SUPPLEMENTARY ONLINE MATERIAL

Metabolism of aceclofenac to diclofenac in the domestic water buffalo *Bubalus bubalis* confirm it as a threat to Critically Endangered *Gyps* vultures in South Asia.

Appendix 1

Methodology to estimate the concentration of aceclofenac and diclofenac in domestic water buffalo *Bubalus bubalis* plasma samples

Aceclofenac

Bioanalytical Method

<table>
<tr><td rowspan="2">Name of compound</td><td>Analyte</td><td>Internal standards</td></tr>
<tr><td>Aceclofenac</td><td>Aceclofenac-D2</td></tr>
<tr><td>Molecular weight of free compound (base / acid)</td><td>354.18</td><td>356.2</td></tr>
<tr><td>Diluent</td><td colspan="2">DMSO</td></tr>
<tr><td>Calibration curve range / Internal standard Working Concentration</td><td>0.765 to 100 ng/mL</td><td>10 µg/mL</td></tr>
<tr><td>Species / Strain</td><td colspan="2">Not available</td></tr>
<tr><td>Matrix</td><td colspan="2">Plasma</td></tr>
<tr><td colspan="3">Chromatographic conditions:</td></tr>
<tr><td>Mobile phase</td><td colspan="2">Pump A: 0.1 % Formic Acid in MilliQ-water
Pump B: Acetonitrile</td></tr>
<tr><td>Gradient conditions</td><td colspan="2">Binary Gradient:
<table><tr><th>Time (min)</th><th>% B concentration</th></tr><tr><td>0.01</td><td>Start</td></tr><tr><td>0.50</td><td>30</td></tr><tr><td>2.00</td><td>90</td></tr><tr><td>3.50</td><td>90</td></tr><tr><td>3.60</td><td>30</td></tr><tr><td>5.00</td><td>Stop</td></tr></table></td></tr>
<tr><td>Column (make)</td><td colspan="2">Zorbax XDB C8, 50*4.6 mm, 5µ</td></tr>
<tr><td>Injection volume (µL)</td><td colspan="2">10</td></tr>
<tr><td>Flow rate (mL/min)</td><td colspan="2">1</td></tr>
<tr><td>Run time (min)</td><td colspan="2">5</td></tr>
<tr><td>Sample cooler temperature (°C)</td><td colspan="2">4</td></tr>
<tr><td>Column oven temperature (°C)</td><td colspan="2">40</td></tr>
<tr><td>Rinsing solution</td><td colspan="2">Acetonitrile:methanol:IPA:water(3:3:3:1)</td></tr>
</table>

Plasma Sample preparation: Protein precipitation method
CC & QC preparation: An aliquot (95 µL) of blank plasma was spiked with 5 µL of analyte working solution. Sample Treatment Procedure: 100 µL of CC / QC/ Study samples were aliquoted into pre-labeled eppendorff tubes and 10 µL of internal working standard solution (10µg/mL of D2-IS) was added. Samples were quenched with 300 µL of acetonitrile and vortexed. All the samples were centrifuged at 14000 rpm for 5 minutes at 4^0C. 200 µL of supernatant was transferred into RIA vial and evaporated to dryness under gentle stream of Nitrogen at 40^0C for 30 mins. Samples were reconstituted with 100 µL of acetonitrile and transferred into HPLC vials for LC-MS/MS analysis.

Mass spectrometric conditions

Instrument ID	API 4000 LC-MS/MS	
Mass parameters	Analyte: ACECLOFENAC	IS: ACECLOFENAC D-2
MRM transitions	352.0 → 75.2	354.0 → 74.9
Resolution – Q1	Unit	Unit
Resolution – Q3	Unit	Unit
Declustering potential (DP) Volts	-42	-27
Entrance potential (EP)	10	
Collision energy (CE) Volts	-15	-20
Collision cell exit potential (CXP) Volts	-12	-12
Ionisation / Polarity	Negative	
Dwell time (mille seconds)	500	
Ionisation Source	ESI	
IS	-4500	
Collision gas (CAD)	6	
Curtain gas (CUR)	25	
GS1	45	
GS2	60	
Temperature	500 °C	

Diclofenac

Bioanalytical Method

<table>
<tr><td rowspan="2">Name of compound</td><td>Analyte</td><td>Internal standards</td></tr>
<tr><td>Diclofenac</td><td>Diclofenac-D4</td></tr>
<tr><td>Molecular weight of free compound (base / acid)</td><td>296.14</td><td>300.17</td></tr>
<tr><td>Diluent</td><td colspan="2">DMSO</td></tr>
<tr><td>Calibration curve range / Internal standard Working Concentration</td><td>5.6 to 5400ng/mL</td><td>10µg/mL</td></tr>
<tr><td>Species / Strain</td><td colspan="2">Not available</td></tr>
<tr><td>Matrix</td><td colspan="2">Plasma</td></tr>
<tr><td colspan="3">Chromatographic conditions:</td></tr>
<tr><td>Mobile phase</td><td colspan="2">Pump A: 5 mM Ammonium Formate Solution with 0.1% Formic acid
Pump B: Acetonitrile with 0.1 % Formic acid</td></tr>
<tr><td>Gradient conditions</td><td colspan="2">Binary Gradient:
<table><tr><td>Time (min)</td><td>% B concentration</td></tr><tr><td>0.01</td><td>Start</td></tr><tr><td>0.50</td><td>25</td></tr><tr><td>2.50</td><td>85</td></tr><tr><td>3.50</td><td>85</td></tr><tr><td>3.60</td><td>25</td></tr><tr><td>5.00</td><td>Stop</td></tr></table></td></tr>
<tr><td>Column (make)</td><td colspan="2">Zorbax XDB C8, 50*4.6 mm, 5µ</td></tr>
<tr><td>Injection volume (µL)</td><td colspan="2">10 µL</td></tr>
<tr><td>Flow rate (mL/min)</td><td colspan="2">1</td></tr>
<tr><td>Run time (min)</td><td colspan="2">5</td></tr>
<tr><td>Sample cooler temperature (°C)</td><td colspan="2">4</td></tr>
<tr><td>Column oven temperature (°C)</td><td colspan="2">40</td></tr>
<tr><td>Rinsing solution</td><td colspan="2">Acetonitrile:methanol:IPA:water(3:3:3:1)</td></tr>
<tr><td colspan="3">Plasma Sample preparation: Protein precipitation method</td></tr>
<tr><td colspan="3">CC & QC preparation: An aliquot (47.5 µL) of blank plasma was spiked with 2.5 µL of analyte working solution.
Sample Treatment Procedure: 50 µL of CC / QC/ Study samples were aliquoted into pre-labeled eppendorff tubes and 10 µL of internal working standard solution (10 µg/mL of D2-IS) was added. Samples were quenched with 150 µL of acetonitrile and vortex mixed. All the samples were centrifuged at 14000 rpm for 5 minutes at 4^0C. 100 µL of supernatant was transferred into HPLC vials and injected to LC-MS/MS for analysis.</td></tr>
</table>

Mass spectrometric conditions

Instrument ID	API 4000 LC-MS/MS	
Mass parameters	Analyte: DICLOFENAC	IS: DICLOFENAC D-4
MRM transitions	296.4 → 215.100	300.5 → 219.00
Resolution – Q1	Unit	Unit
Resolution – Q3	Unit	Unit
Declustering potential (DP) Volts	30	45
Entrance potential (EP)	10	
Collision energy (CE) Volts	28	22
Collision cell exit potential (CXP) Volts	14	20
Ionisation / Polarity	Positive	
Dwell time (mille seconds)	200	
Ionisation Source	ESI	
IS	4500	
Collision gas (CAD)	6	
Curtain gas (CUR)	25	
GS1	40	
GS2	60	
Temperature	500 °C	

Appendix 2

Concentrations of aceclofenac and diclofenac in plasma of domestic water buffaloes *Bubalus bubalis* in relation to time since dosing with aceclofenac.

Sr. No.	Sample ID	Hour Nominal	Concentration (ng mL^{-1})	
			Aceclofenac	Diclofenac
1	A0-1	0	BLQ < 0.77	BLQ < 5.6
2	A0-2	0	BLQ < 0.77	BLQ < 5.6
3	A2-1	2	11.3	2340
4	A2-2	2	10.2	2450
5	A4-1	4	11	2980
6	A4-2	4	10	2620
7	A8-1	8	1.25	2990
8	A8-2	8	6.54	3370
9	A12-1	12	5.43	2270
10	A12-2	12	5.88	2800
11	A24-1	24	7.28	1040
12	A24-2	24	8.75	840
13	A48-1	48	8.52	124
14	A48-2	48	9.5	107
15	B0-1	0	BLQ < 0.77	BLQ < 5.6
16	B0-2	0	BLQ < 0.77	BLQ < 5.6
17	B2-1	2	14.6	2540
18	B2-2	2	11.4	2380
19	B4-1	4	10.6	3040
20	B4-2	4	30.2	3380
21	B8-1	8	8.07	3200
22	B8-2	8	8.74	3040
23	B12-1	12	10.5	2740
24	B12-2	12	6.89	2830
25	B24-1	24	5.2	1040
26	B24-2	24	7.66	1190
27	B48-1	48	5.8	87.8
28	B48-2	48	4.89	79.8
29	C0-1	0	BLQ < 0.77	BLQ < 5.6

Sr. No.	Sample ID	Hour Nominal	Concentration (ng mL^{-1})	
			Aceclofenac	Diclofenac
30	C0-2	0	BLQ < 0.77	BLQ < 5.6
31	C2-1	2	8.12	2050
32	C2-2	2	8.88	2180
33	C4-1	4	8.98	2950
34	C4-2	4	6.74	2880
35	C8-1	8	8.19	3200
36	C8-2	8	8.31	3140
37	C12-1	12	6.32	2430
38	C12-2	12	5.88	2360
39	C24-1	24	9.79	965
40	C24-2	24	7.1	1030
41	C48-1	48	8.85	74.6
42	C48-2	48	5.21	61.8

Note: Lower Limit of Quantification: Aceclofenac, 0.77 ng $mL^{-1}$and Diclofenac, ng mL^{-1}