmaceutical ingredient. With the clinical use of diclofenac and aceclofenac in India being 1 mg/kg and 2 mg/kg, respectively, we

conclude that the use of aceclofenac in cattle is no different to use of pure diclofenac at their respectively indicated dose.

Kinetic homogeneity – that is, the theory that observed concentrations of chemicals in plasma are proportional to those expected in tissues – makes analysis of diclofenac-metabolite in cattle tissue redundant. It can be assumed that all tissues in a cow treated with aceclofenac would absorb and eliminate diclofenac as a metabolite at rates relative to those we report for plasma. It follows that a proportion of vultures feeding on the carcass of a cow given aceclofenac would be poisoned by diclofenac. A residue depletion study of ^{14}C -diclofenac administered (2.5 mg/kg bw/day; 3 days; intramuscularly) to 16 calves, subsequently slaughtered at 3, 7, 14 and 17 days post-administration, showed concentrations of 150-1040 $\mu\text{g}/\text{kg}$ for liver (peak at 7 days), 117-324 $\mu\text{g}/\text{kg}$ for kidney (peak at 3 days), 234-6363 $\mu\text{g}/\text{kg}$ for muscle at the injection site (peak at 3 days) and 0-14 $\mu\text{g}/\text{kg}$ for muscle away from the injection site (peak at 3 days; EMEA 2003). As expected, these concentrations are lower and the periods of absorption and elimination longer than what we found for diclofenac-metabolite in plasma. Diclofenac as a metabolite is eliminated in cattle similarly to pure diclofenac, the proportion poisoned would also be similar to the proportion poisoned at a carcass of a cow recently treated with pure diclofenac (>10%: Green *et al.* 2006), if the mean time between dosing and death is the same for both drugs.

The illegal use of human formulations of diclofenac in livestock remains the greatest threat to *Gyps* vultures in South Asia (Cuthbert *et al.* 2011). A recent strengthening of the ban on diclofenac to include human formulations in vials larger than 3 ml is hoped to make dosing large animals less convenient and more expensive and thereby all but end the use of the vulture-killing drug in livestock. However, it may also elicit an increase in the use of aceclofenac, particularly if pharmaceutical companies seek to promote this drug as an analogue of diclofenac. This risk fortifies

the need for an immediate ban on all aceclofenac formulations that can be used to treat livestock. Since aceclofenac is also used in human health care, such a ban must cover the manufacture, distribution, retail and use of bolus and injectable formulations in doses suitable for large animals (i.e., vials >3 ml) and not just formulations labelled for veterinary use. Without such a ban, the recovery of Critically Endangered vultures across the Indian sub-continent will not be successful.

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Supporting Information

Pharmacokinetic parameters obtained from non-compartmental modelling for aceclofenac and diclofenac-metabolite in six cattle (Appendix S1). LC-ESI-MS/MS ion count trace for the analysis of a mixed calibration standard containing the three target compounds of interest (Appendix S2).

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Table 1. Pharmacokinetic parameters obtained from non-compartmental modelling for aceclofenac and diclofenac-metabolite in six *Bos taurus* cattle. Values are geometric means with standard deviations.

Parameters	Units	Aceclofenac	Diclofenac-metabolite
		Mean \pm SD	Mean \pm SD
C_{max}	$\mu\text{g}/\text{mL}$	1.16 \pm 0.51	1.87 \pm 1.08
T_{max}	H	1.57 \pm 0.75	6.26 \pm 1.03
AUC_{last}	$\mu\text{g}\cdot\text{h}/\text{mL}$	7.88 \pm 4.12	17.40 \pm 9.78
AUC_{inf}	$\mu\text{g}\cdot\text{h}/\text{mL}$	10.51 \pm 5.96	48.34 \pm 17.70
λ	1/h	0.16 \pm 0.05	0.11 \pm 0.05
$AUMC_{last}$	$\mu\text{g}\cdot\text{h}^2/\text{mL}$	39.03 \pm 22.55	117.01 \pm 68.91
$t_{1/2}$	h	4.23 \pm 1.26	6.09 \pm 3.47
MRT	h	6.10 \pm 1.81	8.78 \pm 5.01

Figure 1. Chemical structure of pure aceclofenac and its likely primary metabolites in mammals, diclofenac and 4'-hydroxydiclofenac.

Figure 2. Plasma concentration ($\mu\text{g}/\text{mL}$) over time (h) for (a) aceclofenac and (b) diclofenac-metabolite of aceclofenac in six female *Bos taurus* cattle.

Figure 3. Additive geometric mean plasma concentration ($\mu\text{g}/\text{mL}$) over time (h) for aceclofenac (dark grey), diclofenac-metabolite of aceclofenac (light grey) and 4'-hydroxydiclofenac-metabolite of aceclofenac (black) in six female *Bos taurus* cattle.

Fig. 1

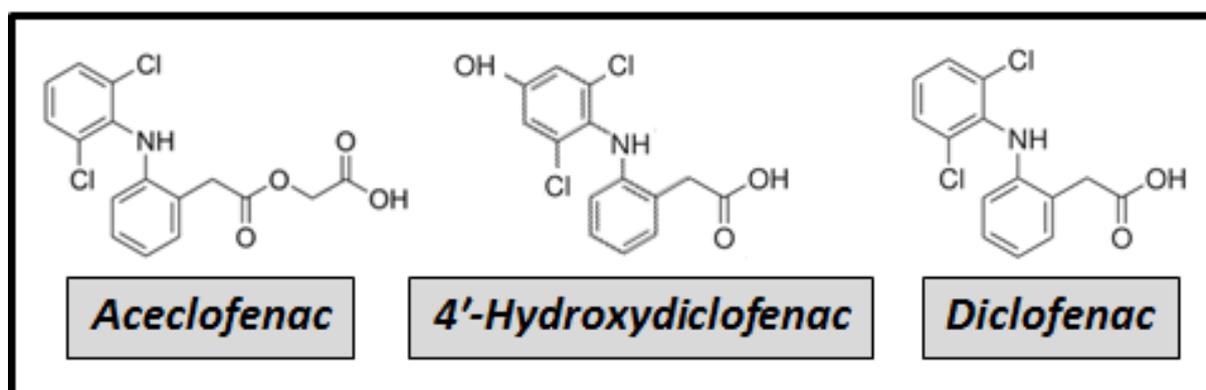


Fig. 2

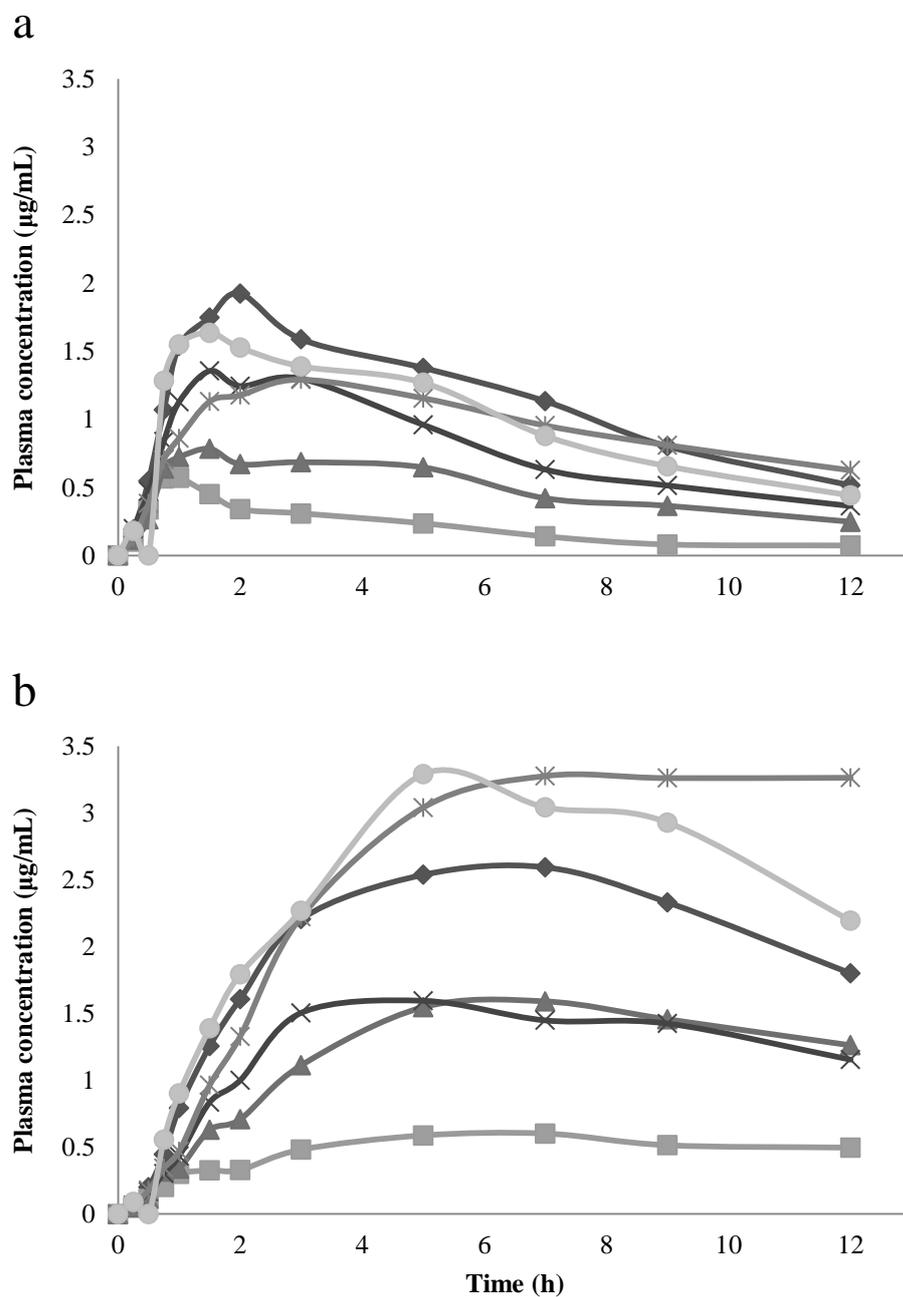


Fig. 3

