Aligning species conservation with animal welfare: formulations of vulture-safe meloxicam manufactured in South Asia and the reaction of goats to their administration

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A publication from the RSPB Centre for Conservation Science'
RSPB Research Report No 52

RSPB's work for the conservation of Asian vultures is undertaken as part of the programme of Saving Asia's Vultures from Extinction (SAVE)

www.save-vultures.org

Published by the Royal Society for the Protection of Birds,
The Lodge, Sandy, Bedfordshire SG19 2DL, UK. © 2014

ISBN 978-1-905601-44-8

Recommended citation:

Cuthbert, R.J., Paudel, S., Chaudhary, A., Kaphle, K., Thapa, J., Subedi, T.R. and Taggart, M.A. (2014). Aligning species conservation with animal welfare: formulation of vulture-safe meloxicam manufactured in South Asia and the reaction of goats to its administration. RSPB Research Report No. 52. Royal Society for the Protection of Birds, Sandy, Bedfordshire, UK. ISBN 978-1-905601-44-8

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Front cover photograph by Richard Cuthbert

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Executive Summary

1. Resident vulture populations in South Asia have undergone catastrophic declines due to their toxicity to the non-steroidal anti-inflammatory drug (NSAID) diclofenac, which was widely used as a painkiller in treating domestic ungulates, the principal food source for vultures. As a result of the critically endangered status of resident vultures, the manufacture of veterinary products of diclofenac was banned in India, Nepal and Pakistan in 2006.
2. Preparations of the NSAID meloxicam are promoted as an alternative to diclofenac, due to its safety to vultures and the wide-scale use of meloxicam in Europe where it is the main NSAID of choice for treating livestock. However, veterinarians in South Asia have raised concerns on the pain and irritation reaction that meloxicam products were causing when injected in goats and cattle.
3. We investigated the pH, osmolarity and formulation of a range of veterinary NSAIDs that were available for purchase in India and Nepal, and undertook an experiment to measure the pain reaction of domestic goats (*Capra hircus*) to South Asian meloxicam products and to Metacam®, the meloxicam product used in Europe.
4. NSAIDs purchased in South Asia exhibited a wide range of pH values and osmolarities, with many products having high pH (strongly alkaline) and high osmolarities (hypertonic), far in excess of those found in mammalian plasma. All products contained the labelled ingredients and concentrations of active ingredient were generally within the stated range. Only Metacam® was found to contain meglumine, an excipient used in many pharmaceutical products.
5. Treatment of goats, undertaken in a replicated and blind experiment, revealed that both meloxicam products manufactured in South Asia caused acute pain reactions following intra-muscular injection. In contrast, there was no difference in the reaction of goats to injections of Metacam® in comparison to injections with saline solution. The degree of pain of South Asian products was related to both high pH and high osmolarity. While short-term pain reactions were observed, no lasting pain or tissue damage occurred with any product.
6. The high pH and osmolarity of South Asian products of meloxicam are a result of the low solubility of meloxicam in water, with solubility increasing in more alkaline solutions. The addition of the excipient meglumine (as found in Metacam®) increases the solubility of meloxicam, without creating concomitant problems of high pH and osmolarity. Boehringer Ingelheim, the German pharmaceutical company that developed and markets Metacam®, has waived copyright to their formulation in India, allowing this to be used to manufacture meloxicam in South Asia. Promotion of a safe, effective and pain-free veterinary meloxicam formulation will benefit vulture conservation, animal welfare and the pharmaceutical industry in South Asia.

1. Background

Populations of *Gyps* vultures endemic to South Asia have shown catastrophic declines due to the use of the non-steroidal anti-inflammatory drug (NSAID) diclofenac in the veterinary treatment of livestock. Diclofenac is highly toxic to vultures, causing death due to kidney failure. Carcasses of domesticated ungulates contaminated with this drug have caused population declines of more than 97% in India, Pakistan and Nepal, leading three vulture species to be classified as critically endangered (IUCN 2007). The safety to vultures of alternative veterinary NSAIDs to diclofenac has been tested. The drug meloxicam has been shown to be safe for vultures and other scavenging birds (Swan *et al.* 2006; Swarup *et al*. 2007). It is also as diclofenac effective for the treatment of livestock, has fewer reported side effects and is manufactured in India (Engelhard et al 1995; Noble and Balfour 1996; Milne et al 2003; Ghosh et al 2004). The other alternative tested, ketoprofen, causes kidney failure and death of vultures at concentrations found in ungulate carcasses (Naidoo *et al.* in press; Taggart *et al.* 2009). Since the ban on the manufacture of veterinary diclofenac in 2006, the governments of India, Nepal and Pakistan and conservation agencies have promoted the replacement of diclofenac with meloxicam and the number of companies manufacturing the drug and its availability in pharmaceutical outlets has increased. However, some concerns have been raised by veterinary practitioners in South Asia about the apparent pain and reaction of livestock following injection with meloxicam products manufactured in South Asia. Such concerns and the perception of vets to this new product may be hampering the switch from diclofenac to meloxicam, and thereby threatening vulture conservation efforts. In order to evaluate whether meloxicam products in use in South Asia are causing pain and irritation to treated animals we first analysed the composition of a range of meloxicam and other NSAID products that were available for purchase in India and Nepal. Following this, we undertook an experiment to measure the reaction of domestic goats *Capra hircus* to a range of veterinary NSAIDs manufactured in South Asia and to a meloxicam product manufactured by the European company (Metacam®, Boehringer Ingelheim, Germany) that first developed meloxicam and markets the compound on a large scale in Europe and North America.

2. Methods

2.1 Ethics Statement

No national veterinary related acts exist in Nepal governing ethical and animal welfare issues for research, however in Nepal research can be undertaken under the auspices of a University, as in this case. The experiment was undertaken by a registered veterinarian and in the presence of the Chief Veterinary Officer, Department of National Parks and Wildlife Conservation, Chitwan National Park, Nepal. An independent ethical review of the research was undertaken by the ethics committee of the International Centre for Birds of Prey, UK. Ethical approval to undertake this work was also provided by the Directors of the Royal Society for the Protection of Birds. All drug products administered were approved veterinary products widely available for sale and use in livestock in Nepal and India [23]. All animals were closely monitored following dosing and the injection sites were rechecked 4 hours after injection and for 4 days thereafter: no lesions or inflammation requiring pain relief or treatment were observed.

2.2 Composition of veterinary products

Veterinary NSAIDs available for purchase over the counter were obtained from veterinary shops and pharmacies in India and Nepal. Products were purchased in the north and western

states of India with shops located in Gujarat (*n*=14 shops), Rajasthan (*n*=9), Maharashtra (*n*=8), Madhya Pradesh (*n*=6), and Andhra Pradesh (*n*=6). Further products were bought from >20 shops in the lowland terai region of Nepal. A total of 116 samples, consisting of 39 different preparations and eight combinations of veterinary NSAIDs were analysed to measure pH, osmolarity (concentration of all solutes in milliosmoles per ml of water) and the concentration of the drug versus the stated concentration of the drug listed on the label. The eight combinations of NSAIDs purchased and tested were meloxicam, meloxicam with paracetamol, diclofenac, analgin (dipyrone), ketoprofen, nimesulide, piroxicam with paracetamol and phenylbutazone with aspirin (sodium salicylate) (Table 1). A full list of the products tested and manufacturing companies is listed in Appendix Table 2. All 39 preparations were manufactured in India.

Each sample was analysed to measure the concentration of the labelled NSAIDs and the presence of other NSAIDs and paracetamol. The 13 NSAIDs tested for were aspirin, carprofen, diclofenac, dipyrone (analgin), flunixin, ibuprofen, indomethacin, ketoprofen, naproxen, nimesulide, phenybutazone, piroxicam and meloxicam. These 13 compounds were selected either on their pre-existing use as veterinary NSAIDs within South Asia, on there potential likely future use within the region, and suitability to be included within a multi-residue technique (Taggart *et al.* 2009). In addition, products were also analysed for the presence of the molecule meglumine (C7H17NO5), an excipient commonly used in the manufacture of several pharmaceutical products including Metacam®. The NSAIDs were determined simultaneously following a validated procedure using an Agilent LC-ESI/MS system with a dual mode (ESI/APCI) ionisation source (see Taggart *et al.* 2009 for details). All NSAID standards were purchased from Sigma-Aldrich and the primary molecular weights and fragments of compounds analysed are reported in Taggart *et al.* (2009). NSAID standards at concentrations between 5 and 1000 g/L were used to calibrate the instrument and assess concentrations in formulations. Stock standards at 500 mg/L were created in 50:50 acetonitrile:Milli-Q water and stored at 4° C in light proof vials. These were then diluted in 100% acetonitrile to create the working standard solutions. Concentrations of NSAIDs in formulations ranged from 1.5 to 500 mg/ml (g/L) and had to be diluted in acetonitrile to up to 500000 times to bring concentrations into the calibrated range. Dilution was successful for all products; however several ketoprofen products began to precipitate upon dilution with acetonitrile and as such were firstly diluted in water, then once at lower concentrations, finally diluted in acetonitrile for analysis. Working standard solutions were stored at -20° C in 2mL liquid chromatography (LC) vials. Limits of quantification (LOQ) for these compounds by LC-ESI/MS have been reported previously (Taggart *et al.* 2009) and since these were concentrated formulations all compounds were analysed at between 125 and 1000 g/L, i.e., well above the respective LOQs. Meglumine was also analysed by LC-ESI/MS in the same analytical run in negative ion mode at 194 m/z. Sensitivity toward meglumine is actually greater in positive ion mode but levels were quantifiable using this technique in one formulation of Metacam® manufactured by Boehringer Ingelheim (in which meglumine is known to be an ingredient).

Product pH was analysed using a benchtop meter (Crison GLP22), which was calibrated with commercially available solutions of an approriate pH (i.e., 4, 7 or 10; Crison). Osmolarity was measured in samples diluted ten times in Milli-Q water (to bring levels within the range for the meter) using a Roebling benchtop Osmometer which was autozeroed using Milli-Q water and calibrated using a Roebling standard solution at 300 mOsmol kg^{-1} of water. In order to check the accuracy of the pH, osmolarity and formulation results, a sub-sample of 20 meloxicam products were also sent to a second laboratory for independent analysis.

2.3 Experimental design for testing meloxicam products

A total of six meloxicam and two diclofenac products sourced from South Asia and one meloxicam product sourced from Europe (Metacam®) were administered to domestic goats in a pair-wise replicated experiment, with six or seven observers each evaluating the pain reaction of all animals following the injection. South Asian manufactured brands of meloxicam included three products with meloxicam as the sole active ingredient and three with meloxicam + paracetamol. Meloxicam products were selected to include a range of pH and osmolarity values. Domestic goats were chosen as the experimental subjects because they exhibit a more obvious reaction to pain and are more prone to vocalise than other livestock (e.g. cattle and water buffalo; NRC 2009). The observers were blind to which product was being used or if the substance injected was a drug or sham-dose. Commercially bought saline solution was used as the sham substance for injections. The manufacturer's recommended dose of meloxicam was calculated, based upon the body mass of each goat, and administered in a single dose. The same volume of saline was injected for the sham dose. Because the European brand of meloxicam had a higher concentration of active ingredient (50 mg ml^{-1}) in comparison to South Asian brands (20 mg ml^{-1}), the correct dose of Metacam® would involved a smaller volume of injection in comparison to South Asian brands. In order to provide an even high test of the tolerance of Metacam®, this formulation was injected at 2.5 times its recommended dose, in order that the volume administered would match that injected for meloxicam manufactured in South Asia.

Twelve healthy female goats were used in the experiment. Eleven were adults ranging in mass from 10 to 20 kg and there was one kid weighing about 5 kg. Animals were individually marked and numbered and were kept for a minimum of 8 days prior to the first trial. All the goats were reared locally and were used to being handled. The 12 goats were held at the National Trust for Nature Conservation Park Headquarters, Sauraha, Chitwan National Park, Nepal. For a given product, each of four animals received two injections, with an interval of two hours between them. One injection was of the product and the other was of saline solution. The sham injection was given first to two animals and the product injection was given first to the other two, to control for a possible treatment order effect. The order of compounds to be injected and the initial order of control and product injections was chosen at random. After 5 days, so that all meloxicam was metabolised and excreted, another compound was tested on each goat, and after a further 6 days a third product was tested. Each goat received a different compound in each of the three trials and the order of the injections (sham or product first) was the opposite of that in the previous trial. For the three trials, intra-muscular injections were administered by a qualified veterinarian to the gluteal muscle (trial 1), neck muscle (trial 2) and again in the gluteal muscle (trial 3). The first of the two injections in each trial was administered to the left side and the second to the right side of the animal. Full details of the experimental protocol and order of injections are indicted in Table 2. Prior to the injection, hair at the site was clipped and shaved so that the skin was visible and the location of the injection was marked with a *ca.* 50 mm diameter circle, using a permanent marker pen, with the injection site at its centre.

2.4 Assessment of pain

Assessing the pain reaction of different compounds was undertaken by evaluating the pain reaction of an animal injected with a compound in comparison to the same animal injected with a sham dose of saline solution: the difference in the compound versus sham score giving an indication of pain. Two assessments of pain were made following each injection, measuring firstly the animal's immediate reaction to the injection of the product, and secondly measuring any continued signs of pain in the five minutes after the injection. The initial

reaction was further broken down in to two stages, with pain assessed for (a) at the insertion of the needle and (b) at the injection of the product. The initial reaction was scored separately by all observers present and recorded on a four-point ordinal scale of, 0 no reaction to injection, 1 small reaction to injection (e.g. flinches, short vocalisation), 2 medium reaction (sustained flinching and/or vocalisation) and 3 extreme reaction to injection (obvious distress and pain). The median of the scores obtained from the observers was taken as a single overall index of pain for each animal treatment. The second pain assessment was reached by agreement among all observers, with 11 categorical pain behaviours (NRC 1992; see Appendix Table 1) recorded as either present or absent during the period of monitoring. The sum of the pain categories recorded was used to produce a total pain score for each animal treatment, ranging from 0 (no pain reactions) to a maximum of 11 (all pain categories observed). Seven observers were present in trial 1, and six present in trials 2 and 3, with five of the same observers present in all three trials. Observers included veterinarians working for the Department of National Parks and Wildlife Conservation and veterinarians working at the Chitwan Veterinary teaching hospital, Nepal, who were all used to injecting and treating goats and other livestock. Other observers were research biologists working for Bird Conservation Nepal and the Royal Society for the Protection of Birds, UK. Following the initial monitoring of pain, the injection site was examined 4 hours after the injection, and then once a day for the following 4 days. Any evidence of swelling, lesions or abscesses or other abnormalities at the site were recorded, measured (max width, breadth and height) and photographed.

2.5 Statistical analysis

Due to the ordinal scale data from the pain assessments and use of medians, non-parametric Wilcoxon signed rank tests were utilised to test if the pain reaction following dosing was significantly different to the pain reaction of the same individual animals sham dosed with saline solution. Because of the small number of repeats available to test each product (n=4), products were grouped as compounds in order to increase statistical power. The four groups were the three South Asian meloxicam products (n=12), three meloxicam + paracetamol products (n=12), the two diclofenac products (n=8) and a single test of Metacam® (n=4). Statistical testing was one-tailed, testing the directional null hypothesis that pain reaction of compounds was not greater than the pain reaction following-sham dosing. Pearson and Spearman rank correlations coefficients were used when appropriate (for normally distributed and ordinal data, respectively). Tests of the relationship between pH and osmolarity of products and subsequent pain reactions were undertaken with multiple ordinary least squares regression and by Kendall rank partial correlation coefficients (Wessa 2008). The dependent variable used in all analyses, was the pain score calculated as the difference between the pain reaction following injection of an active product in comparison with the pain score in the same animal following injection with the sham saline solution. For graphical purposes, figures pain reactions of different compounds utilised the mean ($\pm$ 1 standard deviation) of the medians obtained from four tests of each compound.

Table 1. Veterinary NSAID products purchased from pharmacies in India and Nepal, with the exception of the European brand of meloxicam (Metacam ®) sourced in the UK, indicating the number of preparations of each drug, the total number of samples tested, and the mean ± one standard deviation and range [in parentheses] for pH, osmolarity and concentration of the NSAID as a percentage of that given on the label (as measured with a validated LC-ESI/MS methodology; Taggart *et al.* 2009).

NSAID	*n* preparations	*n* tested	pH	Osmolarity (mOsmol ml^{-1})	NSAID as % of label
Meloxicam all products	15	53	9.45 ± 1.12 [6.5 - 10.9]	6.86 ± 2.75 [1.72 – 10.92]	124 ± 12 [107 - 154]
Meloxicam	9	38	10.05 ± 0.31 [9.6 - 10.9]	5.49 ± 2.18 [1.72 – 8.01]	127 ± 13 [111 - 154]
Meloxicam + Paracetamol	6	15	8.57 ± 1.33 [6.5 -10.3]	9.14 ± 2.01 [5.25 – 10.92]	121 ± 10 [107 - 143]
Meloxicam (Metacam ®)	1	1	8.60	4.34	106
Analgin	4	26	6.34 ± 0.52 [5.5 - 6.7]	3.19 ± 0.32 [2.60 – 3.77]	120 ± 10 [67 - 152]
Diclofenac	12	14	9.14 ± 0.54 [8.0 - 10.1]	3.82 ± 1.45 [0.68 – 6.67]	87 ± 14 [50 -100]
Ketoprofen	5	20	6.56 ± 0.72 [5.0 - 7.0]	6.45 ± 4.99 [0.73 – 14.10]	105 ± 6 [86 - 125]
Nimesulide	1	1	10.0	17.14	107
Piroxicam + Paracetamol	1	1	8.6	7.64	88
Phenylbutazone + Aspirin	1	1	10.0	2.86	70*

* % values are for the concentration of phenylbutazone within this product

Table 2. Order and sequence of injections for veterinary compounds and products injected during the three phases of the trials. The NSAID compound is indicated by M = Meloxicam, M+P = Meloxicam + Paracetemol and D = Diclofenac, with the actual product in parentheses.

	Trial 1, 17/03/2009		Trial 2, 22/03/2009		Trial 3, 29/03/2009	
Goat #	Injection 1	Injection 2	Injection 1	Injection 2	Injection 1	Injection 2
1	M (Zobid-M)	Sham	Sham	M (Melonex)	Sham	M+P (Ampar)
2	Sham	M (Melonex)	M+P (Ampar)	Sham	Sham	D (Diclo-lab)
3	M (Melonex)	Sham	Sham	M+P (Disovet-MP)	Sham	D (Diclo-lab)
4	Sham	D (Fenak)	D (Diclo-lab)	Sham	Sham	M+P (Melonex-Plus)
5	M+P (Ampar)	Sham	Sham	D (Fenak)	Sham	M (Metacam)
6	Sham	M+P (Disovet-MP)	M (Metacam)	Sham	D (Fenak)	Sham
7	D (Diclo-lab)	Sham	Sham	M (Zobid-M)	M+P (Melonex-Plus)	Sham
8	Sham	M (Diclovet-M)	M (Melonex)	Sham	M (Zobid-M)	Sham
9	M (Metacam)	Sham	Sham	M (Diclovet-M)	M+P (Disovet-MP)	Sham
10	Sham	M (Zobid-M)	M (Diclovet-M)	Sham	Sham	M+P (Melonex-Plus)
11	M (Diclovet-M)	Sham	Sham	M+P (Ampar)	D (Fenak)	Sham
12	Sham	M (Metacam)	M+P (Disovet-MP)	Sham	M+P (Melonex-Plus)	Sham

3. Results

3.1 Composition of veterinary NSAIDs

All 39 preparations of NSAIDs were found to contain the labelled active ingredient or ingredients. The pH and osmolarity of the seven NSAIDs varied considerably, both within and between products (Figure 1). Comparison of Indian meloxicam products with other veterinary NSAIDs indicate that products containing only meloxicam had pH values in the upper range of all compounds, whereas meloxicam + paracetamol products were characterised by very high osmolarities. Comparison of these products manufactured in South Asia with a European manufactured brand of meloxicam (Metacam ®), indicate clear differences in pH and osmolarity (Figure 1). The percentage formulation of meloxicam products (meloxicam alone, or meloxicam + paracetamol), were all close to or above the stated range. For analgin, ketoprofen and nimesulide, the drug percentage was either close to the stated range or in excess of the listed drug concentration (Table 1). In contrast, formulations of diclofenac, piroxicam and phenylbutazone revealed less than the stated concentration, although only one product each of piroxicam and phenylbutazone were available for testing. Most of the 14 diclofenac products were within 65-100% of the labelled concentration; however one diclofenac product contained just 50% of the labelled value. Visual inspection of the pH and osmolarity of ketoprofen indicated a very large range of osmolarities, suggesting that this compound is being formulated in different ways (Figure 1). Two of the three ketoprofen products with very high osmolarities formed a white precipitate upon dilution with water, whereas the single ketoprofen product with a low osmolarity formed a white precipitate when diluted with acetonitrile. The excipient meglumine was not found to be present in any of the South Asian meloxicam products, or any of the other NSAIDs analysed. In contrast analysis of Metacam® revealed meglumine at a concentration of between 3.50-4.25 mg/ml. Comparison of values independently analysed in a second laboratory for a sub-sample of 20 meloxicam products indicated that both laboratories were producing consistent and similar results. For both laboratories values of pH, osmolarity and percentage formulation were all significantly and positively correlated (pH, Pearson correlation r = 0.995, P < 0.001; osmolarity, Pearson r = 0.845, P < 0.001; and % formulation Pearson r = 0.765, P < 0.001).

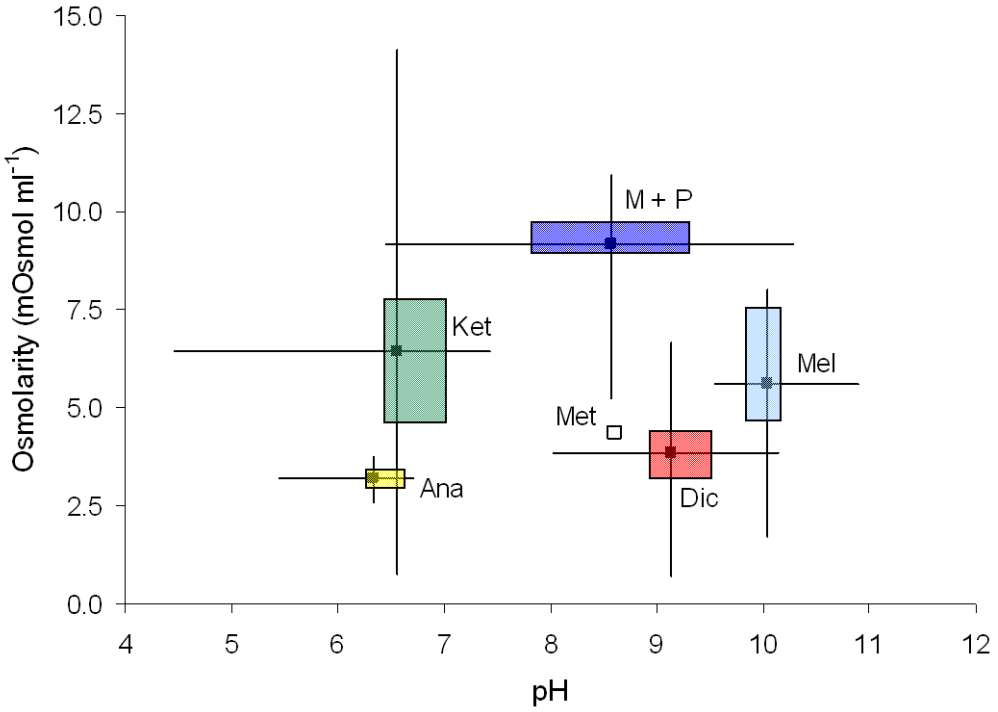

Figure 1. Values of pH and osmolarity for five veterinary NSAIDs on sale in India and Nepal indicating mean levels (filled square), inter-quartile ranges (box plot) and the range (vertical and horizontal line) of values. NSAIDs indicated are meloxicam (Mel, light blue shading), meloxicam + paracetamol (M + P, dark blue), analgin (Ana, yellow), diclofenac (Dic, red) and ketoprofen (Ket, green). Values of pH and osmolarity for the European brand of meloxicam (Metacam®) are indicated by the unfilled square (Met).

3.2 Reaction of goats following treatment

There was no significant difference in pain reaction between sham-dosing and use of an NSAID for any formulation for pain scores on insertion of the needle (Table 3 & 4). However, for differences in pain scores upon injection of the product and saline solutions, one-tailed non-parametric Wilcoxon signed rank tests revealed significant differences between product and sham injection for meloxicam-dosed and meloxicam + paracetamol-dosed animals (Table 3). There was no greater pain reaction in animals following injection with the European manufactured brand of meloxicam than for injection of saline solution, either in the initial reaction to the injection, or in the subsequent five minute monitoring period (Table 3). Similarly, there were no significant differences in pain reaction for the two diclofenac products. The small sample of goats (n=4) meant that there was limited power to detect statistical differences in the Metacam® dosed goats. However, visual inspection of the median pain reaction of goats upon injection, and in the five minutes after injection, indicated that the pain reaction following injection with Metacam® was the same, if not lower, than the reaction after injecting saline solution (Figure 2). As would be expected, there was no significant difference between sham-dosing and any of the compounds for insertion of the needle (Table 3), and significant differences only occurred upon injection of the solution and in the monitoring period after the injection. For the eight products examined, the pain index in the five minutes following the injection was highly correlated with the pain index upon initial injection of the compound (Spearman rank correlation r = 0.79, P < 0.005). There was no significant correlation between pain index associated with insertion of the needle and pain following the injection (Spearman r = 0.18, P = 0.31).

All signs of pain had ceased after five minutes of monitoring. Checks of each animal at two hours indicated no evidence of swelling, lesions or abscesses or other abnormalities at the site for products or sham injections.

Levels of pH and osmolarity were quantified for all nine products used in the pain testing. Multiple ordinary least squares linear regression analysis and Kendall rank correlation of the pain reaction of goats upon injection and in the five minutes following the injection, indicate significant relationships between pH and osmolarity and subsequent pain reaction, as well significant effect of both pH and osmolarity int the overall model (Table 5 and Figure 3). No significant relationship was found for pH and osmolarity, or the combined effect of pH and osmolarity and the insertion of the needle. The data included one outlying meloxicam product, with a very low pH value and high osmolarity, and the significance/non significance of the regressions was estimated both with and without this.

Table 3. Results for one-tailed non-parametric Wilcoxon signed rank tests examining differences between medians for the observed pain response of goats receiving injections of compounds versus sham-dosing with saline. Results are indicated for four groups of compounds, three Indian manufactured brands of meloxicam, three Indian brands of meloxicam + paracetamol, Metacam®, and two Indian brands of diclofenac, with pain scores measured upon insertion of the needle, injection of the compound, and during 5 minutes of after injection monitoring. Wilcoxon statistic and *P* values (for one tailed testing) are presented.

Compound	Products / Goats tested	Insertion of needle	Injection of compound	5 minutes after injection
Meloxicam	3 / 12	$15.5_{1,6}$ $P = 0.173$	$55.0_{1,10}$ $P < 0.005$	$77.0_{1,12}$ $P < 0.005$
Meloxicam + Paracetamol	3 / 12	$23.0_{1,7}$ $P = 0.075$	$53.5_{1,10}$ $P < 0.01$	$76.5_{1,12}$ $P < 0.005$
Metacam®	1 / 4	$2.5_{1,3}$ $P = 0.740$	$6.0_{1,4}$ $P = 0.428$	$6.0_{1,4}$ $P = 0.428$
Diclofenac	2 / 8	$2.0_{1,3}$ $P = 0.789$	$6.0_{1,3}$ $P = 0.091$	$0.0_{1,3}$ 1.0 $P = 0.969$

Table 4. Values of pH and osmolarity and the subsequent mean medium values of the pain reaction (based on 4 repeat trials) upon insertion of the needle, injection of the compound/product and 5 minutes after injection, for the 9 tested NSAIDs. Pain scores were calculated as the difference between the observed pain following injection with a compound/product in comparison to the pain scores obtained from the same animal after sham-dosing with saline solution. No difference in pain reaction of the compound/product in comparison to the saline solution would produce a pain score of 0.

Compound (product)	pH	Osmolarity (mOsmol ml^{-1})	Insertion of needle	Injection of product	5 minutes after injection
M (Diclovet-M)	10.2	5.98	2.00	2.27	4.00
M (Melonex)	10.2	5.81	2.08	3.42	4.75
M (Zobid-M)	10.6	6.16	2.35	3.04	3.50
M+P (Disovet-MP)	9.8	10.05	2.64	2.96	3.25
M+P (Melonex-Plus)	9.4	9.88	2.08	2.83	4.50
M+P (Ampar)	6.4	10.92	1.84	3.12	4.00
M (Metacam)	8.6	4.34	1.58	1.46	0.00
D (Diclo-lab)	9.2	3.22	2.21	1.88	0.25
D (Fenak)	8.5	3.69	1.58	1.54	1.75

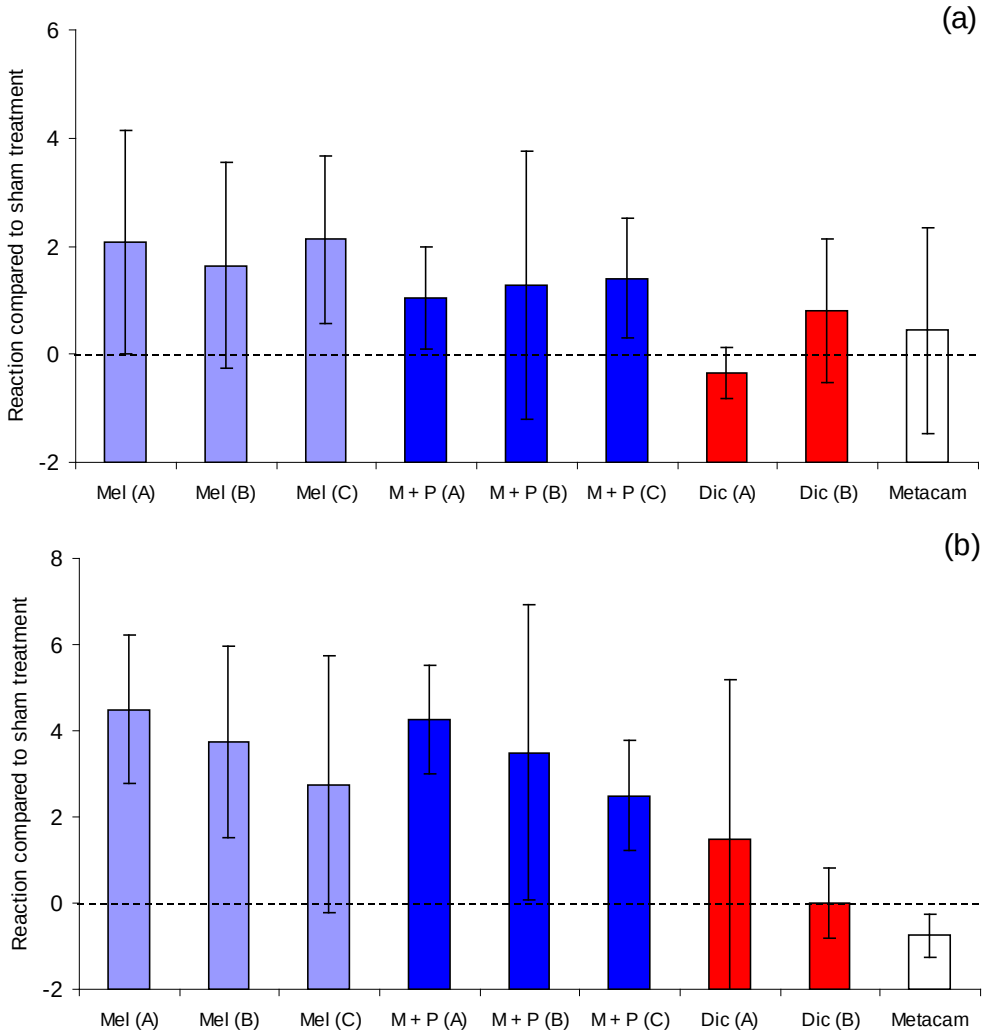

Figure 2. Mean values of the median pain scores for 3 Indian meloxicam products (light blue bars), 3 Indian meloxicam + paracetamol products (dark blue), two Indian diclofenac products (red) and the single European brand of meloxicam (Metacam®) (unfilled), in comparison to the pain scores obtained from the same animal after sham-dosing with saline solution, for (a) pain reaction of goats upon injection of the product/solution, and (b) pain reaction of goats during the five minutes after injection. No difference in pain reaction of the compound in comparison to the saline solution would produce a pain score of 0 (horizontal dashed line). Each product was tested on 4 goats and error bars represent ± 1 standard deviation. The pain reaction of the 3 meloxicam products and 3 meloxicam + paracetamol products in comparison to sham-dosing was statistically significant for both the pain reaction upon injection and the pain reaction for the 5 minutes following injection (see Table 3).

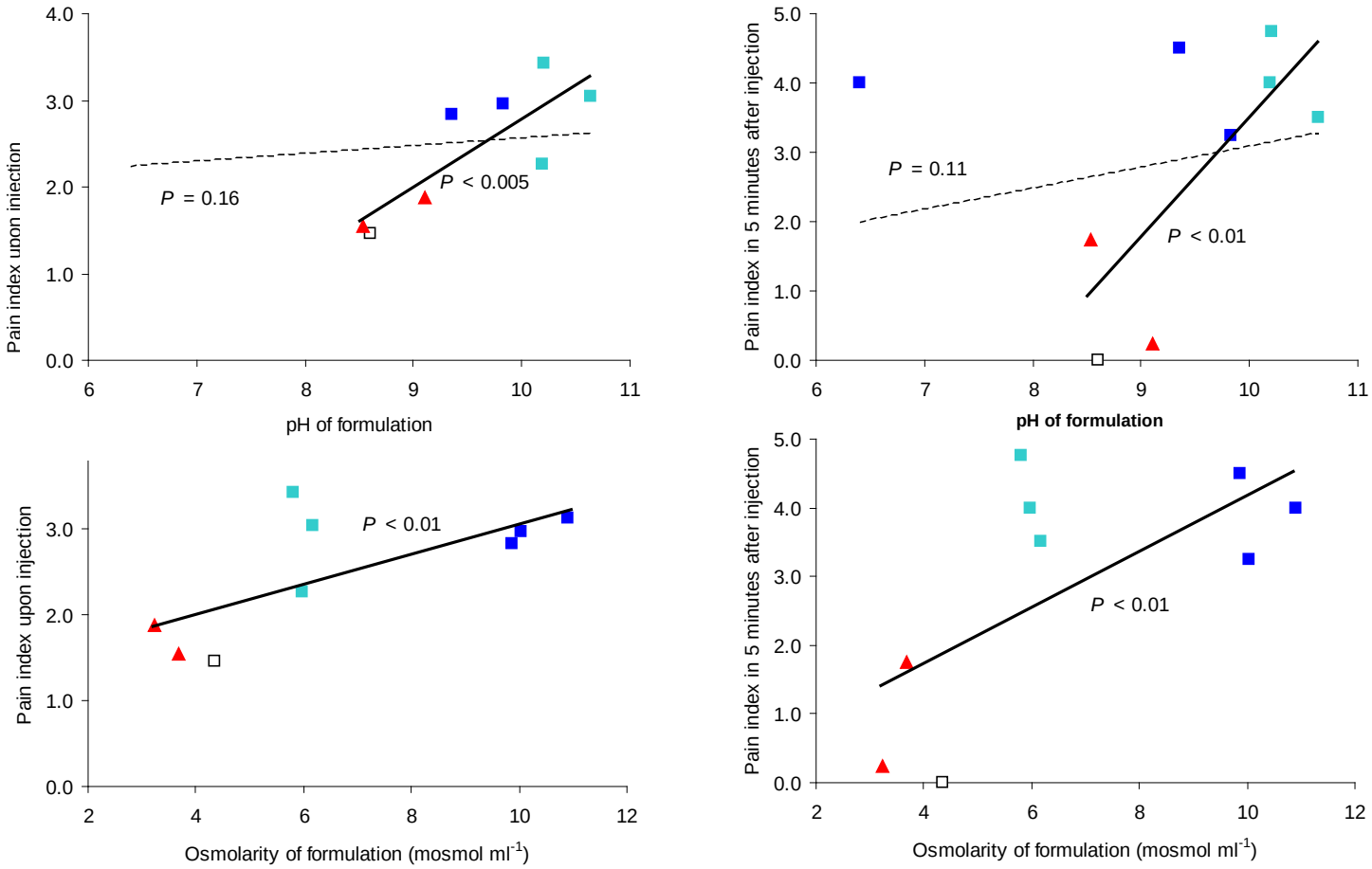

Figure 3. Pain reaction of goats upon injection (left graphs) and during 5 minutes after injection (right graphs) against pH and osmolarity, along with the R^2 value and significance of the fitted ordinary least squares regression lines. Dashed and solid lines indicate the regression including and excluding the outlying product, respectively (the outlier value is clear in the upper two graphs). Values are the mean of the medians of the four repeats. South Asian meloxicam products = light blue squares, meloxicam + paracetamol = dark blue squares, diclofenac = red triangles, Metacam ® = unfilled square.

Table 5. Results of multiple ordinary least squares regression models for pH, osmolarity and the overall model fit (*t* and *F* values), and results of Kendall rank correlation coefficients for pH and osmolarity (τ (tau) values) with pain score (the difference between the pain reaction of the product versus sham treatment in the same animal) as the dependent variable. Statistical tests are presented for the pain scores upon insertion of the needle, injection of the product and subsequent 5 minutes after injection. *P* values are indicated as: N.S. = not significant; * = <0.05; ** <0.01 and *** <0.005

	Insertion of needle		Injection of product		5 minutes after injection	
	Regression	Kendall	Regression	Kendall	Regression	Kendall
With outlier						
pH	$t = 1.03$ N.S.	$\tau = 0.16$ N.S.	$t = 1.44$ N.S.	$\tau = 0.27$ *	$t = 1.66$ N.S.	$\tau = 0.26$ *
Osmolarity	$t = 1.40$ N.S.	$\tau = 0.17$ N.S.	$t = 2.91$ **	$\tau = 0.32$ *	$t = 3.00$ *	$\tau = 0.28$ *
Overall model	$F_{2,33} = 1.34$ N.S.	-	$F_{2,33} = 4.48$ *	-	$F_{2,33} = 4.91$ *	-
Outlier excluded						
pH	$t = 1.22$ N.S.	$\tau = 0.22$ N.S.	$t = 3.31$ ***	$\tau = 0.47$ ***	$t = 2.34$ *	$\tau = 0.40$ **
Osmolarity	$t = 0.85$ N.S.	$\tau = 0.21$ N.S.	$t = 0.89$ N.S.	$\tau = 0.28$ *	$t = 1.66$ N.S.	$\tau = 0.30$ *
Overall model	$F_{2,29} = 1.79$ N.S	-	$F_{2,29} = 8.33$ ***	-	$F_{2,29} = 6.33$ **	-

4. Discussion

The results of this study support reports made by practising veterinarians in South Asia that brands of meloxicam and meloxicam + paracetamol on sale in India and Nepal are causing irritation and pain in treated animals. While the results of this study indicate that pain reactions were short-lived, lasting a matter of minutes, nonetheless treated animals were clearly distressed upon and immediately after injection with these products. In marked contrast, the European brand of meloxicam (Metacam®) produced no noticeable pain reaction and indeed the average pain score for this formulation was no different to the reaction when the same goats were injected with saline solution.

There were marked differences between Metacam® and the other meloxicam and meloxicam + paracetamol products in pH and osmolarity. The Indian manufactured brands of meloxicam either had very high pH values (alkaline) or very high osmolarities. The reaction of goats indicated that pain was related to both increasing pH levels and increasing osmolarity of compounds. Values of pH and osmolarity for other veterinary NSAIDs available for sale and in use in South Asia indicate that these products also encompass a range of high (and low) pH values and a very wide range (including extremely high) osmolarities. While the pain reaction of these other compounds was not tested, the relationship found between pain and increasing pH and osmolarity in our study, strongly suggests that similar pain reactions will be encountered with other products. While our analysis of these NSAID formulations indicated a wide range of pH and osmolarities, analysis of levels of active ingredients indicated that most NSAIDs contained the same or higher levels of active ingredient as labelled. Only preparations of veterinary diclofenac (which it is now illegal to manufacture in India, Nepal and Pakistan) were found to be of poor quality, containing lower levels (50% less in one instance) of active ingredient than labelled. There were no instances of any NSAID containing anything but the labelled active ingredient.

Given the tolerance of goats to Metacam® and the successful use of this drug for veterinary treatment in Europe and North America (and stringent pharmacovigilence for medicinal products in both Europe and North America), why are South Asian products causing pain and how are they being made differently? The pH level of the mammalian body is closely regulated and lies between 7.35 to 7.45, while plasma osmolarity averages around 0.30 mOsmol ml^{-1}. In comparison to these values, it is clear that many NSAID formulations on sale in South Asia have pH values that are very alkaline and highly isotonic. The pH scale is itself logarithmic and products with the highest pH levels (up to pH 10.6; Table 1) will contain more than 1000 times the concentration of hydroxide ions than the body. To put this in to perspective, household ammonia (bleach) typically has pH levels of 11 to 11.5, close to the pH level of the most alkaline meloxicam products analysed. Unsurprisingly, intra-muscular injections with products of pH similar to bleach will cause acute pain and for larger volume injections (as administered to cattle and buffalo) may also be likely to cause tissue damage and necrosis (although this was not observed in our study on goats). Likewise, very hypertonic formulations (in comparison to the mammalian plasma) are also likely to cause acute pain due to the osmotic pressure they create at the injection site. The observed maximum osmolarity for a meloxicam product recorded here was 10.92 mOsmol ml^{-1}, i.e., over 35 times the osmolarity of the plama.

The likely reason for the very high pH levels of meloxicam products in South Asia is that molecules of meloxicam have a relatively low solubility in water (at a neutral pH of 7.0 solubility is 0.26 mg ml^{-1}), however solubility increases with increasing pH , i.e., solubility at pH 8 is reported as 1.55 mg ml^{-1}, and at pH 10 is 2.31 mg ml^{-1}. Hence, it would seem that pharmaceutical manufacturers in India are raising formulation pH levels for meloxicam

formulations in order to create sufficient concentrations for injection. All South Asian brands of meloxicam were found to have concentrations of meloxicam of 20 mg ml^{-1} and pHs >10, whereas Metacam® with a measured pH of 8.6 has a dose concentration of 50 mg ml^{-1}. The other key difference between Metacam® and South Asian brands of meloxicam is the absence of the molecule meglumine from Indian manufactured products. Meglumine is used as an excipient in many pharmaceutical products, including Metacam®. The addition of meglumine creates a meloxicam-meglumine salt, and this greatly increases the solubility of meloxicam in water, enabling Metacam® to have a dose concentration 2.5 times higher than South Asian manufactured products, without a correspondingly high pH level. Details of this formulation are freely available on the internet under the copyright patents lodged by Boehringer Ingelheim. Moreover, Boehringer Ingelheim, the parent company that originally developed meloxicam and markets this drug in Europe and North America, has generously agreed to waive copyright to the formulation for products manufactured and marketed in South Asia, as they are aware of the issues involved for conserving vultures.

We welcome the fact that the number of meloxicam products being manufactured and used in South Asia has risen since the ban on veterinary diclofenac products; in 2004/05 there was only one company manufacturing veterinary meloxicam in India, but this study showed at least 14 companies were manufacturing it in 2008. However, the acute pain reaction that these products are causing is a source of concern with respect to animal welfare. It is also of concern because such a reaction of animals to treatment is leading farmers and veterinarians to continue to use diclofenac products; including diclofenac manufactured for human use (BNHS & BCN unpublished data). This is being done in preference to switching to meloxicam, the only veterinary NSAID confirmed to be safe for vultures and other scavenging birds (Swan *et al.* 2006; Swarup *et al.* 2007). We hope that this study, which outlines some problems with the manufacture of veterinary NSAIDs in South Asia, is of use in firstly identifying the source of the problem, and secondly, that with the offer of Boehringer Ingelheim to waive the copyright to the formulation of meloxicam, it also provides an effective solution to the mutual benefit of vulture conservation and livestock treatment.

5. Acknowledgements

Thanks to the National Trust for Nature Conservation for allowing this study to go ahead at Kasara, Chitwan National Park, and for their staff time in feeding and looking after the goats both before and during the trials. Our thanks go to Juergen Daemmgen and Andrew Routh for advice and helpful suggestions on the experimental design of this study, and to Rhys Green and Juliet Vickery for comments that improved an earlier draft of this report. The project was funded by the Royal Society for the Protection of Birds.

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Appendices

Appendix 1 – Authors, institutions and contributions to the study

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Appendix 2 – Species-specific signs of pain behaviour

Table A1 Species-specific behavioral signs of pain in sheep and goats as criteria for Administering Analgesics (Excerpted and adapted from Recognition and Assessment of Pain, Stress and Distress in Laboratory Animals, Recognition and Alleviation of Pain and Distress in Laboratory Animals, NRC, Washington, DC 1992, updated 05-15-2007)

Pain reactions observed for sheep and goats
1. Dull, depressed, head held low
2. Rapid, shallow respiration
3. Grunting or grinding teeth
4. Persistent licking or kicking at an area
5. Reluctance to move
6. Standing and lying down repeatedly
7. Loud, persistent vocalization
8. Jumping and/or flight behaviour
9. Sudden defecation and/or urination
10. Twitching/trembling at injection site
11. Rigid holding of leg or neck

Appendix 3 – List of NSAIDs purchased and tested

Table A2 NSAIDs purchased from pharmacies in India and Nepal and tested for pH, osmolarity, % formulation and the presence of meglumine, indicating the type of NSAID, product name, manufacturing company, volume, and labelled drug concentrations (where products contained more than one active ingredient the labelled concentrations are listed in same order as listed in the first NSAID column).

NSAID	Name	Manufacturer	Volume (ml)	Drug conc. (mg/ml)
Analgin	NFI	Parkin Remedies	30	500
Analgin	Vetalgin	Intervet India Pvt. Ltd.	30	500
Analgin & 2 Anti-spasmodics	Morgan-Vet	Morvel Laboratories Pvt. Ltd.	30	500 & 2 & 0.02
Analgin & Paracetamol	Bolin	Sellwell Pharmaceuticals Ltd.	30	150 & 150
Diclofenac	Activa - 30	Health Biotech (P) Ltd.	30	25
Diclofenac	Dicloflame	Parenteral Pharma Pvt. Ltd.	30	25
Diclofenac	Diclolab	Laborate Pharmaceuticals India Ltd.	30	25
Diclofenac	Diclovet	Umedica Labs	30	25
Diclofenac	Dicoliv	Ind-Swift Limited.	30	25
Diclofenac	Disic-Vet	SWEGA Laboratories Ltd	30	25
Diclofenac	Doflex	Jagsonpal Pharma	30	25
Diclofenac	Fenak	Nitin Lifescience Ltd.	30	25
Diclofenac	Fenak	Nitin Lifescience Ltd.	30	25
Diclofenac	Valfen	Nitin Lifescience Ltd.	30	25
Diclofenac	Valfen	Nitin Lifescience Ltd.	30	25
Diclofenac	Zobid	Ambalal Sarabhai Enterprises Limited	30	25
Ketoprofen	Butagesic-K	Concept Pharmaceutical Ltd.	30	100
Ketoprofen	Ketolon	Tineta Pharma Pvt. Ltd.	15	100
Ketoprofen	Ketop	Umedica Laboratories Pvt. Ltd.	15	100
Ketoprofen	Neoprofen	Alps Pharmaceuticals (P) Ltd.	15	100

Ketoprofen	Vetoprofen	Shivek Labs Ltd.	15	100
Meloxicam	A3Vet	Brihans Laboratories	30	5
Meloxicam	Biocam	Biovet Pvt. Ltd.	30	5
Meloxicam	Diclovet-M	Phamanza (India)	30	5
Meloxicam	Melonex	Intas Pharmaceuticals Ltd.	30	5
Meloxicam	Meloswift	Integrated Labs	30	5
Meloxicam	Meloswift	Ultra Drugs PVT Ltd	100	5
Meloxicam	Meloxi	Prashanti Formulations Ltd	30	5
Meloxicam	Metacam	Boehringer Ingelheim	50	20
Meloxicam	Oxycam Vet	Vet India Pharmaceuticals Ltd.	30	5
Meloxicam	Zobid-M	Sarabhai Zydus	30	5
Meloxicam & Paracetamol	Ampar MP	Altra Drugs Pvt. Ltd.	30	5 & 150
Meloxicam & Paracetamol	Disovet-MP	Devine Formulations	30	5 & 150
Meloxicam & Paracetamol	Melonex Plus	Insat Pharma	30	5 & 150
Meloxicam & Paracetamol	MP-3	Pharmanza (India)	30	5 & 150
Meloxicam & Paracetamol	Proxyvet-MP	Health Biotech (P) Ltd.	30	5 & 150
Meloxicam & Paracetamol	Restamin Plus	Integrated Laboratories Pvt. Ltd.	15	5 & 150
Nimesulide	Nimovet	Indian Immunologicals Ltd.	15	100
Paracetamol and Piroxicam	PDP	Embark Lifescience PVT Ltd	30	150 & 10
Phenyl Butazone & Sodium Salicylate	Artizone-S	Alved Pharma & Foods Pvt. Ltd.	30	200 & 20