Bull Environ Contam Toxicol (2010) 85:377–380
DOI 10.1007/s00128-010-0109-7

Diclofenac Residues in Blood Plasma and Tissues of Vultures Collected from Ahmedabad, India

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Received: 18 May 2010 / Accepted: 13 September 2010 / Published online: 24 September 2010

Abstract The study reports residues of diclofenac, a non-steroidal anti inflammatory drug, in tissues of 11 White-backed Vulture, *Gyps bengalensis* collected between 2005 and 2007 and blood plasma of 12 White-backed Vulture, four Egyptian Vulture, *Neophron percnopterus* and two Griffon Vulture, *Gyps fulvus* collected during 2005. Samples were analysed using High Performance Liquid Chromatograhy (HPLC) equipped with UV detector. One of the White-backed Vultures collected during 2005 had substantial urate deposits on its viscera and diclofenac was detected in its liver (1.42 ppm wet weight) and kidney (1.18 ppm wet weight), which is suggestive of diclofenac exposure and intoxication. Although uric acid crystals were not observed in the remaining birds received during 2005, the residues of diclofenac detected were at levels higher than the toxic limits (0.25–1 ppm). No residues were detected in any of the tissues of birds collected during 2006 and 2007 (6 birds). About 89% (16 of 18) of plasma samples collected during 2005 had diclofenac residues (White-backed vulture: BDL to 0.17 ppm; Egyptian vulture: BDL to 0.09 ppm; Griffon vulture: 0.07–0.14 ppm). However, plasma diclofenac concentrations were less than the concentrations reported to be toxic. Although use of diclofenac for treating cattle has been banned in India, regular monitoring is recommended to assess the effectiveness of the ban on the drug in support of the conservation of these species.

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Keywords White-backed vulture · Egyptian vulture · Griffon vulture · Diclofenac · Blood plasma

White-backed Vulture *Gyps bengalensis* was one of the most common raptors in the Indian subcontinent (Ali and Ripley 1968). The population decline in White-backed Vulture, Long-billed Vulture *Gyps indicus* and Slender-billed Vulture *Gyps tenuirostris* has been reported from all parts of the country, and it exceeded 92% overall (Prakash et al. 2003), leading the International Union for Conservation of Nature (IUCN) to list all these three species as critically endangered (Hilton-Taylor 2000). The consequences have been severe, not just for the vultures, but also for other supporting ecosystems. Vultures are natural scavengers, cleaning up carrion of wildlife and domestic livestock, and also of cattle, sacred to Hindus, which cannot be consumed by people (Risebrough 2004). Similar population declines in vultures have also occurred in Pakistan and Nepal (Oaks et al. 2004). Although various hypotheses were proposed to explain the population decline in vultures in India and Indian subcontinent, studies by Oaks et al. (2004) and Shultz et al. (2004) in Pakistan, India and Nepal provided evidence that the non-steroidal anti-inflammatory drug, diclofenac was the major cause of the population crash.

Oaks et al. (2004) found that all birds collected dead with visceral gout had detectable diclofenac residues in their kidneys. Furthermore, none of the birds found without visceral gout contained diclofenac residues. These findings were supported by experimental evidence showing dose-dependent mortality of White-backed Vulture fed with tissues of livestock treated with a normal veterinary dose of diclofenac shortly before death. White-backed Vultures

those were gavaged with diclofenac also exhibited visceral gout and died. Similar significant association was found between the presence of gout and contamination of kidney or liver tissues with diclofenac in dead and dying White-backed Vulture and Long-billed Vulture collected in India and Nepal (Shultz et al. 2004). Moreover, a simulation model of vulture demography, described by Green et al. (2004) observed that population decline could be caused by contamination with a lethal level of diclofenac in a small proportion (between 1:130 and 1:760) of ungulate carcasses available to vultures. However, all species of vultures need not be affected as Rattner et al. (2008) explained in Turkey Vulture (*Cathartes aura)*. Although, some studies relating the diclofenac poisoning in Pakistan and Indian subcontinent under established laboratory conditions are available, information on residue levels of diclofenac in free ranging vultures in India is scare. The present study measured the diclofenac residues in blood plasma and select tissues of three species of vultures, namely White-backed Vulture, Egyptian Vulture and Griffon Vulture which were victims of kite flying in Ahmadabad, India during 2005–2007.

Materials and Methods

Between 2005 and 2007 11 individuals of dead or dying White-backed Vultures were collected from Ahmedabad, India (Table 1). During 2005, only blood samples were collected from 12 White-backed Vultures, four Egyptian Vultures and two Griffon Vultures, which were received dying due to kite injury. It should be noted that people fly kites as an entertainment in and around Ahmedabad during an annual festival (*Uthrayan)* in January. The samples were collected with the help of Gujarat State Forest Department and Animal Help Foundation, Ahmedabad on opportunistic basis in all 3 years.

Post-mortem examination on 10 adults and one sub adult was performed and diclofenac residues were determined in tissues (liver, kidney, gut contents, and blood plasma) following the methodology described by Oaks et al. (2004) and Shultz et al. (2004). Tissue samples (0.5 g) were homogenized with 4 mL of acetonitrile and centrifuged (5000 rpm) to pellet the debris. Three milliliters of the supernatant were then passed through solid-phase extraction cartridges (Waters Sep-Pak Plus t-C18) at 1 mL/min. The cartridges were washed with an additional 3 mL of acetonitrile, the eluates pooled, and concentrated to a final volume of 1 mL. The volume of acetonitrile was adjusted for samples weighing less than 0.5 g.

For blood samples, about 5 mL was drawn from the brachial vein and transferred to 200 µL pre-refrigerated saline test tubes and stored at 0–4°C. These samples were centrifuged within 48 h for 10 min at 3000 rpm to separate plasma. Plasma samples were stored in the freezer at −20°C until extraction and purification. About 0.5 mL of thoroughly mixed homogenous aliquot of plasma was transferred to a 15 mL centrifuge tube with a PTFE (Sigma–Aldrich) lined screw cap. About 0.5 mL 5 M HCl and 5 mL of ethyl acetate were added to the sample and extracted using a mixing rotor for 20 min and the extracts were centrifuged at 2500 rpm for 10 min. The supernatant was transferred to a well-cleaned glass test tube and evaporated to dryness under a thin stream of nitrogen at 40°C. The residues were reconstituted with 200 µL of acetonitrile transferred to HPLC auto sampler vials and stored for analysis.

Diclofenac was quantified by high-performance liquid chromatography (Agilent 1100 series) fitted with a Zorbax SB-C18 (4.6 mm × 150 mm, 5 µm) column (Agilent) equipped with diode array detector. Diclofenac standard

Table 1 Diclofenac residues in the tissues of White-backed Vulture collected from Ahmedabad, India between 2005 and 2007

Sample ID	Date of collection	Place of collection	Diclofenac concentration (ppm wet weight)		
			Liver	Kidney	Gut content
WBV1	January' 2005	Shahibang, Ahmedabad	1.42	1.18	BDL
WBV2	January' 2005	Ranip, Ahmedabad	BDL	0.896	BDL
WBV3	January' 2005	IIM campus, Ahmedabad	BDL	1.14	BDL
WBV4	January' 2005	Airport road, Ahmedabad	BDL	1.44	BDL
WBV5	January' 2005	Airport road, Ahmedabad	BDL	BDL	BDL
WBV6	January' 2006	Market, Ahmedabad	BDL	BDL	BDL
WBV7	January' 2006	Airport road, Ahmedabad	BDL	BDL	BDL
WBV8	January' 2007	Airport road, Ahmedabad	BDL	BDL	BDL
WBV9	January' 2007	Market road, Ahmedabad	BDL	BDL	BDL
WBV10	January' 2007	Market, Ahmedabad	BDL	BDL	BDL
WBV11	January' 2007	Ahmedabad	BDL	BDL	BDL

BDL Below detection limit

(Sigma, D6899) was dissolved in 1:1 (v/v) acetonitrile: water to prepare calibration standards of 0.1, 0.05, 0.01 ppm and generate a linear calibration curve with R^2 values equal to 0.999. Samples and standards (10 μL) were subjected to a binary gradient elution profile, which consisted of 0.1% acetic acid in water (solution A) and 100% acetonitrile (solution B) as follows; starting conditions 75% A/25% B for 0.1 min, a 15-min linear gradient from 75% A/25% B to 5% A/95% B, followed by a 5-min column-wash step in 5% A/95% B, and a 10-min re-equilibration step with 75% A/25% B before the next injection. Flow rate was 0.7 mL min-1 and the column temperature was 40°C. Detector wavelength was programmed at 280 nm and lower limit of detection was 0.001 ppm. The mean recovery from fortified samples was approximately 75% and the results were not corrected for per cent recovery.

Results and Discussion

Among the five White-backed vultures received during 2005, one adult bird from Shahibang (WBV-1) died on the third day of its receipt at the lab. During post-mortem examination, apparent urate deposits were found over much of the viscera. Diclofenac residues were detected in both liver (1.42 ppm) and kidney (1.18 ppm) while it was not detected in gut contents (Table 1). Diclofenac residues detected in this bird were higher than the levels reported by Oaks et al. (2004). Similar findings have been reported in several species of *Gyps* vultures naturally exposed to or experimentally treated with diclofenac (Meteyer et al. 2005).

An adult bird collected from Ranip (WBV-2) also died on the same day of collection due to kite string injury and heavy blood loss. Post-mortem examination did not show any abnormalities but the kidney (0.896 ppm) and plasma (0.003 ppm) samples had diclofenac residues which were higher than the levels reported earlier (Oaks et al. 2004). Another bird received from IIM Campus (WBV-3) also died on the third day of its collection and it contained 1.14 ppm of diclofenac in its kidney but diclofenac was not detected in liver and gut contents.

Of the two individuals (1 adult, WBV 4 and 1 juvenile, WBV 5), which were collected from Airport Road, Ahmedabad, the adult bird died on the same day of its collection due to heavy blood loss. It (WBV-4) was dissected immediately and there was no urate deposit on internal organs. However, the kidney (WBV-4) sample contained 1.44 of diclofenac. Kidney diclofenac residue levels were higher than the concentration reported by Oaks et al. (2004) and comparable with the bird that had urate deposits. The juvenile (WBV 5) died the day after its collection. There was no evidence of external injury, although the bird was unable

Table 2 Comparison of diclofenac residues in the tissues of vultures collected from Ahmedabad during 2005–2007

Species	Year	Tissue	No. of samples analysed	No. of samples detected	Diclofenac residues (ppm wet weight)	
					Range	Mean
White-backed vulture	2005	Gut content	3	0	BDL	BDL
		Kidney	5	4	BDL - 1.44	1.17
		Liver	5	2	BDL - 0.42	1.42
		Plasma	12*	11	BDL - 0.17	0.04
	2006	Gut content	2	0	BDL	BDL
		Kidney	2	0	BDL	BDL
		Liver	2	0	BDL	BDL
		Plasma	NA	–	–	–
	2007	Gut content	4	0	BDL	BDL
		Kidney	4	0	BDL	BDL
		Liver	4	0	BDL	BDL
		Plasma	NA	–	–	–
Egyptian vulture	2005	Gut content	NA	–	–	–
		Kidney	NA	–	–	–
		Liver	NA	–	–	–
		Plasma	4*	3	BDL - 0.09	0.05
Griffon vulture	2005	Gut content	NA	NA	NA	NA
		Kidney	NA	NA	NA	NA
		Liver	NA	NA	NA	NA
		Plasma	2*	2	0.07–0.14	0.11

NA Not available, *BDL* Below detection limits

* Plasma samples collected from live individuals

to fly. The post-mortem was unremarkable and no diclofenac residues were detected in its tissues.

Apart from these dead birds, plasma samples from 18 live birds (twelve White-backed Vultures, four Egyptian Vultures and two Griffon Vultures) were also analyzed for diclofenac residues. For White-backed vultures, diclofenac residues in plasma ranged between BDL and 0.17 ppm and for Egyptian vulture values ranged from BDL to 0.09 ppm. Blood plasma from two Griffon vultures contained 0.07 and 0.14 ppm. Although birds collected during 2006 and 2007 did not have detectable levels of diclofenac in the tissues, all the samples collected during 2005 had detectable levels (BDL to 0.17 ppm) of diclofenac except two samples (Table 2). Diclofenac concentrations reported in this study are less than the administrated concentration (0.8 mg/kg body weight) which result in elevated plasma uric acid levels and alanine aminotransferease activities in *Gyps* vultures (Swan et al. 2006). In contrast, Rattner et al. (2008) reported no relationship between administered diclofenac and residues in plasma in Turkey vultures (*Cathartes aura*), a New World vulture that is phylogenetically distinct from *Gyps* vultures. Although Muralidharan et al. (2008) and Dhananjayan et al. (2010) reported varying levels of organochlorine pesticide residues in tissues of White-backed vulture and blood plasma of three species of vultures respectively, no information is available on the levels of diclofenac in blood plasma of free ranging vultures in India.

Although the samples collected were on opportunistic basis, diclofenac residues in liver and kidney were detected in birds which had uric acid crystal deposition in the viscera. Moreover, in four birds without urate deposits, residues of diclofenac were detected at levels exceeding 0.25 to 1 ppm) concentrations associated with toxicity (Oaks et al. 2004). As reported by Shultz et al. (2004), our study area falls within which evidence exists that a high percentage of dead vultures have visceral gout and which is strongly associated with diclofenac contamination. Studies have also reported that the turkey vultures seem to be remarkably tolerant to diclofenac compared to Gyps vulture (Oaks et al. 2004; Shultz et al. 2004; Swan et al. 2006; Rattner et al. 2008). Sensitivity to cycloxygenase-2-inhibitors varies considerably among avian species. Notably, the cyclooxygenase-2- inhibitors carprofen and flunxin have been observed to evoke renal disease and gout in rather diverse species of birds (Cuthbert et al. 2007). Although the use of diclofenac for treating cattle has been banned in India (Government of India 2006), monitoring residue levels in biota and the environment seems warranted as a conservation measure to protect *Gyps* species.

Acknowledgments We thank Mr Pradeep Khanna, PCCF Gujarat State Forest Department, Mr. Rahul Shaghal, Animal Help Foundation and Kartik Sastri, Ahmedabad for their support. Director, Sálim Centre for Ornithology and Natural History, Coimbatore extended financial assistance. We are beholden to Dr Barnett Rattner, Patuxent Wildlife Research Center, US for critically evaluating this manuscript.

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